urines containing the 5.0 g/L concentrations of glucose produced readings of 2.5 g/L. Neither method showed interference from 0.1 or 0.2 g of ascorbic acid per liter.

On the basis of these interference data, we predict that the BM33071 would not present false-negative results under the conditions studied. The effect of ascorbic acid should be minimal, given the normal excreted concentration of ascorbic acid of usually less than 0.25 g/L.

References

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Indican Interference with Six Commercial Procedures for Measuring Total Bilirubin

R. Poon and I. H. Hinberg

We have studied the effect of indican on six commercial procedures for the measurement of total bilirubin in serum. Total bilirubin measured by the Bilirubin A-Gent™ (Abbott) 2,4-dichlorophenyl diazonium procedure increased by 50 mg/L for each 1 mmol/L of added indican. Similarly, total bilirubin measured by the Bilirubin C-System™ (Boehringer Mannheim) 2,5-dichlorophenyl diazonium procedure increased by 33 mg/L per mmol/L of indican. Indican also interfered with the Micro Bilirubin Reagent Set™ (Harleco) Malloy–Evelyn procedure, but to a much lesser extent. The Jendrassik Bilirubin Reagent System™ (American Monitor) and a modified Jendrassik–Grot procedure (Hoffmann-LaRoche) adapted to the Cobas Bio analyzer were unaffected by the presence of indican. The amount of interference with the 2,5-dichlorophenyl diazonium procedure increased significantly with color development time and was twice the initial amount after 30 min. Concentrations of indican as high as 0.38 mmol/L have been found in sera of patients with renal failure, which would increase total bilirubin values measured by the first two procedures above by 19 and 12 mg/L, respectively. Users of these procedures should therefore be suspicious of unexpectedly high bilirubin values obtained with sera from patients with chronic renal disease.

Additional Keyphrases: renal disease · analytical error

Fifty-six years ago, Harrison and Bromfield (1) reported that indican (indol-3-yl sulfate), a natural metabolite that accumulates in the sera of patients with chronic renal failure (2, 3), interfered with the colorimetric determination of total bilirubin. However, except for one report by Ertingshausen et al. (4), that the determination of total bilirubin by a procedure involving 2,4-dichlorophenyl diazonium (2,4-DCPD) gave falsely increased results in the presence of indican, no attention has since been paid to this interferent.

We have studied the effect of indican on six commercial colorimetric procedures for the measurement of total bilirubin in serum and report our findings.

Materials and Methods

Indican was obtained from Sigma Chemical Co., St. Louis, MO 63178; reference grade bilirubin from Pfannstiel Laboratories Inc., Waukegan, IL 60085; bovine serum albumin from Armour Pharmaceutical Co., Kankakee, IL 60901; and 2,4-dichloroaniline and 2,5-dichloroaniline from Aldrich Chemical Co., Milwaukee, WI 53201. All other chemicals used were reagent grade, obtained from Fisher Scientific Co., Ottawa, Ontario, K2E 7L6.

We prepared samples with indican concentrations between 0 and 0.92 mmol/L by adding appropriate volumes of a 47 mmol/L solution of indican to aliquots of pooled human serum. The manual and automated colorimetric bilirubin measurement procedures we studied are described in Table 1. The kit manufacturers’ directions for use were strictly followed. Where calibrators were not provided, we used bilirubin standards in bovine serum albumin, 40 g/L, prepared as described by Perry et al. (5).

Stabilizer-free 2,4-DCPD reagent was prepared at room temperature by adding sodium nitrite (final concentration, 10 μmol/L) to a solution containing 2 mmol of 2,4-dichloroaniline and 69 mmol of sulfamic acid per liter of water/methanol (1/1, by vol). We used the reagent 2 min after adding the sodium nitrite.

To measure absorbance, we used a Beckman DU-8B spectrophotometer (Beckman Instruments Inc., Fullerton, CA 92634); difference spectra were obtained with a Cary 219 spectrophotometer (Varian Associates Inc., Palo Alto, CA 94303).
Results

Our results (Figure 1) show that total bilirubin measured with the Bilirubin C-System, which involves use of 2,5-dichlorophenyl diazonium (2,5-DCPD), increased by 33 mg/L for each millimole of indican added per liter. Similarly, total bilirubin values obtained with the A-Gent bilirubin kit, a 2,4-DCPD procedure, increased by 50 mg/L for each millimole of indican per liter. Indican also interfered, but to a much lesser extent, with the Micro Bilirubin Reagent Set, which involves a Malloy–Evelyn procedure. In contrast, the Jendrassik Bilirubin Reagent System, a Jendrassik–Grof method, was not affected by the presence of indican.

Roche Analytical Instruments has adapted this last procedure to the Cobas Bio Centrifugal Analyzer by omitting the final alkaline reagent addition step; this modified procedure was similarly unaffected by indican. The Cobas Total Bilirubin Reagent procedure, a modified Malloy–Evelyn method in which the accelerating agent is dimethyl sulfoxide instead of methanol, was also unaffected by indican.

We also confirmed the findings of Ertinghausen et al. (4) that the 2,4-DCPD reagent reacts with bilirubin to form a color complex with a maximum absorbance at 540 nm. Reaction of the 2,4-DCPD reagent with indican, on the other hand, produced a color complex with an absorbance band centered around 480 nm (Figure 2, top). The significant absorbance of this band at 540 nm was responsible for the erroneously high total bilirubin values obtained with 2,4-DCPD procedures in the presence of indican. Both color complexes were stable for at least 30 min.

Although the 2,5-DCPD reagent is structurally similar to the 2,4-DCPD reagent, it forms different color complexes with bilirubin and indican (Figure 2, bottom). As shown previously by Wahlefeld et al. (7), the 2,5-DCPD reagent reacted with bilirubin to produce a color complex with maximum absorbance at 520 nm that was stable for at least 30 min. In contrast, the color complex initially formed by reaction of the 2,5-DCPD reagent with indican had absorb-

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Table 1. Bilirubin Test Kits Studied

<table>
<thead>
<tr>
<th>Kit</th>
<th>Conditions for azobilirubin formation</th>
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<tbody>
<tr>
<td>A. A-Gent Bilirubin Test (Abbott Laboratories, Mississauga, Ontario L4W 2S7)</td>
<td>Reaction with stabilized 2,4-DCPD reagent at acidic pH in the presence of methanol</td>
</tr>
<tr>
<td>B. Bilirubin C-System (Boehringer Mannheim Canada, Dorval, Quebec H9P 1A9)</td>
<td>Reaction with 2,5-DCPD reagent at acidic pH in the presence of a detergent</td>
</tr>
<tr>
<td>C. Micro Bilirubin Reagent Set (Harleco, Gibbstown, NJ 08027)</td>
<td>Reaction with p-diazobenzenesulfonic reagent at acidic pH in the presence of methanol (Malloy–Evelyn method)</td>
</tr>
<tr>
<td>D. Jendrassik Bilirubin Reagent System (American Monitor Corp., Indianapolis, IN 46268)</td>
<td>Reaction with p-diazobenzenesulfonic reagent at acidic pH in the presence of caffeine reagent followed by an alkaline reagent addition (Jendrassik–Grof method)</td>
</tr>
<tr>
<td>E. “Cobas” Total Bilirubin (Hoffmann-La Roche Ltd., Etobicoke, Ontario M9C 5J4)</td>
<td>Reaction with a stabilized p-diazobenzenesulfonic reagent at pH 1.6 in the presence of dimethyl sulfoxide (modified Malloy–Evelyn method)</td>
</tr>
<tr>
<td>F. Jendrassik Bilirubin Reagent System adapted to the Cobas Bio analyzer (by Roche Analytical Instruments Inc., Nutley, NJ 07110)</td>
<td>Reaction with p-diazobenzenesulfonic reagent at acidic pH in the presence of caffeine reagent, but without addition of alkaline reagent (modified Jendrassik–Grof method)</td>
</tr>
</tbody>
</table>
bance bands with maximum absorbances at 375, 460, and 530 nm; the absorbance of the 460 nm band decreased with time, while that of the 530 nm band increased to almost twice its initial value after 30 min (Figure 2, bottom). Because it is this band at 520 nm that is responsible for the interference of indican with the measurement of total bilirubin by 2,5-DCPD procedure, the amount of interference will increase significantly as color development is prolonged.

The absorbance spectra in Figure 2 were obtained with commercial 2,4-DCPD and 2,5-DCPD reagents (Table 1). Because the commercial 2,4-DCPD reagent used contained a stabilizer, probably 1,5-naphthalenedisulfonate (4), we also conducted studies with stabilizer-free 2,4-DCPD reagent. This reagent reacted with bilirubin and indican to produce color complexes with absorbance spectra identical to those in Figure 2 (top).

In the Malloy–Evelyn procedure, bilirubin reacts with p-diazobenzenesulfonyl reagent to form a color complex with a maximum absorbance at 560 nm. Indican also reacts with the reagent, to give peak absorbances at 380 and 490 nm. However, the 490-nm peak had near-baseline absorbance at 560 nm and therefore only minimally influenced bilirubin measurement.

In the Jendrassik–Grof procedure, bilirubin reacts to form a broad absorbance band from 400 to 700 nm with peak absorbance at 595 nm. Indican does not react to produce a color complex in this wavelength range.

Discussion

Only the 2,4-DCPD and 2,5-DCPD procedures for determining bilirubin were markedly affected by indican, which reacted with the reagents to form color complexes that absorbed significantly at the wavelengths used for the measurement of bilirubin, thereby producing falsely high results.

Concentrations of indican as high as 0.38 mmol/L have been found in sera of patients with chronic renal failure (4). Our results show that this concentration would increase total bilirubin values measured by the 2,4-DCPD and 2,5-DCPD diazonium procedures by more than 19 and 12 mg/L, respectively. Because a total bilirubin value of 15 mg/L is generally considered abnormally high (8), the interference of indican with these procedures may lead to false presumptive diagnoses of liver abnormalities in these patients, necessitating time-consuming, expensive, and hazardous additional diagnostic procedures.

Because of their relative stability and the speed with which they react with bilirubin, both the 2,4-DCPD and 2,5-DCPD procedures are widely used in automated and manual procedures for total bilirubin (4, 9, 10). Users of these reagents should be suspicious of unexpectedly high values for bilirubin in sera from uremic patients.

References