Enzymic Inhibition Assay for Methotrexate with a Discrete Analyzer, the ABA-100

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We adapted an inhibition assay for methotrexate, involving dihydrofolate reductase from bovine liver, for use with a discrete analyzer (the ABA-100). The analyzer was used both for dilution and a 5-min pre-incubation of the sample with NADPH–enzyme reagent, and for the assay itself. The standard curve was linear between 10 and 120 μg/L. Without pre-incubation the standard curve was nonlinear. The presence of albumin in the NADPH–enzyme reagent enhanced both enzyme activity and stability. Within-run precision (CV) was 2.0% (n = 24), run-to-run precision 7.1% (n = 49). Results obtained on patients' samples (29 sera, 15 urines, 18 cerebrospinal fluids) by the present method and a radioimmunoassay method did not differ statistically (p > 0.05) when the paired data were analyzed by use of the sign test and Wilcoxon's ranked sign test.

Additional Keyphrases: urine, cerebrospinal fluid - monitoring therapy

Methotrexate, used in relatively high doses, is an effective drug for the treatment of various malignancies. The pharmacological action of methotrexate depends on its ability as a folate analog to inhibit the enzyme dihydrofolate reductase (EC 1.5.1.3), which converts dihydrofolate to tetrahydrofolate in the presence of NADPH. After high-dose methotrexate treatment, fatal toxicity to normal cells is prevented by "rescue": administration of citrovorum factor (leukovorin, 5-formyl-tetrahydrofolate), which acts as a metabolic source of tetrahydrofolate.

The clinical management of patients receiving methotrexate can be optimized by monitoring the concentration of the drug in body fluids during therapy. Both the dose of citrovorum factor and the duration of rescue therapy are modified if the methotrexate concentration in serum exceeds a threshold concentration, the dosage of citrovorum factor being increased and the duration of rescue therapy extended until the methotrexate concentration is below the threshold value (1, 2). This individualization of treatment based on the monitoring of serum methotrexate concentration substantially reduces the occurrence of life-threatening toxicity during therapy.

Procedures now available for the routine monitoring of methotrexate concentration in biological fluids include radioimmunoassay, competitive binding radioassay, and enzyme inhibition assay (3-6). We describe here an assay for methotrexate in which commercially available dihydrofolate reductase from bovine liver is used. Our procedure, developed for an automated discrete analyzer, offers improvements over most existing procedures (3, 5). The assay modifications we describe result in a linear standard curve and increased stability of the enzyme reagent.

Materials and Methods

Apparatus

We performed the assay with an ABA-100 (Abbott Bi-chromatic Analyzer; Abbott Labs, Diagnostic Division, Pasadena, CA 91030). We serially diluted the patients' samples with a Micromedic dilutor (Micromedic Systems, Inc., Rohm and Haas, Philadelphia, PA 19105). We used 20-μL "Lancer" (Sherwood Medical Industries, St. Louis, MO 63103) pipets and 5-μL "Micropets" (Clay Adams, Division of Becton, Dickinson and Co., Parsippany, NJ 07054) to dispense reagents.

Reagents

Reaction buffer. Mix together 68.5 mL of a 0.2 mol/L monosodium phosphate solution, 31.5 mL of a 0.2 mol/L disodium phosphate solution, and 100 mL of a 4 g/L solution of bovine albumin. Adjust the pH of this reagent to 6.5 at 25°C. Add 0.2 mL of β-mercaptoethanol, and store the reaction buffer at 4°C. Prepare fresh reaction buffer every two weeks.

Dihydrofolate buffer. Dilute 25 mL of tris(hydroxy-methyl)aminomethane buffer (1.0 mol/L, pH 7.5) and 5.3 mL of β-mercaptoethanol to 250 mL with distilled water. This buffer is stable for at least two months if stored at 4°C.

Dihydrofolate reagent. Wash the contents of a 25-mg ampul of dihydrofolic acid (Sigma Chemical Co., St. Louis, MO 63178; cat. no. D-7006) into a beaker with 10 mL of a solution containing, per liter, 0.5 mmol of HCl and 14 mmol of β-mercaptoethanol. Stir the resulting suspension, and store it in 0.3-mL aliquots, capped and in the dark, at −20°C (stable for at least four months). When an aliquot is needed, thaw it in the dark and add 1.4 mL of dihydrofolate buffer. The diluted dihydrofolate solution is stable for at least 24 h in the pH 7.5 buffer when protected from light and stored at 4°C; it is less stable if stored in the reaction buffer at pH 6.5.

NADPH–enzyme reagent. Prepare the reagent by combining 9 mL of the reaction buffer and 2 μL of the ammonium sulfate suspension of bovine liver dihydrofolate reductase (Sigma, cat. no. D6385) to a 1-mg vial of NADPH (Sigma). Mix the vial by gentle inversion, replace the NADPH vial cap with a reagent vial cap supplied for use with the Analyzer, and store the vial on ice.

Methotrexate standards. Prepare a 100 mg/L stock methotrexate solution by dissolving 10 mg of methotrexate (Sigma, cat. no. A4763; or Aldrich Chemical Co., cat. no. 19, 469-7) in 100 mL of 0.1 mol/L NaOH. Absorption spectra of Sigma and Aldrich methotrexate in 0.1 mol/L NaOH showed absorption maxima at 368, 302, and 255 nm, and the absorptivity value we found was identical to that reported in the literature (7).

Prepare an intermediate working standard (1000 μg/L) by diluting the stock solution 100-fold with distilled water. Prepare six working standards (10, 20, 40, 80, 100, 120 μg/L) by diluting the intermediate working standard sequentially in distilled water. These are stable for at least five months if
stored tightly capped at \(-20^\circ C\). Thaw aliquots of the working standards in the dark.

Procedure

**Preliminary preparations**: Prepare three serial 10-fold dilutions of the patients’ samples with a Micromedic dilutor, set to pick up 100 \(\mu L\) of specimen and dispense 900 \(\mu L\) of saline (NaCl, 9 g/L). Carefully wipe the dilutor tips between each dilution.

**Preincubation**: Prime the 1:51 syringe plate with the NADPH-enzyme reagent and select a 5-min analysis time for the pre-incubation. Add distilled water to the sample cup in the 04 position and fill the remaining sample cups with six methotrexate standards, controls, undiluted patients’ specimens, and the three serial dilutions of each specimen. There is sufficient space on the ABA carousel to assay five patients’ specimens. Start the ABA and then position the probe on the boom arm immediately before pick up of sample 04. The instrument will pick up 5 \(\mu L\) of sample and dispense sample and reagent (250 \(\mu L\)) into the cuvette. Stop the ABA at the end of the 5-min dispense revolution and remove the probe from the boom arm.

**Methotrexate assay**: After the pre-incubation period, run the methotrexate assay with the ABA-100 in the “first revolution read” (FRR) mode, using the following ABA settings:

<table>
<thead>
<tr>
<th>Filter</th>
<th>Temperature</th>
<th>Mode</th>
<th>Reaction direction</th>
<th>Analysis time</th>
<th>Revolutions</th>
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<tr>
<td>340/380</td>
<td>30 °C</td>
<td>Rate, FRR</td>
<td>Down</td>
<td>10 min</td>
<td>2</td>
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Beginning with cuvette 04, add 20 \(\mu L\) of the diluted dihydrofolate reagent to each cuvette manually with a Lancer pipet when the carousel advances to the 00 or reading position. Use a new tip for each cuvette addition to prevent cross contamination of cuvette contents. Wipe the pipet tip carefully to prevent addition of excess dihydrofolate reagent before expelling the dihydrofolate reagent beneath the liquid in the cuvette. With the pipet in the dispense mode, mix the cuvette contents with the pipet tip by moving the pipet in a circular motion. There is adequate time before the initial reading (18 s) to add the dihydrofolate reagent and to mix the cuvette contents. During revolution 2 the change in \(A_4\) (i.e., \(\Delta A_4 = A_{41} - A_{40}\)) will be printed for each cuvette. Final concentrations in the reaction mixture are: dihydrofolate reductase, 7.9 U/L; NADPH, 103.9 \(\mu mol/L\); dihydrofolic acid, 85.4 \(\mu mol/L\); and \(\beta\)-mercaptoethanol, 14.3 \(\mu mol/L\).

**Calculations**: To calculate results, plot \(\Delta A_4\) vs standard methotrexate concentration on linear graph paper and read each control and patient’s sample from this standard curve. Choose a specimen dilution that gives a result that falls within the curve. Multiply the result by the appropriate dilution factor to obtain the specimen methotrexate concentration in micrograms per liter. To express the concentration in moles per liter, divide by 4.54 \(\times 10^8\).

**Comparison of present procedure and radioimmunoassay for methotrexate**: We compared results obtained for serum, urine, and cerebrospinal fluid samples by the enzymic procedure and a radioimmunoassay kit procedure (\(^{125}\)I Methotrexate-RIA Kit; Diagnostic Biochemistry, Inc., Roselle St., San Diego, CA 92121). Most samples were analyzed by both procedures during a working day. The remainder were frozen and analyzed within two weeks by the enzymic assay.

**Controls**: We used two controls during the study, one a patient’s serum, the second prepared by adding a concentrated methotrexate solution to drug-free serum. These controls were stored frozen, in aliquots.

Results and Discussion

**Standard Curve**

**Effect of preincubation**: In the present procedure methotrexate is assayed by exploiting its ability to inhibit dihydrofolate reductase. Figure 1 illustrates the observed inhibition of dihydrofolate reductase activity by increasing methotrexate concentration. In the upper curve of Figure 1, the reaction was started within 18 s after methotrexate and the NADPH-dihydrofolate reductase reagent were mixed. However, if methotrexate was allowed to pre-incubate for 5 min with the NADPH-enzyme reagent (lower curve), progressively greater inhibition of enzyme activity was observed as methotrexate concentration was increased. Pre-incubation times of 5, 10, 30, and 60 min gave superimposable methotrexate inhibition curves.

In Figure 1, enzyme activity decreases linearly up to about 120 \(\mu g\) of methotrexate per liter, at which point there is a sharp break in the rate of decrease of enzymic activity. This phenomenon, which has the characteristics of a titration curve in which methotrexate inhibits enzyme activity stoichiometrically, has previously been observed with dihydrofolate reductase from several sources (7, 8). Williams et al. (7) found that pre-incubation of methotrexate and NADPH with dihydrofolate reductase from *E. coli* resulted in essentially stoichiometric inhibition of enzyme activity. Without preincubation, methotrexate appeared to act as a simple competitive inhibitor of this enzyme with a finite dissociation constant. A stoichiometric inhibition of dihydrofolate reductase purified from bovine liver was observed by Peterson et al. (8) when they preincubated the enzyme with NADPH and methotrexate. In a ligand binding assay for methotrexate (4), binding of methotrexate to dihydrofolate reductase from guinea pig liver was also observed to be essentially stoichiometric in the presence of NADPH, and formed the basis for a sensitive sequential assay for methotrexate in serum.
We chose to pre-incubate in our assay because of the increased linearity and "titration nature" of the standard curve. In contrast to our procedure, Finley and Williams (5), who use the same source of dihydrofolate reductase, did not pre-incubate the enzyme with methotrexate and NADPH before assay and found a nonlinear standard curve over all methotrexate concentrations.

Effect of albumin: Figure 2 (upper curve) shows the effect of including albumin in the reaction buffer used to dilute the ammonium sulfate suspension of dihydrofolate reductase: enzyme activity was enhanced. The NADPH-enzyme reagent prepared with albumin showed no decrease in enzyme activity during 28 h when stored at 4 °C. In contrast to our results, Brooks (9), who prepared his bovine liver dihydrofolate reagent without albumin, detected decreased enzyme activity within 1 h.

Stability and linearity: Figure 3 illustrates the stability of the composite standard curve over 18 analytical runs (three weeks). The zero-methotrexate standard exhibited a run-to-run CV of 4.8% and a within-run CV of 1.4% in enzyme activity. The lowest concentration of methotrexate that could be reliably distinguished from zero was 10 μg/L.

Dihydrofolate was stored in pH 7.5 buffer to increase stability. The final reaction mixture pH was 6.5. The slope of the standard curve was identical at pH 6.0 and 6.5, but slightly less at pH 7.0. This is consistent with the reported pH optimum of 6.2 (10).

Precision

Within-run CV, determined with a patient's serum (mean = 66 × 10^9 μg/L), was 2.0% (n = 24). Run-to-run precision for the same serum was 7.1% (n = 49). This material was diluted 1000-fold before assay, with three subsequent serial 10-fold dilutions, and the reading fell near the midpoint of the standard curve. The second serum control, the one prepared by adding methotrexate to drug-free serum (target value = 94 μg/L), had a run-to-run precision of 3.5% (n = 33) and a mean of 100 μg/L. This control was assayed without serial dilution and gave readings near the upper end of the dynamic range of the standard curve. The between-run precision of a serum sample was calculated from 10 runs, mean = 4.76 μg/L, SD = 1.96 μg/L, CV = 39%. The variability of 18 standard curves was also determined; the CV observed for the 10, 20, and 30 μg/L standards were 5.4, 3.9, and 4.2%, respectively.

Method-Comparison Studies

In Figure 4 we compare results by the present procedure and a specific radioimmunoassay procedure for methotrexate (n = 62) in 29 sera, 15 urines, and 18 cerebrospinal-fluid specimens. Methotrexate concentrations as determined by the radioimmunoassay procedure ranged from 2.6 × 10^{-8} to 4.0 × 10^{-3} mol/L. Four of the specimens analyzed had concentrations by radioimmunoassay of less than 2.2 × 10^{-8} mol/L. Although such low concentrations cannot be measured precisely by the present procedure, they all gave results below that for our lowest standard (2.2 × 10^{-8} mol/L). Statistical analysis of the pooled specimen data by the sign test and

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**Fig. 2. Effect of albumin, added to NADPH-enzyme reagent, on methotrexate standard curve**

**Fig. 3. Methotrexate standard curve**

The graph is the average of 18 methotrexate standard curves. The vertical bars indicate 2 SD.

**Fig. 4. Methotrexate comparison of radioimmunoassay and present method**

29 sera (Δ), 15 urines (O), 18 cerebrospinal fluids (●)

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**Fig. 5.** Methotrexate standard curve

The graph is the average of 18 methotrexate standard curves. The vertical bars indicate 2 SD.

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**Fig. 6.** Methotrexate comparison of radioimmunoassay and present method

29 sera (Δ), 15 urines (O), 18 cerebrospinal fluids (●)
Wilcoxon's signed rank test showed no significant difference \( p > 0.05 \), and no statistically significant difference \( p > 0.05 \) was seen when these tests were performed on the individual data for serum, urine, and cerebrospinal fluid. Linear regression of data shown in Figure 4 yielded a slope = 1.003, intercept = 0.042, \( r = 0.997 \), and standard error of estimate \( = 0.116 \). Our results for sera are in agreement with those reported by Finley and Williams (5), who found excellent correlation between the same radioimmunoassay procedure and an enzyme assay in which bovine liver dihydrofolate reductase was also used.

Conditions which interfere with the enzyme inhibition assay for methotrexate were not re-investigated in this study. Samples which were icteric or demonstrated visible hemolysis or lipemia gave similar results by both radioimmunoassay and enzyme inhibition assay. We did not assay severely icteric, lipemic, or hemolized samples, but they would be expected to exhibit interference similar to that observed by Finley and Williams (5).

Wang et al. (6, 11) commented on the presence of impurities in the methotrexate used for therapy and also on the presence of metabolites of methotrexate in body fluids. The close agreement between the radioimmunoassay and the present enzymic procedure indicates either a negligible concentration of these compounds as compared to methotrexate or, more likely, similar reactivity of these compounds in the two-assay systems.

References