Unmeasurable Total Bilirubin in an 80-Year-Old Man

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CASE DESCRIPTION

An 80-year-old man presented to the hospital with a 1-week history of shortness of breath, lethargy, and poor oral intake. He has a medical history of diabetes mellitus, hypertension, dyslipidemia, and chronic obstructive airway disease (COAD). His medications include subcutaneous insulin 10 units every 8 h, telmisartan 80 mg once daily, and atorvastatin 40 mg nightly. On admission, he had a temperature of 37.8°C, blood pressure of 142/94 mmHg, pulse rate of 91/min, respiratory rate of 24/min, and oxygen saturation of 95% under room air. Clinically, he was not jaundiced. Physical examination revealed wheezing and left lower zone crepitation while a chest X-ray revealed left lower zone consolidation. Blood investigations revealed anemia (hemoglobin = 8.6 g/dL; reference interval [RI]: 13–17 g/dL), thrombocytopenia (platelet = 120 k/μL; RI: 150–410 k/μL), hyperproteinemia (total protein = 100 g/L; RI: 64–83 g/L) and hypoalbuminemia (albumin = 17 g/L; RI: 34–48 g/L).

Total bilirubin (TBIL) was ordered as part of a liver panel in this patient. Interestingly, TBIL could not be quantified on our analyzer (Alinity ci, Abbott Laboratories) due to a reaction check failure.

QUESTIONS TO CONSIDER

1. What are the possible causes of unreportable total bilirubin result?
2. What are the principles of spectrophotometric measurement?
3. How can paraprotein interference in total bilirubin assays be minimized?

Examination of the reaction curve (RC) showed an abnormally high absorbance during reagent blank measurement. The patient denied taking any medication that could potentially interfere with the TBIL assay. His blood sample was collected by an experienced nurse into a standard 5 mL serum tube containing clot activator (VACUETTE®, Greiner Bio-One). The specimen was clear on visual and photometric assessment (Fig. 1). The analyzer's spectrophotometer lamp was functioning well. Assay calibration and internal quality control readings were within limits. Repeat testing of the serum sample showed similar results and serial dilution excluded high analyte concentration (Table 1). An aliquot of the patient's serum was sent for analysis to 3 external

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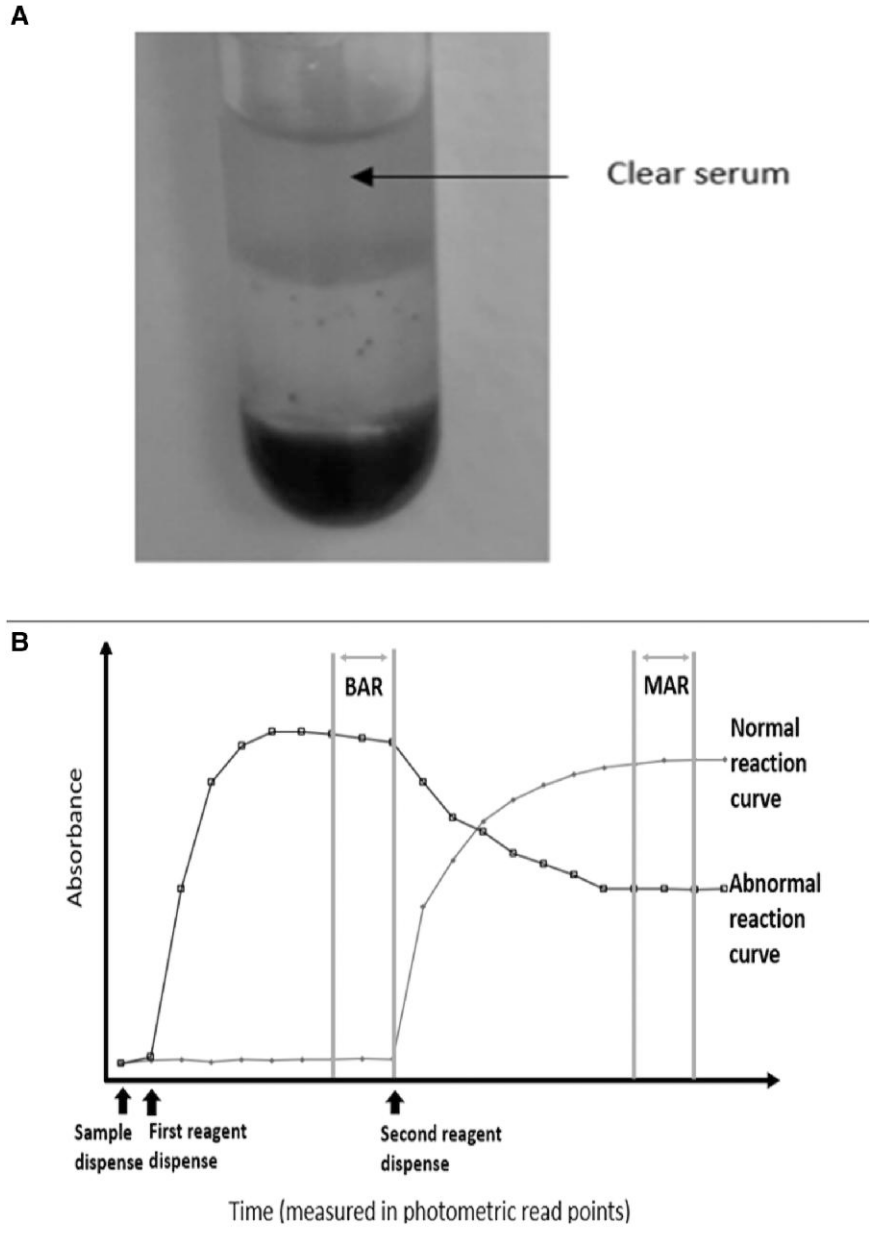

Fig. 1. Patient's serum sample and reconstructed RC of total bilirubin assay on Alinity ci analyzer. (A), The patient's serum sample prior to analysis; (B), Schematic diagram of total bilirubin reaction curve (RC) displaying BAR and MAR and the sample, R1 and R2 dispense times; (C), Normal (RC) in a healthy individual; (D), Abnormal (RC) of the patient observed during analysis of a neat sample; (E), RC in the patient using a 1:10 diluted sample.

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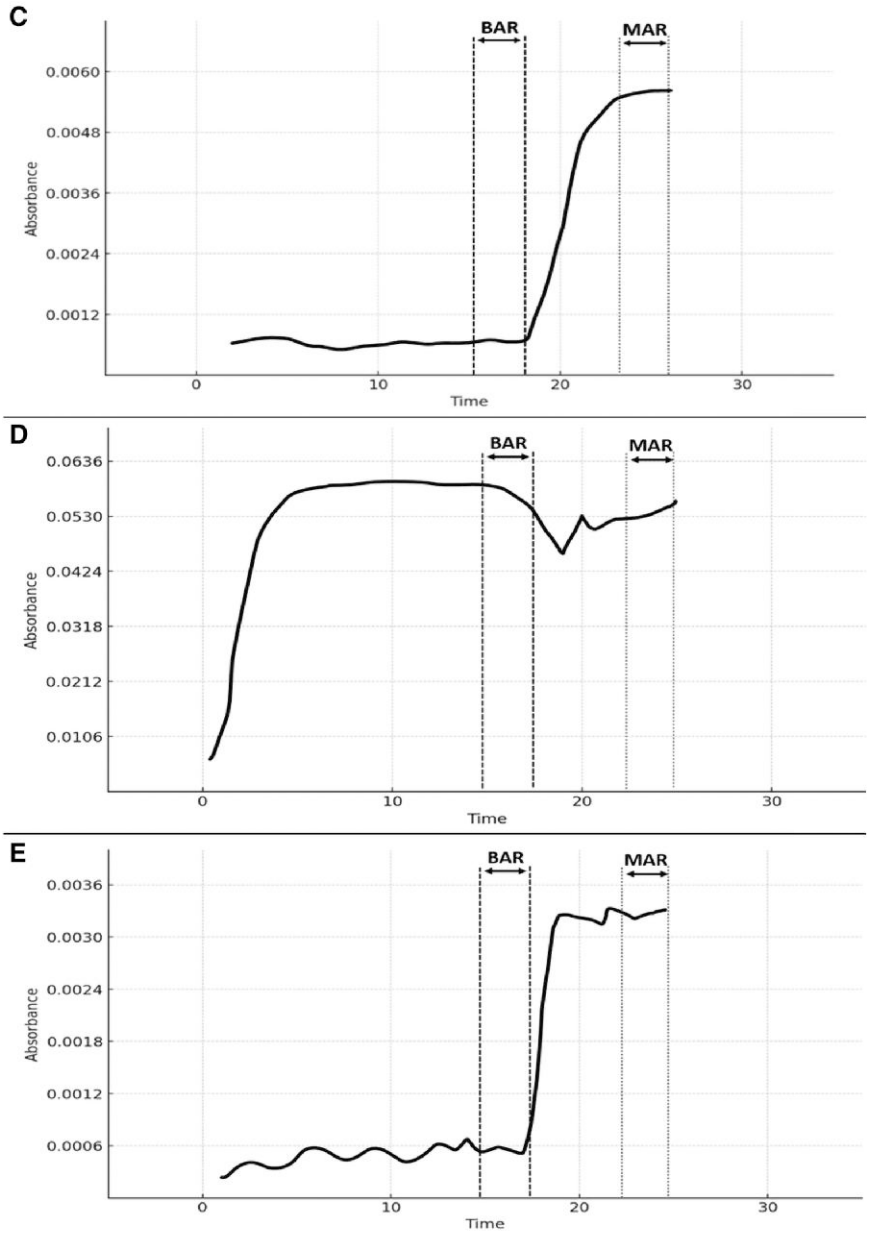

Fig. 1. Continued

platforms (AU5800, Beckman Coulter; Cobas 8000, Roche Diagnostics; Atellica CH930, Siemens Healthineers), 2 of which were able to produce TBIL results while the third platform was unable to do so (Table 1). The initial TBIL result was not reported in this patient due to a suspected paraprotein interference. However, findings from external platforms were communicated to the treating clinician and the laboratory recommended outsourcing future TBIL testing to an external laboratory.

The patient was diagnosed with acute exacerbation of COAD secondary to pneumonia. He was

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Table 1. Comparison of the total bilirubin assays and results on 4 different analytical platforms.

	Alinity ci, Abbott Laboratories	Cobas 8000, Roche Diagnostics	AU5800, Beckman Coulter	Atellica CH930, Siemens Healthineers
Methodology	Diazo reaction	Diazo reaction	Diazo reaction	Vanadate oxidation
Active ingredients in reagent 1	Surfactants (4.53%), HCl (9.33 g/L)	Phosphate (50 mmol/L), detergents, stabilizers, pH 1	Acetic acid (0.1–1%), lithium dodecyl sulphate (2–5%)	Citrate buffer (pH 2.9) (0.1 mol/L), detergent
Active ingredients in reagent 2	2,4,-Dichloroaniline (0.81 g/L), HCl (5.563 g/L), Sodium nitrite (0.345 g/L), surfactant (1.96%)	3,5-Dichlorophenyl diazonium salt (≥1.35 mmol/L)	Caffeine (2.1 mmol/L), 3-5-dichlorophenyl-diazonium-tetrafluoroborate (0.31 mmol/L)	Phosphate buffer (pH 7.0) (10 mmol/L), Sodium metavanadate (4 mmol/L)
Neat concentration	Reaction check failure	Reaction check failure	4.6 µmol/L	<3 µmol/L
Concentration after 1:5 dilution	<8.6 µmol/L	<2.3 µmol/L	Not performed[a]	Not performed[a]
Concentration after 1:10 dilution	<17.1 µmol/L	Not performed	Not performed[a]	Not performed[a]

[a]Dilution was not performed because the analyzers were able to generate results with the neat sample.

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initiated on inhaled ipratropium bromide/albuterol sulfate every 4 h and intravenous amoxicillin/clavulanic acid 1.2 g every 8 h. Considering the patient's clinical presentation, bicytopenia, and interference with TBIL assay, the pathologist suggested further investigations. Serum protein electrophoresis (SPEP) and immunofixation (IF) revealed IgG kappa and IgM kappa monoclonal bands measuring 34.3 g/L and 20.0 g/L, respectively. Serum quantitative immunoglobulin testing for total IgG and IgM was not performed in this case since they are less specific, measuring both normal polyclonal immunoglobulins and myeloma-associated monoclonal proteins. A peripheral blood film showed 77% abnormal lymphoid cells. Bone marrow aspiration and trephine biopsy confirmed the diagnosis of B-lymphoproliferative disorder. He was subsequently referred to the hematologist for further management.

CASE DISCUSSION

The Alinity ci TBIL assay in our laboratory is based on modification of the diazo method. This reaction requires the sequential addition of reagent 1 (R1) and reagent 2 (R2) to the serum sample. R1 consists of surfactants that act as the accelerator and hydrochloric acid (HCl) that acts to alter the pH of the solution to a strongly acidic medium (pH 1). After the sample is solubilized with R1, R2 (the chromogen-containing reagent) is added to the cuvette. R2 consists of a diazonium salt which reacts with bilirubin to form azobilirubin, resulting in a color change. In this end-point assay, the blank absorbance reading (BAR) is taken after R1 is added to the serum sample, whilst the main absorbance reading (MAR) is taken after R2 is added to the serum sample (Fig. 1). The increase in absorbance, measured at 548 nm, with a bichromatic correction at 604 nm, is proportional to the TBIL concentration in the sample.

Paraprotein interferences have been reported to occur in assays which are conditioned at extreme low or high pHs (1), such as the TBIL assay in our case. In contrast to a normal sample whereby the increase in absorbance on the reaction monitor occurs only after addition of the second chromogen-containing reagent, a serum sample with a high concentration of paraproteins demonstrates an increase in BAR immediately after the addition of the acid-containing first reagent (Fig. 1). As a result, subtracting the BAR from the MAR yields a negative figure, which falls outside the defined calculation limits, resulting in a reaction check failure (Fig. 1).

This phenomenon does not occur due to the colorimetric reaction itself, but rather due to the paraprotein precipitation in a strong acidic environment (1). Upon addition of the second reagent, the interfering absorbance from paraprotein precipitation gradually decreases in magnitude (by dilution), resulting in a lower MAR (Fig. 1). Despite being one of the less common types of paraprotein, IgM paraproteins account for a large portion of interferences (2, 3), as observed in our patient. This can be explained by the pentameric structure of IgM, which has an enhanced ability to form polymers, resulting in a decreased solubility (2).

Three platforms in our case utilized a diazo reaction in the measurement of TBIL. Nevertheless, 2 platforms (Alinity ci and Cobas 8000) were unable to produce a TBIL result from our patient's sample (Table 1). Two aspects were considered; first, differences in reagent composition and pH; second, the light-scattering effect of paraprotein aggregates. Both assays above were conducted in strong acidic environments (presence of HCl, pH <1). Stronger acids have been shown to facilitate the formation of large protein aggregates, consequently altering the measurement of transmitted light; whereas weaker acids cause formation of smaller precipitates that may be corrected by background subtraction (4). Although protein stabilizing agents are present in R1 to prevent precipitation (Table 1), the solubilizing capacity of these agents may be insufficient when the concentration of proteins is significantly higher than the usual (5), or when paraprotein aggregates

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continuously form in a low pH environment. In contrast, the AU5800 was able to produce TBIL results. The utilization of a relatively weaker acetic acid in its R1 formulation (Table 1) may be one of the reasons why protein precipitates were less likely to form and interfere with the absorbance reading.

In spectrophotometry, bilirubin absorption at a specific wavelength is measured, allowing its concentration to be determined quantitatively according to the Beer–Lambert Law (6). Similar to lipoproteins, paraprotein precipitates can cause sample turbidity, resulting in light scattering and interference with absorbance measurement (4). Background subtraction, performed by deducting the absorbance at the secondary wavelength from the total absorbance occurring at the primary wavelength, may be able to correct absorbance deviation from small precipitates (4). Nevertheless, background subtraction may not be able to correct absorbance caused by larger aggregates (4). The erratic and unpredictable movement of large paraprotein aggregates, leading to random light scattering, may contribute to the higher BAR compared to MAR observed in our case (4).

Contrary to the three platforms above utilizing a diazo method, the Atellica CH930 TBIL assay is based on a chemical oxidation method whereby bilirubin is converted to biliverdin utilizing vanadate in a mildly acidic environment (7). A similar mechanism is employed in the enzymatic method whereby bilirubin is converted to biliverdin via bilirubin oxidase in an alkaline environment (8). Both of these methods measure the decrease in absorbance during the reaction, which is proportional to the TBIL concentration of the sample. While diazo methods are inexpensive and more widely used, the vanadate oxidation method offers a wider analytical measuring range and less reported interference by paraproteins, hemolysis, and lipemia (7). Although not available in our setting, dry chemistry systems (Vitros, Ortho Clinical Diagnostics) utilizing the unconjugated bilirubin and conjugated bilirubin (BuBc) slide assay have been reported to be the least susceptible to paraprotein interference (5, 9). This is due to the use of solid barriers that filters endogenous interferences such as proteins and lipids before the specimen reaches the reaction layer (5, 9). Besides differentiating bilirubin fractions, this method is also less vulnerable to photodegradation and turbidity interference compared to traditional TBIL assays (5, 9).

Paraproteinemia can interfere significantly with TBIL assays, leading to unreliable results that complicate clinical interpretation. Awareness of this phenomenon may help prevent unnecessary concerns and investigations. As seen in our laboratory, implementing warning flags from the analyzer, checking photometric reaction data, reviewing the delta check on patient results, and correlating results with clinical information are steps that can be performed to detect paraprotein interference (9, 10). Pre-dilution of the sample until the interfering paraproteins are no longer present and selective removal of the paraproteins by ultrafiltration or polyethylene glycol are techniques that can be utilized to lower the incidence of paraprotein interference (2, 9). A third and easy method, which was performed in our scenario, is to analyze the sample on an alternative platform (10).

EDUCATIONAL POINTS

- Paraproteins interfere with total bilirubin assays through reagent interactions, precipitation, increased turbidity and altered optical readings.
- Extreme pH levels can precipitate paraproteins, affecting light transmission measurements.
- Paraprotein interference is idiosyncratic and may not depend on subtype or concentration.
- Unreportable or discrepant total bilirubin results should prompt investigation for paraprotein interference.
- Pre-dilution, ultrafiltration, polyethylene glycol treatment, or analysis on alternative platforms can mitigate paraprotein interference in total bilirubin assays.

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CONCLUSION

Paraprotein interference in TBIL measurement can arise due to reaction between reagent formulation and paraproteins, precipitation of paraproteins, increased sample turbidity and changes in optical readings. The magnitude of paraprotein interference may be independent of its subtype and concentration (7). Additionally, inconsistent results may even be reported between assays using the same method (7). As seen in our case, identification of an unreportable TBIL result led to further investigation and diagnosis of a hematological disorder in the patient. Understanding assay methodologies and recognizing potential interferences allow laboratories to transform analytical challenges into diagnostic opportunities.

Nonstandard Abbreviations: COAD, chronic obstructive airway disease; RI, reference interval; TBIL, total bilirubin; R1, reagent 1; R2, reagent 2; BAR, blank absorbance reading; MAR, main absorbance reading.

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