A Radioimmunoassay for Methotrexate and Its Comparison with Spectrofluorimetric Procedures

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SUMMARY

A radioimmunoassay procedure has been developed for the direct measurement of methotrexate (MTX) in plasma, serum, cerebrospinal fluid, or urine samples. The assay is sensitive to levels of at least 100 pg of MTX and is highly specific for MTX in the presence of folic acid (citrovorum factor), folinic acid, tetrahydrofolic acid, and other folate analogs and known metabolites. Results from this procedure have been compared with those obtained with a spectrofluorimetric method, utilizing the plasma of cancer patients undergoing high-dose MTX treatment with citrovorum factor rescue. Results indicate that the method should be useful in the future in assisting individualization of dosage regimens and in the study of the pharmacokinetics and metabolism of MTX in cancer patients.

INTRODUCTION

The use of high-dose MTX treatment with subsequent "rescue" with CF has been of considerable recent interest (6, 8). Variability of individual patients in their response to such treatment, as well as the variable toxicity involved, is reflected in the large number of differing regimens that have been reported (5, 9, 11, 14, 17, 18, 23, 26, 31). The establishment of a pharmacokinetic model (16, 30) could lead to individualized dosage regimens and an understanding of individual variations in metabolism and toxicity (14). The development of such a model and its clinical application are completely dependent upon a simple and clinically convenient assay giving reliable quantitation of MTX in the presence of CF, folic acid, tetrahydrofolic acid, and possible MTX metabolites. Freedom from interference by such agents in currently used MTX assays, particularly at low levels of MTX, has not been clearly established in many clinical studies reported. Until very recently, MTX levels in serum, plasma, and urine have been estimated in many instances by methods that do not differentiate the administered drug from possible metabolites or other decomposition products, at least without prior separation procedures (1, 15, 16, 19). For example, spectrofluorimetric methods may be subject to errors resulting from the presence of interfering fluorescent materials in plasma, such as folic, tetrahydrofolic, and folinic acids. Fluorimetric methods (7, 10, 13, 21) are also of insufficient sensitivity for quantitation of MTX at the low levels needed for an accurate pharmacokinetic analysis. Enzymatic assay procedures (3, 32), while more sensitive than fluorimetric methods, appear to be more cumbersome than desirable for routine clinical use (6) and may not distinguish MTX from CF and possible metabolites.

Bohoun et al. (4) and Levine and Powers (22) have reported radioimmunoassays for MTX. Subsequently, Raso et al. have reported immunoassays for both MTX (28) and CF (27). Very recently, Myers et al. (24) and Arons et al. (2) have published a competitive protein binding assay based upon commercially available bacterial dihydrofolate reductase and a direct ligand-binding radioassay involving dihydrofolate reductase from L1210 leukemia cells, respectively.

In this report, we wish to describe a fast and reliable radioimmunoassay method for MTX, applicable to the specific determination of MTX to levels of less than 100 pg in plasma, serum, and urine. The method is compared at μg levels with a spectrofluorimetric procedure (7), using the plasma of patients with neoplastic disease undergoing MTX treatment with CF rescue. The time course of disappearance of MTX from the plasma has been followed by immunoassay to levels as low as 2 ng/ml. This method appears to have certain advantages over previously reported immunoassay procedures.

MATERIALS AND METHODS

Materials. The following were used: human serum albumin (crystalline, B grade; Calbiochem, La Jolla, Calif.); poly-L-lysine (average molecular weight, 35,000; Sigma Chemical Co., St. Louis, Mo.); a standard solution containing the sodium salt of 4-aminonitropteroylglutamic acid salt (MTX) in a quantity equivalent to 25 mg free acid per ml (Lederle Labs., Pearl River, N. Y.); a coupling reagent N-ethyl-N'-[3-dimethylaminopropyl]carbodiimide hydrochloride (Aldrich Chemical Co., Inc., Milwaukee, Wis.); Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.); [3',5',9(n)-H]methotrexate, sodium salt (specific activity, 9.5 Ci/m mole), 95 to 97% pure by thin-layer chromatography (Amersham/Searle Corp., Arlington Heights, Ill.); dextran (radioimmunoassay grade; Schwarz/Mann, Orangeburg, N. Y.); charcoal (radioimmunoassay grade) and...
bovine serum albumin (powder; Armour Pharm. Co., Phoenix, Ariz.); octoxynol (Triton X-100), PPO, and 2,2-phenylenebis(5-phenoxazoyle) (Fisher Scientific Co.; Pittsburgh, Pa.); a solution of the calcium salt of 5-formyltetrahydrofolic acid (CF) in a quantity equivalent to 3 mg free acid per ml (leucovorin); solutions of the sodium salt of folic acid and tetrahydrofolic acid in a quantity equivalent to 5 mg free acid per ml (Lederle).

The following materials were kindly supplied by Dr. David G. Johns and Dr. Samuel A. Jacobs of the National Cancer Institute, Bethesda, Md.: 7-hydroxymethotrexate, 4-aminoopteroic acid, methotrexate dimethyl ester, and 4-amino-4-deoxy-N\(^{15}\)-methylpteroylglutamate.

**Coupling of MTX to Human Serum Albumin and Pollysine.** The carbodiimide conjugation procedure described below is a modification of that first reported by Goodfriend et al. (12) and Ricker and Stollor (29) and was utilized subsequently by others (4, 22, 28). Modifications in pH and reaction conditions enabled a high degree of conjugation and an accurate assessment of this was made by both UV spectral and radiotracer techniques.

Two ml of MTX sodium (parenteral formulation, containing the equivalent of 50 mg MTX free acid) were added to 25 ml of phosphate buffer (0.15 M NaCl-0.01 M sodium phosphate; pH 7.4), and then to a solution of 2.4 mg (50 \(\mu\)Ci) of \([3',5',9(n)-3^H]\)MTX in 0.5 ml water. The stirred solution was then adjusted to pH 5.5 with 0.1 n HCl. To this was added a solution of 38 mg of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride in 1.0 ml distilled water and 100 mg human serum albumin in 2.0 ml distilled water. After readjustment of the pH to 5.5, the mixture was stirred for 24 hr at 5°. The clear yellow solution was then transferred to a dialysis bag and dialyzed against 0.01 N HCI for 7 days at 5°, then finally against distilled water, with change of the dialysis medium each day. For calculation of the amount of MTX bound to the albumin, the absorbance (at 308 nm) of aliquots of the dialysates was compared with the absorbance of a standard MTX solution. In addition, the same information was derived by scintillation counting on both aliquots of dialysates and aliquots of the contents of the dialysis bag. Results indicated 45 to 49 moles MTX bound per mole of human serum albumin (molecular weight, 70,000).

For coupling of MTX to pollysine, the same procedure was used, except that 100 mg pollysine (average molecular weight, 85,000) was used in place of the albumin. The conjugate precipitated soon after the beginning of the reaction period and henceforth appeared to be largely insoluble in the aqueous medium. Spectral and tracer studies indicated 81 to 85 moles MTX bound per mole of pollysine.

For both albumin and pollysine conjugates, thin-layer radioscans on silica gel, with methanol as eluant, indicated no trace of noncovalently bonded material within the conjugate. Conjugates were lyophilized and stored in a desiccator at –10°.

**Immunization Procedure and Schedule.** For the production of antibodies, 2 rabbits (2- to 3-kg female New Zealand White) were given injections of a human serum albumin conjugate of MTX, 1 with a pollysine conjugate. For each animal, the finely powdered lyophilized conjugate (0.5 mg) was emulsified in 3 ml of a mixture of 0.9% NaCl solution and Freund’s complete adjuvant. Albumin conjugates were water-soluble, pollysine conjugates were suspended. The emulsion was injected intradermally at approximately 50 sites on the shaved backs of the rabbits. The 1st booster injection (0.1 mg antigen in 2 ml 0.9% NaCl solution) was administered 2 weeks later (injected s.c. at 20 sites). Subsequent boosters (0.1 mg) were given s.c. at 1-month time intervals following the initial injection. Blood samples were drawn from the marginal ear vein 10 days subsequent to each booster injection. Serum was frozen after the addition of thiomersal (final concentration, 0.01%).

**Titer Determination.** Individual serum samples were evaluated by serial dilution for their ability to bind 0.25 ng \([^3H]MTX\) in the absence of added nonradioactive MTX. For this evaluation, 100-\(\mu\)l samples of antisera dilutions were used with the detailed experimental procedures given immediately below. In routine work, antisera giving 40 to 60% binding was chosen as optimum.

**Radioimmunoassay Procedure: Determination of Standard Radioimmunoassay Curve for MTX and Assay of Unknowns.** All samples each day were run in triplicate to minimize error. In a typical experiment, 0.6 ml buffer (0.15 M NaCl and 0.01 M sodium phosphate, pH 7.4) was added to each of sixty 5-ml polystyrene test tubes (12 x 75 mm); that is, 20 sets of triplicates were run. To each tube were added 0.10 ml 5% bovine serum albumin in the phosphate buffer and then 0.10 ml buffer (or buffer containing standard, or test material of plasma, serum, or urine). To obtain a standard calibration curve, 0- to 1-ng quantities of cold MTX in a maximum of 100 \(\mu\)l of buffer, normal plasma, serum, or urine were added to selected tubes in place of the unknown plasma, serum, or urine. To desired tubes was then added 100 \(\mu\)l antiserum, normally freshly diluted 1:1000 with buffer each day (final dilution, 1:10000). Finally, 0.10 ml of buffer containing 0.25 ng of \([^3H]MTX\) was added to each tube, making the total volume 1.0 ml. At a specific activity of 9.5 Ci/mmole, each tube therefore contained about 11,000 dpm or about 3,000 cpm at the normal observed counting efficiency of 25 to 30%.

After addition of the \([^3H]MTX\), the tubes were stirred vigorously (Vortex) for 5 sec and then shaken gently at 25° for 2 hr or left overnight at 5°. After chilling to 0° for 15 min in ice, 0.5 ml of well-mixed dextran-coated charcoal suspension (0°) (The charcoal suspension was prepared immediately prior to use by the dilution of 2 ml of a stock suspension with 18 ml buffer; the stock suspension containing 2.5 g charcoal and 62.5 mg dextran in 25 ml buffer.) was added to each tube, and the tube contents were mixed thoroughly once again. The tubes were left for 15 min at 0° and then centrifuged at 2° for 10 min (5000 rpm). Supernatant (1.0 ml) (two-thirds of the total volume) was then carefully removed with an automatic pipet and mixed with 10 ml of scintillation counting solution [toluene (2 liters), octoxynol (1 liter), PPO (12 g), and 2,2-phenylenebis(5-phenoxazoyle) (300 mg)]. The samples were then counted for 10 min with a Packard Model 3320 scintillation spectrometer.

The channels ratio method was used to monitor counting efficiency, to compute dpm from cpm, and to determine the final percentage of binding figures. Nonspecific binding,
RESULTS AND DISCUSSION

Evaluation of Assay Methodology. As outlined in "Materials and Methods," MTX was coupled to 2 different carriers, human serum albumin and poly-L-lysine. Carboxyl groups of the glutamic acid moiety of MTX were activated at pH 5.5, using a water-soluble carbodiimide reagent. Coupling occurs then on the poly-L-lysine amino groups or primarily the side-chain lysine amino groups of albumin. Tritiated MTX was added in order to facilitate calculation of the ratio of MTX to albumin in the conjugate. Both dialysates and conjugate were counted. Independent measurements of UV absorption of dialysates and conjugates at 308 nm were also made. These results indicate about 45 to 49 moles of MTX bound per mole of albumin (the lysine content of albumin is 58 moles/mole) and 81 to 85 moles of MTX per mole of poly-L-lysine.

This high ratio of MTX to carrier may be quite important with regard to obtaining a more consistent antigenicity of the conjugate and nature of the antibodies developed. To date, only 1 (4) of several papers (4, 22, 28) on MTX clearly defines the extent of conjugation (18 to 23 moles/mole of bovine serum albumin). Immunization of Rabbit A (human serum albumin conjugate) produced a titer of 1:1000 in 6 weeks, rising to 1:20,000 in 3 months, then leveling off at this value and remaining constant for the 10-month observation period. Rabbit B, receiving the albumin conjugate, responded to a maximum titer of about 1:1000 in a 3-month period. Rabbit C, receiving the polylysine conjugate, exhibited a titer of only 1:100 even after 8 months of booster injections. The binding capacity of undiluted antisera was retained completely for the 10-month period tested, although highly diluted antisera upon storage have shown a partial loss in binding ability for MTX.

Results and Discussion

Clinical Protocol. Patients with advanced malignant disease were treated with MTX-CF rescue therapy according to the technique described by Djemassi et al. (9). This particular regimen of escalating doses is most suitable for pharmacokinetic study and has not shown undue toxicity in our hands (25). Chart 2 shows typical MTX plasma levels after a 1000-mg infusion over 6 hr and 9 mg of CF given i.m. every 6 hr for 8 doses, beginning 6 hr after the completion of the infusion (t = 12 hr).
always incorporated into each day’s work. No significant differences were observed with the following variations in the procedure described in "Materials and Methods," i.e., change in the incubation time between 1 and 7 hr at 25 or at 5° overnight (18 hr) or variation in time of incubation with dextran-coated charcoal between 15 and 30 min at 0°. Longer incubation with charcoal (i.e., 1 hr) resulted in slight but significantly increased apparent binding figures (“stripping”). Nonspecific binding in the absence of antiserum amounted to about 3% in all cases, and this amount was subtracted from samples containing antiserum. However, it was found that highly diluted solutions of radiolabeled MTX (such as that used in the assay) must be made up fresh and utilized each day; otherwise figures for nonspecific binding increased over 2 or 3 days to as much as 7 to 8%, even when solutions were stored at 5°. The probable explanation for these observations arises from the fact that the commercially tritiated MTX contains about 29% of the label in the 9-methylene group, which might be subject to exchange to the water of the aqueous buffer. This amount of tritium would then remain in the water of the supernatant solutions and appear as nonspecific binding.

In the assay, the addition of up to 10% (0.1 ml/ml) normal rabbit serum, pooled normal human serum, normal human plasma, human cerebrospinal fluid, or human urine to the assay tubes did not appear to affect the shape or position of the immunoassay curves nor did heparin, EDTA, or citrate in normal concentrations utilized for anticoagulation.

Under the conditions routinely used (Chart 1), the sensitivity of the assay for MTX was about 100 pg; however, this could be increased readily to about one-half this quantity or less by the use of less than 0.25 ng of [3H]MTX, which is of sufficient specific activity (9 Ci/m mole) to give adequate counts. An additional further dilution of antiserum also increased sensitivity.

Studies on specificity or cross-reactivity were carried out with 8 folate analogs or known metabolites of MTX, using antiserum from 2 different rabbits. For these compounds, the concentration required to give an arbitrary 50% binding of 0.25 ng/ml was determined. Results are summarized in Table 1.

The results shown would indicate that, at least with these 2 rabbits and with 3-month bleedings, specificity of the antisera toward the compounds listed was quite similar. As might be expected, the greatest cross-reactivity was found with compounds in which structural differences were nearest to the point of conjugation in the antigen preparation (i.e., dimethyl-MTX and 4-amino-4-deoxy-N10-methylpteroylglutamyl-γ-glutamate). In the case of CF or 5-methyltetrahydrofolic acid, it seems highly unlikely that cross-reactive concentration ranges would be reached clinically, particularly at the dosage levels of CF utilized in the present studies and other reported protocols.

Other common drugs which might be present in plasma samples were not investigated, but interference seems unlikely because of the high structural specificity indicated in Table 1.

Table 1 indicates reproducibility of the assay as measured in a series of prepared standards, giving means and correlation coefficients for amounts found versus the amounts actually added. These results were derived by using the straight line portion of a standard curve such as that shown in Chart 1.

Plasma samples from several patients were assayed using the immunoassay procedure, diluting samples to achieve a concentration range of 100 to 750 pg, and results were compared with those obtained in a spectrofluorimetric

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rabbit A*</th>
<th>Rabbit B*</th>
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<tbody>
<tr>
<td>MTX</td>
<td>0.00025 (1)*</td>
<td>0.00025 (1)</td>
</tr>
<tr>
<td>Folic acid</td>
<td>4.4 (17,600)</td>
<td>4.0 (16,000)</td>
</tr>
<tr>
<td>Tetrahydrofolic acid</td>
<td>6.25 (25,000)</td>
<td>5.16 (20,600)</td>
</tr>
<tr>
<td>CF</td>
<td>8.0 (32,000)</td>
<td>7.6 (30,400)</td>
</tr>
<tr>
<td>5-Methyltetrahydrofolic acid</td>
<td>7.0 (28,000)</td>
<td>5.48 (21,900)</td>
</tr>
<tr>
<td>4-Amino-4-deoxy-N10-methylpteroylglutamyl-γ-glutamate</td>
<td>0.035 (140)</td>
<td>0.029 (116)</td>
</tr>
<tr>
<td>7-Hydroxymethotrexate</td>
<td>1.7 (6,800)</td>
<td>1.3 (5,200)</td>
</tr>
<tr>
<td>Dimethylmethotrexate</td>
<td>0.00132 (5.28)</td>
<td>0.00235 (9.4)</td>
</tr>
</tbody>
</table>

* For Rabbits A and B, antiserum utilized was obtained 3 months after initial immunization. Antisera A was utilized at a dilution of 1:10,000; antiserum B was utilized at a dilution of 1:1000.

* Numbers in parentheses, relative concentration (MTX = 1).
method developed by Freeman (10) as modified by Chakrabarti and Bernstein (7). Results from 1 patient (J. K.) are shown in Chart 2. In each immunoassay of a plasma sample, a blank value for nonspecific binding in the absence of antiserum was determined and was subtracted from the total binding figure. However, it was found that in no case did this value exceed 2% above the nonspecific binding in the absence of test plasma and antiserum. Data in Chart 2 were derived using the same standard reference solution of MTX in both assay procedures.

In addition to absolute values of MTX present in patient samples, recovery studies of MTX from these same samples were conducted. The results are summarized in Table 3 and indicate that the material added to each original plasma sample, over a wide range of concentrations, is recovered in quantities ranging between about 90 and 110%.

Advantages of the Immunoassay Technique Described: Significance of Clinical Data. From observations of Patient J. K., plus similar data from other individuals, the following preliminary conclusions can be drawn. The immunoassay procedure gives information regarding levels of MTX present at times far later after administration than those that can be followed by the fluorometric procedure. Plasma levels as low as 2 ng/ml have been measured, while the fluorescence technique is unreliable below about 1 μg/ml. In addition to lack of sensitivity, the fluorescence technique is probably subject to error (overestimation of MTX) because of the presence of CF (after t = 6 hr; see Chart 2).

After infusion of MTX is stopped (t = 6 hr) and before CF is administered (t = 12 hr), observed MTX values in the immunoassay have been consistently lower than those observed in the spectrofluorometric procedure. This observation would be consistent with the possible presence in this patient of a metabolite not cross-reactive to the antiserum utilized, but undistinguishable from MTX in the fluorimetric assay.

The further utilization of these techniques may enable (a) pharmacokinetic evaluation of data and resultant progress toward individualizing patient therapy and (b) detailed study of the metabolism of MTX in humans and its variation in a group of patients.

The assay described represents a direct method for the determination of MTX specifically, without interference from closely related folate derivatives, particularly CF, in plasma, serum, cerebrospinal fluid, and urine and perhaps will be useful in the measurement of tissue levels. Extractions or chromatographic purification procedures are avoided, and the determination is accurate down to the 100 pg level with an error of about ±10%. Relative to some other reported immunoassay methods, the procedure appears to offer, along with the recently described competitive protein binding methods (2, 24), the advantages of a shorter incubation period and the convenience of a charcoal absorption technique, rather than a double antibody technique (22), and freedom from interference from plasma proteins, thus avoiding extraction procedures (28). The method should be particularly attractive because of its specificity for MTX and perhaps will be able to distinguish this from metabolites, at least those involving metabolic alteration at sites remote from the position of coupling in the antigen preparation (glutamic acid carboxyl groups). This method also appears to be considerably more convenient than enzymatic assay methods (32), less subject to interference, and far more sensitive than both the enzymatic and the fluorimetric procedures (7, 10, 13, 21, 32). Validation of the accuracy of this immunoassay method has been achieved through comparison with the fluorimetric procedure, which is reliable at concentrations above about 1 μg/ml, at least in the absence of CF.

Comparison of the antibody specificity with specificity shown by preparations of folate reductase used in competitive protein binding methods (2, 24) should be of interest in future work on metabolite detection and identification.

At higher plasma levels, our immunoassay suffers the disadvantage of error and inconvenience inherent in multiple high dilutions necessary to obtain concentrations falling within the range of the standard curve and exploratory dilution until the optimum dilutions are achieved. However, the increased accuracy obtained, with a sensitive immunoassay procedure operating over a fairly narrow concentration range, is of special interest in the pharmacokinetic characterization of MTX and subsequent placement of dosage regimens on a rational basis for each individual. This immunoassay methodology may also enable extended tis-
sue measurements of importance to mechanism of action, metabolite, and toxicity studies. Such studies are in progress.

ACKNOWLEDGMENTS

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