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Note

Separation and identification of methotrexate and its metabolites, 7-hydroxymethotrexate and polyglutamates, in human tissues by reversed-phase high-performance liquid chromatography coupled with radioimmunoassay

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The intracellular conversion of methotrexate, 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (MTX), to polyglutamate forms has been demonstrated in a variety of normal and malignant human cells [1]. Results obtained from in vitro studies have shown that the polyglutamation is an important determinant of drug sensitivity in certain malignant cell lines [1]. However, the extent of polyglutamate formation in tumor cells and normal host tissues as well as role(s) of polyglutamates in the antitumor activity in cancer patients receiving MTX therapy have not been fully elucidated.

To investigate the metabolism of MTX, in particular the degree of polyglutamate synthesis in cancer patients receiving MTX therapy, we have examined clinical specimens obtained from a patient who had received high-dose MTX therapy. We report a method of high-performance liquid chromatography (HPLC) coupled with a radioimmunoassay (RIA) to separate and identify MTX and its polyglutamate forms in the specimens. Our results indicate that, in addition to MTX and its metabolite, 7-hydroxymethotrexate (7OH-MTX), small amounts of polyglutamates were also detectable in various tissues shortly after high-dose MTX infusion.

EXPERIMENTAL

Human tissues

The use of human tissues in this investigation has been approved by our Institutional Review Board at the University of Texas System Cancer Center, M.D.

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Anderson Hospital and Tumor Institute (Houston, TX, U.S.A.). Tissues were obtained at the time of autopsy from a patient with disseminated squamous carcinoma who had received a 6-h high-dose MTX infusion (150 mg/kg) and died of the disease 16 h after the infusion. Tissue samples were stored at −70 °C until use.

Chemicals

Both MTX and 7OH-MTX, isolated from rabbit urine after MTX infusion [2], were purified prior to use according to the methods previously described [3,4]. Authentic standards of 4-NH_2-N^{10}-CH_3-$PteGlu_2$ (MTX-G_1) to -$PteGlu_7$ (MTX-G_6) were purchased from Medical Science Foundation, University of Southern Alabama (Mobile, AL, U.S.A.). Hexanesulfonic acid (HSA) sodium salt was obtained from Eastman (Rochester, NY, U.S.A.).

Equipment

A Laboratory Data Control (LDC, Riviera Beach, FL, U.S.A.) HPLC system with a Nova-Pak™ C_{18} column (150×3.9 mm I.D., particle size 5 μm) (Waters Assoc., Milford, MA, U.S.A.) was used. The system was equipped with a UV detector (LDC Spectromonitor III) and an electronic integrator, Model CSI-38 (Columbia Scientific Industries, Austin, TX, U.S.A.). For the analyses of clinical specimens, an C-130B precolumn of dry-packed Perisorb RP-18, 30–40 μm pellicular (Upchurch Scientific, Oak Harbor, WA, U.S.A.) was routinely used.

Chromatographic procedure

Chromatography was performed at room temperature with a linear gradient of 7.25–25.25% methanol in 5 m*M* HSA solution, pH 3.75 over 20, 40, or 60 min as needed. A flow-rate of 1.0 ml/min and a back-pressure of 8–12 · 10^6 Pa were used. The UV absorbance was monitored at 305 nm. The capacity factor (k') of each authentic compound was determined using the following formula: $k' = [V_R(\text{retention volume}) - V_M(\text{void volume})]/V_M$. Tissue extracts were prepared for HPLC analyses using a method described previously [2]. Briefly, 10% (w/v) crude tissue homogenates of clinical specimens were made in 10 m*M* sodium ascorbate, pH 7.2, and boiled for 10 min to denature proteins. The supernatant fluids from the boiled samples were extracted with methanol using C_{18} Sep-Pak cartridges (Waters Assoc.). The methanol extracts were evaporated under nitrogen gas and the dried residues were dissolved in 100–200 μl of distilled water. A volume of 20–200 μl of authentic chemicals or human tissue extracts was applied for each HPLC analysis.

In order to separate and quantitate MTX and its metabolites, clinical specimens were chromatographed over 60 min, and 1-ml fractions of HPLC eluates were collected for RIA. The fractions containing each form of polyglutamates were identified by comparing the retention time of each fraction with that of authentic polyglutamates as well as cochromatography of the specimens with authentic polyglutamates.

Quantitation of MTX and 7OH-MTX

Purified MTX and 7OH-MTX at serial concentrations (100–2000 ng) were cochromatographed, and the areas under the peaks of the drugs at each concentration were computed by an integrator. Experiments were done in triplicate. A linear relation was observed between the amounts of the drugs tested and the areas under the peaks. The amounts of MTX and 7OH-MTX in tissue extracts were determined by plotting the areas under the peaks of MTX and 7OH-MTX obtained by HPLC analyses of the specimens against the areas under the peaks of known amounts of the drugs.

Radioimmunoassay

To quantitate polyglutamates in HPLC eluates, RIA was performed according to the method described previously [5,6], using methotrexate [^{125}I] RIA kits (Diagnostic Biochemistry, San Diego, CA, U.S.A.). The reactivity of each specimen was expressed as percentage radioactivity precipitated by the specimen as compared with the radioactivity precipitated in the control reaction mixtures containing a standard anti-MTX serum and [^{125}I]MTX. Experiments were done in duplicate, and the mean reactivity was obtained for each specimen. The amounts of polyglutamates in HPLC eluates were determined using a standard reaction curve for each of the polyglutamate forms which had been obtained with known amounts of the authentic compounds.

RESULTS

With authentic compounds, a 20-min chromatography yielded a good separation of each compound. This system gave a capacity factor (k') value (mean $\pm$ 1 S.D.) of 27.1 ± 0.4 for MTX and 25.0 ± 0.3 for 7OH-MTX determined by five separate HPLC analyses. The k' values achieved for MTX-G_1–MTX-G_6 were 21.2 ± 0.2, 17.3 ± 0.3, 14.9 ± 0.2, 12.5 ± 0.3, 10.6 ± 0.3 and 9.3 ± 0.2, respectively. Fig. 1A shows a chromatogram of the authentic compounds which were separated over 60 min. Retention times obtained from eight different HPLC analyses (mean $\pm$ 1 S.D.) were 54.7 ± 1.1 min for MTX, 48.5 ± 1.2 min for 7OH-MTX and 40.5 ± 1.5, 31.7 ± 2.2, 26.5 ± 2.6, 22.3 ± 2.7, 18.9 ± 2.2 and 16.5 ± 2.4 min for MTX-G_1–MTX-G_6, respectively. These differences in the retention time among the compounds enabled us to separate each of them when 1-ml fractions of the HPLC eluates were collected.

Fig. 1B illustrates a representative chromatographic profile of a specimen (normal liver). As seen, two major peaks representing 7OH-MTX and MTX were obtained. In addition, the chromatogram revealed numerous minor peaks. In order to quantitate polyglutamate forms in the specimen, the fractions collected were tested by RIA. As further seen in Fig. 1B, various amounts of MTX-G_1–MTX-G_4 were detected by this method. A standard curve for each form of polyglutamates shown in Fig. 2 served to determine the concentrations of the drugs in the eluates. Each of the authentic MTX and its polyglutamates demonstrated variable sensitivities by RIA (MTX, $G_1 > G_2$, G_3, $G_4 > G_5$, G_6). The reactivity of 7OH-MTX was lower than those of polyglutamates (data not shown).

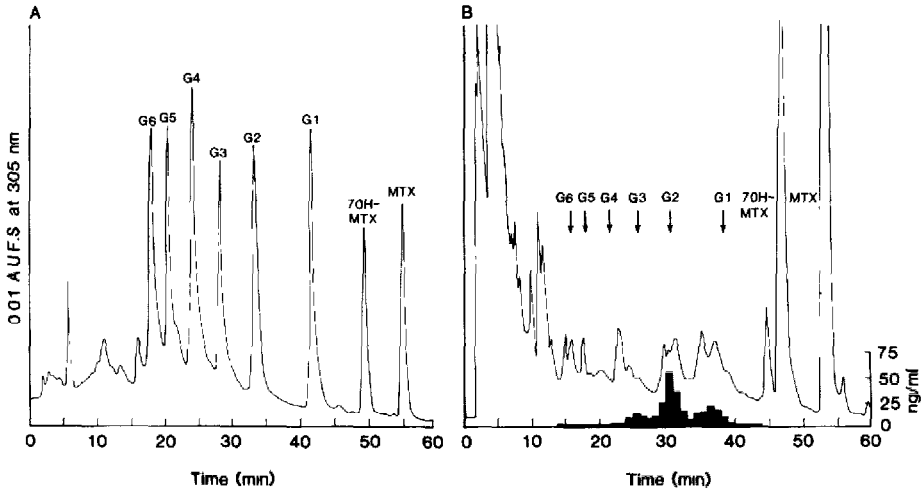

Fig. 1. HPLC profiles of MTX and its related compounds (A) and a human tissue specimen (B). (A) MTX, 7OH-MTX and MTX polyglutamates (G_1–G_6) were cochromatographed over 60 min as described in Experimental. (B) A normal liver tissue specimen was obtained from a patient 16 h after a high-dose MTX infusion and was subjected to HPLC analysis. Fractions (1 ml) of the eluates were collected and examined by RIA to quantitate MTX polyglutamates in the eluates. Arrows (G_1–G_6) indicate the positions of polyglutamates eluted. Bars represent concentrations of polyglutamates in the eluates.

Table I demonstrates concentrations of MTX, 7OH-MTX, and MTX polyglutamates detected in tumors and normal tissues of the patient. The major form of the drugs detected in the tissues was MTX. The amounts of 7OH-MTX detected varied from tissue to tissue. In addition, small amounts of polyglutamates (G_1–G_4) were found in both normal and tumor tissues.

DISCUSSION

In previous studies, MTX polyglutamates, MTX-G_1 and MTX-G_2, were separated using gel filtration or ion-exchange systems [7–9]. These techniques, however, have a limited use due to low resolving powers. Recently, HPLC has been used for the separation of MTX and 7OH-MTX polyglutamates from cultured human cells [10–12].

In the present study, we have developed a highly reproducible method of

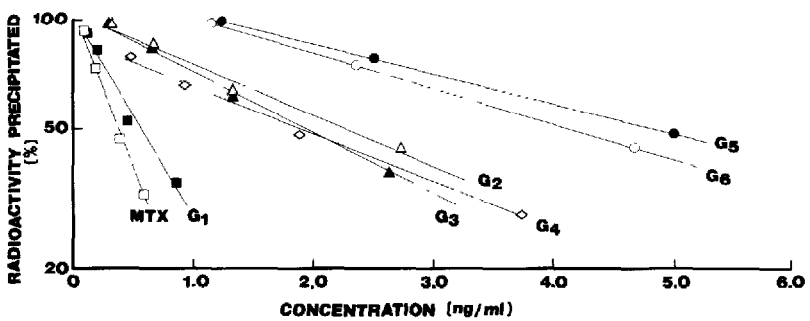

Fig. 2. Reactivity of MTX and MTX polyglutamates (G_1–G_6) by RIA

TABLE I

CONCENTRATIONS OF MTX, 7OH-MTX AND MTX POLYGLUTAMATES DETECTED BY HPLC COUPLED WITH RIA IN TUMORS AND NORMAL TISSUES OF A PATIENT WHO RECEIVED A HIGH-DOSE MTX INFUSION

N.D. = not detected.

Tissue	Concentration (pg/μg of protein)					
	MTX	7OH-MTX	G_1	G_2	G_3	G_4
Liver, normal	71.4	31.7	4.5	11.8	3.3	0.6
Liver, tumor	133.5	3.9	0.9	5.7	8.3	N.D.
Brain, normal	60.2	7.3	0.3	4.0	1.8	N.D.
Brain, tumor	317.3	2.6	0.5	4.0	2.4	N.D.

reversed-phase HPLC with a high resolving power. With this technique, we have been able to separate MTX, 7OH-MTX, and MTX polyglutamate forms (G_1–G_6) over 20–60 min periods as needed. To detect polyglutamates in clinical specimens, we have applied our HPLC system in combination with a commercially available RIA of HPLC eluates. The results have shown the presence of polyglutamates (G_1–G_4) in tumors and normal tissues of a patient who received a high-dose MTX infusion in good agreement with the results reported by others [13,14]. Our approach has provided a means to quantitate each form of polyglutamates present in the tissues of patients who have received MTX infusion for their malignancies.

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