Methotrexate Interferes with Determinations of Conjugated Bilirubin with the Kodak Ektachem 400

Sven Swanson, Theodore E. Mifflin, and James C. Boyd

Immediately after intravenous infusion of a high dose (concentration in serum >1000 μmol/L) of methotrexate, the apparent conjugated bilirubin (Bc) concentrations in serum of two osteosarcoma patients, as measured by the Kodak Ektachem 400 analyzer, were greater than the corresponding total bilirubin concentrations, but decreased as the concentrations of methotrexate in serum decreased. In an interference study we found that methotrexate added to sera containing a wide range of basal Bc concentrations increased the measured Bc concentration in a linear and dose-related fashion. Methotrexate also interfered negatively with measurements of unconjugated bilirubin (Bu). The source of the interference appears to be an overlap in the absorption spectrum of methotrexate with Bc and Bu at 400 nm.

Additional Keyphrases: analytical error • spectrophotometry • multilayer film analysis

Methotrexate is widely used in the treatment of various neoplastic diseases, including osteosarcoma (1). Because of the potential hepatotoxicity with high-dose methotrexate therapy (1), various measures of hepatic function (including aminotransferases and total and conjugated bilirubin) are monitored before and at intervals after infusion of methotrexate. Recently, we identified two osteosarcoma patients whose concentrations of conjugated bilirubin (Bc), as measured in the Ektachem 400 (Eastman Kodak Co., Rochester, NY 14650), were several times higher than their concentrations of total bilirubin (TBil) in samples drawn immediately after methotrexate infusion.1 One of the two patients had a measured Bc concentration of 66 mg/L and simultaneously a TBil concentration of 9 mg/L. The other patient's bilirubin concentrations were of similar magnitude and error. Because these increased Bc concentrations were associated with each patient's highest measured methotrexate concentration (1190 μmol/L in one patient, 1290 μmol/L in the other), we undertook the following study to document the suspected interference of methotrexate with the determination of Bc in the BuBc slides used in the Ektachem 400.

Materials and Methods

We prepared a stock methotrexate 2000 μmol/L solution by dissolving methotrexate powder (+l-amethopterin; Sigma Chemical Co., St. Louis, MO) in pooled human serum containing no Bc as determined by the Ektachem 400. Solubility was enhanced by adjusting to a mildly basic pH with 6 mol/L NaOH solution. Aliquots of pooled serum were supplemented with the stock methotrexate solution to yield final methotrexate concentrations of 0, 125, 250, 500, 1000, and 1500 μmol/L. Bc, Bu, and TBil concentrations were determined with the Ektachem 400, calibrated according to the manufacturer's specifications. We also determined the "direct" bilirubin (DBil) concentrations by the modified Jendrassik–Gröf method (2), using a DU-7 spectrophotometer (Beckman Instruments, Irvine, CA).

In addition, we mixed aliquots of serum samples from six individual patients (not including the osteosarcoma patients) whose Bc values were previously determined to range from 0 to 250 mg/L with an equal volume of the stock methotrexate solution or of plain pooled serum (control). Bu, Bc, and TBil were measured for each sample to quantify the interference when the methotrexate concentration was 1000 μmol/L.

Results

The concentration of Bc measured with the Ektachem and the methotrexate concentration were linearly related in pooled serum samples supplemented with methotrexate (r = 0.9993; [Bc, mg/L] = 0.036 × [methotrexate, μmol/L] – 2). TBil concentrations were similar in all seven samples and averaged 7 mg/L. All Bu concentrations were reported by the Ektachem 400 as less than –3 mg/L except at the two lowest concentrations of methotrexate. With the modified Jendrassik–Gröf method, the DBil concentrations in these samples were all less than or equal to 1 mg/L. Similarly, the DBil concentrations from the specimens obtained from the two osteosarcoma patients at the time of the falsely elevated Bc concentrations were measured to be 4 and 1 mg/L. The six individual patients' samples when supplemented with the methotrexate stock solution had Bc concentrations that were consistently higher than when the samples were supplemented with control pooled serum. The average bias between the Bc measured in the samples with and without methotrexate was 33 mg/L (Table 1). TBil concentrations were not affected by the addition of methotrexate. All Bu concentrations were reported by the Ektachem 400 as less than –3 mg/L.

<table>
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<tr>
<th>Table 1. Bc Concentration as Measured in the Ektachem 400 In Methotrexate-Supplemented and Control Samples from Six Patients</th>
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<td>Methotrexate-supplemented* serum</td>
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*With 1000 μmol of methotrexate per liter.

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1 Nonstandard abbreviations: Bc, conjugated bilirubin; Bu, unconjugated bilirubin; TBil, total bilirubin; DBil, direct bilirubin (in the modified Jendrassik–Gröf method).

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Discussion

Methotrexate is a highly protein-bound (3) yellow compound, which has a molar absorptivity of about 4700 L·mol⁻¹·cm⁻¹ at 400 nm, and essentially zero at 460 nm (4). At 400 nm, Bu and Bc have similar absorbances but Bu has a much greater absorptivity than Bc at 460 nm (5). In determining the concentrations of Bu and Bc, the Kodak Ektachem applies two simultaneous equations that utilize this difference in spectral response (6). Inspection of these two equations suggests that increased absorbance at 400 nm (due to the presence of methotrexate) would falsely increase the reported Bc concentration and simultaneously depress the Bu concentration. We could not quantify the decrease of Bu at different concentrations of methotrexate because the Bu concentrations were reported as less than −3 mg/L.

This hypothesized effect of methotrexate on the calculated concentrations of Bu and Bc is consistent with our experimental observation of the linear positive interference of the drug with Bc. Methotrexate’s lack of absorbance at wavelengths greater than 460 nm explains why no interference was noted in the TBil determinations on the Ektachem TBil slide or with the DBil determinations in the Jendrassik-Gröff method. In the former method, spectral measurements were made at 540 and 460 nm; in the latter method, at 600 nm.

Because methotrexate is largely bound to serum proteins, it may become separated from these proteins in the BuBc slide by a mechanism similar to that which releases Bu (personal communication with Terry Shirrey, Kodak, November 12, 1985). Free methotrexate thus would gain access to the reaction layer and be measured simultaneously with Bu and Bc at 400 nm. Whether methotrexate attaches to the mordant in the reaction layer of the BuBc slide is unknown; however, the free drug’s absorbance at 400 nm is sufficient to cause the interference.

Kodak has also recorded an interference with the BuBc slide due to methotrexate (7). The 6 mg/L interference they report for Bc at a methotrexate concentration of 50 mg/L (110 μmol/L) is similar to the interference we noted. High concentrations of methotrexate (220 μmol/L) have also been demonstrated to cause a positive interference with the cerebrospinal fluid (CSF) protein assay on the DuPont acs (8). A spectral interference similar to ours was thought to be responsible for the observed differences.

References

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