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A BIOLUMINESCENT IMMUNOASSAY FOR METHOTREXATE AT THE SUBPICOMOLE LEVEL

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Summary: A bioluminescent immunoassay has been developed for measuring methotrexate. Methotrexate was covalently linked to firefly luciferase resulting in a product containing two methotrexates per mole of luciferase. This derivatized enzyme retained 60% of its catalytic activity. The luciferase-methotrexate was bound by antibody raised against methotrexate-hemocyanin. The rate of binding was essentially the same as binding of free methotrexate. When the enzyme conjugate was incubated with varying concentrations of free methotrexate and the antibody, the amount of the enzyme-conjugate bound was inversely proportional to the concentration of free methotrexate. Using this procedure it is possible to detect 2.5 pmoles of methotrexate. A double antibody technique had an increased sensitivity and as little as 0.5 picomoles could be measured reproducibly.

INTRODUCTION

The therapeutic value of methotrexate in both neoplastic (1-3) and non-neoplastic disorders (4-7) has been demonstrated. High dose methotrexate therapy is used for the treatment of certain types of cancer but this may produce severe toxic side effects (8-9). To achieve optimum therapeutic effects with the minimum of toxic side effects, it may be necessary to monitor the serum methotrexate levels.

Various assay techniques for methotrexate are available (10-15). The radioimmunoassay is by far the most sensitive and specific assay for methotrexate. The problems encountered with using such an assay includes the use of expensive β or γ counters, the short shelf life of [^{125}I] or [^{75}Se] labelled methotrexates, and the handling and disposing of radioisotopes.

In this paper we describe a bioluminescent immunoassay for methotrexate which is as sensitive as the radioimmunoassay.

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MATERIALS AND METHODS

Crystalline firefly luciferase was prepared and assayed as previously described (16). Luciferin was synthesized according to the method of Seto (17). Methotrexate was kindly supplied by Lederle Laboratories. [^{3}H] methotrexate was a gift from Dr. R. Howell and B. Chau, University Hospital, San Diego, California. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCL (EDAC)[1] was obtained from Bio-Rad Laboratories. The anti-goat IgG fraction from rabbit serum was obtained from Miles Laboratories. ATP was obtained from Boehringer Mannheim. Sephadex G-50 and Sepharose 4B were obtained from Pharmacia. Cyanogen Bromide was purchased from Eastman Kodak, Rochester, New York. The preparations and serologic characteristics of goat antibody to methotrexate has been reported (14).

Conjugation of Luciferase and Methotrexate:

500 nmoles of methotrexate was incubated with 3.5 μmoles of EDAC in 0.3 ml of 0.1 M sodium phosphate pH 7.0 for 15 minutes at 25°C. Then 2 μmoles of ATP and 10 nmoles of firefly luciferase were added to give a final volume of 0.5 ml. The reaction mixture was incubated at 4°C for 16 hours. It was dialyzed against 0.1M phosphate pH 7.0 and then passed through a Sephadex G-50 column (1x30 cm) to remove any free methotrexate. The amount of bound methotrexate was determined by the absorption at 302 nm, assuming a molar absorption of 22,000 at this wavelength (18). Protein concentration was determined by the Coomassie (19) or Lowry (20) procedures. The amount of methotrexate linked to luciferase varied from 1.3-2.0 moles per mole luciferase.

Fractionation of the Anti-Serum:

Ammonium sulfate was added to the anti-methotrexate goat serum to give 25% saturation. The serum was centrifuged at 7000 rpm for 10 minutes and the pellet discarded. Additional ammonium sulfate was added to 35% saturation, centrifuged and the pellet was dissolved in 0.01M phosphate, 0.15M NaCl pH 7.8 (PBS).

Immobilization of Anti-methotrexate on Sepharose 4B:

Sepharose 4B was activated by standard procedures (21). 1 gram of the activated Sepharose was suspended in 1 ml of 0.2M $NaHCO_3$ pH 8.5 and 1 ml of the immunoglobin preparation of 30 mg/ml goat anti-methotrexate was added. This was stirred for 16 hours at 4°C. The derivatized Sepharose was washed with PBS until the absorbance of the supernatant was less than 0.03 at 280 nm. 80% of the added protein was covalently linked to the Sepharose. The Sepharose-anti-methotrexate was suspended in 10 ml of PBS and stored at 4°C.

Binding of [^{3}H] methotrexate to the Immobilized Antibody:

50 μl of the Sepharose-antibody and 100 μl of underivatized Sepharose (1gm/5ml) and increasing concentrations of [^{3}H]-methotrexate were incubated in a 0.5 ml volume for 3 hours at room temperature. The tubes were centrifuged and the supernatant was counted. The amount of luciferase-methotrexate that bound to the Sepharose-antibody was determined in the same way except the bound luciferase was determined by suspending the Sepharose in buffer and assaying for luciferase activity. The competitive binding studies were performed in a similar procedure where the incubation mixture contained a constant amount of luciferase-methotrexate and increasing concentrations of free methotrexate.

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[1] Abbreviations

EDAC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl

PBS: phosphate buffered saline

Double Antibody Assay:

6 pmoles of luciferase-methotrexate and increasing concentrations of free methotrexate (0-30) pmoles, and 25 µl of 2.7 mg/ml goat anti-methotrexate were incubated in a final volume of 250 µl. After two hours at room temperature, 50 µl of 2.1 mg/ml specific rabbit antigoat IgG was added to the tubes. After 1 hour at room temperature the tubes were centrifuged and the pellets were washed with PBS until no more luciferase was detected in the wash. The pellets were then assayed for luciferase activity.

RESULTS

The amount of [^{3}H]methotrexate that bound to the Sepharose-antibody is shown in Figure 1. The maximum amount of [^{3}H]methotrexate bound to 50 µl of immobilized antibody is 1.7 pmoles. The same amount of immobilized antibody bound 0.8 pmoles

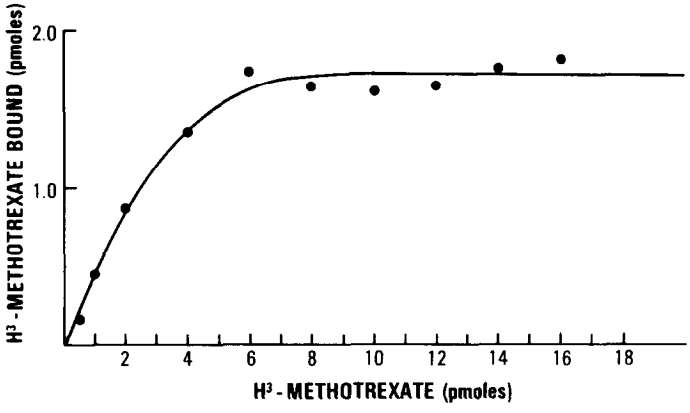

Figure 1. Binding curve of [^{3}H]methotrexate to Sepharose-antibody. 50 µl of Sepharose-antibody was incubated with increasing concentrations of [^{3}H]-methotrexate. The amount bound was determined as described in Materials and Methods. The non-specific binding was only 0.5-1.0% of the total counts.

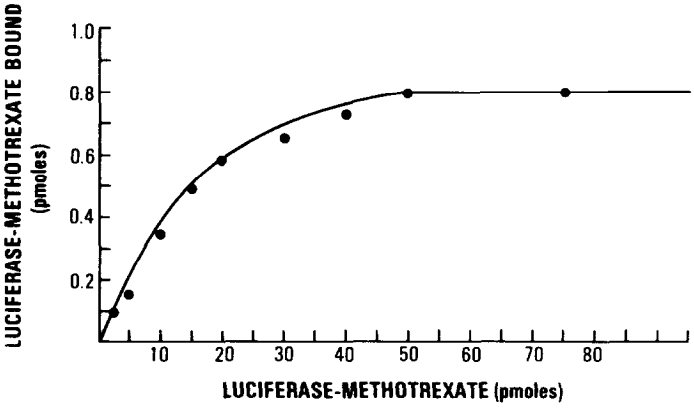

Figure 2. Binding curve of luciferase-methotrexate to Sepharose-antibody. Conditions are described in Materials and Methods. There was no detectable non-specific binding.

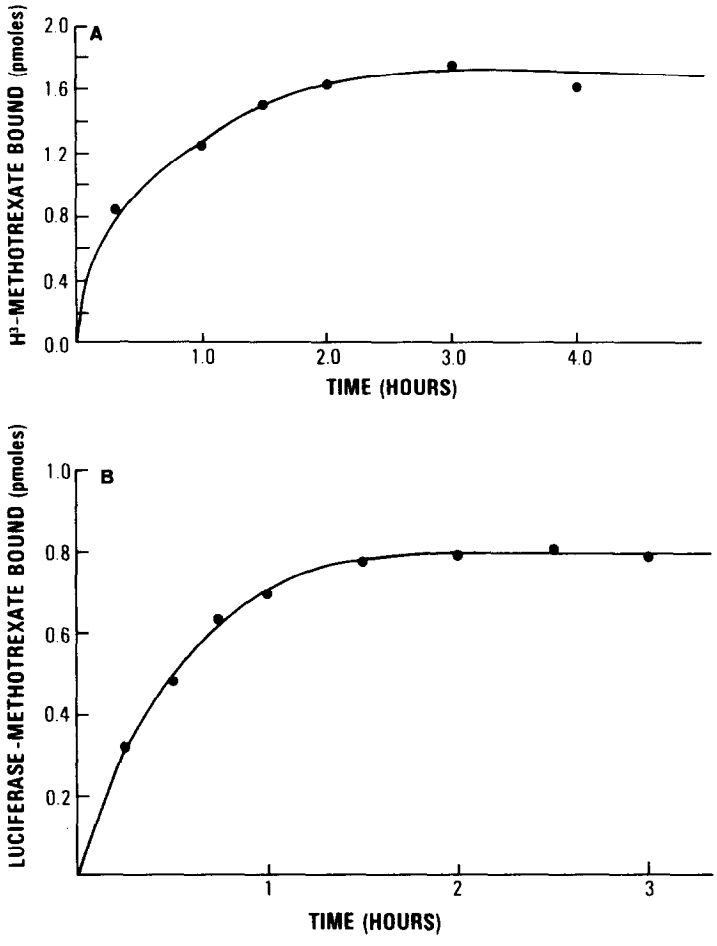

Figure 3. Time course of binding of methotrexate to Sepharose-antibody. 50 µl of Sepharose-antibody was incubaged with 25 pmoles of [^{3}H]-methotrexate (A) or 25 pmoles of luciferase-methotrexate (B) in 0.4 ml final volume. Binding was determined as described in Materials and Methods.

of luciferase-methotrexate as shown in Figure 2. This luciferase contained 2 moles of methotrexate per mole of luciferase. The rate of binding of [^{3}H]methotrexate (Figure 3A) or luciferase-methotrexate (Figure 3B) is essentially the same. At 25^0 C they both reach maximal binding within approximately 2 hours.

Figure 4 shows the amount of luciferase-methotrexate bound to the Sepharose-antibody as a function of increasing concentrations of free methotrexate. The amount of bound luciferase decreases with increasing concentrations of free methotrexate in the range 0-10 pmoles.

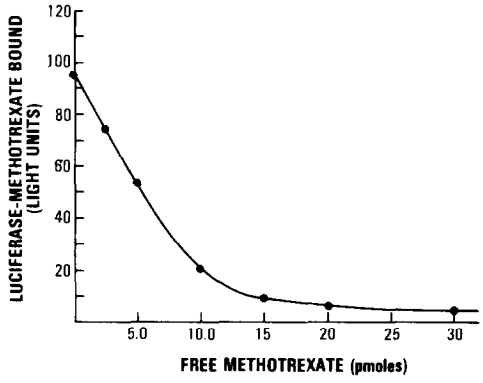

Figure 4. Competitive binding curve of free methotrexate with luciferase-methotrexate to Sepharose-antibody as measured by bioluminescence.

Utilizing a second heterogeneous immunoassay technique, the double antibody precipitation procedure, the sensitivity was greatly increased as shown in Figure 5. The largest decrease in bound luciferase-methotrexate occurs between 0-1.0 pmoles of free methotrexate. With this procedure it is possible to reliably measure 0.5 pmoles of free methotrexate. As little as 50 femtomoles can be detected.

DISCUSSION

Techniques used for detection of methotrexate include a spectrophotometric method (12) which has a detection limit of 220 pmoles/ml; the enzyme immunoassay

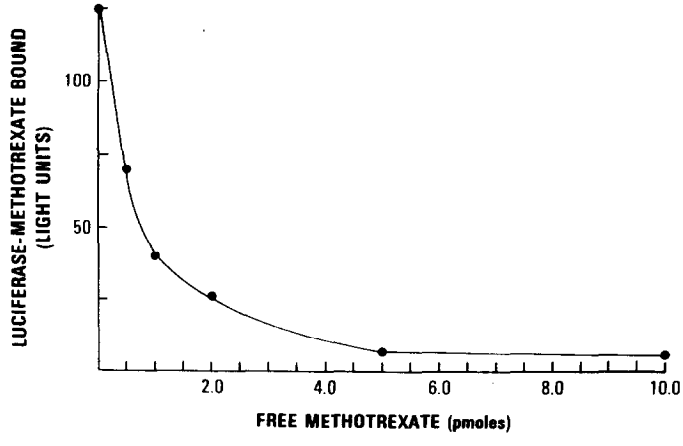

Figure 5. Competitive binding curve of free methotrexate with luciferase-methotrexate using the double antibody technique. Conditions are described in Materials and Methods.

using β-galactosidase (10) which can measure 2.2 pmoles/ml, and the radioimmunoassay with a detection of 0.2 pmoles/ml (13). The bioluminescent immunoassay described here is comparable in sensitivity with the radioimmunoassay but eliminates the problems associated with the use of radioisotopes.

The sensitivity of this assay like that of the radioimmunoassay is dependent upon the concentration of the antibody and the concentration of the luciferase-methotrexate used. Therefore, it is essential that a standard curve be obtained whenever new Sepharose-antibody or luciferase-methotrexate is prepared.

Figures 1 and 2 illustrate that the total binding capacity of the antibody for free methotrexate and luciferase-methotrexate is similar. It requires a higher concentration of luciferase-methotrexate to obtain maximal binding (40 pmoles) than with the free methotrexate (8 pmoles). There are two possible explanations for this: (1) The affinity of the antibody may be much greater for the free methotrexate; or (2) All of the luciferase molecules may not have methotrexate linked to them. The fact that the antibody does bind to the luciferase-methotrexate is probably due to the similar orientation of the methotrexate when bound to luciferase and to the antigenic carrier protein, hemocyanin (14). In both cases, the methotrexate is covalently bound by the formation of amide bonds between the glutamate carboxyl groups of methotrexate and the amino groups of the protein. Thus, the 4-amino end of methotrexate is the most distant from the protein and would be expected to be the immunodominate region for antibody formation and therefore the immunospecific region for antibody binding.

This paper clearly illustrates the use of bioluminescence as a suitable replacement for radioisotopes to detect low concentrations of methotrexate. It is most likely that this type of assay can be extended to many other molecules of biological interest.

Acknowledgements

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