Cancer Chemother Pharmacol (1983) 10: 96–99

Cancer Chemotherapy and Pharmacology

Radioimmunoassay for Methotrexate Using Hydroxyethylmethacrylate Hydrogel

Kevin J. Tracey[1], Richard Mutkoski[1], Jose A. Lopez[2], William Franzblau[1], and Carl Franzblau[1]

[1] Department of Biochemistry, Boston University School of Medicine, and
[2] Section of Medical Oncology, The Evans Memorial Department of Clinical Research, Division of Medicine, Boston University Medical Center,
80 East Concord Street, Boston, MA 02118, USA

Summary. *A sensitive, rapid method for the measurement of methotrexate in biologic fluids has been developed using a solid-phase radioimmunoassay (RIA). Hydroxyethylmethacrylate monomer was polymerized in the presence of rabbit antimethotrexate antisera and the resultant gels lyophilized, ground to a fine powder, and aliquoted into 3-ml syringes fitted with a fritted filter disc. Control gels without incorporated antibodies did not bind radiolabeled methotrexate, whereas a dose-response curve expressing percent bound methotrexate against antiserum concentration could be readily constructed for appropriate gels. Drug concentrations of less than 1ng/ml can be measured. The fate of a bolus IV injection of methotrexate (100 mg) into an adult female baboon was evaluated by the HEMA-RIA system and compared with a previously published enzyme assay for the drug. These studies describe a stable and efficient system for rapid separation of bound antigen by simple filtration.*

Introduction

The folic acid antagonist, methotrexate (MTX, amethopterin, 4-amino-10-methyl-pteroylglutaminic acid), has been an established antineoplastic agent in clinical use for over 25 years. Efficacy against childhood acute leukemia, choriocarcinoma, breast cancer, lung cancer, and head and neck cancer has been consistently described [5, 12]. In recent years, a renewed interest in enhancing the therapeutic index of this drug has resulted in the clinical application of high-dose treatments. The use of high doses of MTX is made possible by the administration of leukovorin (citrovorum factor, 5-formyl-tetrahydrofolate), which acts as a source of tetrahydrofolate and 'rescues' normal cells from irreversible damage [2, 6, 8, 10]. Both the clinical efficacy and the undesirable toxicity of MTX have been shown to be related to its serum concentration [7, 11]. Careful monitoring of drug levels has transformed high-dose MTX into a relatively safe and non-toxic therapy [9].

Methods for measuring MTX concentrations include biologic assays, chromatographic techniques, and immunoassays [1]. The solid-phase radioimmunoassay (RIA) described in this report incorporates anti-MTX serum into a hydroxyethylmethacrylate (HEMA) polymer, known as a hydrogel. The resulting lyophilized hydrogel offers improvements over most existing procedures for measuring MTX. There is rapid and efficient separation of free from bound antigen, incubation periods are short, and arduous pipeting steps are eliminated.

Reprint requests should be addressed to C. Franzblau

Materials and Methods

Rabbit MTX antiserum (titer of 1 : 50,000) was obtained from Seragen, Inc., Dorchester, MA, USA. Ophthalmic grade 2-hydroxyethylmethacrylate (HEMA) and divinylbenzene were products of Polyscience, Inc., Warrington, PA, USA; ammonium persulfate and TEMED (*N,N,N,'N'*-tetramethylethylenediamine) from Bio-Rad, Inc., Richmond, CA, USA; 75 × 50 mm microscope slides and 22-mm plastic coverslips from Fisher Scientific, Co., Pittsburg, PA, USA; 3-ml syringes from Becton-Dickinson and Co., Rutherford, NJ, USA; plastic filter frits from Amicon, Inc. and I^{125} MTX tracer from The Enzyme Center, Inc., Boston, MA, USA. All samples were counted in a Packard Gamma Counter. The Methotrexate Radioassay I^{125} Kit was used according to the manufacturer's instructions (The Enzyme Center, Inc., Boston, MA, USA).

Preparation of HEMA-Antiserum Hydrogels. Hydrogels of HEMA were prepared in the following manner: 0.5 cm^3 HEMA, 0.25 cm^3 antiserum, 0.25 cm^3 distilled water, 6 µl divinylbenzene, 10 µl TEMED, and 100 µl 6% (wt/vol.) ammonium persulfate were added sequentially and mixed thoroughly in a Vortex mixer. The resulting solution was allowed to polymerize between two microscope slides (separated by two pairs of coverslips) at 37° C for 45 min. The hydrogels were then thoroughly rinsed in each of three beakers of distilled water for 5 min and then dialyzed exhaustively against 4 l 50 m*M* Tris, 0.15 *M* NaCl, pH 7.35, at 4° C. The hydrogels were then placed on microscope slides, frozen at −4° C and lyophilized overnight. The resulting brittle plastic was ground to a fine powder in a covered mortar and pestle. Hydrogels containing less antiserum were prepared by diluting the antiserum with distilled water so that the aqueous phase was maintained at a volume of 0.5 cm^3.

Preparation of Assay Chambers. Circular discs (9 mm in diameter) of plastic filter frit were wedged into the bottom of the 3-ml syringe chambers. The hydrogel-antiserum powder was suspended in distilled water at a concentration of 25 mg/ml. Aliquots (100 µl) of the well-stirred solution were dispensed into the syringe chambers.

Radioimmunoassay Procedure. All samples were run in duplicate in 13 × 100 mm disposable culture tubes at room

Table 1. Procedure for RIA with HEMA-antiserum

Reagents	Blank tube (µl)	Zero tube (no HEMA) (µl)	Sample tube (µl)
Buffer	1,000	1,000	900
Standard/sample	–	–	100
MTX tracer	100	100	100
Buffer	400	400	400

Step 1: Buffer, standard or sample, MTX tracer drawn into assay chamber (syringes)
Step 2: Rinsing buffer drawn into assay chamber
Step 3: Incubation at room temperature for 40 min with intermittent mixing in a Vortex
Step 4: Supernatant dispersed from the chamber into a clean test tube
Step 5: Determination of radioactivity by gamma counter

temperature. The buffer used throughout the procedure was 0.1 *M* NaCl, 0.05 *M* potassium phosphate, pH 7.4, containing 0.1% gelatin. The protocol for the assay procedure is shown in Table 1. The reagents were added in the order indicated and drawn into the assay chambers (syringes), which could then be mixed in a Vortex after inserting the entire syringe into a clean 13 × 100 mm test tube. The original sample tube was rinsed with 400 µl buffer, which was then drawn into the syringe as well. Any air inadvertently drawn into the syringe was squeezed out of the inverted chamber. The solutions were intermittently mixed in a Vortex for 40 min. The supernatant was squeezed into a clean test tube for determination of the radioactivity of each sample.

Standard Curve. The concentration of MTX in an unknown sample is determined by the degree to which it displaces the binding of the tracer to the hydrogel antiserum powder. The standard curve is obtained by plotting $[1-(B/B_0)] \times 100$ as a function of the concentration of unlabeled MTX where: B = (sample count − blank) and B_0 = (zero count − blank).

Assay of MTX in Baboon Serum. A 25-kg female baboon (*Papio anubis*) was given a 100 mg IV bolus injection of MTX. Six blood samples were collected from a femoral vein during the subsequent 25 h. Methotrexate serum concentrations were determined simultaneously by the HEMA-antibody (HEMA-AB) method and by the competitive enzyme method. Two, three, and four 10-fold dilutions were prepared for each serum sample. This series of dilutions covers variations through the range of drug concentrations obtained during MTX therapy. Samples were stored at −4° C if not assayed within 24 h of collection.

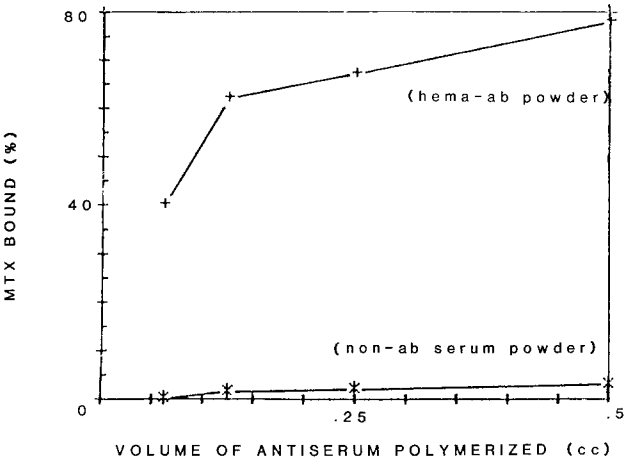

Fig. 1. Specific binding of tracer to HEMA-AB and control

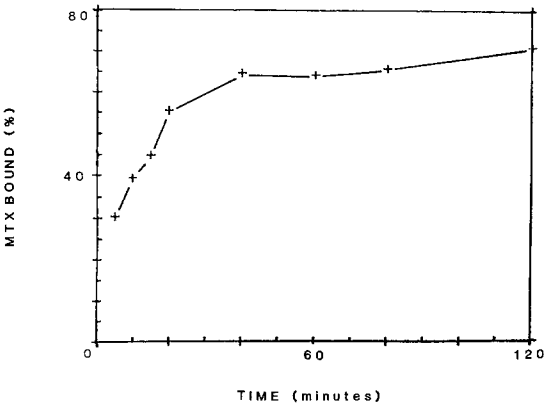

Fig. 2. Binding of antigen to HEMA-AB as a function of time

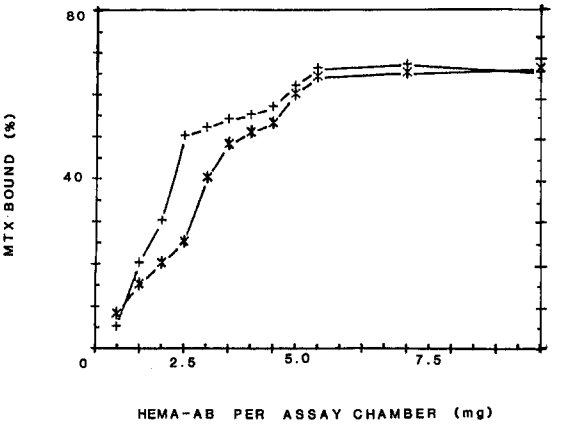

Fig. 3. Binding of MTX to HEMA-AB as a function of the quantity of powder. (+) standard assay; (*) assay with 0.25 ng unlabeled MTX/ml

Results

To construct a HEMA-antiserum hydrogel that would optimally bind specific radiolabeled antigen, HEMA monomer was polymerized in the presence of an aqueous phase containing antiserum. Maximum binding of tracer to powder was obtained for a given concentration of antiserum when the ratio of monomer to aqueous phase was 1 : 1. This formula was routinely used in the preparation of all HEMA-antiserum powders as described above.

Binding of a constant amount of tracer to a series of powders containing increasingly dilute antiserum decreased as illustrated in Fig. 1. Hydrogels prepared with non-immunized rabbit serum did not bind MTX to any significant degree.

The kinetics of MTX binding to the hydrogel-antiserum powders was examined. Approximately 65% of the tracer was bound in 40 min (Fig. 2). This incubation time was chosen for all assay procedures.

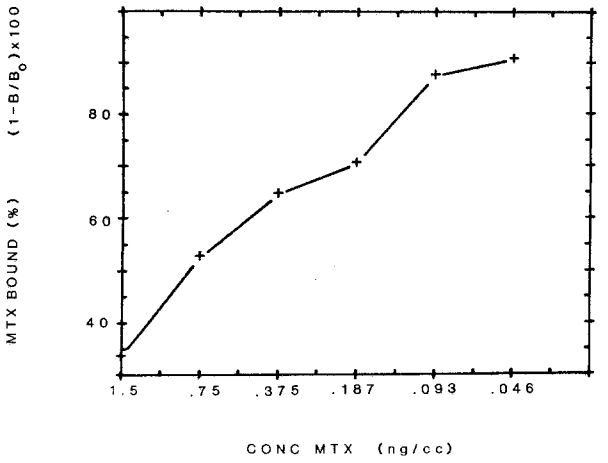

Fig. 4. Standard curve of a routine MTX assay using 2.5 mg HEMA-AB powder

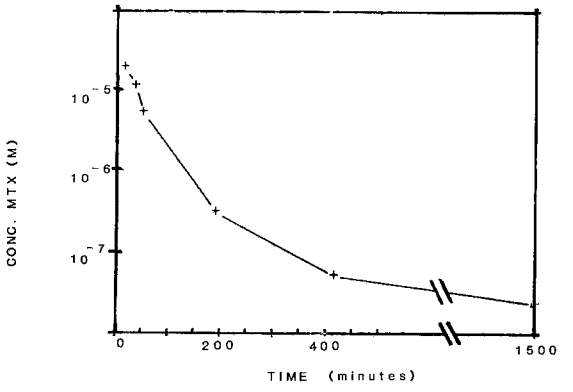

Fig. 5. Plasma clearance of MTX determined by the HEMA-AB RIA

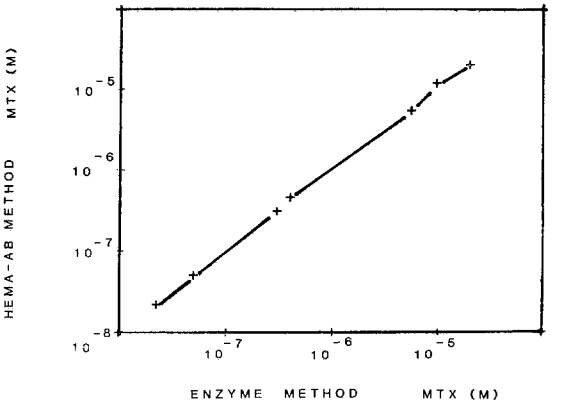

Fig. 6. Plasma (MTX) determined by the HEMA-AB RIA and the competitive enzyme technique

The quantity of powder required for maximum sensitivity of the assay was also determined. Varying quantities of powder were placed into separate syringes. The assay was carried out as above, except that 0.25 ng unlabeled MTX/cm^3 was added to each assay tube. The results were then compared with those obtained when the entire series was performed with labeled MTX present, as shown in Fig. 3. Maximum assay sensitivity will be obtained at that concentration of powder for which the inhibited curve is displaced approximately 50%. A powder concentration of 2.5 mg/ml was therefore routinely used for all assay procedures. A standard curve of a routine HEMA-antiserum RIA utilizing 2.5 mg powder is shown in Fig. 4.

The plasma clearance of MTX was determined in the baboon by measuring drug concentrations with the HEMA-AB assay (Fig. 5). Following the initial distribution phase, two phases of disappearance were obtained, as previously described [4]. A comparison between the HEMA-AB assay and the competitive enzyme method was performed in the baboon serum samples. Methotrexate concentrations determined for each sample were plotted against those obtained by the competitive assay. Analysis of variance revealed no significant difference in the concentration range studied (10^{-5}–10^{-9} M).

Discussion

Numerous methods for solid-phase RIA have been reported for a variety of hormone and drug antigens. These have included the utilization of plastic test tubes and discs, agarose and plastic beads, porous glass, and polyacrylamide gels [13, 14]. In the present RIA the controlled entrapment of antiserum into a hydrogel matrix proved to be simple, inexpensive, and stable. In addition, the hydrogel is not toxic or dangerous to prepare.

The utility of the HEMA-hydrogel as a solid phase appears to be related to the porosity of its polymeric form. The porosity of hydrogels is dependent upon both the kinetics of polymerization and the concentration of cross-linking agent (divinylbenzene). Polymerization conditions described above provided the optimal HEMA-AB hydrogel for use in RIA.

A shortcoming of many solid-phase techniques is the inability to control antibody binding to the solid phase. By polymerizing monomeric HEMA in the presence of diluted antiserum of known concentrations one can entrap antibodies in a controlled manner. This allows for long-term stability of the antibody and accurate dispensing of the material. The specificity of the HEMA-AB is dependent upon the antibody specificity, as no appreciable binding of tracer occurred to control serum powders. A recent evaluation of current assay techniques for serum MTX found no significant differences between results obtained from enzyme immunoassay and from RIA [3]. The simplification of the specific and sensitive RIA technique appears desirable.

The MTX HEMA-antiserum RIA has proved to be reliable and reproducible. Because the antibodies are entrapped less than 1 h is required for incubation, and any centrifugation steps are eliminated. Precise timing is less critical and dozens of samples may be assayed simultaneously with the results available within 1 h. With few modifications and the incorporation of different antisera, this method could be readily adaptable for determination of a variety of antigens. Expensive laboratory equipment is not required for this technique and the practical simplicity of the assay procedure will prove to be useful in the clinical laboratory.

References

1. Aherne GW, Quinton M (1981) Techniques for the measurement of methotrexate in biological samples. Cancer Treat Rep [Suppl 1] 65: 55
2. Bertino JR (1977) Rescue techniques in cancer chemotherapy: Use of leucovorin and other rescue agents after methotrexate treatment. Semin Oncol 4: 203

3. Buice RG, Evans WE, Karas H, Nicholas CA, Paramjeet S, Straughn AB, Meyer MG, Crom WR (1980) Evaluation of enzyme immunoassay, radioassay and radioimmunoassay of serum methotrexate as compared with liquid chromatography. Clin Chemistry 26: 1902
4. Chabner BA, Donehower RC, Schilsky RL (1981) Clinical pharmacology of methotrexate. Cancer Treat Rep [Suppl 1] 65: 51
5. Ensminger WD, Grindley GB, Hoglind JA (1979) Antifolate therapy: Experimental approaches using rescue, selective host protection and drug combination. In: Rosowsky P (ed) Advances in cancer chemotherapy. Marcel Dekker, New York, p 61
6. Frei E, Jaffe N, Tattersall HNM, Pitman S, Parker L (1975) New approaches to cancer chemotherapy with methotrexate. N Engl J Med 292: 846
7. Goldie JH, Price LA, Harrap KR (1972) Methotrexate toxicity. Correlation with duration of administration, plasma levels, dose and excretion pattern. Eur J Cancer 8: 404
8. Goldin A (1978) Studies with high-dose methotrexate – historical background. Cancer Treat Rep 62: 307
9. Isacoff WH, Eilber F, Tabbarah H, Klein P, Dollinger M, Lemkin S, Sheehy P, Cone L, Rosenbloom B, Sieger L, Block JB (1978) Phase II clinical trials with high-dose methotrexate therapy and citrovorum factor rescue. Cancer Treat Rep 62: 1295
10. Levitt M, Mosher MB, DeConti RC, Farber LR, Skeel RT, Marsh JC, Mitchell MS, Papac RJ, Thomas ED, Bertino JR (1973) Improved therapeutic index of methotrexate with "leucovorum rescue". Cancer Res 33: 1729
11. Nirenberg A, Mosende C, Mehta B (1977) High-dose methotrexate with citrovorum factor rescue: Predictive valve of serum methotrexate concentrations and corrective measures to avert toxicity. Cancer Treat Rep 61: 779
12. Porter R, Wilthshaw E (1962) Methotrexate in the treatment of cancer. Williams & Wilkins, Baltimore
13. Smith KO, Gehle WD (1980) Semiautomation of immunoassay by use of magnetic transfer devices. In: Van Vunakis H, Langone J (eds) Methods in enzymology, vol 70. Academic Press, New York, p 388
14. Updike SJ (1979) Automation and simplification of radioimmunoassay by gel entrapment of antibody. Antibiot Chemother 26: 67

Received June 8, 1982/Accepted October, 1982