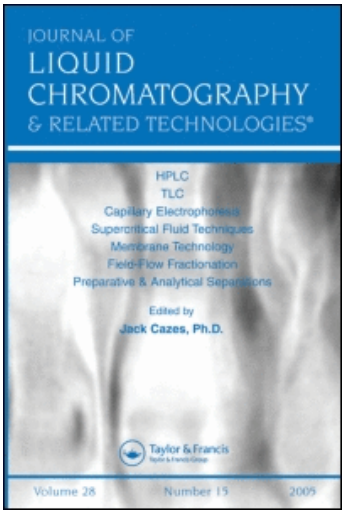

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Paired-Ion High Pressure Liquid Chromatography of Methotrexate and Metabolites in Biological Fluids

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PAIRED-ION HIGH PRESSURE LIQUID CHROMATOGRAPHY OF METHOTREXATE AND METABOLITES IN BIOLOGICAL FLUIDS

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ABSTRACT

A paired-ion high pressure liquid chromatography procedure (HPLC) is described for the separation of methotrexate and its major metabolites (7-hydroxymethotrexate, 4-amino-4-deoxy-N^{10}-methylpteroic acid, methotrexate diglutamate, methotrexate triglutamate), and therapy-related compounds (Diamox and 5-methyltetrahydrofolate). The mobile phase consisted of a 70% solution of 5 mM hexanesulfonic acid, pH 3.75, and 30% methanol eluted on a reverse phase column or columns and monitored at 305 nm or 280 nm UV absorption. The lower limit of sensitivity for methotrexate and 7-hydroxymethotrexate in plasma was 2 ng/ml.

INTRODUCTION

Methotrexate (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid), an antifolate, has been used for more than 20 years for the treatment of acute leukemia, osteosarcoma, and several other neoplastic diseases. In the last decade, the concept of high-dose methotrexate

therapy concurrent with citrovorum factor rescue in cancer chemotherapy has been developed (1) and extensively used in the management of a variety of malignant disorders (2). Methotrexate doses of up to 500 mg/kg have been administered with varying degrees of response. The high-dose methotrexate regimen with citrovorum factor (5-formyltetrahydrofolate) rescue and treatment details have been published (3).

The metabolism of methotrexate in humans was originally reported to be minimal. However, metabolites such as 7-hydroxymethotrexate and 4-amino-4-deoxy-N^{10}-methylpteroic acid have been reported in human urine and plasma after the infusion of high doses of methotrexate (4,5). This prompted us to develop a rapid method for quantitating methotrexate and its metabolites in order to investigate the pharmacology of these compounds.

The introduction of high pressure liquid chromatography (HPLC) has resulted in a great advancement in separatory science. The development of the reverse phase column and paired-ion solvent systems (6) extends the application of this technology greatly and makes possible the separation of such structurally similar compounds as methotrexate and 7-hydroxymethotrexate. This communication reports results of the use of paired-ion HPLC for the separation of methotrexate, its metabolites, and the support drugs of methotrexate therapy.

MATERIALS AND METHODS

A Glenco HPLC System 1 (Glenco Instrument, Houston, Texas) with a Whatman Partisil PXS 10/25 ODS column(s) (4.6 x 250mm) was used. An electronic integrator, Model CSI-38, (Columbia Scientific Industries, Austin, Texas) was used to determine peak areas and retention times. The system was equipped with a UV absorbance detector 254 nm and 280 nm or a variable wavelength UV detector (LDC Spectromonitor III: Laboratory Data Control, Riviera Beach, Florida).

The mobile phase was a solution of 70% 5mM hexanesulfonic acid, pH 3.75, and 30% methanol. The solution was filtered through

a 0.20 μm pore size membrane filter (Millipore; Bedford, Massachusetts) prior to use. The flow rate was 1 ml/min and UV absorbance was monitored at 280 nm and 305 nm. The latter wavelength was used to avoid the UV-absorbing background in plasma and urine samples. Samples of from 10 μl to 50 μl plasma extract or urine were injected into the HPLC system. The plasma extract was prepared by treating 1 ml of plasma with an equal volume of 6% perchloric acid, neutralizing the supernatant with 1 M KOH, removing potassium perchlorate by centrifugation, and extracting the supernatant with ethyl acetate:2-propanol (10/1). The organic layer was evaporated under nitrogen and the residue was then dissolved in 100 μl of water.

Glass-distilled methanol was obtained from Burdick and Jackson Laboratories (Muskegon, Michigan). Hexanesulfonic acid sodium salt was obtained from Eastman Organic Chemicals (Rochester, New York). Methotrexate (94% pure) was obtained from the National Cancer Institute, Division of Cancer Treatment, Bethesda, Maryland. The 7-hydroxymethotrexate and 4-amino-4-deoxy-N^{10}-methylpteroic acid were gifts from Dr. Ti Li Loo, and the methotrexate polyglutamates were kindly provided by Dr. Charles M. Baugh, University of Southern Alabama, Mobile, Alabama. The 5-methyltetrahydrofolate was purchased from Sigma Company, St. Louis, Missouri.

RESULTS AND DISCUSSION

Figure 1a shows a separation of methotrexate (MTX), 7-hydroxymethotrexate(7-OH MTX), methotrexate diglutamate (MTXG), methotrexate triglutamate ($MTXG_2$), and 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) using paired-ion chromatography reagant at pH 3.75. This system gave a difference of retention times between methotrexate and 7-hydroxymethotrexate of approximately 180 s with two connected Whatman Partisil PXS 10/25 ODS columns. The capacity factor (K') values achieved for methotrexate triglutamate, methotrexate diglutamate, 7-hydroxymethotrexate, methotrexate, and 4-amino-4-deoxy-N^{10}-methylpteroic acid were 1.08, 1.56, 2.03, 2.51, and 8.36, respectively.

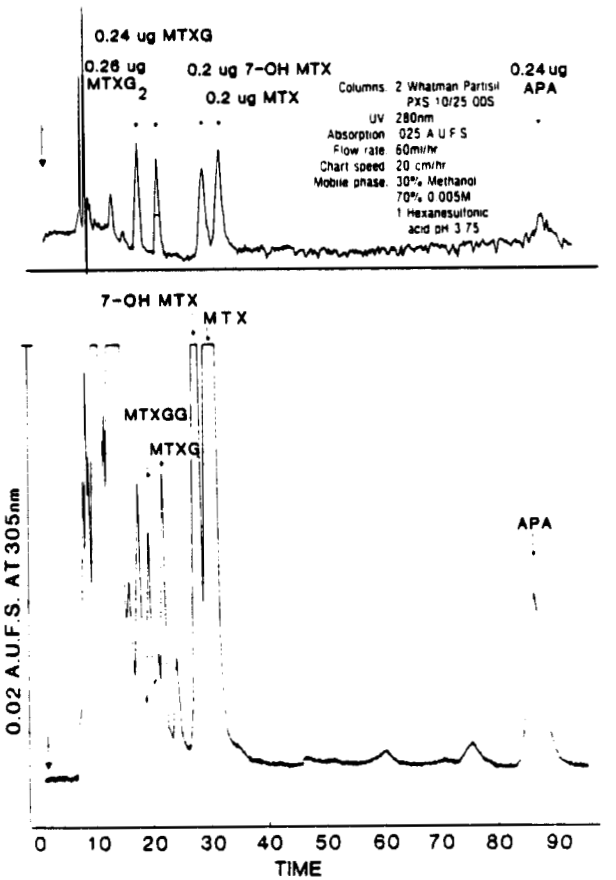

Figure Ia: Isocratic elution of methotrexate and related compounds with two connected Whatman partisil ODS columns.

Figure Ib: A 15 µl injection of patient urine in the same system.

In comparison, the best achieved separation of methotrexate and 7-hydroxymethotrexate with methanol and ammonium formate (0.005 M) at pH 3.5 was 30 s with a flow rate of 1 ml/min.

Standard curves for quantitation of methotrexate and 7-hydroxymethotrexate were derived by adding known quantities of the two compounds to plasma samples, extracting the samples using the procedure outlined, and quantitating the extract on HPLC. The standard curve derived for 7-hydroxymethotrexate was also used for quantitation of 4-amino-4-deoxy-N^{10}-methylpteroic acid since extractions for two concentrations of 4-amino-4-deoxy-N^{10}-methylpteroic acid corresponded to values on the 7-hydroxymethotrexate standard curve. Standard curves for 7-hydroxymethotrexate and methotrexate were linear over the concentration range from 2 μg/ml to 100 μg/ml:R=0.97 for 7-hydroxymethotrexate and R=0.99 for methotrexate.

Reproducibility of the extraction procedure was determined by extracting a plasma sample containing 50 μg/ml methotrexate daily over a 5 day period with the following results: n=7, CV=11%. Another standardization sample containing 10 μg/ml 7-hydroxymethotrexate and methotrexate in water was directly injected daily producing the following values: 7-hydroxymethotrexate n=7, CV=4.6%; methotrexate n=7, CV=6.7%.

Figure 1B illustrates the chromatographic profile of a human urine sample after high-dose methotrexate infusion (200 mg/kg). Since a large quantity of methotrexate appeared in the urine, paired-ion reagent was necessary to give an adequate separation of 7-hydroxymethotrexate and methotrexate. Usually less than 20 μl of untreated urine sample is needed for a suitable chromatogram. The urinary profile showed the prescence of methotrexate, 7-hydroxymethotrexate, and 4-amino-4-deoxy-N^{10}-methylpteroic acid. Under the conditions employed, both methotrexate diglutamate and triglutamate retention areas were superimposed in areas where normal urinary or plasma components appeared. To improve the detection of these metabolites, a variable wavelength UV detector was used at a wavelength of 305 nm. Figure 2 shows comparative chromatographic profiles of a urine sample at 280 nm and 305 nm. These chromatograms clearly demonstrate the advantage of a higher wavelength. For urine samples collected 72 h postinfusion, lyophilization was necessary to increase concentrations.

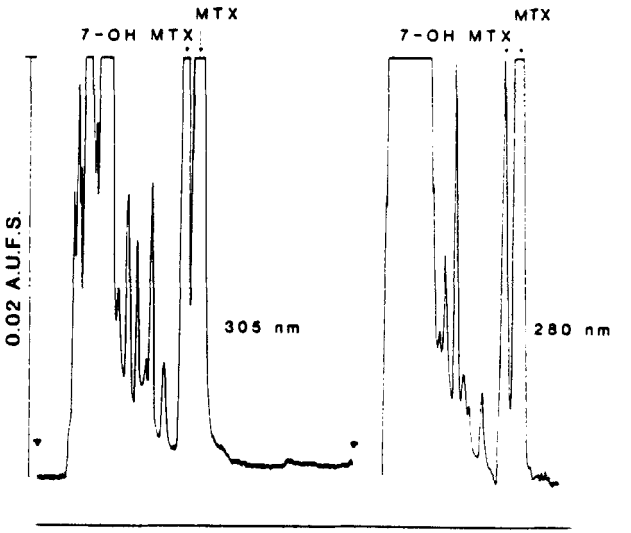

Figure 2: Comparison of urine samples monitored at 280 nm and 305 nm.

In chromatograms of patient urine, in addition to methotrexate and metabolites one can also identify acetazolamide (diamox) and 5-methyltetrahydrofolate. The former was introduced in the treatment regimen as an alkalinizing agent and the latter is the metabolite of citrovorum factor, an agent used in rescue therapy. A chromatographic profile of a human plasma sample after methotrexate is shown in Fig. 3. A single Whatman Partisil ODS column was utilized. Significant amounts of methotrexate, 7-hydroxymethotrexate, and 4-amino-4-deoxy-N^{10}-methylpteroic acid are evident. However, the separation among MTXG, $MTXG_2$ and the therapeutic additives was inadequate.

It has been suggested that the metabolism of methotrexate is dose dependent (7). This has been confirmed by us. With increased methotrexate dosage from 50 mg/kg to 200 mg/kg in children with osteosarcoma, plasma and urinary 7-hydroxymethotrexate increased accordingly (8). The highest detectable 7-hydroxymethotrexate concentration was found to be 2.74×10^{-4} mol/L in plasma and 2.2×10^{-4} mol/L in urine. 4-amino-4-deoxy-N^{10}-methylpteroic acid

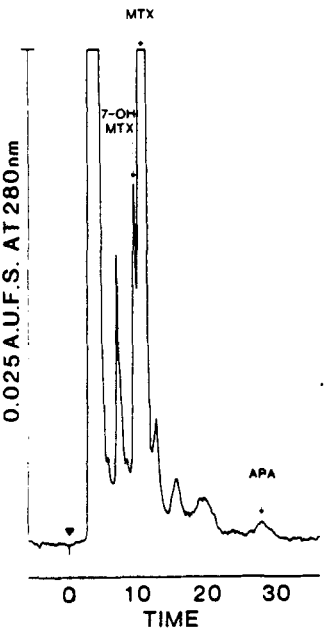

Figure 3: Chromatogram of patient plasma extraction. Single partisil ODS column used in separation. Retention times: 7-OH MTX 545 S., MTX 631 S., APA 1684 S.

does not normally appear in either plasma or urine; however, in these patients with prolonged methotrexate clearance, 4-amino-4-deoxy-N^{10}-methylpteroic acid was identified in both plasma and urine at peak concentrations of 1.6×10^{-5} mol/L and 6.1×10^{-5} mol/L. Initial identification of methotrexate, 7-hydroxymethotrexate and 4-amino-4-deoxy-N^{10}-methylpteroic acid was made utilizing co-chromatography with known samples of the three compounds. Further identification of methotrexate, 7-hydroxymethotrexate, 4-amino-4-deoxy-N^{10}-methylpteroic acid and diamox in the chromatogram has been made by mass spectrometry (Smith, R. G., et al., unpublished). 4-amino-4-deoxy-N^{10}-methylpteroic acid may be a methotrexate metabolite produced by intestinal flora (9).

It is apparent that the paired-ion HPLC system is useful in the pharmacologic study of methotrexate and other folate analogs. Separation of methotrexate and 7-hydroxymethotrexate has been previously achieved with reverse phase ion-paired HPLC, although the retention time for 7-hydroxymethotrexate was greater than 45 min (10). This ability to separate methotrexate from its metabolites may also prove to be important in the understanding of methotrexate toxicity.

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