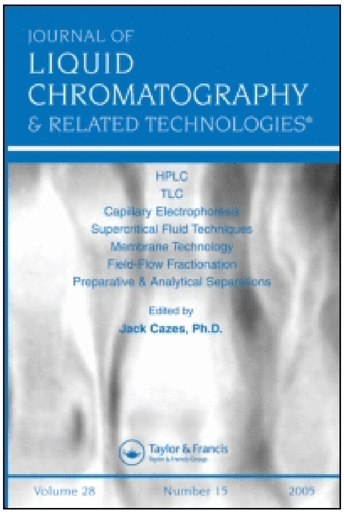

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A RAPID AND SENSITIVE METHOD FOR DETERMINATION OF TRIMETREXATE FROM BIOLOGICAL FLUIDS

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INTRODUCTION

Trimetrexate (2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino) methyl]quinazoline), (Fig. 1), a potent inhibitor of dihydrofolate reductase (DHFR), has demonstrated antitumor activity against murine and human cell lines both *in vitro* and against a spectrum of murine tumors *in vivo* (1,2).

The pharmacology and toxicity of this agent has been examined previously by Weir *et al.* (3) in dogs. They monitored the drug levels in biological fluids and tissues by two methods of

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determination: a dihydrofolate reductase inhibition assay and a high pressure liquid chromatographic (HPLC) method using electrochemical detection. Difficulties were reported in the recovery of trimetrexate using several sample preparation procedures for the HPLC analysis.

In conjunction with Warner Lambert/Parke Davis the Vermont Regional Cancer Center has recently begun trimetrexate Phase I clinical trials. Initial studies in our laboratory demonstrated the value of an enzyme-based methodology for measuring this agent in mouse serum (4). However, to eliminate the possibility of interference from a metabolite possessing similar enzyme inhibition properties, we also pursued an HPLC method. This method employs Bond Elut mini extraction columns which expedited and simplified previous approaches to sample cleanup. It yields both high recoveries and reproducible results, and therefore is applicable for biological samples.

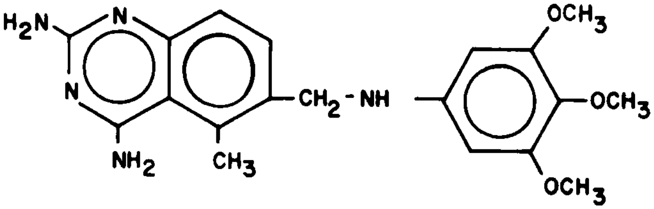

FIGURE 1

Structure of trimetrexate.

MATERIALS AND METHODS

Standards and Reagents

Trimetrexate was obtained from Warner Lambert/Parke Davis (Ann Arbor, MI, USA). HPLC grade water, methanol and acetonitrile were purchased from Burdick and Jackson Labs (Muskegon, MI, USA). Sodium citrate was purchased from Mallinckrodt (St. Louis, MO, USA): sodium acetate from Baker (Phillipsburg, NJ); phosphoric acid and triethylamine from Fisher Scientific, (Pittsburgh, PA). The serum, plasma and urine routinely used in the analytical methodology development were either donated by healthy volunteers or obtained from Pel-Freeze (Rogers, AR, USA). The C_{18} Bond Elut sample preparation columns, with a 500 mg capacity column bed, were purchased from Analytichem (Harbor City, CA). A vacuum manifold with the capacity of holding ten sample preparation columns (also obtained from Analytichem, Harbor City, CA) allowed for simultaneous sample extractions.

Chromatographic Apparatus and Conditions

All methodology development and drug analyses were performed on a Spectra-Physics Model 8700 liquid chromatograph which was fitted with a u-Bondapak C_{18} (10um) analytical column (Waters Assoc. 0.39 x 30 cm) and an RP-18 Spherisorb guard column (Rainin Instr., 0.46 cm x 3 cm). The Kratos Model 757 variable-wavelength U.V. detector was set at a wavelength of 241 nm. The mobile phase was comprised of 60% HPLC grade water containing 0.02% phosphoric acid and 0.08% triethylamine, with 40% HPLC grade acetonitrile. The flow was 2 ml/min. Integration of all resulting peaks, reported as peak areas, was performed by a Spectra-Physics 4200 computing integrator.

Sample Preparation

A C_{18} Bond Elut column was pre-wet by aspirating three column reservoir volumes of methanol followed by three column reservoir volumes of water through the column prior to sample loading. Only the excess water was pulled through the column before adding the sample. It was necessary to centrifuge plasma samples, before processing, for approximately 3-5 min. in order to remove insoluble lipids from the samples.

One ml of serum or plasma was loaded onto the C_{18} Bond Elut column, aspirated, and washed sequentially with 6 ml of water, 1.5 ml acetonitrile, and 0.5 ml of water. Again, only the excess solvent was removed. The trimetrexate was eluted from the C_{18} Bond Elut with 1 ml of a 95% methanol and 5% 0.08M sodium citrate solution. The eluent was filtered through a 0.45u Acrodisk (Gelman) before a 10ul sample was injected, in order to preserve the life of the analytical column, as well as the guard column.

A similar procedure was used for the extraction of trimetrexate from urine. The sample preparation column was pre-wet as previously described. One ml of urine was loaded onto the column and washed with 6 ml of water, 1 ml of acetonitrile, and 1 ml of a 75% 0.02M sodium acetate (pH 4.5) 25% methanol solution. Trimetrexate was recovered with 1.25 ml of the 95% methanol, 5% 0.08M sodium citrate solution.

RESULTS AND DISCUSSION

The Bond Elut methodology for the extraction of trimetrexate described here proved to be a consistent, sensitive and efficient method for our purposes. Figure 2 compares chromatograms of patient plasma and urine blanks to the chromatograms of the same patient following administration of trimetrexate. As demonstrated in Fig. 2, there is not any interference with the trimetrexate peak using this mini column sample preparation method under these HPLC conditions.

Prior to sample extraction, a daily standard curve (trimetrexate in mobile phase) is run to determine the peak areas of various drug concentrations. Spiked plasma samples in the concentration range used for the standard curve are extracted concurrently with the patient samples, providing an external standard for our method. A daily calibration curve was generated by plotting the concentrations of the standards vs the areas of the corresponding peaks. This curve was linear over at least a 250 fold concentration (100 ng/ml - 25 ug/ml) and has a correlation coefficient of 0.999 using the least squares regression method. The concentrations of the extracted spiked plasma samples vs the peak areas had a correlation coefficient

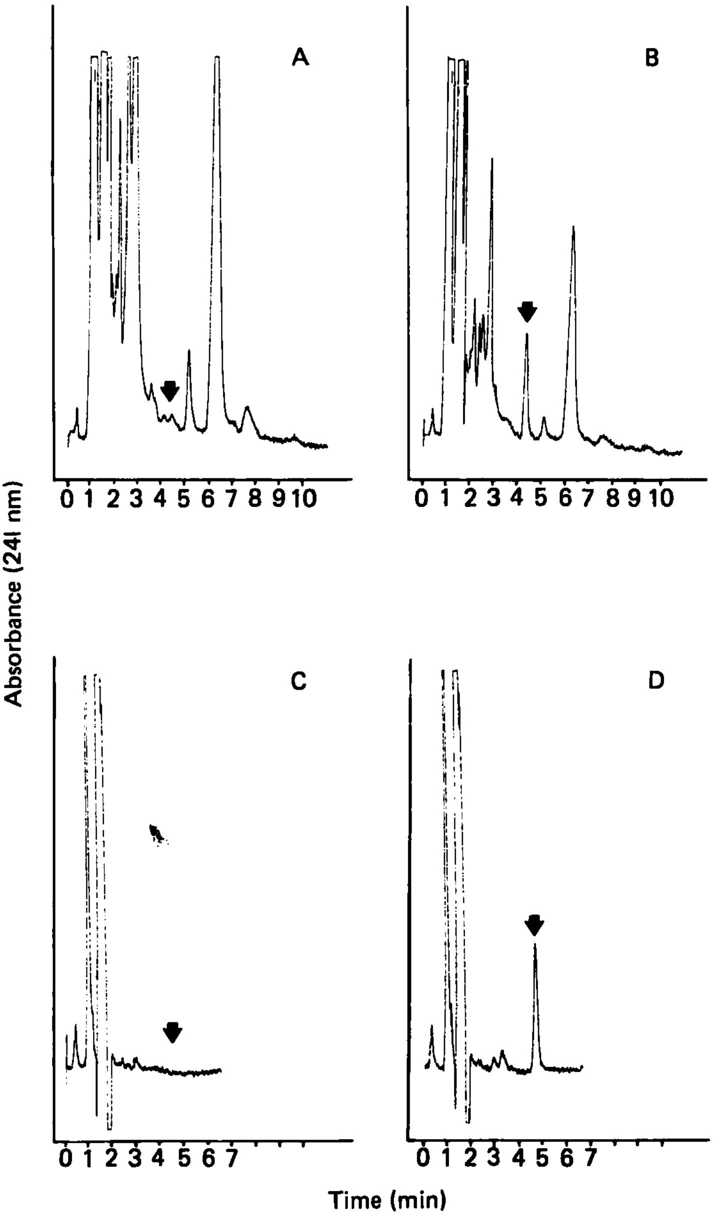

FIGURE 2

Chromatograms of urine and plasma from patient receiving 0.5 mg/m^2 of trimetrexate. (A) Patient urine collected before administration of trimetrexate, (B) urine collected over the first four hours following drug administration, (C) patient plasma before drug, (D) plasma 10 min. following drug administration. Arrows indicate expected retention time of the drug.

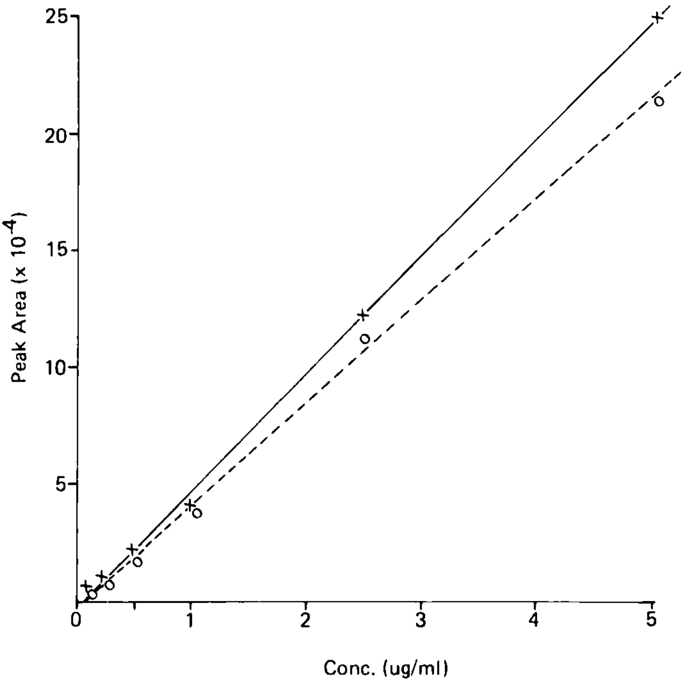

FIGURE 3

Standard curve for trimetrexate. The solid line indicates directly injected standards. The dashed line indicates trimetrexate extracted from plasma.

of 0.996. The standard curve and the extracted sample curve were similar showing that recovery problems have been eliminated in this system (see Fig. 3).

The average intraday recovery is 96% ± S.E. 1.8% (n=10) with a coefficient of variation of 5.8%. The average interday recovery is 99.2% ± S.E. 1.0% (n=10) with a coefficient of variation of 3.2%.

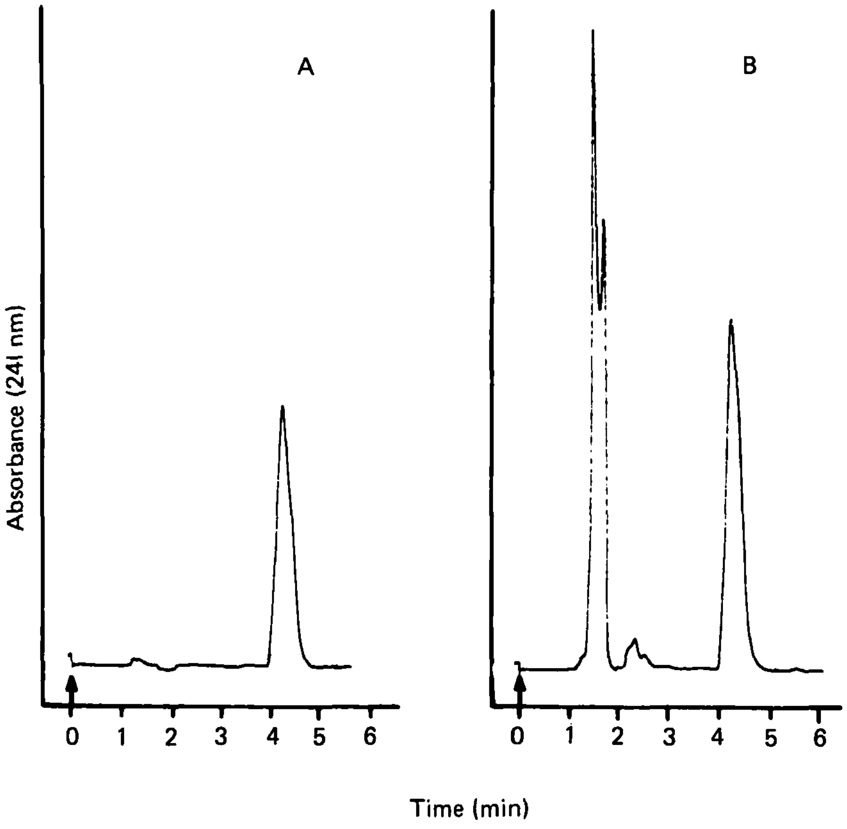

FIGURE 4

(A) Chromatogram of trimetrexate standard. (B) Chromatogram of trimetrexate standard and mouse metabolite injected simultaneously. Metabolite peak(s) are seen in solvent front.

Recent work in this laboratory has shown trimetrexate in mice to be metabolized to a more polar compound that also inhibits DHFR. The mouse urine metabolite (5) was obtained and injected into this system to ascertain whether or not it interfered with the trimetrexate peak. In figure 4, elution of the metabolite is seen in the solvent front, separate from the trimetrexate peak. Elimination of interference from metabolites gives a more precise quantitation of trimetrexate than an enzyme inhibition assay.

More extensive studies will be required to establish the presence of a polar metabolite of trimetrexate in man. However, at this time we are able to quantitate trimetrexate drug levels as low as 20 ng/ml in plasma, and 50 ng/ml in urine. This is sensitive enough to quantitate patient samples at our first Phase I dose: 0.5 mg/m^2. The sensitivity could be increased by increasing the injection size or by sample concentration. Data for pharmacokinetics of trimetrexate in man will be documented as the Phase I trial progresses.

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