Enzymatic Assay for Methotrexate in Serum and Cerebrospinal Fluid

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We describe an assay for methotrexate in biological fluids. The assay is based on inhibition by methotrexate of dihydrofolate reductase from Lactobacillus casei. The lower limit of sensitivity in serum is 2 × 10⁻⁸ mol (about 10 μg) of methotrexate per liter. Within-run precision is ±5% (coefficient of variation) and day-to-day variation is 18%. Advantages of the assay are ease of manipulation, high sensitivity, and specificity.

Additional Keyphrases: cancer treatment • psoriasis treatment • drug assay

Methotrexate (MTX) is a folic acid antagonist widely used in cancer chemotherapy (1-4) and sometimes in severe psoriasis (5). In recent years, high doses of MTX followed by citrovorum factor “rescue” have been used in treating certain tumors (6). The citrovorum factor restores the body pools of tetrahydrofolate cofactors, which are depleted in the presence of MTX. MTX blocks the reduction of folic acid and dihydrofolate to tetrahydrofolic acid by inhibiting the enzyme tetrahydrofolate dehydrogenase (EC 1.5.1.3). Although it is thought that MTX damages sensitive tumors more than other rapidly proliferating tissues (1), such selectivity is not complete, and serious toxicity— including skin rash, myelosuppression, mucosal ulceration, and renal damage—can result. Monitoring serum methotrexate to predict toxicity after high-dose MTX therapy is reported (7-9). In addition, a correlation has been demonstrated between cerebrospinal fluid MTX concentrations and neurotoxicity after intrathecal administration of MTX (10). Use of serum MTX values to adjust the citrovorum factor dose and avert impending toxicity has also been reported (7). Such monitoring is likely to assume a greater role in the treatment of cancer and psoriasis with MTX in the future.

The enzyme-inhibition assay for MTX has been available for over a decade (11, 12) and has been used in studies of the pharmacology of MTX (1, 8, 9, 13). Unfortunately, descriptions of this assay have not been sufficiently detailed to enable clinical laboratories without expertise in enzyme kinetics to adopt the method. In addition, handling and storage of dihydrofolate, the substrate for the method, has required special care. The commercial availability of the enzyme and dihydrofolic acid has prompted us to develop a simple procedure for measuring MTX. The method is accurate, fast, sensitive, and precise, and can be used with any recording spectrophotometer capable of monitoring absorbance at 340 nm, although the instrument described below has proved particularly efficient. We have been using this assay to monitor high-dose therapy with MTX in children since September 1974 in a drug-assay laboratory staffed by medical technologists.

Materials and Methods

Instrumentation

Spectrophotometer. Absorbances were measured with a Model 300N Microsample Spectrophotometer with a 3017-T aspirating thermocuvette (Gilford Instrument Laboratories, Inc., Oberlin, Ohio 44074). The wavelength was 340 nm; the cuvette was maintained at 30 °C, and was set to aspirate 0.9 ml.

Data presentation. Changes in absorbance (ΔA) were measured and recorded by a Timer/Printer (Syva Corp., Palo Alto, Calif. 94304). The program was set on “ΔA”, the delay time at 20 s, and the read time at 120 s. We found this Timer/Printer ideally suited to this task, although the assay can be performed by any instrument, including a strip-chart recorder, capable of accurately recording ΔA.

Dihydrofolate dispenser. A PB 600-1 Repeating Dispenser (Hamilton Co., Reno, Nev. 89502) was used, fitted with a 1.0-ml Hamilton gas-tight syringe, so that it dispensed precise 20-μl aliquots.
Reagents

*Reaction buffer.* A mixture of 0.7 ml of β-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y. 14650), 50 ml of tris(hydroxymethyl)aminomethane buffer (1 mol/liter, pH 7.5) and 20 ml of ethylenediaminetetraacetic acid (50 mmol/liter) was diluted to 1 liter. The reaction buffer is stable for at least two months when stored at 4 °C.

*Methotrexate calibrators.* Sodium methotrexate (supplied by the National Cancer Institute, NIH, Bethesda, Md. 20014) was diluted with water to obtain five concentrations between $2.0 \times 10^{-8}$ and $3.0 \times 10^{-7}$ mol/liter (calculated as the free acid). Stored frozen in 1-ml aliquots, these calibrators were stable for at least seven weeks.

*Dihydrofolate buffer.* A mixture of 25 ml of tris(hydroxymethyl)aminomethane buffer (1.0 mol/liter, pH 7.5) and 5.3 ml of β-mercaptoethanol was diluted to 250 ml with distilled water. This buffer is stable for at least two months at 4 °C.

*Dihydrofolate acid.* This was purchased from Sigma Chemical Co., St. Louis, Mo. 63178, in 25-mg ampules. The contents of an ampule were washed into a beaker with 10.0 ml of a mixture of 5 mmol/liter HCl and 14 mmol/liter β-mercaptoethanol. The resulting suspension was stirred while 0.3 ml was withdrawn and dissolved in 2.7 ml of the dihydrofolate buffer. When 20 μl of this solution was added to 1.0 ml of reaction buffer, the absorbance in the 1-cm pathlength cell of the spectrophotometer was 0.075 ± 5 A. (If necessary, the concentration of the suspension was adjusted to bring the absorbance into the proper range.) The suspension was stored in 0.3-ml aliquots in plastic culture tubes. Stored capped and frozen at −20 °C in the dark, this preparation is stable for at least two months.

*Dihydrofolate solution.* An aliquot of dihydrofolate acid for the assay was prepared each day by thawing a tube of the suspension and diluting it with 2.7 ml of the dihydrofolate buffer. The resulting solution was then loaded into the 1.0-ml Hamilton Dispenser. Both the filled dispenser and the tube containing the unused dihydrofolate acid solution were stored in crushed ice. The dispenser was refilled from the tube as needed, and the unused portion was discarded at the end of the day.

*Dihydrofolate reductase (EC 1.5.1.3).* We used an enzyme preparation from *Lactobacillus casei.* (This enzyme is now available from the New England Enzyme Center, Boston, Mass. 02111.) The specific activity was 90 μmol/h per milligram of protein. The enzyme is stored in a desiccator at −20 °C. Individual tubes containing 0.25-mg samples of the enzyme for use in the assay were prepared by dissolving 25 mg of the powder in 10 ml of distilled water, and dividing the solution into 100-μl aliquots in 12 × 75 mm disposable plastic culture tubes. Stored capped and frozen, this preparation is stable for at least two months.

*NADPH* was purchased from Sigma Chemical Co. in vials containing 1.0 mg each.

*Reaction mixture.* This was prepared freshly each day. Seventy milliliters of reaction buffer was measured into a graduated cylinder. The contents of one 1-mg vial of NADPH and one tube of enzyme were rinsed into a 100-ml amber-colored bottle with the buffer. The bottle was capped and inverted gently several times to mix the contents. This mixture was kept in crushed ice and any unused portion was discarded at the end of the day.

*Patient samples.* Serum, cerebrospinal fluid, and plasma (containing heparin, fluoride, oxalate, or EDTA anticoagulants) were used. Serum specimens were stable for at least six months when stored frozen at −20 °C, but only for three days at room temperature.

**Procedure**

A set of calibrators and a methotrexate control sample were thawed in a beaker of water at room temperature. The reaction mixture and dihydrofolate solution were prepared as above. Twenty microliters of a calibrator was pipetted with an Eppendorf pipet or a suitable dilutor into a 12 × 75 mm disposable glass culture tube. One milliliter of the reaction mixture was then added. The spectrophotometer cuvet was rinsed with distilled water, then purged with air for 5 s. Dihydrofolate solution, 20 μl, was dispensed into the reaction tube from the Hamilton Dispenser. The tube was then vortex-mixed briefly (1–2 s) and the entire solution was aspirated into the cuvet of the spectrophotometer. This aspiration automatically started the timer and, after a delay of 20 s, the initial absorbance was printed. The change in absorbance (ΔA) was printed 120 s later. The five calibrators were each assayed once, and a “zero calibrator” of distilled water was included. The values of ΔA were plotted vs. the methotrexate concentration (Figure 1) to obtain a calibration curve. Patients’ samples (serum or cerebrospinal fluid), and the methotrexate control were assayed in the same way. Serial tenfold dilutions with water were made to obtain a ΔA value which fell within the range of the calibrators.

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**Fig. 1. Calibration curve for MTX assay**
Results for patient samples were checked by making a further dilution with water to obtain another value of $\Delta A$ for each sample that fell within the range of the calibrators. A separate calibration curve was run with each batch of reaction mixture and dihydrofolate acid. A control containing $5.0 \times 10^{-7}$ mol of MTX per liter of plasma was diluted 10-fold and assayed daily.

A calibration curve was stable for 12 h. Individual patient's samples could be analyzed in about 5 min, once the calibration curve was established.

Results

The assay measures MTX in concentrations of $2.0 \times 10^{-8}$ to $3.0 \times 10^{-7}$ mol/liter. Samples were diluted in 10-fold steps with water to obtain a concentration in this range. Serum samples were obtained from 50 hospitalized patients receiving various drugs other than MTX. Included were icteric, lipemic, and hemolyzed specimens. Each sample gave the expected result when analyzed as a blank (no MTX) and with MTX added to a concentration of $1.0 \times 10^{-7}$ mol/liter.

Within-run precision was measured by 20 replicate assays of a plasma standard that contained $8.0 \times 10^{-6}$ mol/liter MTX. The range of values obtained was $6.8 \times 10^{-6}$ to $8.0 \times 10^{-6}$ mol/liter (corresponding to ±0.001 A) with an average of $7.5 \times 10^{-6}$ mol/liter and a standard deviation (SD) of $0.43 \times 10^{-6}$ mol/liter. Day-to-day precision was monitored by running a plasma control standard containing $5.0 \times 10^{-7}$ mol/liter. These were run daily under routine assay conditions by six different technologists during 29 days. The mean of the 29 determinations was $6.8 \pm 1.2 \times 10^{-7}$ mol/liter.

Samples were sent to two other laboratories for comparison analysis. The results are indicated in Table 1. The range of MTX concentrations encountered in monitoring high-dose MTX therapy of osteosarcoma in children is from approximately $10^{-3}$ mol/liter to less than $10^{-8}$ mol/liter, depending on the dose and time of sampling (7–9).

Discussion

Methotrexate has found increasing application in cancer chemotherapy and continues to be used in the treatment of severe psoriasis. Laboratory evidence indicates that serum methotrexate concentrations in mice can be correlated with inhibition of DNA synthesis in tissues (13). Clinical studies indicate that serum methotrexate determinations can help predict toxicity and guide supportive therapy during methotrexate treatment (7–10). These studies and the expanding use of this drug underscore the need for a simple assay suitable for routine use by clinical laboratories.

At least five assays are currently available for MTX quantification: fluorometric (16), bacteriological (15), radioimmunological (17), direct ligand binding (18), and enzyme inhibition (11, 12, 19). Fluorometric methods require difficult manipulations and are at least an order of magnitude less sensitive than enzyme inhibition techniques. The microbiological assay, although sensitive, requires a 36-h incubation period and suffers from a large variability that limits its clinical usefulness. The relatively new radioimmunoassay of MTX has good inherent sensitivity for aqueous solutions, however, interference from serum constituents currently limits its usefulness. The direct ligand binding assay, while sensitive, requires time-consuming manipulation, complicated calculations, and expensive equipment. In existing enzymatic methods, the stability and availability of reagents has been a serious problem. These difficulties have precluded its use as a routine, fast, analytical method.

The method described in this paper suffers from none of the above limitations. It is sensitive, precise, accurate, and specific. It uses a common clinical laboratory instrument and commercially available reagents. Details are given for the manipulation and storage of reagents. We believe that this method can be adopted by most clinical chemistry laboratories and that it is a vital adjunct to MTX therapy.

Table 1. Concentration of Methotrexate in 18 Sera, as Measured in Three Laboratories

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<th>Our lab.</th>
<th>Lab. 1</th>
<th>Lab. 2</th>
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<tr>
<td>$8.5 \times 10^{-6}$</td>
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<td>$3.92 \times 10^{-6}$</td>
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* Similar enzymatic method: Linear-regression analysis of the logs of the results yields $y = 0.974x - 0.0316; r = 0.998$

* Fluorometric method (16): Linear-regression analysis of the logs of the results yields $y = 0.990x - 0.026; r = 0.990$

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References


788 CLINICAL CHEMISTRY, Vol. 22, No. 6, 1976