Liquid-Chromatographic Monitoring of 5-Methyltetrahydrofolate in Plasma

Paolo Giulidori, Marzia Galli-Kienle, and Giorgio Stramentinolli

The liquid-chromatographic measurement of 5-methyltetrahydrofolate in biological fluids is described. The sensitivity of the spectrophotofluorometric detector used allows direct evaluation of basal concentrations of the compound in plasma. Because it is resolved from the other common folates and from methotrexate, the procedure is suitable for monitoring it in plasma of patients receiving high-dose therapy with methotrexate.

Additional Keyphrases: monitoring therapy • cancer • folate

Methotrexate in high doses, with 5-THF "rescue," has been used clinically for several years in the treatment for various cancers (1-5). Recently, a "rescue" effect on the side effects of HDMT in tumor treatment has been also demonstrated with 5-MTHF, the predominant circulating form of folate (6-8), in experimental animals (9-11) and man (12, 13). As compared with 5-THF, 5-MTHF was found to be more effective, and less toxic, in Walker 256 carcinosarcoma-bearing rats and in CDF/1 mice with leukemia L 1210 (11). The life span in CDF/1 mice with MTX-treated lymphatic leukemia was also more prolonged after 5-MTHF than after 5-THF (11). In man, the protection against MTX toxic effects afforded by 5-MTHF was demonstrated in various malignant neoplasms (12, 13), and it exceeded that obtained by 5-THF (12).

An adequate concentration of 5-MTHF in the blood is required in the first phase of "rescue," to inhibit entry of the antifolic agent into the cell. For monitoring 5-MTHF in plasma in the presence of MTX or other folates, we describe a simple HPLC method, which allows evaluation even of the basal concentrations normally present in plasma. This procedure may also be useful when 5-THF "rescue" is used, because 5-THF is converted to 5-MTHF in vivo (7, 9).

Materials and Methods

Reagents and standards. All reagents were of analytical or chromatographic grade and the solutions were prepared with freshly distilled water.

Tetrahydroammonium phosphate solution (PIC A) was from Waters Associates, Milford, MA. DHF, THF, and p-ABG standards were obtained from Sigma Chemical Co., St. Louis, MO; calcium 5-THF was from Bracco S.p.A., Milan, Italy; FA from Roche S.A., Basel, Switzerland; sodium MTX was from Lederle Laboratories, American Cyanamid Co. Calcium 5-MTHF was synthesized according to Blair and Saunders (14); the structure of the synthetic compound was confirmed by nuclear magnetic resonance analysis.

BioResearch Co., Research Laboratories, 20060 Liscate (Milan), Italy.

1 Institute of Chemistry, University of Milan School of Medicine, Milan, Italy.
2 Address correspondence to this author.
3 Nonstandard abbreviations used: 5-MTHF, 5-methyltetrahydrofolate; DHF, dihydrofolate; THF, tetrahydrofolate; p-ABG, p-aminobenzoylglutamate; 5-THF, 5-formyltetrahydrofolate; FA, folate; MTX, methotrexate; HDMT, high-dose methotrexate; and HPLC, "high-performance" liquid chromatography.

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All standard folates were dissolved in phosphate buffer (0.1 mol/L, pH 7.4) containing 5 mL of mercaptoethanol per liter, and analyzed by HPLC under three different conditions: (a) paired-ion chromatography, as described later; (b) reversed-phase chromatography in a Perkin-Elmer HC ODS Sil-X (2.6 × 25 cm) column, eluting with 1 mL/min of a mixture of 0.1 mol/L acetic acid/methanol/mercaptoethanol (9/18/0.5 by vol), with final pH adjusted to 3.5 with 1 mol/L NaOH; (c) ion-exchange chromatography in a Whatman Partisol SCX (0.46 × 25 cm) column, eluting with a linear gradient of ammonium formate buffer, pH 4.5 to 500 mmol/L in 20 min, flow rate 1 mL/min.

Under the conditions here described, only THF showed any measurable decomposition. FA, DHF, p-ABG, 5-THF, and 5-MTHF, recovered from the HPLC eluates (system a) and re-injected, each gave a single peak with the same retention time as in the first analysis.

Animal treatment. Four male beagle dogs (14–20 kg), fasted overnight, were treated with 1 mg of 5-MTHF per kilogram body weight, intravenously. Blood was sampled 0, 2, 5, 10, 20, and 40 min, and 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24 h after the injection.

Sample preparation. Heparinized blood was stored at 0–4 °C before centrifugation, also at 4 °C. Ascorbic acid (5 mg/mL) was added to the plasma, and the specimens were frozen and kept at −20 °C until analysis. Then, 4 μL of 1 mol/L Na2CO3 was added to 0.3 mL of plasma and the mixture was stirred and heated in boiling water for 5 min (15). After cooling on ice and breaking the protein clot with a stainless-steel needle, we centrifuged the specimens in a Beckman Microfuge for 5 min. Aliquots (10–50 μL) of the supernatant fluid were injected into the chromatograph.

Under these conditions, mean analytical recovery of added 5-MTHF from plasma was 95% (SEM, 3%, n = 10), ensuring that there was no decomposition of the compound during boiling the sample in the presence of ascorbate.

Chromatographic separation. We used a Perkin-Elmer liquid chromatograph equipped with two pumps, series 3, a variable-wavelength ultraviolet detector LC 75, and a spectrophotofluorometer 650–10 LC. The detector was set at 295 and 350 nm for excitation and emission, respectively. The separation was performed at room temperature on a Lichrosorb RP-18 5μm, 0.4 × 25 cm column (Merck) by eluting at 1 mL/min with a mobile phase, used also for the column conditioning, that we prepared as follows: a 25 mmol/L solution of tetrahydroammonium phosphate in methanol was filtered through a 0.2-μm membrane (Sartorius GmbH, Göttingen, F.R.G.). To 200 mL of the filtrate 0.1 mol of H3PO4 and 680 mL of freshly glass-distilled water were added. The pH was adjusted to 4.5 with 1 mol/L NaOH and the solution was diluted to a final volume of 1 L, filtered through a 0.2-μm membrane, and degassed under reduced pressure. Such a solution gives noticeably less baseline noise.

Results

Figure 1 illustrates the resolution of the tested folates from one another and from MTX under the chosen conditions. The fluorometric analysis (Figure 1) shows that MTX, 5-THF, and FA are not revealed at the wavelengths used when 5-MTHF gives a very intense signal. THF gives rise to three
peaks; the only one detectable by spectrophotofluorometry was at the retention time of p-ABG, shorter than that of 5-MTHF. To check the linearity of the method, we analyzed human plasma samples with known amounts of 5-MTHF added. Figure 2 shows the results obtained for two concentration ranges, 10 to 100 μg/L and 2 to 10 mg/L.

The slopes of the lines obtained by regression analysis for calibration curves in the lower concentration range, prepared by using plasma from two subjects, were: 5.03 (SEM 0.23, r² = 0.996) and 5.79 (SEM 0.57, r² = 0.972), respectively. The slope of the line for the higher concentration range (Figure 2b) was 5.31 (SEM 0.72, r² = 0.999). Basal 5-MTHF concentrations of the two plasma samples used for preparation of the standard curves were found to be 7.4 and 7.5 μg/L, in good agreement with those resulting from the regression lines calculated for calibration curves on the same plasma samples: 6.7 ± 2.7 and 8.2 ± 2.5 (Figure 2). Basal 5-MTHF concentrations, measured in nine ostensibly healthy subjects, were 5.12 ± 0.44 μg/L, in accordance with those determined by authors (7) who used microbiological assay (4.8 ± 0.28).

By the present method, 5-MTHF plasma kinetics were determined after intravenous administration of the drug to four dogs. Figure 3 shows the concentrations in plasma and the mean plasma decay curve. Results obtained for 5-MTHF for some persons being treated with HDMT are reported in Table 1.

Discussion

HPLC separation of 5-MTHF from other folates has been described (15-19). Both ion-exchange (15-17, 19) and ion-pair HPLC (15, 18) have been reported, the latter yielding a better separation than the former. For the detection and quantitative evaluation of folates in biological fluids Allen and Newman (18) suggest the use of C₁₈ reversed-phase ODS 5-μm columns and a gradient of methanol/water containing PIC A for elution of the compounds. These authors could separate 5-MTHF from p-ABG, 5-fTHF, DHF, and FA, with a detection limit of 15-50 ng for the various folates; MTX was not eluted. Adding anti-oxidants to the sample before analysis avoids 5-MTHF decomposition, but THF is still unstable, giving rise to several peaks. To obtain high sensitivity for folate detection

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**Table 1. 5-MTHF Concentration in Plasma of Six Male Patients on HDMT with 5-MTHF “Rescue” a**

<table>
<thead>
<tr>
<th>Patient's age, yr</th>
<th>Diagnosis</th>
<th>MTX dose, g</th>
<th>5-MTHF concn, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>non Hodgkin's lymphoma</td>
<td>2</td>
<td>980</td>
</tr>
<tr>
<td>56</td>
<td>non Hodgkin's lymphoma</td>
<td>2</td>
<td>1193</td>
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<td>non Hodgkin's lymphoma</td>
<td>2</td>
<td>467</td>
</tr>
<tr>
<td>38</td>
<td>non Hodgkin's lymphoma</td>
<td>1.5</td>
<td>756</td>
</tr>
<tr>
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<td>non Hodgkin's lymphoma</td>
<td>2</td>
<td>837</td>
</tr>
<tr>
<td>35</td>
<td>acute lymphocytic leukemia</td>
<td>1</td>
<td>120</td>
</tr>
</tbody>
</table>

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*a* 5-MTHF (25 mg) was administered i.v. 24 h after the start of a 6-h continuous infusion of MTX. Blood for the analysis was collected 6 h after 5-MTHF injection and processed as described in the Methods section. (These results are part of data to be published elsewhere.)
in plasma and spinal fluids, the sequential use of two columns has been suggested (19); 5-MTHF is retained at the top of an RP8 column after washing with a buffered solution; the compound is then transferred by countercurrent elution to an ion-exchange column. 5-MTHF is detected by an electrochemical detector, which allows the evaluation of concentrations in plasma of a little as 0.9 µg/L. If an ultraviolet detector is used in addition to the electrochemical one, FA and 5-fTHF can also be measured, but they are not resolved from each other or from MTX.

The conditions we used in the present study were chosen with a view to monitoring 5-MTHF in the plasma of MTX-treated patients. To avoid decomposition of the tested compound, we added ascorbate to the samples, which were then heated according to the procedure described by Chapman et al. (15). 5-MTHF was resolved from all the other folates considered in the present study as well as from MTX. Three peaks arising from THF decomposition did not interfere with measurement of 5-MTHF. As little as 1–2 µg of 5-MTHF per liter could be evaluated with good precision. The measurement was unaffected by the presence of very high MTX concentrations, because this compound was not revealed by fluorometric detection.

On the other hand, use of both fluorometric and ultraviolet detection allows simultaneous measurement of 5-MTHF and MTX, the detection sensitivity for MTX being 0.5 mg/L. Our results seem reasonably to indicate the usefulness of this method for monitoring 5-MTHF in plasma when either this compound or 5-fTHF represents a “rescue” in treatments with HDMT.

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References