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# HIGH-PRESSURE LIQUID CHROMATOGRAPHY ANALYSIS OF METHOTREXATE POLYGLUTAMATES IN CULTURED HUMAN BREAST CANCER CELLS

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Methotrexate (MTX) is an antineoplastic agent commonly used in the treatment of acute lymphoblastic leukemia, choriocarcinoma, osteogenic sarcoma, breast cancer and other tumors. Recently, poly-γ-glutamyl metabolites of MTX (MTXGn) have been found in a variety of human tissues including liver (1), cultured skin fibroblasts (2), human breast cancer cells (3), and bone marrow cells (4). Although these derivatives act as inhibitors of dihydrofolate reductase intracellularly (5,6), the extent of their synthesis *in vivo*, transport properties, precise identification and precise role in drug action remain uncertain. Conventional analytical systems for MTXGn's which employ either gel filtration or ion-exchange chromatography are often time-consuming and of limited resolving power. Indeed, poly-γ-glutamyl derivatives greater than glutamyl-glutamyl-methotrexate ($MTXG_2$) have yet to be described for any human cell type. High-pressure liquid chromatography (HPLC) assays have allowed precise identification of several MTX metabolites, including 7-hydroxymethotrexate (7,8) and 2,4-diamino-$N^{10}$-methyl-pteroic acid (DAMPA) (9). This paper describes the isolation and partial characterization of multiple MTXGn's in human tumor cells through the use of HPLC analysis.

## MATERIALS AND METHODS

### Propagation of cells in culture

MCF-7 cells, a line of human breast cancer cells in continuous monolayer culture, were provided by Dr. Marc Lippman (National Cancer Institute, Bethesda, MD). The human derivation, hormonal dependency, and growth characteristics of these cells have been described (10). The cells were grown in 75 $cm^2$ plastic flasks in improved minimal essential medium (NIH Media Unit, Bethesda, MD) supplemented with 10% fetal calf serum, L-glutamine at 584 μg/ml, penicillin at 124 μg/ml, and streptomycin at 270 μg/ml under 95% $O_2$/5% $CO_2$ at 37°. Approximately 1 x $10^7$ cells were used for each experiment.

### Chemicals

[3',5',9-$^3$H]MTX was purchased from Amersham (Arlington Heights, IL). The drug was further purified by DEAE-cellulose chromatography with elution along a linear gradient of 0.1 to 0.4 $NH_4HCO_3$, pH 8.3 (11). Purified synthetic $MTXG_1$, $MTXG_2$ and $MTXG_6$ were provided by Drs. John Montgomery (Southern Research Institute, Birmingham, AL) and C. M. Baugh (Department of Biochemistry, University of South Alabama, Mobile, AL). Acetonitrile (ACN) and tetrabutyl ammonium phosphate (PicA) were obtained from Waters Associates (Milford, MA), and Aquassure liquid scintillation counting fluid from the New England Nuclear Corp. (Boston, MA). All other chemicals were of reagent grade and purchased either from Fisher Scientific Co. (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO).

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Preparation of sample

MCF-7 cells were incubated in medium, without serum or folic acid containing 2 μM [$^3$H]-MTX, 2 mM L-glutamine, 10 μM thymidine, and 10 μM deoxyinosine. This medium allowed cell proliferation to proceed unperturbed in the presence of MTX. After a 24-hr incubation, the medium was aspirated from the flasks and the cells were washed once with ice-cold phosphate-buffered normal saline (PBS), pH 7.4. Cells were scraped off the flask surface with a rubber policeman and, after an additional wash in iced PBS, were resuspended in 1 ml of distilled $H_2O$. Following this, 4 ml of ice-cold 10% trichloroacetic acid (TCA) was added and cellular debris was pelleted by centrifugation at 10,000 g for 15 min. A Sep-Pak $C_{18}$ cartridge (Waters Associates) was prepared by injecting 2 ml of 100% ACN followed by 3 ml of $H_2O$. The cell extract was then injected, followed by 3 ml of $H_2O$ to remove remaining protein. MTX and its metabolites were then eluted with 2 ml of 100% ACN. The sample was evaporated to dryness under nitrogen and resuspended in 200-500 μl of the HPLC mobile phase buffer. Of the radioactivity present in the TCA extract, 87 ± 14% percent was recovered in the ACN eluate.

High-pressure liquid chromatography assay

Assays were done using a model 6000A pump, U6K injector, and 660 solvent programmer from Waters Associates. Retention times of authentic MTX, $MTXG_1$, $MTXG_2$ and $MTXG_6$ were determined by monitoring ultraviolet absorbance at 254 nm on a Waters model 440 absorbance detector. Sample fractions of 25-50 μl were injected on a 30 x 0.39 cm $C_{18}$ μBondapak column (Waters Associates) and eluted at 1 ml/min along a gradient of 30 to 40% ACN in 5 mM PicA for 15 min, followed by 40% ACN/5 mM PicA for the final 20 min of the separation. One-min fractions were collected directly into scintillation vials using an LKB 2112 Redirac fraction collector and assayed for radioactivity by liquid scintillation counting; 80 ± 11 percent of the injected radioactivity was recovered.

Correlation between retention time and the number of glutamyl residues

Using an HPLC method to separate polyglutamate derivatives of folic acid, Cashmore et al have demonstrated a linear correlation between the logarithm of the adjusted retention time and the square root of the number of glutamyl residues in the molecule. The adjusted retention time, $t'_R$, is given by the relationship $t'_R = t_R - t_O$, where $t_R$ and $t_O$ are respectively the retention times of the eluting compound and of an inert substance not retained by the column (12). We found that such a relationship existed for MTXGn's and could thus be used to estimate the γ-glutamyl chain lengths of unknown radioactive compounds detected in the MCF-7 cell extracts. The standard curve for MTXGn's was constructed by isocratic elution of the synthetic MTXGn standards using a mobile phase of 40% ACN/5 mM PicA (Fig. 2). Radioactive compounds purified from cell extracts were eluted under the same conditions and their adjusted retention times plotted on the standard curve from which the γ-glutamyl chain length was determined.

## RESULTS

The chromatography of the MCF-7 cell extract following a 24-hr incubation with 2 μM [$^3$H]-MTX is shown in Fig. 1A. Six radiolabeled peaks were resolved. The first small peak, which does not inhibit dihydrofolate reductase (13), was unidentified while the next three peaks co-chromatographed

with MTX, $MTXG_1$, and $MTXG_2$. The last two peaks, at 27 and 30 min, both inhibited dihydrofolate reductase in a spectrophotometric assay (13) and, on incubation with conjugase (3), reverted to a migration pattern identical to that of MTX, suggesting that they were higher MTXGn's. For comparison, Fig. 1B shows the results of chromatography of the same MCF-7 cell extracts on a Sephadex G-15 column (3). Following a 24-hr incubation with 2 μM [$^3$H]-MTX, a single radiolabeled early-migrating peak that co-chromatographed with authentic $MTXG_2$ was resolved from MTX. By comparison with Fig. 1A, this peak must contain multiple polyglutamate derivatives. Other HPLC systems, including an anion-exchange system with a sodium phosphate buffer gradient and a reverse-phase system with an ACN gradient, failed to resolve the same number of peaks. To more specifically identify the radioactive peaks at 27 and 30 min in Fig. 1A, the γ-glutamyl chain lengths of these compounds were estimated using the standard curve shown in Fig. 2. The peak at 27 min (point x) corresponded to a compound containing 4.2 glutamyl residues ($MTXG_3$) and the peak at 30 min (point y) to a compound containing 5.2 glutamyl residues ($MTXG_4$).

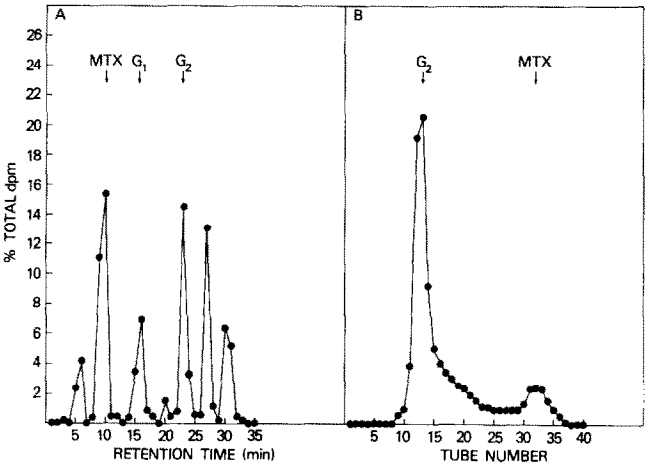


FIGURE 1. Separation of MTX polyglutamates. After incubation with 2 μM [$^3$H]-MTX, MCF-7 cell extracts were chromatographed on the HPLC system (A) or on a Sephadex G-15 column (B).

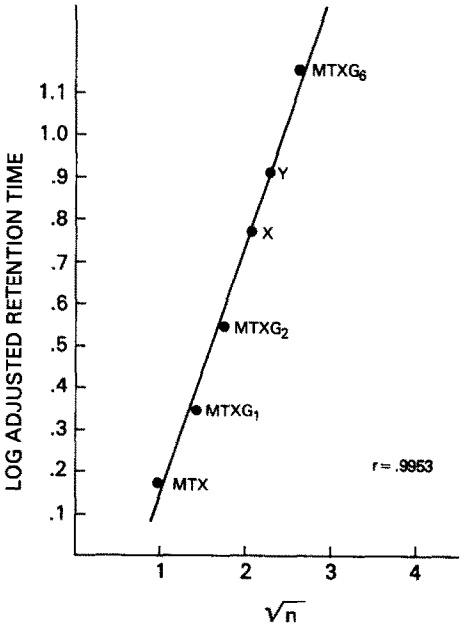


FIGURE 2. Structure-retention relationships of the MTX polyglutamates. Plot of the adjusted retention time versus the square root of the number of glutamyl residues for synthetic MTXGn's. Points x and y represent, respectively, the adjusted retention times of the peaks at 27 and 30 min in Fig. 1A.

## DISCUSSION

In recent years, considerable interest has developed over the intracellular metabolism of MTX to polyglutamate forms. Previous studies have established that these metabolites are synthesized by a variety of human and animal tissues (1-6,14-16), that their synthesis is dose- and time-dependent (2-4,6) and tissue-specific (15), that they bind readily to dihydrofolate reductase intracellularly (3,6), and that their formation is associated with prolonged inhibition of DNA synthesis (5,6). Despite this intense interest, considerable controversy still exists concerning their capacity to be retained intracellularly, the mechanisms involved in regulating their formation, and the extent of polyglutamate synthesis by human tumor cells and normal host tissues. Many previous studies of MTX polyglutamate synthesis have been hampered by the lack of a rapid, sensitive, and highly reproducible assay system.

The ion-pair HPLC analytical system described in this paper has enabled us to demonstrate the synthesis of multiple polyglutamate forms of MTX in human breast cancer cells. While the formation of $MTXG_1$ and $MTXG_2$ has been demonstrated in a number of cell types using gel filtration or ion-exchange systems (2-6,15,16), precise identification of metabolites was previously not possible due to the limited resolving powers of these techniques. The demonstration of at least four distinct MTXGn's formed by human tumor cells requires a reappraisal of many conclusions regarding the pharmacologic properties of these compounds. Important questions remain concerning the exact types of MTXGn's synthesized by normal and malignant tissues. Precise characterization of the structure and pharmacologic properties of the intracellular MTXGn's should now be possible using the discrete peaks obtained in the ion-pair system.

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