A 2,4-Dichlorophenyl Diazonium-Based Method for Total Bilirubin without Interference from Indican in Uremic Sera

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Comparison of total bilirubin quantification by a 2,4-dichlorophenyl diazonium method (2,4-DCPD) with a Jendrassik–Grof type of method showed excellent correlation for randomly selected sera. However, sera from uremic patients on chronic hemodialysis showed a marked positive bias for the 2,4-DCPD result as compared with the Jendrassik–Grof result. The mean difference was 5.3 mg/L, and resulted in about 20% of the hemodialysis patients having bilirubin values >13 mg/L, the upper limit of our reference range. Indican in uremic sera reportedly reacts with certain diazo reagents, so we investigated indican’s reactivity in the above methods. In vitro addition of indican caused no interference in the Jendrassik–Grof method, but produced a significant positive interference in the 2,4-DCPD method, 1 mmol of indican per liter appearing as about 36 mg of total bilirubin per liter. Long reaction times with the 2,4-DCPD reagent accentuate the problem. By shortening the reaction time with the 2,4-DCPD reagent to 1.7 min, we find that the indican interference can be eliminated, without affecting quantification of total bilirubin in either normal or uremic sera.

Since van den Berg and Snapper first described the reaction of bilirubin in human serum with Erlich’s diazo reagent (diazotized sulfanilic acid), a multitude of bilirubin methods have been described (1). Currently, most clinical laboratories use methods for total bilirubin based on that described by Jendrassik and Grof in 1938 (2). In their procedure diazotized sulfanilic acid is reacted with bilirubin in the presence of sodium benzoate and caffeine. These and other compounds—including methanol, ethanol, urea, dimethyl sulfoxide, and surfactants—have been termed “accelerators,” because they release unconjugated bilirubin from albumin binding sites, such that total bilirubin may be measured.

In 1962, Rand and diPasqua (3) described an alternative diazo reagent, 2,4-dichlorophenyl diazonium (2,4-DCPD), which is more stable in solution and has been adapted to several automated instruments, including the Olympus "Demand." While evaluating this instrument, we compared total bilirubin as measured by the 2,4-DCPD method with that by a modified Jendrassik–Grof method (4). For random specimens the comparisons were excellent, but, when we compared results for patients from our renal dialysis unit a significant positive bias to the 2,4-DCPD method was discovered. We found that indican (indol-3-yl sulfate) is the interfering compound in sera of patients with chronic renal failure. During the preparation of this manuscript, Poon and Hinberg (5) reported similar findings that indican interferes with several commercial bilirubin procedures involving 2,4-DCPD, and with a closely related analog, 2,5-dichlorophenyl diazonium (5). They cautioned that one should be suspicious of unexpectedly high bilirubin values in uremic patients. We have extended these studies, and describe here a method involving 2,4-DCPD that may be used to quantify total bilirubin in uremic sera without indican interference.

Materials and Methods

Standard Reference Material (SRM no. 916) bilirubin was obtained from the National Bureau of Standards, Washington, DC 20234; bovine albumin from Calbiochem-Behring, La Jolla, CA 92037; and indican from Sigma Chemical Co., St. Louis, MO 63178. Other reagents were A grade or better. All methods were standardized with a bilirubin solution prepared from the SRM bilirubin dissolved in sodium carbonate followed by addition of 40 g/L bovine albumin (1). This standard was stored in portions at −70 °C.

Serum specimens were selected without conscious bias from those submitted for routine bilirubin analysis. They were from randomly selected patients or from uremic patients on chronic hemodialysis. The specimens were stored protected from light at 4 °C for up to 24 h or at −20 °C for up to one month. Sera from uremic patients on propranolol were excluded, as falsely elevated bilirubin concentrations are measured by Jendrassik–Grof methods (6–8).

We used a slight modification of a Jendrassik–Grof total bilirubin method for centrifugal analyzers (9). We used the same volumes and composition of reagents that (9) describe, but we reduced the sample volume from 50 μL to 20 μL, because we found the standard curve was not linear to 200 mg/L as originally reported. We performed the Jendrassik–Grof analyses and spectrophotometry with the 2,4-DCPD reagent as described below, using either a "Rotochem II" (American Instrument Co., Silver Spring, MD 20901) or a "Multistat III" Instrumentation Laboratory, Lexington, MA 02173 centrifugal analyzer. The method of Cross et al. (4) is serum blanked, but does not incorporate the alkaline sodium tartrate reagent to convert azobilirubin to azobilirubin blue as Jendrassik and Grof originally described (2).

Reagents for use in the Olympus "Demand" discrete analyzer were from Worthington Diagnostics Systems, Inc., Freehold, NJ 07728, and were used as described by the manufacturer. The 375 μL of final reaction mixture contains 15 μL of sample in 0.83 mmol/L 2,4-DCPD and 40 mmol/L hydrochloric acid plus an unspecified amount of a "surfactant" and sodium 1,5-naphthalene disulfonate in the "sample" cuvette, and 15 μL of sample in 40 mmol/L hydrochloric acid in the "blank" cuvette. The time from sample addition to spectrophotometric measurement in the Demand (A440/A340 bichromatic) is ordinarily 8.3 min, but was shortened as described below. We measured indican in the uremic sera by the spectrophotometric method of Monias and Schapiro (9).

Results and Discussion

Figure 1 shows the correlation for total bilirubin as measured by the 2,4-DCPD method in the Demand with the
original reaction time of 8.3 min compared with that measured by the Jendrassik–Grof method. The two methods show excellent correlation with randomly selected, non-uremic sera, but for sera from patients on chronic hemodialysis, the 2,4-DCPD method shows a significant positive bias. As indican has been reported to react with certain diazo reagents (10, 11), we measured the indican in the uremic sera and plotted the difference in measured total bilirubin in uremic sera by the 2,4-DCPD method and Jendrassik–Grof method vs the measured indican concentration in serum. As shown in Figure 2, there was good correlation between these variables, the slope of the linear regression line being 34 mg of bilirubin per liter positive bias per millimole of indican per liter. This value is very close to the 36 mg/L increase in apparent bilirubin concentration with the 2,4-DCPD method per mmol/L indican that we found on in vitro addition of indican to pooled non-uremic serum. Thus, we believe that indican is the substance accumulating in uremic serum that is responsible for the observed methodological bias.

The visible spectrum of the reference-grade bilirubin in bovine albumin after reaction with either 2,4-DCPD reagent or with diazotized sulfanilic acid reagents shows a single broad absorbance, peaking at about 525 to 530 nm. When we added aqueous indican solutions to diazotized sulfanilic acid reagent, we observed no absorbance in the 450 to 600 nm range. However, when indican was added to 2,4-DCPD reagent, we found three peaks—at approximately 540, 460, and 370 nm—similar to results reported by Poon and Hinberg (5). As shown in Figure 3, the 540-nm chromophore from indican’s reaction with 2,4-DCPD develops much more slowly than does azobilirubin.

Based on the reaction rate data, we decreased the reaction time for the Olympus Demand with the 2,4-DCPD reagent from 8.3 min, as originally recommended by the manufacturer, to 1.7 min. As shown in Figure 4, comparability of the 2,4-DCPD method and Jendrassik–Grof method for total bilirubin in the near-normal range is very good, and indican interference is eliminated. On comparison of the two methods over a broader range of 0 to 220 mg/L total bilirubin, the correlation is excellent (r = 1.00; slope, 1.03; intercept, −1.3 mg/L). Doumas et al. (12) suggested that reaction times as long as 10 min are required for complete reaction of bilirubin in human serum with the Jendrassik–Grof reagent. However, we find that such long reaction times are not necessary to form azobilirubin from bilirubin in serum or
albinm-based standards with surfactant-activated 2,4-DCPD reagent. Thus, a long reaction time offers no advantage for 2,4-DCPD-based methods, and magnifies indican interference in uremic sera.

References


Corrections

Vol. 30

p 1553, Insert the symbol "O" in the sublegend to Fig. 1, third line, to read "... method: O, activity ..."

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p 572, middle of right column: delete the three repeated lines of type.

p 575, in "Table 1 (continued)," the first column heading should read "Sample size, Ny = No."

p 616, left column, first text paragraph: the second sentence should read, "This nonlinear protein binding explains the dependence of renal clearance of disopyramide on total disopyramide concentration in serum and the lack of dependence of renal clearance on the concentration of free disopyramide (5). Furthermore, the pharmacological response to this drug is better correlated with the concentration of the free drug in serum than with its total concentration (4, 6); we thus conclude that both the free fraction and the total concentration should be measured when one is monitoring arrhythmic patients who are being treated with this drug."

p 617, left column: delete the two lines of text following the first paragraph of Results, and delete the three lines following the second paragraph.

p 648: Three lines are missing in the section on Ferritin standards, as follows: "Ferritin from human liver, 9.7 µg per bottle, i.e., 9.1 mg/L (National Institute for Biological Standards and Controls, Hampstead–Holly Hill, London; reference 80/602)."

p 641, left column, third line of text at bottom: "3983" should read "398.8" and "4140" should read "414.0."

p 647, middle column, preceding the 10th line from bottom, insert: "Using this method, we found the ..."

p 655, right column: the concluding paragraph (preceding the references) should read as follows: "Our results indicate that assay of serum SHBG is of value in the analysis of risk factors for mortality in post-menopausal women. Whether low SHBG concentration in these women primarily reflects androgen excess, estrogen deficiency, or other endocrine or metabolic changes remains a subject for further study, as does the relationship to adipose tissue distribution."

Ed. note: Most of the above corrections result from inexpertness of new layout personnel. We hope this will be corrected and we apologize for these aberrations.