HPLC of Folinic Acid Diastereoisomers and 5-Methyltetrahydrofolate in Plasma

Marie-Christine Etienne, Nathalie Speziale, and Gérard Milano

We present a rapid, sensitive, and automated HPLC method with direct resolution of l-folinic acid (l-FA), d-folinic acid (d-FA), and 5-methyltetrahydrofolate (5MTHF) from plasma samples. Plasma (500 μL) is first extracted on solid phase (RP-18 cartridge). The d-FA peak is collected on-line from a reversed-phase column (RP-8, 119 x 2 mm, 4 μm: HPLC 1) and then automatically loaded onto a chiral stationary phase (human serum albumin, 150 x 4.6 mm, 7 μm: HPLC 2). The same mobile phase flows in both systems (0.2 mol/L Na₂HPO₄:1-propanol, 98:2, pH 6.2). HPLC 1 allows quantification of 5MTHF by absorption at 313 nm; HPLC 2, the quantification of l-FA and d-FA by electrochemical detection in the oxidation mode (total run time 18 min). Recoveries are >80%. CVs for intra- and interassay reproducibilities are <5% and 15%, respectively. Linearity of the response (0.1–1 μmol/L and 1–50 μmol/L, r = 0.99, P < 0.01) is satisfactory. The sensitivity limit is 50 nmol/L for 5MTHF and 20 nmol/L for l-FA and d-FA. This assay is substantially improved over existing methods regarding feasibility and is being used in pharmacokinetic investigations in cancer patients.

Additional Keyphrases: chromatography, reversed-phase • chiral HPLC • leucovorin • pharmacokinetics

Folinic acid (FA; leucovorin) is a vitaminic factor widely used in oncology (1). FA has been used for >20 years as a rescue agent for high-dose methotrexate therapy (2). Recent studies have shown that FA increases the in vitro cytotoxicity (3) and in vivo therapeutic efficacy (4) of 5-fluorouracil. This new synergistic concept has renewed interest for FA in clinical oncology. Commercially available FA is prepared from (S)glutamic acid, resulting in a racemic mixture of the two diastereoisomers, l-FA (S,S-FA) and d-FA (R,S-FA) (Figure 1). Biological activity of the racemic mixture is supported by the natural l-FA, with d-FA having little or no activity (5). In addition to stereoeoisomeric differences in biological activity, FA has stereospecific pharmacokinetics (6–8), with plasma half-life of l-FA being significantly shorter than that of d-FA. This results from the rapid biotransformation of l-FA into the active cofactor 5-methyltetrahydrofolate (5MTHF), whereas d-FA does not undergo metabolism. Consequently, d-FA tends to accumulate in plasma relative to l-FA. Regarding FA pharmacological activity, previous studies failed to demonstrate any interference of d-FA with the activity of l-FA (9, 10; R. L. Schilaty, K. E. Choi, M. A. Liebner, E. E. Vokes, A. Quaspari, unpublished abstract, 1989).

Stereospecific pharmacokinetic information on l-FA, d-FA, and 5MTHF is important for optimizing the administration of FA (route, dose, and frequency). This information, however, is incomplete, largely because of the complexity of the current analytical procedures. Until the recent development of chiral stationary phases, quantification of the active FA isomer was achieved through microbiological assays with folate-dependent microorganisms (11–13). These bioassays may lack specificity because of cross-reactions with other biologically active folates (7, 14). Moreover, quantification of the active isomer in patients receiving a cytotoxic agent may require collection of the dl-FA from an HPLC column before the microbiological assay (15). In all cases, d-FA evaluation has required conventional HPLC so that d-FA quantification could be estimated from the difference between dl-FA measured by HPLC and l-FA measured by microbiological assay (6, 7, 14). Although complex, these bioassays led to very sensitive detection (sensitivity limit ~1 nmol/L) (13, 15).

The availability of an immobilized bovine serum albumin (BSA) stationary phase allowed FA diastereoisomer resolution to be performed by HPLC, as described by Wainer and Stiffin (16). This assay included conventional HPLC followed by the appropriate switching of the dl-FA peak onto a BSA column, but it lacked sensitivity, mainly because of the low efficiency of the BSA column. This assay was improved by Silan et al. (17), who performed the chiral HPLC on the BSA column and then used postcolumn peak compression and elution on two RP-18 HPLC columns so that the diastereoisomers were trapped and compressed. The sensitivity was improved (~10 nmol/L), but the complexity of the method and the need for a gradient elution led to a total run time >70 min (17). Other HPLC procedures include a conventional separation, with the collection of the dl-FA peak followed by the concentration of the dl-FA and its reinjection onto a BSA stationary phase; the concentration of dl-FA is achieved either by a single evaporation (18) or by an additional extraction on an

[Fig. 1. Structure of FA and 5-methyltetrahydrofolate diastereoisomers]
RP-18 cartridge (19). An alternative approach, recently developed by Priest et al. (20), is based on the enzymatic cycling of the d-FA to methylenetetrahydrofolate followed by its entrapment in a stable ternary complex with thymidylate synthase and triitated fluorooxuryridylate. This radioenzymatic assay is very sensitive (limit ~1 nmol/L) but is time consuming and requires the use of radiolabeled products. The complexity of these procedures may restrict their clinical application. We have developed a rapid (total run time 18 min), sensitive, automated chiral HPLC assay for direct resolution of l-FA, d-FA, and 5MTHF from plasma samples.

**Material and Methods**

**Chemicals**

d-FA (racemic mixture 50/50, purity 98.8%), d-5MTHF (racemic mixture 50/50, purity 91.2%), and l-ascorbic acid were from Sigma (la Verpillière, France). l-FA (purity 97.3%) and d-FA (purity 98.9%) were kindly supplied by Lederle Laboratories (Rungis, France). Standard solutions of 10 mmol/L were prepared in doubly distilled water and portions were stored at −20 °C. Phosphoric acid (85%) was purchased from Carlo Erba (Paris, France). All other solvents and reagents were of analytical grade: disodium hydrogen phosphate (Merck, Nogent sur Marne, France), methanol and 1-propanol (Prolabo, Paris, France), tris(hydroxymethyl)aminomethane phosphate (TrisP; Fluka, Mulhouse, France). Doubly distilled water was used for preparing the mobile phase.

**Sample Processing**

Ascorbic acid (1 g/L) was added to blank pooled blood donor plasma. Original and published extraction procedures for folic acid analogs were tested, including both organic and solid-phase extraction. All the organic extraction procedures tested, including those of Wainer and Stiffin (16), Straw et al. (6), and Stout et al. (21), and the perchloric acid method of Van Tellingen et al. (22) were excluded because of significant background interference from injected blank plasma. Similarly, the solid-phase extraction that we developed with the RP-18 cartridge (Sep-Pak; Millipore Waters, Bedford, MA) for FA and 5MTHF nonstereospecific HPLC analysis (23) resulted in interference with 5MTHF in the present HPLC system. We tested different extraction cartridges, including Quat-Amine SPE (Baker, Sanford, ME); Adsorbex RPCI, Adsorbex RP-18, and Polypher RP-18 (Merck, Nogent sur Marne, France); and Bond Elut RP-18 (Varian, Sunnyvale, CA).

The optimized procedure was as follows: 500 μL of plasma was diluted six fold in 9 g/L NaCl containing 1 g/L ascorbic acid. To this, 200 μL of 100-fold-diluted phosphoric acid [i.e., 8.5 g/L H₃PO₄] was added (final pH 4.5). Samples were extracted on Bond Elut RP-18 cartridges (no. AI 1210-2028) that had first been conditioned with methanol (4 x 2 mL) and 100 mmol/L TrisP, pH 4.7 (3 x 2 mL). The cartridge was rinsed with 0.5 mL of 10 mmol/L TrisP, pH 4.7, and eluted with 1.5 mL of methanol:10 mmol/L TrisP (75:25, vol:vol), pH 7, containing 0.15 g/L ascorbic acid. The eluates were evaporated at 37 °C under a nitrogen stream for 30 min. Dry residues were dissolved in 250 μL of 9 g/L NaCl containing 1 g/L ascorbic acid (two-fold-concentrated samples). Forty microliters was injected onto the chromatographic system. Quantification of plasma samples was done by regression analysis based on a calibration curve in plasma. The calibration curve was prepared for each run by adding pure dl-FA (racemic mixture, 50/50) and 5MTHF from Sigma to blank plasma.

**Apparatus**

The chromatographic system (Figure 2) consisted of two HPLC columns connected through a Rheodyne 7010 switching valve (equipped with a 600-μL sample loop). On-line collection of the dL-FA peak from a reversed-phase column (HPLC 1) into the loop of the switching valve is followed by automatic injection of the peak onto the chiral stationary phase (HPLC 2). The choice of the reversed-phase column was dictated by the need to minimize the volume of the dL-FA peak so that it could be injected onto the chiral phase. The use of a narrow-bore column (Superspher RP-8, 119 x 2 mm, 4-μm particle, Merck no. 16861, flow rate 0.25 mL/min) allowed the dL-FA to be eluted in 500 μL, whereas with a conventional column (250 x 4.6 mm, flow rate 1 mL/min), the elution volume was 2 mL (resolution on the chiral phase was satisfactory up to 600 μL injected). [Note: Superspher RP-8 performance is rapidly reduced if pure aqueous phase is used, and the column must be conditioned with methanol:water (30:70 by vol). This allows 100-200 injections to be done.] Thermostatic control of the chiral column was optimized (35 °C) to improve the chromatographic profile, i.e., to decrease the retention factor and the peak area: peak height ratio. The BSA bonded silica phase (BSA-BP; Resovsil-BSA, 150 x 4 mm, 7-μm particles; Macherey Nagel, Düren, Germany) used by Silan et al. (17) and Schilasky and Ratain (19) and the newly available human serum albumin BP (HSA-BP; chiral protein 2, 150 x 4.5 mm, 7 μm) packed by Société Française Chromato Colonne (Neuilly Plaisance, France) (24) were compared. HSA-BP led to a better resolution than BSA-BP. The background noise recorded by electrochemical detection increased as a function of the tem-
perature applied to the chiral column; this phenomenon was strongly marked with BSA-BP, thus decreasing the signal-to-background ratio. HSA-BP was thus selected to improve the sensitivity of the analysis. (Note: HSA-BP must be stored at 4°C under 50 mmol/L Na₂HPO₄, pH 7, containing 0.1 g/L NaN₃. This column allows >500 injections to be performed without impairing the quality of resolution.)

HPLC 1 included a Model 590 pump (Millipore Waters), an automatic temperature-regulated injector set at 5°C (WISP 710 B, Millipore Waters), and an ultraviolet detector (Spectroflow 783; Kratos Analytical, Ramsey, NJ). HPLC 2 included a Model LC9A pump (Shimadzu, Kyoto, Japan), an electrochemical detector (Coulonchem 5100 A; ESA, Bedford, MA) equipped with a Model 5020 guard cell, a Model 5010 analytical cell, and a column heater (Model CH-30; Fiotron, Oconomowoc, WI). Both integrators were Hewlett-Packard (Palo Alto, CA) Model 3390A. The significant volume injected on the chiral phase and the great sensitivity of the electrochemical detection necessitated the use of the same mobile phase in the two HPLC systems to minimize the background noise on HPLC 2. The choice of phosphate buffer and 1-propanol was dictated by the HSA-BP requirements. Buffer strength was tested between 0.1 and 0.2 mol/L (optimal range for electrochemical detection), 1-propanol content was tested between 0 and 50 mL/L, and pH was tested between 5.5 and 7.5. An increase in the buffer strength, the 1-propanol content, or the pH induced a decrease in the l-FA and d-FA retention factors, thus improving the peak area:peak height ratio. The optimal mobile phase was 0.2 mol/L Na₂HPO₄:1-propanol (98:2 by vol), pH 6.2 [adjusted with concd. (8.5 g/L) phosphoric acid], which had to be filtered twice through the 0.2-μm filter.

HPLC 1 (flow rate 0.25 mL/min) allowed 5MTHF quantification by absorption at 313 nm (0.002 A full scale). Quantification of l-FA and d-FA on HPLC 2 (flow rate 1 mL/min) was achieved by electrochemical detection in the oxidation mode. The signal was recorded on the second electrode (E2) of the analytical cell, the first electrode (E1) being used as a cleaning electrode to improve the selectivity of the detection. Optimal potentials for the dual-electrode analytical cell were 0.30 V for E1 and 0.55 V for E2. Response time was 0.4 s. The analysis was automated through the ultraviolet detector (HPLC 1), which controlled the switching valve. (Note: Pure standard (1000 nmol/L each of d-l-FA and 5MTHF) was injected onto HPLC 1 to evaluate the start and the end times of the d-l-FA peak. The dead volume between the outlet of the detector and the switching valve was taken into account to program the collection time for the d-l-FA peak. Typical width of the d-l-FA peak on HPLC 1 is 2 min (500 μL), and 0.2 min (50 μL) is added at the start and the end of the collection time so that the total volume collected is 600 μL.)

Run time for the entire analysis was 18 min. Chromatographic peaks were quantified by area.

Results and Discussion

Injection of the racemic mixture of d-l-FA results in very good resolution of the two FA diastereoisomers (Figure 3). Identification of l-FA and d-FA peaks was achieved through separate injections of the pure standards, which demonstrated that l-FA eluted first. With the blank plasma, no significant peaks eluted at l-FA and d-FA retention times. A shoulder was observed at the 5MTHF retention time in the majority of blank plasma samples, but this never corresponded to a measurable peak. Given the physiological concentrations of l-5MTHF encountered in normal subjects, ~10 nmol/L (25), it is likely that the shoulder corresponds to endog-
enous l-5MTHF. With our protocol (using ascorbic acid and maintaining the samples at 5 °C), we did not observe any degradation of l-FA, d-FA, or 5MTHF peaks for analyses of ≤48 h.

Assay characteristics are presented in Table 1. Recoveries were satisfactory, between 81% and 89%. We tested different pteridine analogs as potential internal standards, including lumazine, pterin, pterin-6-carboxylic acid, pteric acid, aminopterin, trimetrexate, and p-aminocetophene (the internal standard used in our previous nonstereospecific HPLC assay (23)). Except for aminopterin interfering with 5MTHF and p-aminocetophene being eluted at 40 min, all other compounds tested eluted too quickly (retention time <4 min for HPLC 1), thus leading to interferences with blank plasma. The rapidity of the assay (18 min) and the simplicity of an isocratic elution are two improvements in the present method compared with other published procedures (16–19). Thus, we tested the precision of the assay without an internal standard, and found a CV of <5% for the intra-assay reproducibility and, as predicted, a higher interassay reproducibility (CV <15%).

Electrochemical detection of FA was initially reported by Birmingham and Greene (26), who used amperometry. We chose coulometric detection to improve both sensitivity and selectivity of the analysis. The oxidation curve (plot of the signal as a function of the potential applied on E2 with E1 set at 0 V) suggests that FA exhibits at least two oxidizable functional groups, one fully oxidizable at ~0.65 V (probably the primary amine), the other being fully oxidizable at much higher potential (>1 V) (Figure 4). Optimization of the potential applied on E1 is shown in Figure 5. Plot of the signal recorded on E2 as a function of the potential applied on E1 shows that optimal potential for E1 is 0.30 V, allowing 96% of the signal to be detected on E2. Finally, we optimized the potential applied on E2 to get the highest signal-to-background ratio (results not shown), and thus fixed E2 at 0.55 V. This optimization of electrochemical detection enables a detection limit of 20 nmol/L to be reached for both l-FA and d-FA. The detection limit for 5MTHF was 50 nmol/L.

Linearity of the response was demonstrated for two different calibration curves, 0.1–1 and 1–50 μmol/L, depending on the concentrations in the analyzed samples (Table 1). The specificity of the assay was tested with drugs currently administered in association with FA, i.e., 5-fluorouracil and its circulating catabolites (dihydrofluorouracil and fluoro-β-alanine), methotrexate and its metabolite 7-hydroxymethotrexate, as well as acetaminophen and acetysalicylic acid. Plasma containing added acetaminophen (50 mg/L) or acetysalicylic acid (50 mg/L) exhibited a measurable interfering peak at the retention time of 5MTHF (HPLC 1). Methotrexate and 7-hydroxymethotrexate peaks were detected in drug-supplemented plasma at 27 and 33 min, respectively, on HPLC 1. Thus in patients receiving FA treatment during methotrexate therapy, the analysis should be lengthened at least to 35 min. 5-Fluorouracil and its catabolites were not detected in the assay.

Two examples of chromatographic profiles of plasmas obtained from patients illustrate the feasibility of the assay (Figure 3). One is from a child receiving methotrexate therapy plus FA treatment 6 h after oral administration of 12 mg · m⁻² of dl-FA (plasma concentrations are 200, 40, and 560 nmol/L for 5MTHF, l-FA, and d-FA, respectively); the other is from an adult under continuous venous infusion of 200 mg · m⁻² · day⁻¹ of dl-FA (plasma concentrations are 2980, 1070, and 19 600 nmol/L for 5MTHF, l-FA, and d-FA, respectively). In patients receiving dl-FA, l-FA exhibits lower plasma concentrations than do d-FA and 5MTHF (6–8, 27). Regarding intravenous administration of dl-FA, Newman et al. (7) reported steady-state plasma concentrations of l-FA of >1000 nmol/L during a standard continuous infusion of 500 mg · m⁻² · day⁻¹ for 5.5 days; Priest et al. (27) reported l-FA plasma concen-

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**Table 1. Validation of the Assay**

<table>
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<tr>
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<th>l-FA</th>
<th>d-FA</th>
<th>5MTHF</th>
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<tr>
<td>Recovery, %</td>
<td>81 ± 8</td>
<td>85 ± 7</td>
<td>81 ± 6</td>
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<td>(n = 13)</td>
<td></td>
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<tr>
<td>5000 nmol/L</td>
<td>84 ± 9</td>
<td>88 ± 6</td>
<td>89 ± 4</td>
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<tr>
<td>(n = 7)</td>
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<td>Reproducibility, %</td>
<td>1.8</td>
<td>1.7</td>
<td>2.9</td>
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<tr>
<td>Intra-assay (n = 6)</td>
<td>6.7</td>
<td>10.3</td>
<td>14.0</td>
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<tr>
<td>Inter assay (n = 9)</td>
<td></td>
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<tr>
<td>Sensitivity limit</td>
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<td>20 nmol/L</td>
<td>50 nmol/L</td>
</tr>
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<td>Linearity, r²</td>
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<tr>
<td>Conc range  A</td>
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<td>0.999</td>
<td>0.994</td>
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<tr>
<td>Conc range  B</td>
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<tr>
<td>Resolution factor, R</td>
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* Recoveries were calculated for plasma containing 500 and 5000 nmol/L each of dl-FA and 5MTHF; mean ± SD.
* Reproducibility (CV%) was measured with plasma containing 500 nmol/L each of dl-FA and 5MTHF.
* The concentration corresponding to 2.5-fold the baseline height of a blank plasma chromatographic profile.
* A:0–10⁻⁷–5×10⁻⁷–10⁻⁶ nmol/L, B:0–10⁻⁸–5×10⁻⁸–10⁻⁷–5×10⁻⁸–10⁻⁹ nmol/L.
* Concentrations correspond to the concentration of the racemic form (d-FA) and to the concentration of 5MTHF. In each case, P <0.01.
* Calculated between l-FA and d-FA.

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Fig. 4. Oxidation curve of l-FA.

E1 was set at 0, and E2 varied between 0.1 and 1.0 V. For each point, 50 μL of l-FA 0.1 nmol/L was directly injected onto the BSA-BP column.
trations of >20 nmol/L 7 h after a single administration of 125 mg·m⁻². These data demonstrate the applicability of the present assay to intravenous pharmacokinetic investigations. The application of this assay to oral pharmacokinetic investigations may be limited because of the low concentrations encountered after oral administration of dl-FA. This is mainly due to saturation in absorption as low as 50 mg per intake (6, 27), and some limitation of the analysis because of sensitivity may occur mainly for single administration. Yet, Priest et al. (27) reported peak concentrations of l-FA as low as 21 and 27 nmol/L after single oral intakes of 25 and 125 mg, respectively, whereas Schilsky et al. (8) reported plasma steady-state concentrations of l-FA of >100 nmol/L during repeated oral administration of 100 mg of dl-FA every 4 h for 5 days.

In conclusion, the present stereospecific HPLC assay represents a substantial improvement over existing methods for FA diastereoisomer analysis. This assay is particularly suitable for large-scale analysis and thus is fully applicable to pharmacokinetic investigations currently being undertaken in patients in our center.

References