**A Rapid and Specific Radioimmunoassay for Methotrexate**

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**SUMMARY**

A sensitive and precise radioimmunoassay for methotrexate has been developed using antibody induced in rabbits, tritium-labeled methotrexate, and a nitrocellulose membrane separation technique. Antibody specificity was characterized by comparing the effectiveness of various related compounds to displace labeled methotrexate from the antibody-binding site. Assay of serum samples from persons receiving the drug was rapid and easy to perform. In a pharmacokinetic study of methotrexate, corresponding results were obtained when measurements were made by either enzymic assay or by radioimmunoassay. Drug concentrations could also be monitored in the cerebrospinal fluid and urine of patients on high-dose methotrexate therapy followed by citrovorum factor rescue. The system measured as little as 0.1 to 1 pmole of methotrexate, depending upon the antiserum used, and naturally occurring folates did not interfere with these determinations.

**INTRODUCTION**

A number of sensitive microbial (4), fluorometric (5, 9), and enzymatic (1, 12) assays have been devised to quantitate the 4-amino analogs of folic acid. Although these procedures are adequate for determinations in the research laboratory, each has certain limitations that have discouraged large-scale clinical use.

Previous workers have shown that antibodies specific for folic acid (8, 10) and methotrexate (8) can be induced in rabbits when these compounds are covalently coupled to protein carrier molecules. Using such antibodies, a radioimmunoassay has been developed for the determination of folic acid concentrations in serum (3).

The details of an assay permitting specific quantitation of the chemotherapeutic folate antagonist methotrexate are now presented.

**MATERIALS AND METHODS**

**Chemicals and Equipment.** Methotrexate was a gift from Drug Research and Development, National Cancer Institute. Methylated bovine serum albumin, folic acid, tetrahydrofolic acid, and 5-methyltetrahydrofolic acid were products of Sigma Chemical Co., St. Louis, Mo. Aminopterin was from Biochemical Laboratories, Inc., Gardena, Calif.; 1-cyclohexyl-3-(2-morpholinoethyl)carboxidiimide metho-p-toluenesulfonate was from Aldrich Chemical Co., Milwaukee, Wis.; folic acid was from Lederle Laboratories, Pearl River, N. Y.; p-aminobenzoylglutamate was from Frinton Laboratories, Vineland, N. J.; and complete Freund's adjuvant was from Difco, Detroit, Mich. Tritium-labeled methotrexate (13.7 Ci/m mole) was purchased from New England Nuclear, Boston, Mass., and nitrocellulose membrane filter discs were obtained from Schleicher and Scheull, Inc., Keene, N. H. Multiple filtration manifolds (Millipore Corp., Bedford, Mass.) were used for the assays. Dr. M. Chaykovsky and Dr. A. Rosowsky generously provided 7,8-dihydmethotrexate, 3'-5'-dichloromethotrexate, 2,4-diamino-6-methylpteridine, and p-[(2,4-diamino-6-pteridinyl)methyl]methylamino[benzoic acid.

**Preparation of the Antigens.** Methotrexate was covalently coupled to methylated bovine serum albumin following the method of Ricker and Stollar (10). The procedure uses a water-soluble carbodiimide reagent, 1-cyclohexyl-3-(2-morpholinoethyl)carboxdiimide metho-p-toluenesulfonate, to form an amide bond between the carboxyl groups of methotrexate and protein amino groups. A typical reaction mixture contained 50 mg of methylated serum albumin plus 20 mg of methotrexate in 2.5 ml of H2O. To ensure optimal coupling, the pH was adjusted to 4.7 (7) before 25 mg of carbodiimide reagent were added. The reaction was allowed to proceed at room temperature for 2 hr and then the conjugate was dialyzed in the cold against two 6-liter changes of 0.2 M NaHCO3 to remove unreacted materials. After extensive dialysis, a yellow and predominantly insoluble product was obtained.

**Immunization Procedure.** Antigen, emulsified in complete Freund's adjuvant, was administered to female New Zealand White rabbits as multiple intradermal and s.c. injections once a week for 3 consecutive weeks. The conjugate, suspended in 0.15 M NaCl, was given i.v. on the 4th week. Two rabbits received a total of 0.5 mg at each interval, while the remaining 2 were given 5-mg doses of the antigen. Sera were collected weekly, beginning 7 days after the end of the immunization schedule, and were stored at -20°. Even diluted serum samples retained full binding activity under these conditions.

**Radioimmunoassay Procedure.** The nitrocellulose membrane radioimmunoassay procedure was adapted from Gershman et al. (6). The standard assay mixture, containing 5000 to 7000 cpm of [3H]methotrexate and 1 µl of antiserum...
in a final volume of 0.4 ml of 0.14 mM NaCl:0.01 mM Tris, pH 7.4, was incubated at room temperature for 30 min. Separation of unbound from antibody-bound radiolabeled methotrexate was achieved by filtration on a nitrocellulose membrane disc followed by washing with 8 ml of buffer. The amount of radioactivity on the dried filter was determined by liquid scintillation counting, and the overall counting efficiency was about 40%. Typically, 2000 to 3000 cpm were retained on the disc in the presence of a specific antiserum, while a background of about 50 cpm was found when serum from unimmunized rabbits was tested.

Competitive analogs or physiological fluid samples containing unlabeled methotrexate were delivered to the standard binding mixture just prior to addition of antiserum. Any decrease in the amount of radioactivity bound to the filter disc reflected competitive replacement of [3H]methotrexate at the antibody-combining site and was expressed as the percentage of displacement. Inhibition of binding was determined as a function of the amount of unlabeled compound added. Data were plotted on log (concentration) /logit (% displacement) graph paper, and the relative effectiveness of each analog was determined from the concentration required to achieve 50% inhibition. A standard displacement curve for unlabeled methotrexate served to relate inhibition to methotrexate concentration in specimens containing unknown amounts of the drug. Assays were performed in triplicate, and the S.D. of such methotrexate determinations was 10% or less.

Extracts were prepared in a small test tube by diluting 0.5 ml of serum or plasma with an equal volume of 0.15 mM NaCl:0.01 mM phosphate, pH 7, and heating it on a steam bath for 30 min. Coagulated protein was broken up in the tube and was then centrifuged at 2500 x g for 15 min to yield a clear supernatant.

RESULTS AND DISCUSSION

Antibody Specificity. Specific antibody and [3H]methotrexate combine in an equilibrium reaction to form a complex that can be detected upon passage through the nitrocellulose filter (Chart 1). When added to the reaction mixture at appropriate concentrations, chemically related molecules can competitively displace tritiated methotrexate from the antibody-combining site. The inhibitory effectiveness of each analog reflects its complementarity with the site, and comparisons serve to define the specificity of the radioimmunoassay system.

The specificity of an early serum from 1 of the rabbits immunized (Ra 73-7b) is illustrated by the displacement curves shown in Chart 2. Methotrexate, the most potent inhibitor, can be reliably measured in the 1- to 100-pmole range. Folic acid and 1 of its reduced forms, folinic acid, are required at 40,000- and 80,000-fold higher concentrations, respectively, to produce comparable displacement of [3H]methotrexate (Chart 2, Table 1). Chemical differences between folates and methotrexate involve substitution of an amino group for a hydroxyl at the 4 position and addition of a methyl group to the N10 region. The sharp immunochemical distinction is almost solely based upon the nature of the substituent in the 4 position, since comparison of methotrexate and aminopterin (which lacks the N10-methyl group) indicates that no large loss of inhibitory effectiveness results when the methyl group is absent (Table 1).

Modifications of other regions of the molecule result in more subtle changes when tested in this system. Thus, 7,8-dihydromethotrexate is 5-fold less effective than is methotrexate, while 3',5'-dichloromethotrexate or an analog that has no glutamate portion (2) each display a ∼20-fold reduction in potency.

Optimal binding depends upon the complete methotrexate molecule, but both the 2,4-diamino-6-methylpteridine and p-aminobenzoylglutamate halves of the structure can react with the antibody sites. Interaction of these methotrexate-related compounds with antibody can be detected due to the extreme sensitivity and range of the assay. While these determinations are of theoretical interest, the systems remain functionally specific for methotrexate.

Of the 4 animals immunized, each provided sera suitable for the radioimmunoassay procedure and each displayed similar but not identical specificity characteristics. One animal produced serum antibodies that could easily detect 0.1 pmole methotrexate, while that from another showed a 250,000-fold differential binding of methotrexate versus folic acid. No correlation has been made between antigen dose and the nature of the antibodies elicited.

Clinical Assays. Stability and simplicity of operation make this radioimmunoassay particularly suited to clinical application. The protein-binding capacity of nitrocellulose membranes is limited (6), and specimens containing excessive amounts can nonspecifically interfere with binding of antimethotrexate antibodies. The capacity of the filters is about 300 μg of protein. For clinical determination of serum drug levels, volumes of whole serum up to 2 μl or 50 μl extract could be directly added to the assay system without appreciable interference. Comparison with normal human serum or extract controls can be used if these or larger volumes are to be assayed.

Excellent recovery (85 to 100%) was achieved when known quantities of methotrexate were added to serum and
Radioimmunoassay for Methotrexate

Chart 2. Competitive inhibitors of the binding of tritium-labeled methotrexate to antibody sites. 7,8-Dihydro, 7,8-dihydromethotrexate; -Glut., \( p\) -[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoic acid. Serum, 1 µl, from rabbit Ra 73-7b was used for this assay.

Table 1

<table>
<thead>
<tr>
<th>Analog</th>
<th>pmoles required for 50% inhibition of (^{3}H)-methotrexate binding</th>
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</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>5</td>
</tr>
<tr>
<td>7,8-Dihydromethotrexate</td>
<td>25</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>50</td>
</tr>
<tr>
<td>( p) -[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoic acid</td>
<td>80</td>
</tr>
<tr>
<td>3',5'-Dichloromethotrexate</td>
<td>120</td>
</tr>
<tr>
<td>2,4-Diamino-6-methylpteridine</td>
<td>45,000</td>
</tr>
<tr>
<td>5-Methyltetrahydrofolic acid</td>
<td>160,000</td>
</tr>
<tr>
<td>Folic acid</td>
<td>200,000</td>
</tr>
<tr>
<td>Folic acid (citrovorum factor)</td>
<td>400,000</td>
</tr>
<tr>
<td>Tetrahydrofolic acid</td>
<td>700,000</td>
</tr>
<tr>
<td>( p) -Aminobenzoylglutamate</td>
<td>120,000,000</td>
</tr>
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assayed either directly or from an extract. In addition to serum and plasma samples, assays have been routinely run on urine and cerebrospinal fluid specimens. Determination of methotrexate levels in tissues has not yet been investigated by the radioimmunoassay method; however, this appears perfectly feasible providing that excessive amounts of protein are not added to the reaction mixture.

Folic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, and folinic acid (citrovorum factor) are relatively poor inhibitors. This fact permits measurement of methotrexate in the presence of these naturally occurring folates even when they are artificially elevated. In a time course study of plasma levels of the drug in patients receiving high-dose methotrexate treatment combined with citrovorum factor rescue (11), corresponding values were obtained with both radioimmunoassay and enzyme measurements (Chart 3). No deflections were observed in the methotrexate level after i.v. and p.o. administration of citrovorum factor. This correlation between these 2 different assays further substantiates the applicability of the radioimmunoassay, since the enzyme technique precludes interference by reduced folates.

Methotrexate radioimmunoassay is currently being used in conjunction with a newly developed radioimmunoassay specific for citrovorum factor (V. Raso, R. H. Blum, M.
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The ability to monitor concentrations of these drugs in patients undergoing treatment should foster a more effective and safer regimen of dosage and will provide a meaningful basis for judging the potency and toxicity of any given drug schedule.

ACKNOWLEDGMENTS

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REFERENCES

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