Radiochemical Method for Measuring Plasma Clearance and Urinary Excretion of Pteroylglutamic Acid

Maria da Costa, Sheldon P. Rothenberg, and Zoltan Rosenberg

A radiochemical procedure is described for specific determination of pteroylglutamate in serum and urine. This method depends on denaturation of methylenetetrahydrofolate with peroxide and measurement of the residual folate by a ligand-binding radioassay. The binding determinant for the radioassay is a folate-binding protein, partially purified from chronic myelogenous leukemia cells, that has low affinity for the reduced folates and thus will preferentially measure residual pteroylglutamate rather than any non-denatured residual methylenetetrahydrofolate. We used this assay to measure the clearance from plasma and the urinary excretion of pteroylglutamate and a small fraction of serum folate that is stable to this oxidation procedure. The plasma clearance after intravenous injection is characterized by an initial rapid distribution phase followed by a second, slower metabolic phase; after about 2 h all of the administered pteroylglutamate has been cleared from the blood. The peak concentration of total folate in serum 1–2 min after administration of pteroylglutamate exceeded the sum of the endogenous stable and baseline serum folate, indicating that a reduced labile folate was released from the liver and perhaps from other tissues. This reduced folate had a slower metabolic clearance rate and was excreted to some extent in urine. Only 2.3 and 7.9% of the pteroylglutamate administered to two normal subjects was excreted as stable folate.

Additional Keyphrases: "stable" folate • folate-binding protein • radioassay • release of a reduced labile folate from liver • tool for studying handling of folate.

The major form of folate in plasma is N-5-methylenetetrahydrofolate (methylFH4) (1); this can be measured either microbiologically with Lactobacillus casei (2) or by ligand-binding radioassay (3, 4), but both of these procedures will also measure pteroylglutamic acid (PGA). Accordingly, studies to determine the specific plasma clearance and metabolic turnover of PGA have required the application of microbiological assays with use of L. casei, which measures methylFH4 and PGA, and Streptococcus faecalis, which measures PGA but not methylFH4 (5).

To circumvent the problem of using two tedious microbiological assays in order selectively to measure the plasma clearance and metabolic turnover of PGA, we have developed a procedure to selectively oxidize endogenous methylFH4 in serum, which then permits the specific determination of the residual, more stable PGA by use of a ligand-binding radioassay. This method has also identified a small amount of endogenous stable folate in serum. This stable folate is probably the same folate previously identified as bound to the folate-binding protein in serum, because it cannot be extracted by charcoal (6); it is also not destroyed by autoclaving in the absence of ascorbate (6) and is probably the small compartment of serum folate measured by S. faecalis (7) and by radioimmunoassay (8).

Materials and Methods

[3H]PGA with specific activity ranging from 10 to 30 Ci/mmol was purchased from Amersham/Searle, Inc., Arlington Heights, IL 60005. Its purity has generally ranged from 80 to 90%, as determined by coprecipitation with unlabeled PGA and ZnSO4 (9).

We used crystalline PGA and methylFH4 (Sigma Chemical Co., St. Louis, MO 63178) as the standards for measuring stable folate and total folate in serum, respectively. The macromolecular binder of folate used in the radioassay for stable folate3 was partially purified folate-binding protein prepared from chronic myelogenous leukemia (CML) cells (10); folate binder partially purified from milk was used in the radioassay for total folate (4).

Extraction of Serum

To measure the PGA (and endogenous, similarly stable folate) in serum, the serum must be extracted by a procedure that will (a) denature the endogenous methylFH4, (b) denature the endogenous folate-binding protein so that it will not interfere in the subsequent radioassay for the PGA, and (c) release any endogenous stable folate bound to the serum folate-binding protein(s).

Several methods have been proposed for the cleavage of folates, and Maruyama et al. (11) have reviewed the problems encountered with either oxidative or reductive treatments. We found that simple autoclaving or boiling the serum, even in the absence of ascorbate, resulted in variable destruction of either methylFH4 or endogenous folate-binding protein. Because it has been shown that methylFH4 is acid labile (12), we used the following extraction procedure to achieve our objectives listed above: Dilute one volume of serum or urine samples (usually 0.5–1.0 mL), collected fresh or stored at −20 °C without ascorbate, with one volume of acetate buffer (35 mmol/L, pH 3.8) and one volume of a 10 mL/L solution of hydrogen peroxide. Incubate for 60 min at 37 °C to denature the reduced folates. Place the mixture in a boiling water bath for 1 min to precipitate the proteins, and adjust the pH to 7.5–8.0 by adding one volume of sodium borate, 20 mmol/L, pH 10.7. Shake the tubes vigorously to disperse the coagulated

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2 Nonstandard abbreviations used: methylFH4, N-5-methylenetetrahydrofolate; PGA, pteroylglutamic acid; and CML, chronic myelogenous leukemia.

3 Hereafter, the term "stable folate" refers to both PGA and the endogenous folate that resists oxidation.

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proteins and thus solubilize any of the stable folate that may have adsorbed to the precipitate. Boil the mixture for another 15 min, separate the precipitate by centrifugation, and assay the supernatant solution for residual folate. Store the extracts at -20°C and assay within a day or two after extraction.

**Radioassay Procedures**

We measured the total folate in the serum before extraction according to the sequential noncompetitive radioassay we previously described (4), using the folate binder prepared from milk and methylFH4 standards. To measure the stable folate remaining in the extracts, we used a competitive system with the folate binder partially purified from CML cells (10) and PGA for the standard curve. This minimizes the measurement of any residual methylFH4 because the affinity of this CML folate binder for reduced folates is much lower than for non-reduced forms of folate (10).

For the competitive radioassay procedure, add the diluted binder preparation (the amount that will bind about 50–60% of the [3H]PGA) to the mixture of [3H]PGA (200 pg) and PGA standards or to an aliquot of the extract to be assayed. The buffer used contains 50 mmol of borate per liter, pH 8.0, and the total volume of the reaction mixture is 0.5 mL.

After 30 min of incubation, stop the reactions by adding 0.4 mL of a 50 g/L suspension of Norit A charcoal in a 1.25 g/L solution of bovine hemoglobin. The charcoal adsorbs all free folates. After centrifugation, determine the protein-bound radioactivity by counting the radioactivity of an aliquot of the supernatant solution in 15 mL of scintillation cocktail containing 5 g of diphenylhexazon and 50 mL of BBS-3 solubilizer (Beckman Instruments, Inc., Mountain-Ke, NJ 07091) per liter of toluene.

Run a blank reaction containing only buffer and [3H]PGA, similarly treated with the charcoal suspension, to determine how much of the nonfolate radioactivity in the tracer preparation did not adsorb to the charcoal. Obtain a value for the net radioactivity, representing bound folate, by subtracting this blank radioactivity from the assay samples. We accumulated sufficient counts (10 000 cpm) to assure a statistical error of ±3% or less.

Construct a dose–response standard curve by plotting the ratio of bound to free [3H]PGA (B/F) vs. the unlabeled PGA in the standard reactions. Refer to this standard curve to obtain the equivalent amount of PGA in the aliquot of extract assayed. If the folate concentration in the test sample is too high to be measured on the curve, dilute the extract with bo- rate buffer before assay.

**Results**

To determine the effect of the extraction procedure on PGA, its efficiency in releasing endogenous protein-bound stable folate, and the denaturation of the folate-binding protein, we added [3H]PGA to normal serum, to serum containing a high concentration of endogenous unsaturated folate-binding protein, and to two normal sera to which the unsaturated folate-binding protein from CML cells was added, and then extracted the sera as described. An aliquot of the extract of the normal sera was then incubated with an excess of folate binder to determine whether, after extraction, the [3H]PGA would still react with the folate-binding protein used for the radioassay. The extracts of the sera containing den- eogenously unsaturated folate binder to which [3H]PGA was added were treated with the charcoal suspension to adsorb free [3H]PGA and thereby determine the efficiency for the release of bound folate. Finally, the extracts were tested for residual unadentated, unsaturated binder by determining if freshly added [3H]PGA would be bound.

The results in Table 1 show that 93% of the [3H]PGA added to normal sera was still reactive with the folate-binding protein after extraction of the serum. Of the bound [3H]PGA in the sera containing endogenous unsaturated folate binder and sera to which unsaturated folate-binding protein from CML cells was added, 0 and 12%, respectively, of the [3H]PGA remained bound after the extraction procedure.

One hundred percent and 84% of the [3H]PGA released

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**Table 1. Effect of Extraction Procedure on [3H]PGA and Folate-Binding Protein in Serum**

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. expts.</th>
<th>Serum + [3H]PGA</th>
<th>Serum containing endogenous unsatd folate-binding protein</th>
<th>Serum + unsatd folate-binding protein from CML cells + [3H]PGA</th>
<th>In extract bound by additional folate-binding protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bound in serum before extraction</td>
<td>Remaining bound in serum extract</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Bound [3H]PGA was determined after removal of free [3H]PGA by charcoal adsorption. Folate-binding protein from CML cells was added to the serum extracts and the [3H]PGA bound was measured in the supernate after charcoal adsorption of the free [3H]PGA. [3H]PGA was added to the charcoal-treated serum extracts and the [3H]PGA bound was measured in the supernate after charcoal adsorption of the free [3H]PGA.

b 1 ng of [3H]PGA was added to 1 mL of serum. Lower specific activity [3H]PGA was used for the sera containing folate-binding protein.

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**Table 2. Analytical Recovery of PGA and Methyl-FH4 Added to Serum**

<table>
<thead>
<tr>
<th>Endogenous stable folate</th>
<th>Folate added</th>
<th>Folate found after extraction</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum + [3H]PGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>—–</td>
<td>100.0</td>
<td>117</td>
<td>117</td>
</tr>
<tr>
<td>—–</td>
<td>50.0</td>
<td>49.0</td>
<td>98</td>
</tr>
<tr>
<td>0.21</td>
<td>2.0</td>
<td>2.5</td>
<td>113</td>
</tr>
<tr>
<td>0.32</td>
<td>0.5</td>
<td>0.8</td>
<td>80</td>
</tr>
<tr>
<td>Mean (CV) = 101 (14)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Serum + methyl FH4      |              |                                |             |
| —–                      | 5            | 0.5                            | 10.0        |
| —–                      | 10           | 0.8                            | 8.0         |
| —–                      | 20           | 0.2                            | 1.0         |
| —–                      | 40           | 0.8                            | 2.0         |
| Mean (CV) = 5.2 (84)    |              |                                |             |

* Insignificant value in comparison to amount of folate added to the serum.
Table 3. Urinary Excretion of Folate after Administration of PGA

<table>
<thead>
<tr>
<th>Subject</th>
<th>Dose, mg</th>
<th>2-h urine vol, mL</th>
<th>Pre-injection folate concn, μg/L</th>
<th>Post-injection folate excretion, μg of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Stable</td>
</tr>
<tr>
<td>A</td>
<td>1.00</td>
<td>456</td>
<td>2.8</td>
<td>0.15</td>
</tr>
<tr>
<td>B</td>
<td>0.86</td>
<td>100</td>
<td>2.0</td>
<td>1.40</td>
</tr>
</tbody>
</table>

a Difference between total and stable folate.

b This represents the total folate in the urine specimen collected during the 2-h time period, minus the endogenous folate excretion calculated from the pre-injection concentration per liter. No. in parentheses indicate percent of dose.

from the unsaturated endogenous and added CML folate binder, respectively, in serum was still reactive with additional folate-binding protein used in the radioassay. After charcoal adsorption of the released [3H]PGA, these serum extracts did not bind additional [3H]PGA, indicating that the folate-binding protein in serum had been denatured.

Table 2 summarizes the analytical recovery of unlabeled PGA and methylFH4 added to normal serum and then subjected to this extraction procedure. We measured the concentration of methylFH4 by using the reference curve prepared with PGA in order to underestimate the true concentration of the methylFH4, because the objective of the procedure is to measure only stable folate. Only 5.2% of the methylFH4 added to the serum in a concentration range found in most normal sera was assayable after extraction, whereas 80 to 117% of the PGA added to serum was accounted for.

The endogenous stable folate measured in serum from normal subjects ranged in concentration from 160 to 510 ng/L with a mean (±SEM) of 243 ± 40 ng/L. This stable folate component in serum approximated 2.8% of the total folate (4).

We determined the clearance of PGA from the plasma of two fasting normal subjects after administration of 15 μg/kg body weight. The total folate in whole serum was measured by the two-phase sequential noncompetitive radioassay (4) and stable folate was measured in the sera after extraction as described. The results are shown in Figure 1. For both subjects, there was an initial rapid decrease in the stable folate concentration, which represents the distribution of the PGA, followed by a slower clearance phase, representing metabolic turnover.

Within 1 to 2 min after the injection of PGA, there was an increase in total serum folate that exceeded the sum of PGA assayed and the fasting preinjection concentration of total folate. This difference in peak concentration of total and stable folate probably represents displacement of hepatic (or other tissue) methylFH4 by the injected PGA as described by Chanarin and McLean (13).

The difference between the concentration of total folate and PGA at each time period is the reduced labile methylFH4 in serum; the rate of decrease of this pool, which is also shown in Figure 1, is considerably slower than the clearance rate of either the PGA or total folate.

We measured the urinary excretion of total folate and stable folate before and after the administration of PGA (Table 3). There was very little stable folate in the control urine of both subjects; therefore, the stable folate measured in the 2-h urine collection represents the excretion of the administered PGA, which was 2.3 and 7.9% of the administered dose. However, there was also a significant increase in the urinary excretion of reduced folate, some of which reflects exchange of the PGA with tissue methylFH4 (13) and some representing conversion of the administered PGA to methylFH4, which was then excreted.

Discussion

This report describes a radiochemical method for measuring, selectively, PGA and other stable folate(s) in serum in which the reduced labile methylFH4 is the major component

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4 In this radioassay procedure the folate-binding protein prepared from milk is used; the assay does not distinguish PGA and methylFH4 because it is a sequential noncompetitive type of radiometric assay. Therefore, it measures total folate in serum.

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Fig. 1. The plasma clearance of PGA (15 μg/kg body weight) administered intravenously to two normal fasting subjects x, total serum folate; ○, stable folate measured; △, reduced folate, calculated from the difference between the total and stable folate at each time period.
This small component of stable serum folate is difficult to quantify by microbiologic methods because these procedures lack the specificity to separate these classes of naturally occurring folates. Though *S. faecalis* and *L. casei* are the organisms most widely used to differentiate these classes of folate because the former organism cannot utilize methylFH₄ for growth (2), both of these organisms will grow on virtually all other monoglutamate-folate derivatives.

The previously described radioimmunoassay for PGA can also measure this small fraction of stable folate in serum (8), but the difficulty of consistently obtaining high affinity antiserum to PGA has limited the value of this procedure.

This radiochemical method utilizes an extraction procedure in which methylFH₄, the major component of serum folate, is denatured by peroxide oxidation and the remaining stable folate is measured by radioassay and compared with a PGA standard curve, with use of the folate binder from CML cells (10). This folate binder has low affinity for methylFH₄ so that, even if a small fraction of endogenous methylFH₄ is not destroyed by the peroxide treatment, it will not contribute significantly to the value for stable folate.

Using this method, we have been able to measure a small concentration of stable folate in normal sera, the identification and significance of which require further study. This method has, however, been used to measure selectively the urinary excretion and plasma clearance of PGA from the serum of two fasting normal subjects. By measuring separately the PGA and total folate in serum, we observed that the peak of total folate was greater than the sum of the PGA and circulating endogenous folate, indicating that there must have been a rapid release of additional folate from the tissues, and this was probably methylFH₄. The difference in the values of PGA and total folate represents reduced folate (again, methylFH₄) and the plasma clearance of this folate was slower than the administered PGA. The re-entry of nascent methylFH₄ synthesized from the administered PGA could decrease the rate of clearance of the reduced folate. However, there is also likely to be a basic difference in the cellular uptake of these folates, because Chanarin (14) also observed that methylFH₄ was cleared more slowly from the plasma than was PGA after their separate administration. In this regard, studies of the cellular transport of folates by a variety of mammalian cells have shown that PGA and methylFH₄ utilize different membrane carrier systems (15).

The folate excreted in the urine during the 2 h of the clearance study was primarily a reduced folate, there being only 2.3 and 7.9% of the administered PGA excreted as stable folate. This observation is also consonant with the studies of Chanarin and McLean (13), which demonstrated that after the administration of isotope-labeled PGA there was an increase in the excretion of nonisotope-labeled folate active for *L. casei* and not *S. faecalis*.

This radiochemical assay method, which selectively measures stable folate, can be used to study this minor but possibly metabolically significant fraction of serum folate. In combination with the radioassay to measure total folate, it can be a useful tool for studying the metabolic conversion of PGA into reduced folate and for estimating the hepatic and other tissue storage pools of methylFH₄ by the displacement and urinary excretion of reduced folate. Such studies are now in progress in normal subjects and in patients with disturbances of folate and vitamin B₁₂ metabolism.

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References


