A Direct Ligand-binding Radioassay for the Measurement of Methotrexate in Tissues and Biological Fluids

Elliot Arons, Sheldon P. Rothenberg, Maria da Costa, Craig Fischer, and M. Perwaiz Iqbal
Division of Hematology-Oncology, Department of Medicine, New York Medical College, New York, New York 10029

SUMMARY

A direct ligand-binding radioassay for methotrexate (MTX) has been developed using dihydrofolate reductase, contained in the lysate of L1210 leukemia cells, as the binding determinant. The procedure is a two-phase reaction system where standard MTX concentrations or the sample being assayed is incubated with the reagent lysate in the first phase, and [3H]MTX is then added in the second phase to titrate the remaining unoccupied binding sites on the enzyme. This method eliminates the need for measuring the residual catalytic activity of the enzyme.

The sensitivity of the radioassay is limited only by the specific activity of the [3H]MTX and now approximates 10 pg of the drug. Folic acid, methylenetetrahydrofolate, formyltetrahydrofolate, and dihydrofolate in concentrations that are physiological do not interfere in the radioassay. Both mercaptoethanol and reduced nicotinamide adenine dinucleotide phosphate increase the binding capacity of the lysate for MTX; but the reduced nucleotide also increases the affinity of the enzyme for the inhibitor.

MTX added to serum can be assayed without extraction if the concentration is greater than 300 pg/ml and recovery of the drug added to serum is about 92%. MTX has been assayed in serum, spinal fluid, and urine of patients who were treated with this drug. It has also been assayed in the lysates of L1210 cells from C57BL x DBA/2 F1 mice treated with MTX. The procedure is simple, rapid, and accurate and should permit better correlation of the therapeutic and toxic effects of MTX with blood concentrations over long-term treatment periods.

INTRODUCTION

The pharmacokinetics and tissue distribution of MTX has been studied in animals and man (1, 7, 10, 12, 13, 15, 18, 24) and has provided fundamental information about plasma clearance, tissue uptake, intestinal absorption, and urinary excretion of this drug. The experimental model for many of these studies has been the administration of a single or a few multiple doses of the drug by a specific route after which the pattern of excretion and distribution was measured over a finite time period. With respect to the clinical use of MTX, there is a dearth of information relating the long-term low blood concentration with either therapeutic or toxic effects (10). Indeed, the importance of monitoring drug concentration may be appreciated from a recent report by Bleyer et al. (5), which showed that the neurotoxicity of intrathecal MTX could be correlated with a cerebrospinal fluid concentration higher than that observed in patients without neurotoxic complications.

One reason for the inadequate information correlating concentration with long-term administration of MTX has been the lack of a sensitive assay method that could be adapted for routine clinical use. The fundamental principle for the assay of these drugs by enzymatic physicochemical methods is based on the stoichiometric inhibition of the enzyme dihydrofolate reductase (EC 1.5.1.3) (23). Whether the enzyme activity is measured by monitoring the oxidation of NADPH (3) or by radioenzymatic analysis (22), the assay for the antagonists has depended on 2 reactions: (a) the binding of the inhibitor to the enzyme; and (b) the reaction of the residual enzyme with the assay substrate (folic acid or dihydrofolic acid). Consequently, conditions that perturb either the inhibitor-enzyme reaction or the enzyme-substrate reaction may affect the results of the assay procedure. This report describes a radiometric assay for MTX that circumvents this disadvantage by directly measuring the binding of [3H]MTX, eliminating the need to measure residual enzyme activity with a 2nd reaction. The absolute sensitivity of this radioassay is limited only by the specific activity of the [3H]MTX and can approximate 10 pg of the drug.

MATERIALS AND METHODS

[3H]MTX with specific activity greater than 5 Ci/m mole was purchased from Amersham/Searle Corp., Arlington Heights, Ill. The purity of this product was greater than 95%, as was determined by descending paper chromatography.
The reaction is stopped after the 2nd-phase incubation by the addition of 0.4 ml of a suspension of 1% neutral Norit A charcoal (Fisher Scientific Co., Springfield, N. J.) in 0.5% dextran of an average molecular weight of 10,000. After it is gently shaken for a few min, the charcoal is precipitated by centrifugation and the [3H]MTX remaining in the supernatant, which has bound to dihydrofolate reductase, is determined with a liquid scintillation counter. Usually, a 0.3-ml aliquot of the supernatant solution is added to 15 ml of a scintillation solution containing 5 g of PPO and 75 ml of BBS-3 solubilizer (Beckman Instruments, Inc., Palo Alto, Calif.) per liter of toluene, and sufficient counts are accumulated for a counting error of 3% or less.

A reaction blank containing no binding lysate is simultaneously determined and the radioactivity not removed by the charcoal is subtracted from the reactions containing the binding lysate. This blank is usually 5% or less of the total radioactivity used. The FB is calculated as the ratio of net cpm remaining in the supernatant to the total cpm used in the reaction. For convenience, the same volume of [3H]MTX as was used in the assay reactions is diluted in a total volume of 0.9 ml, and the radioactivity in 0.3 ml of this mixture represents the total radioactivity standard.

Standard Curve. A dose-response curve representing the saturation of available binding sites by unlabeled MTX is obtained by preincubating standard concentrations of MTX with the diluted binding lysate (Phase 1). The addition of [3H]MTX to the reaction mixture for Phase 2 then determines the remaining unoccupied binding sites on the enzyme. A standard curve is obtained by plotting 1/FB as a function of the pg of unlabeled MTX.

Assay of MTX in Biological Fluids or Cell Extracts. The MTX concentration in serum, spinal fluid, or urine can be assayed directly in an aliquot of the sample without deproteination. If MTX is to be measured in aliquots of undiluted serum greater than 0.05 ml, a similar volume of normal serum should be added to the standard MTX reactions. Alternatively, the serum can be diluted 3-fold with the phosphate buffer and boiled for 5 min, and the MTX can be assayed in 0.2 to 0.3 ml of the supernatant after centrifugation of the precipitate. This procedure is appropriate when the concentrations of MTX in the serum are expected to be very low (i.e., less than 0.5 ng/ml), and it avoids the necessity of adding normal serum to the standard reaction mixtures.

MTX can also be assayed in cells or tissues. For these assays, the cell lysates or homogenized tissue should be boiled with the buffer in order to release bound drug and to denature any cellular dihydrofolate reductase that would interfere in the assay by binding the [3H]MTX.

An aliquot of the samples to be assayed is incubated with the binding lysate for the 1st-phase reaction and is followed by the 2nd-phase incubation with [3H]MTX as described for the standard curve. The MTX concentration is then obtained by referring the experimental 1/FB to the standard curve determined simultaneously.
RESULTS

The sensitivity of the dose-response curve is inversely related to the concentration of both the [3H]MTX and the binding lysate. Examples of 2 typical standard curves are shown in Chart 1. When the concentration of [3H]MTX was 130 pg and the concentration of the lysate was adjusted to bind about 50% of the tracer, a dose-response curve with an absolute sensitivity of approximately 10 pg of unlabeled MTX was obtained (Chart 1A). This level of sensitivity could be used to assay the low concentrations of MTX in tissue or cell extracts, or in biological fluids up to 7 days following administration of the drug. Where greater concentrations of the drug are expected in the samples to be assayed, for example, in clearance studies, the less sensitive dose-response curve may be satisfactory. The standard curve in Chart 1B was obtained using 330 pg of [3H]MTX. In this instance, the absolute sensitivity approximates 25 pg of MTX.

Although it was anticipated that greater precision would be obtained with the less sensitive assay, the experimental plot for both assays resulted in a dose-response curve approaching the plot obtained by the least-squares analyses (r = 0.98 and 0.97). Thus, just a few experimental points could be used to derive the linear standard curve using the least-squares method for determining the equation of the line. We have also observed that linearity of the standard curve ceases when the binding of the [3H]MTX decreases below 12% (1/FB > 8).

The conditions necessary to stabilize the binding properties of the lysate and to obtain the maximum sensitivity of the dose-response curve were investigated. The data in Chart 2 show the effects of NADPH and 2-mercaptoethanol. To appreciate better the comparative effects of these additions to the dose-response curve, the ratio of percentage of [3H]MTX bound to percentage of [3H]MTX free was plotted as a function of the unlabeled MTX. This is a standard method of expressing such data in ligand-binding radioassays. Not only was maximum binding of the [3H]MTX observed when the reaction contained both NADPH and 2-mercaptoethanol, but NADPH also increased the slope of the dose-response effect of the unlabeled MTX. It can be inferred from these observations that 2-mercaptoethanol increases slightly the stability of the enzyme and that NADPH also increases the affinity between the drug and lysate-binding determinant(s).

Other buffer ions such as citrate or acetate were tested and in the range of pH 6.0 appeared to result in somewhat greater variability of binding of the [3H]MTX. The most consistent results were obtained with potassium phosphate at pH 6.0, and variations less than ±0.2 unit did not appear to alter the dose-response curve. At pH greater than 7.0, there is lower affinity for the binding of MTX and the dose-response curve is consequently less sensitive.

The effect of physiological folate analogs on the binding of [3H]MTX by the lysate was also studied since these compounds will be present in the biological fluids and tissue extracts to be assayed for this drug. The results are shown in Chart 3. In the concentration range from 2.2 to 17.6 pmol/reaction mixture neither folic acid, nor 5-formyltetrahydrofolate, nor 5-methyltetrahydrofolate, nor dihy-
drofolate had any effect on the binding of the \[^{3}H\]MTX. In the sample aliquot of biological material to be assayed for MTX, it is not likely that the concentration of physiological folate will exceed this range (up to 17.6 pmoles/ml).

The recovery of MTX added to serum is shown in Table 1. For this study the concentrations of MTX for the standard curve were prepared in normal serum so that the concentration of protein in all reaction mixtures (for standard and assay reactions) was essentially similar. The range of recovery was 70 to 110% with a mean of 91.8%. The greatest errors were at the lowest concentration of MTX. Thus, values of MTX between 200 and 400 pg/ml should be considered as those measured with the greatest coefficient of variation.

Table 2 summarizes the MTX concentration in serum, spinal fluid, and urine of patients treated with this drug. The time each sample was taken following drug administration is shown. It is evident that even 7 days following the administration of MTX i.v. or i.t. the drug could still be assayed in the serum and spinal fluid, respectively. The excretion of 74% of an i.t. dose of MTX in the 1st 24 hr following administration is consistent with the known rapid transit of this drug from brain to blood.

Chart 4 shows the progressive increase in concentration of MTX in L1210 leukemic cells following the s.c. administration of the drug at a dose of 3 mg/kg to BD2F1 mice with the ascitic form of this tumor. The decrease in the activity of folate reductase in the cells measured by radioenzymatic assay (22) occurs simultaneously with the rise in concentration of drug.

An important quality control procedure with radiometric ligand-binding radioassays is to assay 2 dilutions of a sample. If there is no stoichiometric decrease in the concentration of substrate assayed, there are interfering factors in the assay procedure. Table 3 shows samples of serum, spinal fluid, urine, and leukemic cell lysates assayed at 2 dilutions. The mean concentration of the samples assayed after a 2-fold dilution was 46% of the concentration measured in the undiluted sample.

Chart 5 compares the sensitivity of this radioassay for MTX with the spectrophotometric method described by Bertino and Fischer (3), which monitors the decrease in absorbance at 340 nm as NADPH is oxidized to NADP as a consequence of the reduction of dihydrofolate to tetrahydrofolate. Dihydrofolate reductase was prepared for this assay from guinea pig liver (3). The enzyme concentration was adjusted to give the recommended maximum \(\Delta A\) of 0.15 in 5 min. With this assay, inhibition was not evident.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>MTX added (pg/0.1 ml)</th>
<th>MTX recovered (pg/0.1 ml)</th>
<th>% recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>320</td>
<td>308</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>160</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>88</td>
<td>110</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>44</td>
<td>110</td>
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<tr>
<td>5*</td>
<td>320</td>
<td>290</td>
<td>90</td>
</tr>
<tr>
<td>6*</td>
<td>240</td>
<td>200</td>
<td>83</td>
</tr>
<tr>
<td>7*</td>
<td>160</td>
<td>138</td>
<td>86</td>
</tr>
<tr>
<td>8*</td>
<td>80</td>
<td>82</td>
<td>103</td>
</tr>
<tr>
<td>9*</td>
<td>40</td>
<td>28</td>
<td>70</td>
</tr>
<tr>
<td>10*</td>
<td>20</td>
<td>14</td>
<td>70</td>
</tr>
</tbody>
</table>

* Mean, 91.8%.

* MTX was added to a pool of normal serum.

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>MTX concentration (pg/ml)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (M. L.)</td>
<td>10.6 µg/ml</td>
<td>5 min after 25 mg i.v.</td>
</tr>
<tr>
<td>Serum (M. L.)</td>
<td>410 pg/ml</td>
<td>7 days after 25 mg i.v.</td>
</tr>
<tr>
<td>Serum (A. C.)</td>
<td>1.3 mg/ml</td>
<td>7 days after 25 mg i.v.</td>
</tr>
<tr>
<td>Serum (A. C.)</td>
<td>2.4 mg/ml</td>
<td>7 days after 25 mg i.v.</td>
</tr>
<tr>
<td>Spinal fluid</td>
<td>28.5 mg/ml</td>
<td>6 days after 15 mg i.t.</td>
</tr>
<tr>
<td>Spinal fluid</td>
<td>19.0 mg/ml</td>
<td>3 days after 15 mg i.t.</td>
</tr>
<tr>
<td>Urine (J. M.)</td>
<td>18.7 mg/24 hr</td>
<td>24-hr excretion follow- ing 25 mg MTX i.t.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>A Concentration/ aliquot assayed (pg/ml)</th>
<th>B Concentration in 1:2 dilution of aliquot (pg/ml)</th>
<th>B/A*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal fluid</td>
<td>680</td>
<td>330</td>
<td>0.48</td>
</tr>
<tr>
<td>Spinal fluid</td>
<td>285</td>
<td>160</td>
<td>0.56</td>
</tr>
<tr>
<td>L1210 lysate</td>
<td>345</td>
<td>150</td>
<td>0.43</td>
</tr>
<tr>
<td>L1210 lysate</td>
<td>360</td>
<td>185</td>
<td>0.51</td>
</tr>
<tr>
<td>Serum</td>
<td>100</td>
<td>40</td>
<td>0.40</td>
</tr>
<tr>
<td>Serum</td>
<td>270</td>
<td>130</td>
<td>0.48</td>
</tr>
<tr>
<td>Urine</td>
<td>126</td>
<td>50</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* Mean, 46%.
On the other hand, the spectrophotometric assay would be difficult to measure contrast, initial inhibition of the binding of \([3H]\)MTX by the spectrophotometric method (x) for measuring MTX. By the spectrophotometric method, it would be evident at approximately 15 pg of unlabeled MTX in the reaction mixture.

In contrast, initial inhibition of the binding of \([3H]\)MTX by the L1210 enzyme preparation was evident at approximately 15 pg of unlabeled MTX.

With this dose-response sensitivity observed for the spectrophotometric assay, it would be difficult to measure MTX concentrations in the range of 1 to 5 ng/ml and virtually impossible to measure levels in the pg/ml range. On the other hand, the spectrophotometric assay would be sufficiently sensitive to measure the concentrations in blood, urine, and spinal fluid that occur within a few hr following the administration of MTX.

**DISCUSSION**

There is a dearth of information relating the concentration of MTX to therapeutic and toxic effects over prolonged periods of treatment because of a lack of an assay procedure both sensitive and easily adapted by clinical services. Consequently, the dosage schedules for the folate antagonists for most therapeutic protocols have, for the most part, been derived from empiric observation over short-term treatment periods. The importance of knowing the concentration of the drug, however, has recently been demonstrated by Bleyer et al. (5), who showed that toxicity to the central nervous system following the administration of MTX could be correlated with high spinal fluid concentrations of the drug.

Unlike previous methods for assaying MTX, which depend on monitoring the residual activity of the target enzyme, dihydrofolate reductase, the method described in this report measures directly the effect of unlabeled MTX on the binding of \([3H]\)MTX. It is not truly a competitive inhibition assay system because the binding lysate is not added to a mixture of labeled and unlabeled MTX. Rather, this sequential system has been considered a noncompetitive.

radioassay and has the advantage of greater sensitivity, particularly when there is little reversibility of the binding reaction. In addition to the sensitivity of this radioassay, which could be adjusted to meet specific needs, the procedure can measure MTX in biological fluids without the necessity of extracting the drug or deproteinating the sample. Where the MTX is bound, as in cells, then extraction is necessary to free the drug and denature any binding sites (e.g., dihydrofolate reductase).

It has generally been considered that the sole binding site for intracellular MTX is dihydrofolate reductase. With this consideration, and with the demonstrated stoichiometric inactivation of enzyme activity by MTX (2), we anticipated that a standard curve would not be necessary for this radioassay. Under such circumstances, the \([3H]\)MTX displaced from its binding determinant by a sample of material containing this drug should be sufficient to measure the concentrations in the pg/ml range.

The linearity of the standard curve obtained by plotting the 1/FB as a function of the unlabeled MTX is apparent from these studies that this cofactor also enhances binding of nonsubstrate folate analogs such as MTX. This enhanced binding is not simply a stabilization of the enzyme protein because the sharper slope of the dose-response curve obtained with unlabeled MTX increases sharply. NADPH had a marked effect of increasing the binding of \([3H]\)MTX by the lysate, but 2-mercaptoethanol had a lesser effect, and the combination of both agents appeared to be synergistic. It is known that NADPH binds to and stabilizes the catalytic function of dihydrofolate reductase (17, 19). It is apparent from these studies that this cofactor also enhances binding of nonsubstrate folate reductase even with \([3H]\)MTX as the tracer. It is necessary only to substitute standard concentrations of the inhibitor for the MTX standards. What will vary with each inhibitor is the slope of the dose-response standard curve since this is a function of the relative affinity of the inhibitor and \([3H]\)MTX for the enzyme. Inhibitors weaker than MTX will
yield curves with lesser slopes than equimolar concentrations of MTX.

Although we have not specifically studied the effect of metabolites of MTX on the assay system, it can generally be concluded that if the metabolite binds to the enzyme it will be included in the assay. This is not likely to be a significant factor since such metabolites will usually have a lower affinity than the parent MTX compound for the binding site on the enzyme, and the measured effect would be low. On the other hand, from a pharmacological consideration, if such metabolites are measurable, then it is best to have them included in the assay since they would be effective inhibitors of the catalytic activity of dihydrofolate reductase.

Most pharmacological studies to correlate clinical toxicity and dosage have been designed as acute experiments. A recent report of such a study by Goldie et al. (14), confirmed the clinical observation that acute toxicity (bone marrow, mucous membranes) was related to the duration of administration of an i.v. infusion and not to the dose. On the other hand, there is good experimental evidence by Chabner and Young (6) that shows that the suppression of DNA synthesis in L1210 leukemia and normal mouse tissues is a direct function of the plasma concentration of MTX. Irrespective of the dose administered, de novo thymidylate synthesis on the enzyme, and the measured effect would be low. On the other hand, from a pharmacological consideration, if such metabolites are measurable, then it is best to have them included in the assay since they would be effective inhibitors of the catalytic activity of dihydrofolate reductase.

Finally, closer observation of blood concentration and tissue accumulation of MTX may be of value in minimizing the toxicity of MTX, particularly when the drug is administered over long treatment periods. Nonacute MTX toxicity has been recognized in the brain (20), liver (16), kidney (8), lung (11), and bone (21). Although individual characteristics such as age, extent of malignant disease, and renal function can alter the metabolism of MTX, much may be learned by correlating these toxicities with the determination of blood and, when possible, tissue concentration of this drug.

REFERENCES

A Direct Ligand-binding Radioassay for the Measurement of Methotrexate in Tissues and Biological Fluids

Elliot Arons, Sheldon P. Rothenberg, Maria da Costa, et al.