Trimethoprim Interferes with Serum Methotrexate Assay by the Competitive Protein Binding Technique

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Administration of Bactrim® (a combination of trimethoprim and sulfamethoxazole) to a patient who also was receiving methotrexate caused a significant increase in apparent plasma methotrexate concentrations as apparent by competitive protein binding assay with use of dihydrofolate reductase (EC 1.5.1.3) from Lactobacillus casei as the binding protein. This spurious increase was caused by trimethoprim in the patient's plasma. A plasma trimethoprim concentration of 0.1 mg/L inhibited binding of radiolabeled methotrexate to dihydrofolate reductase by 50%. In contrast, radioimmunoassay for methotrexate was not affected by concomitant administration of trimethoprim. The competitive protein binding assay for methotrexate should not be used in patients being treated with Bactrim or Septras® (a similar combination). However, the L. casei competitive protein binding assay technique can be used to assay plasma trimethoprim concentrations with sensitivity to 0.02 mg of trimethoprim per liter.

Additional Keyphrases: radioimmunoassay • drug assay • dihydrofolate reductase • assay of trimethoprim

Methotrexate is an effective antineoplastic agent used to treat leukemia, lymphoma, choriocarcinoma, and other malignancies (1). Methotrexate binds to and inhibits the enzyme dihydrofolate reductase (EC 1.5.1.3) and prevents conversion of dihydrofolate to tetrahydrofolate, an essential cofactor in DNA synthesis. The toxicity of methotrexate can be prevented by the administration of leucovorin (N5-formyltetrahydrofolate). This ability to "rescue" normal bone marrow cells from toxicity, has allowed use of high doses of methotrexate (200-500 mg/kg), along with leucovorin, in an attempt to improve the therapeutic effectiveness of the drug (1, 2).

Therapy with high-dose methotrexate has been complicated by severe myelosuppression and fatalities (3). Several reports (4–6) indicate that routine monitoring of methotrexate concentrations in plasma facilitates early detection of patients who are at high risk of toxicity, who can then be treated with increased and prolonged doses of leucovorin to prevent such toxicity. Many assay techniques are currently available for measuring this drug in plasma (7–13). Of these, radioimmunoassay and the competitive protein binding assay have received the widest clinical acceptance because of their simplicity, rapidity, and availability from commercial sources.

Here we illustrate that naturally occurring enzymes or binding proteins may not be as specific as are antibodies in ligand displacement assays. We present a patient in whom trimethoprim, a component of the widely used antibacterial combinations Bactrim® (Roche) and Septras® (Burroughs-Wellcome) interfered with a competitive protein binding assay for methotrexate and caused false increases in apparent plasma methotrexate concentrations.

Case Report

A 19-year-old man was hospitalized for relapse of acute lymphoblastic leukemia. He was treated with a combination chemotherapy regimen, including a weekly dose of methotrexate, 2 g/m² body surface, followed by leucovorin "rescue." Two courses of methotrexate therapy were given without complication. Three days before the third course of methotrexate, Bactrim, two tablets every 12 h, was started for presumed infection. After receiving the third dose of methotrexate, the patient left the hospital against medical advice. Upon his return to the hospital four days later, a competitive dihydrofolate reductase binding assay (CPBA) for methotrexate indicated his plasma methotrexate concentration to be 4.4 × 10⁻⁸ mol/L. Leucovorin rescue was continued. Plasma methotrexate concentrations of 4 and 6 × 10⁻⁸ mol/L were measured by CPBA at eight and 15 days after the above-mentioned third dose of methotrexate, although the patient showed no evidence of mucositis or renal failure. Possible drug interactions were reviewed and it was noted that the patient had been receiving Bactrim (trimethoprim/sulfamethoxazole). This drug was discontinued and a week later the CPBA showed no evidence of methotrexate in the patient's plasma.

Materials and Methods

Methotrexate for clinical use was obtained from Lederle Laboratories, Pearl River, NY 10965. Methotrexate and trimethoprim for laboratory investigations were purchased from Sigma Chemical Co., St. Louis, MO 63178. Bactrim for both clinical and laboratory use was obtained from Roche Laboratories, Nutley, NJ 07110.

Two assays for methotrexate were used. CPBA was performed by the method of Myers et al. (9). The assay is based on competition between [³H]methotrexate and unlabeled methotrexate for binding to dihydrofolate reductase, with subsequent removal of unbound drug by charcoal absorption.

The assay was performed at 23 °C and involves rapid sequential addition of the following: (a) 4.85 nCi of [³H]methotrexate; (b) 200 μL of a serially diluted patient's sample or standard solution of unlabeled methotrexate; and (c) 100 μL of dihydrofolate reductase in 0.5 μmol/L potassium phosphate buffer, pH 6.2, this final solution containing 0.5 pmol of methotrexate-binding capacity and including 0.24 μmol of freshly prepared NADPH. Immediately after the enzyme-NADPH solution is added, the contents of the tubes are mixed by vortex agitation and 25 μL of a charcoal slurry is added. Samples are again vortex-mixed and centrifuged at 700 X g.
for 30 min. The radioactivity of a 200-μL aliquot of the supernate is counted in a liquid scintillation spectrophotometer.

Maximal binding was 41% and nonspecific binding less than 5% of total counts added using this assay. This assay system is commercially available from the New England Enzyme Center, Boston, MA 02111.

The radioimmunoassay (RIA) for methotrexate used in these studies was purchased as a kit, from Diagnostic Biochemicals Inc., San Diego, CA 92121. In this assay, rabbit antiserum against methotrexate is used as the binding protein and is similar to the assay procedure described by Raso and Schriever (7). This assay, performed at 23 °C, involves the sequential addition of (a) 100 μL of a serially diluted patient’s sample (standard stock solution of unlabeled methotrexate, (b) 100 μCi of [125I]-labeled methotrexate, and (c) 100 μL of methotrexate antiserum. After incubation at 4 °C for 45 min, 1.0 mL of isopropanol is added to each tube, the contents are mixed by vortex agitation, and the tube is centrifuged at 1000 X g for 10 min. The supernate is discarded and the radioactivity in the precipitate is counted in a gamma counter.

Maximal percent binding was 42% and nonspecific binding less than 10% of total counts added.

Standard curves for both the CPBA and the RIA were constructed by plotting the log of unlabelled methotrexate added vs the ratio of radiolabeled methotrexate bound in the experimental determination (B) to the radiolabeled-methotrexate bound in the control assay (B0). Transformation of the ordinate to logit form, logit = ln[(B/B0)/(1 − B/B0)], yielded a linear standard curve.

Results

Plasma from this patient, taken on day 15 after the third dose of methotrexate therapy was administered, was assayed for methotrexate by both the CPBA and by the RIA. No methotrexate was detected in the sample analyzed by the RIA but a methotrexate concentration of 6 × 10⁻⁶ mol/L was measured by the CPBA.

Plasma samples from four additional patients receiving trimethoprim/sulfamethoxazole but not methotrexate were also assayed for methotrexate by the CPBA. Apparent methotrexate concentrations of 3 to 9 × 10⁻⁶ mol/L were measured in the plasma of all four patients.

Bactrim tablets were then crushed, dissolved in plasma, and various concentrations were added to plasma, which was then assayed for methotrexate by the CPBA and the RIA. As shown in Figure 1, Bactrim in concentrations of 0.1–100 mg/L interfered with the binding of radioactive methotrexate to L. casei dihydrofolate reductase. We saw no interference with the RIA until saturation concentrations of Bactrim were reached at 20–100 mg/L.

Trimethoprim is an inhibitor of bacterial but not of mammalian dihydrofolate reductase (14). Trimethoprim caused interference with methotrexate binding to L. casei dihydrofolate reductase at concentrations in plasma as low as 0.01 mg/L (Figure 1). In contrast, concentrations greater than 0.1 mg of trimethoprim per liter of plasma interfere with binding of radiolabeled methotrexate in the RIA (Figure 2).

Discussion

The combination of trimethoprim and sulfamethoxazole is marketed under the trade names Bactrim and Septa. The Medical Letter (15) has recently listed trimethoprim/sulfamethoxazole as primary or alternative treatment for infections caused by 13 different microorganisms. Because of the increased risk of infection in patients receiving chemotherapy for malignant disease, concomitant use of trimethoprim/sulfamethoxazole with methotrexate may not be uncommon.

Fig. 1. Effect of unlabeled methotrexate, trimethoprim, and Bactrim on binding of radiolabeled methotrexate in the L. casei competitive protein binding assay.

Curves relate concentration of unlabeled drug to the logit B/B0.

Single doses of Bactrim or Septa produce trimethoprim concentrations of 1 mg/L in plasma, which increase by 50% with chronic drug administration (16). Such plasma trimethoprim concentrations can interfere with measurement of plasma methotrexate concentrations by the commercially available CPBA.

Both the CPBA and the RIA for methotrexate measure the competition between radiolabeled methotrexate and unlabeled methotrexate for binding to a ligand.

With the RIA, this ligand is an antibody generated by immunizing a rabbit against methotrexate. With this assay, the Ig9 (the molar concentration of unlabeled compound which decreases the binding of radiolabeled methotrexate to 50% of the counts bound in the absence of competing compound) for trimethoprim is 20 000-fold greater than for methotrexate. Therefore, although some methotrexate metabolites compete with methotrexate binding in RIA (17), little or no interference of plasma methotrexate determinations would be expected from trimethoprim.

In the CPBA, labeled methotrexate competes with unlabeled drug for binding to dihydrofolate reductase. Both trimethoprim and methotrexate bind tightly to bacterial dihydrofolate reductase (14). Because the commercially available CPBA for methotrexate involves dihydrofolate reductase from L. casei, trimethoprim causes significant interference in measured methotrexate concentrations. The Ig9 for trimethoprim is only 15-fold that of methotrexate with the CPBA; thus this assay system for methotrexate should not be used in patients being treated with Bactrim or Septa. Similar interference would be expected with enzymic (13) or radiometric assays for methotrexate involving bacterial dihydrofolate reductase. Because the concentration of trimethoprim needed to inhibit binding of methotrexate to mammalian dihydro-
folate reductase by 50% is over 10,000-fold that needed to inhibit bacterial reductases (14), trimethoprim would not be expected to interfere with a CPBA using mammalian dihydrofolate reductase.

The tight binding of trimethoprim to bacterial dihydrofolate reductase causes interference with methotrexate determinations, but this binding can be exploited as a method for determining serum trimethoprim concentrations. In most pharmacological studies of trimethoprim, fluorometric assay techniques sensitive to concentrations of 50 μg of trimethoprim per liter were used. This assay method is not specific for unaltered drug, because certain trimethoprim metabolites contribute to the fluorescence measured (18). The commercially available methotrexate CPBA can measure concentrations of 20 μg of trimethoprim per liter (Figure 1). This assay will measure only active drug or metabolites binding to and inhibiting bacterial dihydrofolate reductase. The CPBA is simple, requiring only 2 h to perform several determinations, and depends only upon commercially available reagents.

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References