
Characterization of Sulfasalazine’s Interference in the Measurement of Conjugated Bilirubin by the Ektachem Slide Method

Douglas W. Franquemont,1 James L. Sutphen,2 David A. Herold,1 and David E. Bruns1

We describe the cases of four patients who were taking sulfasalazine for inflammatory bowel disease, whose conjugated bilirubin concentrations in serum exceeded their corresponding total bilirubin concentrations as measured with a multilayer film analyzer, the Ektachem 400. Sulfasalazine added to pooled human serum at therapeutic concentrations increased the apparent conjugated bilirubin, as measured with the Ektachem, in a linear and dose-related fashion. Measured unconjugated bilirubin was simultaneously decreased to values less than -3 mg/L. The same interference occurred on the Ektachem 700, but an algorithm prevented the instrument from reporting the results. The major metabolites of sulfasalazine in blood did not interfere with analysis for those fractions of bilirubin. Sulfasalazine’s strong absorbance at 400 nm explains its interference with determination of conjugated bilirubin in this instrument.

Additional Keyphrases: inflammatory bowel disease · multilayer film analysis · analytical error

Sulfasalazine is used commonly to treat and prevent relapses of chronic ulcerative colitis and to treat acute exacerbations of Crohn’s disease (I). Total bilirubin (TBil), conjugated bilirubin (BC), and unconjugated bilirubin (Bu) often are measured in patients with inflammatory bowel disease, to monitor the potential development of hepatobilary complications of the disease, including sclerosing cholangitis, pericholangitis, cholecystitis, and chronic active hepatitis (I). We recently learned of a patient who was taking sulfasalazine for inflammatory bowel disease, whose BC concentration as measured by the Ektachem 400 was 9 mg/L, whereas his TBil was only 3 mg/L. The TBil was corrected by analysis with a different type of the instrument. The product labeling that accompanies the Ektachem BuBc slide mentions sulfasalazine as a potential

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Departments of 1 Pathology and 2 Pediatrics, University of Virginia Health Sciences Center, Charlottesville, VA 22908.
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8 Nonstandard abbreviations: TBil, total bilirubin; BC, conjugated bilirubin; and Bu, unconjugated bilirubin.
interfere with absorbance measurements, but no characterization of this interference has been published.

Here we characterize the positive interference of sulfasalazine in determination of Bc with the BuBc slide in the Ektachem 400 and 700 analyzers. We show a direct correlation between the concentration of sulfasalazine added to serum and the reported Bc, and we demonstrate that none of the major metabolites of the drug in blood contribute to this interference. Finally, the ultraviolet–visible spectra of sulfasalazine and its metabolites confirm and explain these findings.

Materials and Methods

We obtained serum samples from the patient described above and from three additional patients who were being treated with sulfasalazine for inflammatory bowel disease.

A 100 mg/L stock solution of sulfasalazine was prepared by dissolving sulfasalazine powder (Sigma Chemical Co., St. Louis, MO) in pooled human serum that contained no Bc detectable with the Ektachem 400 and 700. Aliquots of pooled human serum were supplemented with the stock sulfasalazine solution to yield final concentrations of 0, 5, 10, 25, 50, 75, and 100 mg/L. Similarly, 100 mg/L solutions of the sulfasalazine metabolites were prepared by dissolving sulfapyridine powder (Sigma Chemical Co.), N-acetyl-sulfapyridine powder (a gift from Pharmacia, Uppsala, Sweden), and 5’-OH-sulfapyridine powder (a gift from Pharmacia) in pooled human serum that contained no detectable Bc.

Bc, Bu, and TBil were determined with the Ektachem 400 and 700 analyzers, calibrated according to the manufacturer’s specifications. The Ektachem calculates Bc and Bu by measuring the reflectances of the BuBc slide at 400 nm and 460 nm (2, 3). TBil is determined by measuring the reflectance of the TBil slide at 540 nm (3-5).

For determination of ultraviolet–visible spectra, we prepared separate 100 mg/L solutions of sulfasalazine and its metabolites in 0.5 mmol/L NaOH. Aliquots of each of these solutions were diluted with distilled water to yield concentrations of 10 mg/L. Ultraviolet–visible spectra were obtained with a Model DU-7 spectrophotometer (Beckman Instruments, Irvine, CA).

Results

Analysis of serum from each of the four patients taking sulfasalazine revealed a value for Bc greater than that for TBil as measured by the Ektachem 400 (Table 1). In each case, the Bu was reported as less than −3 mg/L. We prospectively followed the case of patient four, a seven-year-old girl, who began on sulfasalazine for colitis. Before therapy with sulfasalazine, her TBil value was 1 mg/L, her Bc was 0, and her Bu was 0.5 mg/L as measured in the Ektachem. Two days after starting sulfasalazine, however, her TBil remained at 1 mg/L, her measured Bc increased to 12 mg/L, and her Bu was less than −3 mg/L (Ektachem 400).

Sulfasalazine added to pooled human serum at therapeutic concentrations (5–100 mg/L) (6, 7) increased the Bc concentration, as measured in the Ektachem 400 or 700, in a linear and dose-related fashion: Bc (mg/L) = 0.95×sulfasalazine (mg/L) − 2.7 (n = 7, r = 0.9992). In other words, each 1 mg/L of sulfasalazine increased the apparent Bc by 0.95 mg/L. Measured Bu was simultaneously decreased to values less than −3 mg/L, and TBil was unaffected. The same interference occurred in the Ektachem 700, but an algorithm prevented the instrument from reporting the Bc results. The Bu, however, was reported as less than −3 mg/L. Sulfapyridine, N-acetyl-sulfapyridine, and 5’-OH-sulfapyridine did not interfere with Bc or Bu analysis, even at concentrations up to 100 mg/L (Table 2).

The ultraviolet–visible spectrum of sulfasalazine consisted of absorption peaks at 238 nm and 358 nm and a local absorption minimum at 285 nm, in agreement with data reported by McDonnell (8). Sulfasalazine showed strong absorbance at 400 nm (ε = 8937 L/mmol·cm), less absorbance at 460 nm (ε = 2071 L/mmol·cm), and negligible absorbance at 540 nm (ε = 57 L/mmol·cm). The metabolites showed negligible absorbance at or above 400 nm (molar absorptivities, <110 L/mmol·cm).

Although it did not affect Bc or Bu analysis, the standard 100 mg/L serum solution of 5’-OH-sulfapyridine increased the measured TBil to 98 mg/L from a serum blank value of 5 mg/L (Table 2). An ultraviolet–visible absorption spectrum of this solution, with the blank serum as a reference, showed minimal absorbance at 540 nm (ε = 141 L/mmol·cm), the primary wavelength used by the Ektachem to determine TBil.

Discussion

This study demonstrates a direct linear relationship between the concentration of sulfasalazine in serum and the apparent Bc measured in the Ektachem. The much greater absorbance of sulfasalazine at 400 nm compared with 460 nm explains this positive bias. When bound to the mordant within the Ektachem BuBc slide, Bc and Bu have similar molar absorptivities at 400 nm, whereas Bu has a greater molar absorptivity than Bc at 460 nm (2). The Ektachem measures reflection densities at these two wavelengths and then solves simultaneous equations to calculate Bc and Bu. Inspection of these equations reveals that increased absorbance at 400 nm would produce an increased Bc result and a decreased Bu (see Appendix). TBil is primarily measured at 540 nm, and sulfasalazine’s minimal absorbance at this wavelength did not cause measurable interference.

The major metabolites of sulfasalazine in blood showed no interference with the Ektachem’s analysis of Bc or Bu.

Note: All concentrations are reported in mg/L.

Table 1. Fractionated Bilirubin Analyses in Four Patients Taking Sulfasalazine

<table>
<thead>
<tr>
<th>Weight, kg</th>
<th>Dose, g/day</th>
<th>TBil</th>
<th>Bc</th>
<th>Bu</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.5</td>
<td>3</td>
<td>9</td>
<td>&lt;−3</td>
</tr>
<tr>
<td>37</td>
<td>2</td>
<td>2</td>
<td>9</td>
<td>&lt;−3</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>1</td>
<td>12</td>
<td>&lt;−3</td>
</tr>
</tbody>
</table>

Table 2. Effects of Sulfasalazine and Its Metabolites on Fractionated Bilirubin Analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bc</th>
<th>Bu</th>
<th>TBil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfasalazine</td>
<td>↑</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Sulfapyridine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-acetyl-sulfapyridine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5’-OH-sulfapyridine</td>
<td>0</td>
<td>0</td>
<td>1  *</td>
</tr>
</tbody>
</table>

* Effect seen only with concentration 100× greater than found in vivo.
About 10% of orally ingested sulfasalazine appears in serum intact, and its median concentration in serum is only 12 mg/L (6, 7). Colonic bacteria convert most of the drug into sulfinpyridine and 5-aminosalicylic acid. Sulfinpyridine is subsequently absorbed and hepatically metabolized to N-acetylsulfinpyridine and 5'-OH-sulfinpyridine, which are then glucuronidated and rapidly excreted via the kidneys. The median concentration of sulfinpyridine and its metabolites in serum is about 50 mg/L (6, 7), and that of 5'-OH-sulfinpyridine alone has been reported as <1 mg/L (9). Although thought to be the active moiety, owing to local effects on prostaglandin metabolism in the intestine (1), very little 5-aminosalicylic acid is absorbed, and its concentrations in serum are extremely low, <2 mg/L (6). The ultraviolet-visible spectra of sulfinpyridine and N-acetylsulfinpyridine showed no detectable absorbance at 400, 460, or 540 nm, which explains their lack of interference in the fractionated bilirubin analysis.

An aqueous solution of 5'-OH-sulfinpyridine absorbed minimally at 400 nm (ε = 110 L/mol·cm), not at all at 460 nm. Accordingly, it did not demonstrate measurable interference with Bc or Bu analysis. Moreover, the low concentrations of 5'-OH-sulfinpyridine in serum, as well as those of 5-aminosalicylic acid, would make any potential interference negligible. Our standard 100 mg/L 5'-OH-sulfinpyridine serum solution, however, did falsely increase TBII, but this interference was not observed in any of our patients. This reflects the reported very low concentration of 5'-OH-sulfinpyridine in serum in vivo (9). Spectra of 5'-OH-sulfinpyridine, dissolved in water or serum, showed little absorbance at 540 nm (ε = 0 L/mol·cm in water, 141 L/mol·cm in serum), demonstrating that, alone or in combination with serum proteins, 5'-OH-sulfinpyridine would not be expected to interfere with TBII analysis. This suggests that 5'-OH-sulfinpyridine may be interacting with substances within the TBII slide to produce an increased result.

The Ektachem 700 recognized when Bc exceeded TBII—a physiological impossibility—and it notified the operator by an appropriate coded message. It did, however, report the Bu results as less than ~3 mg/L, which strongly suggested an interfering substance with significant absorbance at 400 nm, as documented in this study for sulfasalazine and reported for methotrexate (10). The Ektachem 400 reported all values.

Recognition of Bc falsely increased by sulfasalazine is important because of the potential hepatobiliary complications associated with inflammatory bowel disease. The prevalence of inflammatory bowel disease is 200 000 to 400 000 in the United States, and many of these patients are treated with sulfasalazine (1). Thus, clinical laboratories may need to have alternative methods available for determining Bu and Bc. Moreover, a falsely increased Bc may be accepted as a true value by clinicians, which could lead to unnecessary diagnostic procedures such as endoscopic retrograde cholangiography to diagnose sclerosing cholangitis. Clinicians should act cautiously when interpreting increased Ektachem Bc results in patients taking sulfasalazine.

Appendix

The following equations represent simplified forms of the fundamental relationships used by the Ektachem to determine Bc and Bu (2):

\[
Bc = \frac{K_1D_{400} - K_2D_{460}}{K_5}
\]

\[
Bu = \frac{K_3D_{460} - K_4D_{400}}{K_5}
\]

where D represents the transmission density (mathematically transformed from reflection density) at 400 nm or 460 nm. The K values are constants determined from calibration plots at each wavelength for Bc and Bu.

We thank Hubert Agback of Pharmacia, Uppsala, Sweden, for providing N-acetylsulfinpyridine and 5'-OH-sulfinpyridine.

References