Improved Double-Antibody Enzyme Immunoassay for Methotrexate

Mohammad N. Al-Bassam, Michael J. O'Sullivan, James W. Bridges, and Vincent Marks

We report an enzyme immunoassay procedure for methotrexate measurement that takes less than 3 h to perform. β-o-Galactosidase (EC 3.2.1.23) from Escherichia coli was conjugated to methotrexate by means of the mixed anhydride reaction. Bound and free labeled drug were separated by a preincubated cubic complex of first and second antibody. The enzyme activity of the bound fraction was measured with o-nitrophenyl-β-o-galactopyranoside as substrate. The standard curve covered the range 1 to 10 μg of methotrexate per liter. One microgram of methotrexate per liter inhibited binding of the tracer by 17%. The assay is specific for methotrexate in the presence of folic acid (citrovorum factor), folic acid, tetrahydrofolic acid, and other methotrexate metabolites. Intra- and inter-assay CVs were less than 5 and 10%, respectively. Results obtained with this enzyme immunoassay method agreed well with those obtained with an established radioimmunoassay method.

A folic acid antagonist, methotrexate (MTX), has proved to be of considerable therapeutic value in various clinical conditions. Since its introduction for the treatment of acute lymphoblastic leukemia in 1948 (1), MTX has become increasingly important in cancer chemotherapy, and is widely used as an anti-neoplastic and immuno-suppressive agent. However, the toxic effects of MTX include bone marrow depression (2), hepatotoxicity (3), nephrotoxicity (4), pulmonary complications (5), and neurotoxicity (6). Although the etiology of these toxic manifestations is not well understood, in some cases they appear to be associated with delayed elimination of drug from cerebrospinal fluid (6) or the systemic circulation (7). Pharmacokinetic monitoring of MTX concentration in plasma may therefore be helpful in limiting MTX toxicity (7, 8). Monitoring of MTX plasma concentrations requires a convenient, sensitive, and specific assay method. Fluorometric (9–12), microbiological (13, 14), and enzymic (15–17) methods have been developed for assaying MTX, but these are rather complex and are not generally specific or sensitive enough to be suitable for routine clinical use.

Several radioimmunoassays for MTX have been described (18, 19). Although these appear to be specific and sensitive enough to monitor the drug in biological fluids without initial sample work-up, an enzyme immunoassay for MTX has the potential advantages over radioimmunoassay of being non-hazardous, of having end-point measurement with relatively inexpensive and readily available equipment, and of involving a labeled reagent that is easily synthesized and stable. Recently, a homogeneous enzyme-immunoassay for MTX has been reported (20), involving a competitive heterogeneous MTX enzyme-immunoassay. Free MTX competes with enzyme-labeled MTX for a limited amount of MTX-specific antibody. A second antibody with specificity for the first is used to separate free MTX from antibody-bound MTX. The enzyme activity in the bound phase is inversely proportional to the concentration of free MTX.

In a previous preliminary communication we described an enzyme immunoassay for MTX in which a conventional double-antibody separation phase was used (21). This separation procedure has the disadvantage of requiring an overnight incubation. In the method reported here we use a pre-precipitated complex of first and second antibodies, which markedly speeds the assay. Furthermore, we have optimized the degree of incorporation of MTX into the enzyme, with the result that free MTX more readily displaces the label from the first antibody, giving in a more sensitive assay.

Materials and Methods

Reagents

Methotrexate, aminopterin, 4-amino-N10-methylpteroyl acid, N10-methylfolic acid, and 2,4-diamino-6-methylpteridine were kindly supplied by Lederle Laboratories, Division of American Cyanamid Co., Pearl River, NY 10965. 7-Hydroxymethotrexate was a generous gift from Dr. A. Jacobs, NIH, Bethesda, MD. Folic acid and its analog, tri(hydroxy-methyl)aminomethane (Tris), were purchased from Sigma, London, England. β-D-Galactosidase (EC 3.2.1.23), from Escherichia coli, was purchased from Boehringer Mannheim, Mannheim, F.R.G., as a suspension in 2.2 mmol/L ammonium sulfate. We also used Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ 08854), and bovine serum albumin as a 300 g/L solution from Armour Pharmaceutical Co., London, England. The methotrexate antisera (HP/S/3 11A), which was supplied by Guildhay Antisera, Guildford, Surrey, U.K., by Dr. G. W. Aherne (19), was raised in a sheep, the antigen being a methotrexate–ovalbumin conjugate prepared by the carbodiimide procedure. Donkey anti-sheep second antibody was purchased from Guildhay Antisera.

Diluent buffer contained sodium phosphate (50 mmol/L, pH 7.4), sodium chloride (100 mmol/L), and bovine serum albumin (1 g/L) in distilled water. Tris buffer was prepared from Tris (50 mmol/L, adjusted with acetic acid to pH 7.5) and contained, per liter, 10 mmol of magnesium chloride, 100 mmol of sodium chloride, and 10 mmol of 2-mercaptoethanol. Sodium bicarbonate buffer (100 mmol/L, pH 9.5) also contained magnesium chloride (10 mmol/L). The β-D-galactosidase substrate solution contained o-nitrophenyl-β-D-galactopyranoside (2.3 mmol/L), sodium phosphate buffer (100 mmol/L, pH 7.0), magnesium chloride (1 mmol/L), and 2-mercaptoethanol (10 mmol/L).

Plasma samples collected from patients receiving MTX treatment were assayed by radioimmunoassay (19) by Dr. G.

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The 9-D.galactosidase-MTX Conjugate preparations, resistant impurities was serum activity the fl-D-galactosidase formate of amine dure contained.

The first diluent, antiserum, of albumin with 2.8 mg; 15 μmol) was dissolved sequentially in 0.1 mL of dimethylformamide and cooled to 10 °C. Isobutyl chloroformate (2 mg; 15 μmol) was added and the solution was stirred for 30 min at 10 °C before mixing with a solution of β-D-galactosidase (3 mg; 6 nmol) in 2 mL sodium bicarbonate buffer. The reaction was allowed to proceed for 4 h at 10 °C. The solution was then left overnight at 4 °C. To separate compounds of low relative molecular mass from the enzyme, the solution was applied to a Sephadex G-25 column and eluted with Tris buffer. The fractions containing enzyme activity were collected and pooled, and sodium azide and bovine serum albumin were added to give 200 mg/L and 1 g/L solutions, respectively. The conjugate was stored at 4 °C.

Immunoreactivity of the MTX–β-D-Galactosidase Conjugate

The proportion of β-D-galactosidase conjugated to MTX was determined by incubating a portion of the enzyme conjugate (equivalent to three units) with excess MTX antiserum that had been pre-incubated with second antiserum. The complex was centrifuged (2500 rpm) for 20 min, the supernate was aspirated, and the precipitate was washed once with 2 mL of diluent buffer. Substrate, 1 mL, was mixed with the washed precipitate and held at 40 °C for 1 h, and the reaction was stopped with 1.5 mL of 0.2 mol/L sodium carbonate. The absorbance of the mixture was measured at 420 nm.

Determination of MTX Concentration

The assay protocol is illustrated in Table 1. All dilutions of antiserum, label, standards, and samples were made with diluent buffer. The pre-incubated complex of first and second antibody used for phase separation was prepared in bulk by incubating first antiserum and second antiserum for at least 24 h at 4 °C and storing it until required at 4 °C. The first and second antisera were used at final dilutions of 1 in 5000 and 1 in 30, respectively. Three units of enzyme-labeled MTX were added to each tube. The standards were prepared by dissolving 1 mg of methotrexate in 10 mL of diluent buffer. This was further diluted to give the 1 to 10 μg/L range of concentrations used in the assay. The reagents were added to the tubes in the order indicated in the protocol. Calibration curves were plotted as the ratio of the fraction of the enzyme-labeled MTX bound at a given MTX concentration to the fraction bound when no methotrexate was present, i.e., B/B₀, where B = enzyme activity bound at given MTX concentration, B₀ = enzyme activity bound at zero MTX concentration.

Validation of the Assay

We studied the effect of plasma on the assay by adding known amounts of MTX to pooled plasma and determining the analytical recovery of MTX. The precision and the reproducibility of the assay were assessed by repeatedly assaying several serum samples over a range of MTX concentrations, 50 to 10 000 μg/L. We compared the results obtained with the above enzyme immunoassay procedure with those obtained by radioimmunoassay. The specificity of MTX antiserum was

Table 1. Protocol for Enzyme Immunoassay of MTX

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Total tube</th>
<th>Non specific binding tube</th>
<th>Zero tube</th>
<th>Standard or sample tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex b</td>
<td>700</td>
<td>700</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Standard or sample</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Label</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Diluent buffer</td>
<td>700</td>
<td>700</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Second antibody</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* * Incubate for 60 min at 30 °C, then add 1.5 mL of diluent buffer, centrifuge for 20 min. Aspirate the supernate and wash with 2 mL of diluent buffer, centrifuge, and aspirate. Add 1 mL of substrate solution to the precipitate, mix, and leave for 1 h at 40 °C. End the reaction by adding 1.5 mL of 0.2 mol/L carbonate and measure the absorbance at 420 nm.

* Pre-incubated first antibody and second antibody complex in diluent buffer.

We have calculated that the binding ratio (B/T) of the MTX–β-D-galactosidase–methotrexate conjugate by methotrexate antiserum effect of varying the molar ratio between methotrexate and the enzyme on the formation of the enzyme–methotrexate conjugate. (A) 5000:1; (B) 2500:1, and (C) 1000:1 for methotrexate:enzyme ratio. B = enzyme activity bound at given concentration. T = total enzyme activity.
assessed by investigating the ability of other drugs to interfere in the MTX enzyme immunoassay.

Results

Preparation of the Enzyme–MTX Conjugate

Figure 1 shows antibody dilution curves for conjugates prepared by using different molar ratios of MTX to enzyme. At the highest ratio tested, 96% of the enzyme was bound to immunologically active MTX. However, the titer obtained with this label was relatively low and the MTX standard curves obtained by using this label were relatively insensitive. At a ratio of 2500 to 1, 81% of the enzyme was conjugated to immunologically active MTX. With this label a considerably higher titer and more sensitive standard curves were obtained. At lower ratios of MTX to enzyme, considerably less MTX was incorporated into the enzyme, without any marked in-
crease in titer. The label prepared at a ratio of 2500 to 1 was therefore used in all subsequent investigations. Ninety-five percent of the enzyme activity was retained during the conjugation reaction.

Methotrexate Standard Curve

Figure 2 shows the MTX standard curve obtained by using the enzyme-labeled MTX; 1 µg of MTX per liter inhibits binding of the label by 17%.

Other Analytical Considerations

Specificity. The cross reactivity of some other drugs is shown in Table 2. Cross reactivity ranged from 40% for 4-amino-N\textsuperscript{10}-methylptericoic acid and 20% for aminopterin to <0.001% for folic acid, folinic acid, and di- and tetrahydrofolic acid. The cross reactivities as determined by enzyme immunoassay and radioimmunoassay were very similar.

Analytical recovery of MTX. Table 3 shows the effect of plasma on the recovery of methotrexate. All the added methotrexate was accounted for at plasma concentrations likely to be encountered in the MTX assay.

Precision and reproducibility. The results (Table 4) indicate that the interassay CV was less than 10%, and the intra-assay CV was less than 5%, for any of the samples tested.

Stability of reagents. The enzyme activity of the enzyme label decreased by <10% when it was stored for six months in solution at 4 °C or lyophilized and stored at −20 °C. The immunoreactivity of the label decreased by <8% during this period. The preincubated control of first and second antibody was stable after one month in solutions at 4 °C.

Accuracy. The enzyme immunoassay method was compared with a radioimmunoassay (19), with use of plasma samples from 50 patients treated with methotrexate alone or with folinic acid “rescue”. There was a good agreement between results by the two methods. The correlation coefficient (r) for enzyme immunoassay and radioimmunoassay was 0.99, with confidence limit of 99% (p > 0.0001). The regression line was y = 1.03x + 0.66 where y = results obtained with enzyme immunoassay. The scattergram is shown in Figure 3.

Table 3. Effect of Plasma on Analytical Recovery of MTX

<table>
<thead>
<tr>
<th>Added of methotrexate, µg/L</th>
<th>Recovery of methotrexate, %, ±CV (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>105 ± 4</td>
</tr>
<tr>
<td>50</td>
<td>105.4 ± 3</td>
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<tr>
<td>500</td>
<td>98 ± 4</td>
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<tr>
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<tr>
<td>10 000</td>
<td>104.4 ± 4</td>
</tr>
<tr>
<td>10 000</td>
<td>103.1 ± 3</td>
</tr>
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</table>

Table 4. Precision of MTX Enzyme-Immunoassay

<table>
<thead>
<tr>
<th>Mean amount of MTX measured, µg/L</th>
<th>CV, %</th>
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</thead>
<tbody>
<tr>
<td>Intra-assay (n = 15)</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>4.7</td>
</tr>
<tr>
<td>506</td>
<td>3.0</td>
</tr>
<tr>
<td>11 440</td>
<td>4.0</td>
</tr>
<tr>
<td>Inter-assay (n = 5)</td>
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<tr>
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<td>7.7</td>
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<tr>
<td>11 500</td>
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</table>
from patients undergoing methotrexate treatment with or without folic acid rescue.

We thank Dr. G. W. Ahene and Guildhay Antisera for the generous gift of the methotrexate antiserum, and for supplying many of the methotrexate analogs used in this work. We are indebted to Dr. G. Mould for assaying methotrexate in plasma samples by radioimmunoassay. M. N. Al-Bassam thanks the University of Baghdad, Iraq, for providing financial support during this project. We also thank Mrs. C. Reynolds for secretarial assistance.

References


Discussion

The importance of monitoring MTX concentrations in plasma to limit toxic effects has been emphasized by Salasao (23) and others (26). The enzyme immunoassay described above allows quantitation of MTX in plasma and can measure concentrations of MTX as low as 1 μg/L. Bound and free label are separated by a preincubated complex of first and second antibody, a procedure previously described for radioimmunoassay (27) but one that has not been applied previously to enzyme immunoassay. Folic acid, tetrahydrofolic acid, folinic acid (citrovorum factor), and 7-hydroxymethotrexate do not cross react substantially with MTX antibody. This fact permits measurement of MTX in the presence of these naturally occurring folic acids even when they are present in artificially increased concentrations. The enterohepatic bacterial metabolite 4-amino-N10-methylpteroyl acid does cross react to about 40%. This metabolite may be found in the urine of patients for 24 h or more after the intravenous administration of MTX as well as during long-term treatment with the drug (27–29). However, this metabolite has not been detected in plasma.

The accuracy and reproducibility of the analytical recovery of methotrexate in the presence of various amounts of plasma likely to be used in practice indicates that methotrexate measurements are unaffected by the natural constituents of plasma. The assay has a precision that is within acceptable immunoassay standards, with an intra-assay CV of less than 5% and an inter-assay CV of less than 10%. Storage of the label in solution at 4 °C for six months did not affect the performance of the assay.

MTX plasma concentrations determined by this procedure agreed well with those determined by an established radioimmunoassay method.

The enzyme immunoassay method for MTX described above has several advantages over our previously reported methotrexate enzyme immunoassay (21). It is more sensitive, has better precision and reproducibility, and is considerably more rapid. The present method is amendable to large-batch handling and at least semi-automation. Thus it is suitable for the routine measurement of methotrexate in plasma samples.

Fig. 3. Correlation between results of enzyme immunoassay and radioimmunoassay methods for methotrexate determination.


