Adaptation of the Enzyme-Multiplied Immunoassay for Methotrexate to the Centrifugal Analyzer

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We adapted the homogeneous enzyme-multiplied immunoassay for methotrexate to the centrifugal analyzer. The sensitivity of the assay has been extended from 200 nmol/L down to 12.5 nmol/L. Several protocols may be followed, depending on whether the samples to be tested are from patients on low- or high-dose methotrexate regimens. Precision, accuracy, and sensitivity were satisfactory. Comparison with the enzymic-inhibition method (x) gave the following least-squares regression: y = 1.002 x - 0.013 μmol/L; r² = 0.9721; S_y|x = 0.014 μmol/L.

Additional Keyphrases: drug assay · enzymic-inhibition method compared

The measurement of methotrexate by radioimmunoassay (1), enzymic inhibition (2), and "high-pressure" liquid chromatography (3) is well-established. The lower limit of detection with any of these methods is about 0.01 μmol/L.

The technique of homogeneous enzyme-multiplied immunoassay (EMIT; Syva Corporation, Palo Alto, CA 94304) provides a convenient method for measuring methotrexate, but the sensitivity of the assay according to the standard protocol extends only to 0.2 μmol/L. If the treatment of the patient is confined to administration of large doses of methotrexate, together with citrovorum factor given to diminish the toxic effects of the drug, the usual resulting plasma concentration of methotrexate will be such, after 24 to 48 h, that the EMIT assay will provide adequate sensitivity. However, if the doses of methotrexate are small, and no citrovorum factor is given concurrently (low-dose methotrexate), the plasma concentration in such patients measured after 24 to 48 h will be much lower than the usual concentration after the standard protocol of therapy. Therefore, we modified the method of assay, using EMIT reagents and the centrifugal analyzer, so that the lower limit of detectability was extended to 12.5 nmol/L.

We compared results by the proposed method with those by the enzymic-inhibition technique (2), which in turn had been compared with those by radioimmunoassay.

Materials and Methods

Apparatus

We performed the assay with a centrifugal analyzer (Amicon Rotochem IIa; American Instrument Co., Silver Spring, MD 20920).

The reduction of data was done with our own computer system (DEC 1170; Digital Equipment Company, Maynard, MA 01754) and with a program we have written in MUMPS (Massachusetts General Hospital Utility Multiprogramming Systems) involving a cubic least-squares curve fitting.

Reagents

We used the commercially available reagents (Syva) for methotrexate assay by homogeneous enzyme-multiplied immunoassay: Reagent A (antibodies against methotrexate, glucose 6-phosphate, NAD*1) and Reagent B [glucose-6-phosphate dehydrogenase (EC 1.1.1.49), chemically coupled to methotrexate]. Reconstitute each with 3.0 mL of H₂O. Store at 25 °C 1 h before use. Store at 4 °C when not in use. Stable 12 weeks.

Buffer, tris(hydroxymethyl)aminomethane (Tris)-HCl, 55 mmol/L, pH 8.0, supplied as a 10-mL concentrate. Dilute to 150 mL with distilled water (final concentration, 55 mmol/L). Store at 25 °C. Stable 12 weeks.

Calibrators. Supplied individually in lyophilized form; reconstitute with 1.0 mL of distilled water. Stable for 12 weeks at 4 °C.

Procedure

Prepare suitable standards from the 0.2, 0.5, 1.0, 1.5, and 2.0 μmol/L standards provided by Syva, diluting with a 55 g/L solution of albumin to the following concentrations: 12.5, 25, 50, and 100 nmol/L. The full range of standards need not be used if the patients' samples are expected to contain low concentrations of methotrexate (see Results). The standards, after reconstitution, are stable for three months at 4 °C.

Dilute Reagents A and B sixfold with the Tris buffer to make enough for the assay. Load the sample cups as follows: cup 1, water; cups 2–35, standards, controls, and patients' samples. Set the Rotofil Automatic Sampler-Diluter (American Instrument Co.) as follows: serum/standard pump, 10 μL; flush pump buffer, 200 μL; reagent pump A (Reagent A), 200 μL; reagent pump B (Reagent B), 200 μL. Automatically deliver sample, buffer flush, and Reagent A to the larger, middle well of the transfer disc. Deliver 200 μL of Reagent B to the smaller, inner well of the transfer disc.

As an alternative loading schedule for monitoring low-dose therapy, use standards from 12.5 to 200 (or 500 if necessary) nmol/L. Set the serum/standard pump at 40 μL instead of 10 μL. All other settings remain the same.

Place the transfer disc on the centrifugal analyzer. Begin assay immediately. The analyzer is controlled with the Kinetic Rate III tape, a program supplied by the American Instrument Co., which allows the user to take initial and final absorbance readings at any time chosen. The printout shows initial and final readings, and the delta absorbance for each position. Analyses are made at 30 °C. Set the filter wheel at 340 nm to read the increase in absorbance of NADH. Set the first reading at 15 s, the final at 180 s later.

Calculation. The delta absorbances of the standards (except the 0 standard) are entered into the cubic least-squares fit program via the terminal. The unknown sample delta absorbances are then entered. Figure 1 shows a representative curve.

Results

Standard curve. The delta absorbances of the standards

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Fig. 1. Methotrexate standard curve (computer-produced cubic least-squares fit)

(excluding the 0 standard) are entered into the computer, which calculates a cubic least-squares regression curve. The printout (Figures 2 and 3) provides the following: presentation of the concentration of each point (observed) as calculated from the regression curve; the deviation of that point from the theoretical value, both in concentration units and percent (negative or positive); and a summation of those errors. The analyst may then enter the delta absorbance of the unknown samples, and the computer instantly calculates the concentration from the stored curve. Figure 1 shows a typical computer curve, which can be produced by the program if the user desires, although we do not routinely request the curve.

If the patients' samples are always expected to contain low concentrations of methotrexate (for example, under conditions of low-dose therapy), we use the alternative assay procedure (see Procedure). The range of the standard curve will be from 12.5 nmol/L to 200 or 500 nmol/L, whichever upper limit is found to be suitable by experience. If only this portion of the standard curve is used, the sensitivity of the dose response increases. That is, the absorbance change for each incremental increase of concentration becomes greater, in the region of low concentrations. When a fuller range of standards is used (up to 2.0 μmol/L), the portion of the curve from 25 to 200 nmol/L shows somewhat less incremental response to dose than when a less extensive range is used.

Precision. Within-day precision was evaluated by analyzing four patients' samples 20 times on a single rotor, during the same day. Results (in nmol/L) were for the lower-range curve (12.5–500 nmol/L): mean, 35 (SD, 2.5; CV, 7.1%); mean, 180 (SD, 12; CV, 6.7%); for the usual-range curve (100–2000 nmol/L): mean, 330 (SD, 21; CV, 6.36%); mean, 760 (SD 40; CV, 5.26%).

The same samples were dispensed into small aliquots and frozen at −20 °C. Between-day precision was then assessed by assaying the samples on 20 separate days during a month. The results (in nmol/L) for the lower-range curve were: mean, 33 (SD, 2.8; CV, 8.48%); mean, 190 (SD, 14; CV, 7.37%). For the usual-range curve, results were: mean, 340 (SD, 23; CV, and mean 740 (SD, 43; CV, 5.8%).

**Accuracy.** Analytical recovery, tested by adding methotrexate standard to sera that contained no drug, ranged from 95 to 101%. Serial dilutions of sera of high concentration showed suitable linearity down to the lowest limit of detectability (12.5 nmol/L). There is no carryover with the proposed method.

**Clinical samples.** We previously reported (2) that the correlation of the enzymic-inhibition method to radioimmunoassay was satisfactory. We compared the results of analysis of 50 patients' samples (range of concentration: 0.02–15.5 μmol/L) obtained by the proposed methods and by enzymic inhibition. The regression equation (y = enzymic inhibition, y = proposed homogeneous enzyme immunoassay) was: y = 1.002x −0.013 μmol/L, r² = 0.9721, S₀/₀ = 0.014 μmol/L.

**Discussion**

The proposed adaptation of the EMIT methotrexate assay to the centrifugal analyzer provides a rapid, precise, and accurate method. The alternative protocol is more efficient when samples with low concentration must be tested (plasma concentration after low dosing, or after intrathecal injection). The use of 40 μL of sample (instead of 10 μL as in the regular assay protocol proposed) has the effect of increasing the sensitivity of the method at very low concentrations, and providing a lower limit of detectability to 12.5 nmol/L. The curve so constructed is suitable only up to 0.5 μmol/L, after which there is antigen excess. If samples are expected to be not only very

**FUNCTION: CA**

**CALCULATION PROGRAMS**

1 HEMATOLOGY CALCULATIONS
2 CHEMISTRY CALCULATIONS
3 CHEMISTRY REPORTS
4 HEMATOLOGY REPORTS

**SELECT OPTION * 2,18**

**HOW MANY DATA PAIRS : 6**

FAIR 1 X : .6249 Y : .1
FAIR 2 X : .6551 Y : .2
FAIR 3 X : .7239 Y : .5
FAIR 4 X : .8285 Y : 1.0
FAIR 5 X : .9102 Y : 1.5
FAIR 6 X : .9771 Y : 2.0

**THE CUBIC LEAST SQUARES FUNCTION IS**

\[-4.09 +13.22 x -16.27 x **2 +9.33 x**3 = Y\]

**Fig. 2. Printout of program showing delta absorbances entered for each concentration**

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**ENTER X FOR EVALUATIONS**

X = .6249 Y = .094 DELTA = -.0059
X = .6551 Y = +.21 DELTA = .010
X = .7239 Y = +.49 DELTA = -.0065
X = .8285 Y = +1.00 DELTA = .0015
X = .9102 Y = +1.50 DELTA = .00063
X = .9771 Y = +1.99 DELTA = -.00050

**MEAN VALUE OF DIFFERENCES**

DELTA = 0

X DIFF = 2.11

**Fig. 3. Computer evaluation of standard curve (left four columns), with calculation (right two columns) of two control samples (in duplicate) and one patient’s sample (in duplicate)**
low but in the range of 1.0-2.0 μmol/L, the higher samples may be prediluted before the assay is performed.

If the regular assay protocol is used (10-μl sample, range of standards 100 to 2000 nmol/L), the low concentrations will not be measured accurately (<100 nmol/L). If the range of standards used is from 25-2000 nmol/L (with a 10-μL sample size), the curve is very sensitive above 100 nmol/L but less so from 25 to 100 nmol/L. For example, the absorbance change with the full-range curve is 0.006 for concentrations from 25 to 50 nmol/L, whereas with the low-range curve the absorbance change is 0.026.

As many as 28 samples can be assayed in 3 min. The alternative protocol allows the efficient and rapid measurement of samples obtained from patients receiving low-dose methotrexate therapy.

The influence of metabolites on the assay remains undetermined. In particular, the metabolites 7-hydroxymethotrexate, 4-amino-4-deoxy-N\(^{10}\)-methylpteroic acid, diglutamate, and triglutamate methotrexate should be examined for their effect on the assay, especially at very low concentrations of methotrexate. Our experience has been largely the assay of samples from patients on usual or high-dose therapy, and we have found very low methotrexate concentrations in those patients after many days. This observation suggests that metabolites have no appreciable influence on the assay. However, patients receiving low-dose therapy with no concurrent citrovorum factor should be examined for metabolites to see if these influence the assay when the standard curve is deliberately set to detect very low concentrations. When methotrexate is in very high concentration, its metabolism is extensive (4); perhaps in patients on low-dose therapy, however, metabolism may not be so extensive, so that metabolites would be less likely to interfere in the assay technique.

References

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Two Agarose Electrophoretic Systems for Demonstration of Oligoclonal Bands in Cerebrospinal Fluid Compared

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Demonstration of oligoclonal bands by electrophoresis of cerebrospinal fluid is an important aid in establishing the diagnosis of multiple sclerosis. Electrophoretic systems vary in their effectiveness in doing so. We compared two systems in this respect. For a thin-layer agarose system, sensitivity was less (47%) than for a high-resolution agarose system (87%). Each system had good specificity (92 and 85%, respectively). Interpretation of electrophoretic patterns for cerebrospinal fluid should be available in clinical laboratories. Further, the best available system should be used for demonstration of oligoclonal bands.

Additional Keyphrase: multiple sclerosis

The role of the clinical laboratory in aiding in the diagnosis of multiple sclerosis has changed in recent years because of three procedures: demonstration of oligoclonal bands by agarose electrophoresis of cerebrospinal fluid (CSF) (1), quantitation of myelin basic protein in CSF (2), and quantitation of immunoglobulin G in CSF (3). The incidence of oligoclonal bands in the agarose electrophoretic pattern of the CSF of selected patients with demyelinating diseases is in the range of 70% to 100% (2, 4), although oligoclonal bands in the CSF are not specific for demyelinating diseases (1, 5).

Methods used to demonstrate the oligoclonal bands may differ in sensitivity. Agarose is the electrophoretic support medium used most commonly, but the several commercially supplied variants of agarose electrophoretic techniques differ in many features. Previously, we assessed the performance characteristics of a commercial agarose electrophoretic system used to demonstrate oligoclonal bands (2). Here, we compare the performance of that system with that of a high-resolution agarose electrophoretic system.

Cellulose acetate is known to be an inappropriate support medium for the demonstration of oligoclonal bands (6).

Materials and Methods

Samples. Patients were selected only on the basis of availability of adequate CSF for examination by each method and availability of clinical history. Samples were obtained by standard lumbar puncture techniques.

CSF was concentrated 80-fold by selective permeability (Minicon CS 15; Amicon, Lexington, MA 02173).

Thin-layer agarose-gel electrophoresis. A 2-μL sample of the concentrate was applied, in two applications, to thin-layer agarose gel (Universal Electrophoresis Film; Corning Medical, Medfield, MA 02502). A 1-μL sample of each patient’s serum was applied to a position adjacent to his CSF sample. Elec-