Plasma Methotrexate as Determined by Liquid Chromatography, Enzyme-Inhibition Assay, and Radioimmunoassay after High-Dose Infusion

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Three techniques for measuring methotrexate show various cross reactivities with methotrexate-related compounds: "high-pressure" liquid chromatography, by principle, is virtually specific for methotrexate; the enzyme-inhibition assay quantitates methotrexate, methotrexate diglutamate, and methotrexate triglutamate equally well, but has a 10% cross reactivity with 4-amino-4-deoxy-N\(^{10}\)-methylpteroic acid and 1% with 7-hydroxymethotrexate; radioimmunoassay shows an equal cross reactivity with methotrexate, 4-amino-4-deoxy-N\(^{10}\)-methylpteroic acid, methotrexate diglutamate and triglutamate, and a 5 to 10% cross reaction with 7-hydroxymethotrexate. Radioimmunoassay almost always yielded the highest values for methotrexate, followed by enzyme-inhibition assay then liquid chromatography. The presence of two methotrexate-related compounds, 7-hydroxymethotrexate and 4-amino-4-deoxy-N\(^{10}\)-methylpteroic acid, was confirmed in human urine samples and quantitated in patients' plasma by liquid chromatography, the respective maximum plasma concentrations being 250 and 16 \(\mu\)mol/L. Materials cross reacting with methotrexate in radioimmunoassay of chromatographic fractions from plasma were also noted in fractions corresponding to methotrexate diglutamate and triglutamate peaks, in quantities estimated to be 47 and 30 nmol/L methotrexate equivalents, respectively. 7-Hydroxymethotrexate is eliminated more slowly than methotrexate and its production increases with dosages of methotrexate.

Additional Keyphrases: drug assay - drug metabolism - cancer

The concept of high-dosage methotrexate therapy concurrent with citrovorum-factor rescue in cancer chemotherapy has been developed in the past decade (1). Methotrexate in doses up to 500 mg/kg of body weight has been infused into patients for the treatment of several malignant diseases, with various degrees of response (2). Because severe, even fatal, toxicity can result, quantitation of methotrexate in the patient's biological fluids, particularly plasma or serum, is mandatory (3).

There are several methods for measuring the drug concentrations: fluorometry, radioassay, radioimmunoassay (RIA)\(^{2}\), enzyme-multiplied immunoassay technique, enzyme-inhibition assay (EIA), and microbiological assay (4). Recently, the use of "high-pressure" liquid chromatography (HPLC) after oxidation of plasma samples has also been suggested for quantitation of this drug (5).

Several investigators (4, 6, 7) have studied the metabolism of methotrexate in humans. 7-Hydroxymethotrexate has been identified as a metabolite in plasma (8) and urine (4) by column or liquid chromatography and ultraviolet spectrophotometry, and methotrexate diglutamate has been detected in erythrocytes, leukemic cells (6), and liver tissue in humans (9). Methotrexate triglutamate has been also reported in human erythrocytes and leukemic cells (6), and 4-amino-4-deoxy-N\(^{10}\)-methylpteroic acid is present as a metabolite in plasma and urine of patients receiving high doses of methotrexate (10). Using HPLC, we quantitatively determined the metabolites after high-dose therapy with methotrexate. We also investigated the influence of these metabolites on EIA and RIA.

Materials and Methods

Materials

Methotrexate diglutamate and triglutamate were furnished by Dr. C. M. Baugh, Department of Biochemistry, the University of Southern Alabama Medical School, Mobile, AL. 7-Hydroxymethotrexate was a gift from Dr. T. L. Loo. 4-Amino-4-deoxy-N\(^{10}\)-methylpteroic acid was prepared by treating methotrexate with carboxypeptidase G (14). 1-Hexanesulfonic acid sodium salt was obtained from Eastman Organic Chemicals, Rochester, NY 14650. "Ultraviolet" grade methanol was purchased from Burdick and Jackson Labs., Muskegon, MI 49442. Sources of methotrexate, dihydrofolate reductase, and the RIA kit for methotrexate quantitation were as reported previously (11).

Clinical Aspects

Samples of whole blood were obtained from children with osteosarcoma who were being treated with high-dosage methotrexate and citrovorum-factor rescue. Informed consent for both therapy and sampling was obtained from the patient or the parents, or both. Patients' ages ranged from six to 22 years (median, 15 years). Methotrexate (50 to 250 mg/kg of body weight), dissolved in 500 or 1000 mL of aqueous dextrose (50 g/L), was administered by continuous intravenous drip during 6 h. Urinary alkalization with acetazolamide (Diamox) was started 12 h before infusion, concomitant with a single dose of vincristine (2 mg/m\(^2\) of body surface). Citrovorum factor was given intravenously every 3 h for a total of nine doses, then intramuscularly every 6 h for the next 48 h. Intravenous fluid with sodium bicarbonate in doses of 50 mmol/L was started immediately after the infusion and continued for 48 h. Urinary pH was monitored closely and was kept within the range 7.0 to 7.5 for 72 h after the infusion. Use of concomitant medications that might increase renal or hepatic toxicity was avoided.

Venous blood, 2 to 3 mL, was sampled into evacuated blood-collection tubes containing ethylenediaminetetraacetate before and immediately after methotrexate infusion.

Determination of Methotrexate and Its Metabolites

The concentration of methotrexate in plasma was determined by EIA and RIA as described previously (11). We separated components in the extract of plasma by HPLC, using the Glenco HPLC system (Glenco Scientific, Inc.,

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\(^{2}\) Nonstandard abbreviations used: RIA, radioimmunoassay; EIA, enzyme-inhibition assay; HPLC, "high-pressure" liquid chromatography.

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Results

Identification of metabolites and methotrexate was first based on retention time on HPLC as compared with authentic methotrexate, 7-hydroxymethotrexate, methotrexate diglutamate, methotrexate triglutamate, and 4-amino-4-deoxy-N\textsuperscript{10}-methylpteroyl acid, and co-chromatography with an authentic compound. Concentrations of materials with retention times corresponding to those of methotrexate diglutamate and methotrexate triglutamate were estimated byRIA after separating methotrexate and related materials by HPLC. The eluate was collected in fractions, and methotrexate RIA was subsequently used to quantitate the active materials in them. The urinary counterparts of 7-hydroxymethotrexate and 4-amino-4-deoxy-N\textsuperscript{10}-methylpteroyl acid, isolated by chromatography on diethylaminoethyl-cellulose (12), were confirmed by ultraviolet spectrophotometry and by mass spectrometry. Details of results have been presented (13).

Fig. 1. Chromatograms of representative plasma samples before (left) and 6 h after (right) methotrexate (MTX) infusion
7 OHMTX, 7-hydroxymethotrexate; APA, 4-amino-4-deoxy-N\textsuperscript{10}-methylpteroyl acid

Fig. 2. Plasma 7-hydroxymethotrexate and methotrexate clearance in patients
Plasma methotrexate was quantitated by both HPLC and EIA; 7-hydroxymethotrexate was quantitated by HPLC alone. n = 3 for all points except for 48 h after methotrexate, where n = 1

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Addition of 1-hexanesulfonic acid to the mobile phase made it possible to separate methotrexate and 7-hydroxymethotrexate through a paired-ion mechanism.

This method allowed a separation of methotrexate and 7-hydroxymethotrexate in 1 to 1.25 min, under the conditions we used. However, the methotrexate diglutamate and triglutamate peaks, although separated from the 7-hydroxymethotrexate and methotrexate peaks, were still obscured by normal plasma components. Under our experimental conditions the retention times (in seconds) were: methotrexate triglutamate, 375; methotrexate diglutamate, 460; 7-hydroxymethotrexate, 545; methotrexate, 631; and 4-amino-4-deoxy-N\textsuperscript{10}-methylpteroyl acid, 1684; the CV was <9% for each retention time.

In the case of plasma extracts, we used HPLC to quantitate methotrexate, 7-hydroxymethotrexate, and 4-amino-4-deoxy-N\textsuperscript{10}-methylpteroyl acid in amounts up to 0.1 µg, with CV’s of 10 to 20%. Methotrexate diglutamate and triglutamate could be quantitated to a comparable amount by adding another column to the one in use. The two-column system can substantially increase the retention times for methotrexate diglutamate and triglutamate and thus move these two peaks from the areas occupied by polar components of normal urine.

Figure 1 shows the chromatograms for plasma samples before and 6 h after the start of the infusion. The latter clearly demonstrates the presence of 7-hydroxymethotrexate, methotrexate, and 4-amino-4-deoxy-N\textsuperscript{10}-methylpteroyl acid. Figure 2 shows the clearance of plasma methotrexate and the 7-hydroxymethotrexate for patients who had received 200 mg of methotrexate per kilogram of body weight. The slower
Methotrexate; 7-hydroxymethotrexate served as a quantitating antibody, but 7-hydroxymethotrexate showed a 5 to 10% cross reactivity (Figure 5).

Because of these variations, we compared results by EIA and RIA for samples obtained at four different intervals after infusion. The correlation was best ($r = 0.973$) at 72 h (Table 1). The slopes of the correlation curves at 6, 24, and 48 h were greater with correlation coefficients of 0.566, 0.8403, and 0.8702, respectively. Evidently RIA almost always yields higher values for methotrexate than does EIA. The results in Figure 2 suggest that, for the same plasma samples, HPLC is more specific than EIA for methotrexate, further confirming that both EIA and RIA are inferior for specifically quantitating methotrexate itself.

### Discussion

In some early clinical trials with high-dose methotrexate and citrovorum-factor rescue, the fatality rate was 6% (15). Because of this severe toxicity, quantitation of drug in the blood became mandatory. Our earlier results with EIA indicate that if methotrexate concentrations in plasma are $>1 \mu\text{mol/L}$ 48 h after an infusion, one of every 200 infusions will result in severe toxicity (3). Similar experiences have been reported by others (16).

Toxicity after high-dose infusions is not dose-related (up to 250 mg/kg body weight), but it is age-related (3); patients older than 15 years had a higher frequency of severe to moderate toxicity than patients who were younger (3). Further, patients receiving more than 10 infusions experienced symptoms of toxicity significantly more frequently than those receiving fewer infusions (3).

Because age and frequency of administration affect metabolism (11, 18), we undertook investigations of the metabolism of the drug. Our results by HPLC were almost always lower than those by EIA. Although HPLC is more specific in quantitating methotrexate per se, its metabolites may induce a different type of toxicity. Thus, the comparative usefulness of these various methods needs to be carefully assessed. For instance, 7-hydroxymethotrexate is less soluble in aqueous solution than is methotrexate, and so precipitation in the kidney is more likely, with concomitant renal dysfunction. However, 7-hydroxymethotrexate and 4-amino-4-deoxy-N<sup>10</sup>-methylpteroyl acid inhibit dihydrofolate reductase (EC 1.5.1.3) less effectively than does methotrexate. This enzyme has been generally accepted as the target protein for methotrexate antitumor activity. There is no further direct evidence that humans produce 4-amino-4-deoxy-N<sup>10</sup>-methylpteroyl acid as a metabolite of methotrexate. 4-Amino-4-deoxy-N<sup>10</sup>-methylpteroyl acid was, however, found as a contaminant in certain batches of clinical-grade methotrexate (19).

Further, a number of pharmacokinetic analyses of high-dose methotrexate need to be re-evaluated because of the poor amino-4-deoxy-N<sup>10</sup>-methylpteroyl acid is only 10% as sensitive and of 7-hydroxymethotrexate only 1% as sensitive. With our RIA method, methotrexate, 4-amino-4-deoxy-N<sup>10</sup>-methylpteroyl acid, and methotrexate diglutamate and triglutamate all cross reacted equally with the methotrexate antibody, but 7-hydroxymethotrexate showed a 5 to 10% cross reactivity (Figure 5).

### Table 1. Serum Methotrexate Concentrations as Determined by Enzyme-Inhibition Assay (EIA) and Radioimmunoassay (RIA)

<table>
<thead>
<tr>
<th>Time, h</th>
<th>N</th>
<th>EIA (A) Methotrexate concn, μmol/L (SD)</th>
<th>RIA (B) Methotrexate concn, μmol/L (SD)</th>
<th>P (A vs B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>35</td>
<td>5.71 (2.90)</td>
<td>10.73 (7.15)</td>
<td>0.0005</td>
</tr>
<tr>
<td>24</td>
<td>33</td>
<td>7.85 (8.91)</td>
<td>14.18 (14.09)</td>
<td>0.025</td>
</tr>
<tr>
<td>48</td>
<td>33</td>
<td>0.69 (0.59)</td>
<td>1.61 (1.49)</td>
<td>0.0025</td>
</tr>
<tr>
<td>72</td>
<td>35</td>
<td>1.10 (1.83)</td>
<td>1.46 (2.23)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

*Interval, in hours, from the start of methotrexate infusions.
specificity of the methods, particularly in the case of patients with prolonged drug clearance after high-dose methotrexate infusion. In one infusion eliciting excessive toxicity, only 7-hydroxy methotrexate (10 μmol/L) was detected in plasma 144 h after the start of infusion.

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References