Radioisotopic Assay for Measurement of Serum Folate Levels

By Samuel Waxman, Carol Schreiber, and Victor Herbert

A radioisotopic assay is reported that accurately measures an almost identical serum folate to that measured by Lactobacillus casei and can separate low, borderline, and normal folate levels. This assay utilizes a powdered milk available in any grocery. The assay takes less than 4 hr and can be run in unlimited quantity. This radioassay should simplify diagnosis of folate deficiency and be particularly useful in patients receiving antibiotics and folate antagonists, where the microbiological assay gives false low results.

The reports of several workers suggest that cow's milk contains at least two binders for pteroylglutamic acid (PGA), one of which also binds N-5-methyl tetrahydrofolic acid (methyl THFA). Efforts to develop a radioisotope assay for serum folate by Metz et al. and Ghitis et al., using 3HPGA and milk, and Rothenberg, et al., using 3HPGA and antibody, did not provide results consistent with the established microbiologic assay using Lactobacillus casei. This may relate to the fact that the dominant serum folate is methyl THFA, on which L. casei grows, but which competes relatively weakly as compared to PGA for the same milk folate binders. In the current study, we used 3H methyl THFA instead of 3HPGA. Using this material, and commercially available powdered cow milk as a binder, a rapid, practical radioisotope assay was developed that provides measurements of serum folate levels generally consistent with L. casei assay, but not subject to certain pitfalls of microbiologic assay.

Materials and Methods

Folate Materials

L-1-N-methyl THFA-9,3',5'-3H (3H methyl THFA) was prepared enzymatically from...

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Table 1.—Serum Folate Assay Protocol (Values in Milliliters)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Unknown Serum (0–100 ng/ml)</th>
<th>Ethyl THFA (0–100 ng/ml)</th>
<th>Saline ME*</th>
<th>0.1 M PO4 Buffer pH 7.4</th>
<th>3H Methyl THFA (5 ng/ml)</th>
<th>Milk</th>
<th>HGB Coated Charcoal 1:20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>2.1</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>1.1</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard curve</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Unknown serum</td>
<td>0.4</td>
<td>0</td>
<td>0.1</td>
<td>0.6</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.4</td>
<td>0</td>
<td>0.1</td>
<td>0.7</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>control (serum)</td>
<td></td>
<td></td>
<td></td>
<td>Mix; 30 min at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 0.85% NaCl containing 3.7 × 10⁻⁷ M 2-mercaptoethanol.

³HPGA* and stable d,l-methyl THFA synthetically¹⁰ in Dr. Joseph Bertino’s laboratory by Dr. Peter Nixon. The ³H methyl THFA had a specific activity of 11.5 Ci/mM and a purity of 98% by paper chromatography. Working concentrations of ³H methyl THFA and stable d,l-methyl THFA were prepared in saline containing 2-mercaptoethanol (3.7 × 10⁻⁷ M) (saline-ME), stored at −60°C in convenient aliquots, and thawed just before use. The reducing agent and low temperature are essential to preserve the otherwise unstable material, just as less stringent similar measures preserve serum folate.

**Milk Folate Binder**

Carnation brand Instant Powdered Milk was used as the source of milk folate binder because of its universal availability and long shelf life at room temperature in the powdered form. A 50-ml quantity of 10 g/100 ml aqueous milk solution was dialyzed against 3.7 ml of 0.85% NaCl containing 3.7 × 10⁻⁷ M 2-mercaptoethanol for 60 hr at 4°C and maintained as a stock solution. Although subsequent studies suggest that the aqueous milk solution does not require dialysis prior to use, such dialysis was used in the studies here reported.

**Hemoglobin-coated Charcoal**

Hemoglobin concentrate from outdated human red blood cells was prepared¹¹ by lysing washed red cells with an equal volume of distilled water, followed by one half volume of toluene, shaking for 5 min, centrifuging, and preserving the red bottom layer (hemoglobin) after passing it through Whatman No. 1 filter paper. It is stored at −20°C for use in the assay; 1.25 g of hemoglobin is mixed with 25 g of Norit “A” pharmaceutical grade neutral charcoal (1:20 ratio hemoglobin : charcoal) in a final volume of 1 liter of water and stored at 4°C.

**Assay Protocol for Binding Curves, Standard Curve, and Serum Folate**

The incubation mixture for assay of serum folate (Table 1) consists of 0.1 ml ³H methyl THFA (0.5 ng), 0.1 ml saline-ME, 0.4 ml serum with 0.6 ml 0.1 M Na-KPO₄ buffer (phosphate buffer) pH 7.4 and 0.1 ml milk (concentration determined from binding capacity curves). The incubating volume of 1.3 ml is allowed to equilibrate at room temperature for 30 min. When milk binding curves or standard curves are constructed, serum is replaced by phosphate buffer and unlabeled methyl THFA in appropriate quantities.

* High specific activity, obtained from Amersham/Searle, Des Plaines, Ill. Nonradioactive methyl folate can be obtained from Sigma Chemical, St. Louis, Mo.
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Fig. 1.—The methyl THFA binding capacities of increasing quantities of dialyzed powdered milk solution. From this curve the quantity of milk needed to bind 50–60% of the test amount of $^3$H methyl THFA is determined.

Separation

At the end of the incubation period, the free (unbound) $^3$H methyl THFA is separated from that which is bound by 15-min contact with 1.0 ml of hemoglobin-coated charcoal suspension. Following centrifugation, an aliquot of the clear supematant containing the bound $^3$H methyl THFA is added to 20 ml of scintillation mixture* and counted.

The standard is the amount of radioactivity present when the test amount (0.5 ng) of $^3$H methyl THFA is measured in the absence of milk and of charcoal. A supematant control is run with each sample to determine any radioactivity that is not adsorbable to charcoal in the absence of milk; this value is subtracted from the counts of each sample.

Determination of Milk Binding Capacity for Methyl THFA

For each new preparation of milk binder, a quantity is selected that has a maximal capacity to bind approximately 50–60% of the standard amount of $^3$H methyl THFA used in each test. A fixed amount of $^3$H methyl THFA (500 pg [0.5 ng] prepared in saline-ME) is incubated for 30 min at room temperature with increasing amounts of milk binder. This is followed by batch separation of free folate from bound folate using coated charcoal and measurement of the quantity of $^3$H methyl THFA bound (see Table 1). A curve constructed from the results of such an experiment is illustrated in Fig. 1, which shows that 2 mg of milk powder in solution bound approximately 50% of 500 pg of $^3$H methyl THFA.

Each preparation of dialyzed milk solution shows reproducible folate binding curves for

Fig. 2.—Effect of pH on the binding of $^3$H methyl THFA by milk.

* Toluene containing 0.7% 2,5-diphenyloxazole (PPO) and 10% bio-SOLV BBS-3 (Beckman Instruments, Fullerton, Calif.).
approximately a month if kept at 4°C. The milk binding of 3H methyl THFA is pH-dependent, stable from pH 6.8 to 7.4, with a rapid decrease in binding below pH 6.6 (Fig. 2). The binding does not appear to be appreciably influenced by temperature changes from 4°C to 37°C.

If one wishes, one can also determine the maximal methyl-THFA binding capacity of the milk binder preparation. A fixed quantity of milk in each of 6–12 test tubes is incubated for 30 min at room temperature with a quantity of 3H methyl THFA that is progressively greater in each test tube. Hemoglobin-coated charcoal is then used to separate free from bound 3H methyl THFA. Figure 3 shows 12-test-tube run demonstrating the maximal methyl-THFA binding capacity of dialyzed powdered milk solution (containing 2 mg of powder) to be in the range of 1.8 ng of methyl THFA, with the ascending limb straight-line portion of the curve breaking at about 1 ng.

**Standard Curve**

A standard curve must be prepared at the time of assay and serves as the basis for

**Sample calculation**

cpm standard-bk. = 1364
cpm supernatant control-bk. = 43
cpm O-bk. = 879

\[
\begin{align*}
879 - 43 & = 836 \\
1364 - 43 & = 1321 \\
\frac{836}{1321} & = 0.632 \\
63.2 & = 1.72
\end{align*}
\]

**Fig. 4.—Standard curve prepared after radiodilution of 0.5 ng of 3H methyl THFA by varying quantities of d, l methyl THFA. The bound to free ratio of 3H methyl THFA is plotted against added cold d, l methyl THFA.**
calculation of serum folate for all of the sera assayed at that time. The standard curve is constructed with concentrations of stable d,l-methyl THFA ranging from 0.5 to 10.0 ng (Table 1). The B/F (bound/free) ratios are calculated, and a curvilinear plot, which is the standard curve, constructed as illustrated in Fig. 4. This figure shows sensitive competition at levels of 0.5 ng of d,l methyl THFA. A representative calculation of bound/free ratio is also illustrated in Fig. 4.

The effect of protein on the standard curve was studied, as indicated in Fig. 5. There is little difference in the curve sensitivity when prepared in phosphate buffer as compared to preparation in pooled dialyzed serum or dialyzed 5% albumin. Standard curves are therefore prepared in buffer, since no correction is necessary when different sera are assayed. A single standard curve can be used to assay a large group of different sera.

Calculation of Serum Folate Level

The B/F ratio is calculated for each serum assayed and the serum concentration of methyl THFA is determined by reference to the standard curve.

RESULTS

Figure 6 shows a comparison of 80 serum folate levels measured by both
the aseptic addition L. casei method and the present method, separated into "low," "probable low," and other areas, based on results of the radioisotope assay. Each serum was tested in duplicate by both methods. The folate levels ranged from <1 ng to 25 ng/ml. There is a close correlation in sera so far studied, with a definite separation, without overlap, between low (0-4 ng/ml) and normal (>9 ng/ml with the milk assay) serum folate levels. There are ranges of serum folate levels (4-7 ng/ml) in the milk assay and (3-5 ng/ml) in the L. casei assay that represent possible deficiency. An indeterminate range is also present in both assay methods (6-9 ng/ml for the milk assay). The folate levels obtained from each serum by the two assays were sufficiently similar to permit the same diagnostic interpretation, except that at some of the values less than 3 ng/ml by the L. casei assay, the isotope assay was consistently slightly higher. This may be due to decreased competition by stable d,L-methyl THFA in constructing the standard curve, perhaps due to failure of the d-form to bind to milk. Some of the normal values do appear somewhat lower by the isotopic than by the microbiologic assay. Whether the observation proves generally so remains to be determined, as does any possible significance thereof.

Daily fasting blood samples obtained for 4 days from a normal subject, when assayed for serum folate twice at intervals of 2 wk, are reproducible within a 10% variation (Fig. 7). Serum dialyzed overnight against 0.1 M phosphate buffer pH 7.4 has no folate activity when measured in this system, and stable methyl THFA added to serum is completely recovered.

The competition by various folates and folate analogues for milk binding sites for 3H methyl THFA is shown in Fig. 8. There is no competition by methotrexate or folinic acid. However, PGA competes with methyl THFA for the milk binding of 3H methyl THFA, suggesting that 3HPGA could be used instead of 3H methyl THFA to assay serum folate, provided an appropriate

Fig. 7.—Reproducibility of daily serial folate levels obtained when assayed twice at 2-wk intervals.
Fig. 8.—The competition by various folates and folate analogues for milk binding sites when added to milk prior to \(^{3}H\) methyl THFA: MTX, methotrexate; Folinic acid (Leucovorin); PGA, pteroylglutamic acid. Ordinate is \(1/\%\) of \(^{3}H\) methyl THFA (0.5 ng) bound by 2 mg milk powder in aqueous solution.

Correction is made for the relative affinity of PGA to that of natural folate for milk binder.

Sera obtained from patients receiving antibiotics were measured for folate levels by both assay methods (Fig. 9). In eight such sera, where L. casei folate levels were less than 1 (presumably antibiotic interference with L. casei growth)\(^{13}\) low normal or normal levels were measured by the milk \(^{3}H\) THFA assay. In one patient receiving methotrexate, the serum folate was predictably not measurable by L. casei, but a normal serum level was obtained with the isotope assay.

Fig. 9.—Serum folate levels measured by L. casei assay as compared to isotope assay of patients receiving antibiotics or methotrexate. MTX, methotrexate.
**DISCUSSION**

Metz et al.³ and Ghitis et al.⁴ demonstrated that milk has a fixed binding capacity for PGA. Da Costa and Rothenberg⁵ assayed PGA with antibody to PGA and ³HPGA, but their serum measurements gave results much lower than expected serum *L. casei* folate levels.

The present procedure uses: (1) ³H methyl THFA of high specific activity, with which there is effective competition by serum folate (mainly methyl THFA) for the milk binder; (2) A powdered milk product of long shelf life and easy availability as folate binder; (3) A stable standard curve not influenced by different sera or proteins.

Microbiologic assay of serum folate cannot be used easily with bacterially contaminated or turbid serum and gives false low results if the serum contains a high level of certain antibiotics¹² or antifolates. Moreover, microbiologic assay requires overnight incubation and the maintenance of a special laboratory for such procedures. The present assay does not have these disadvantages. It is specific for methyl THFA, PGA, and closely allied folates and is not affected by methotrexate, folinic acid, or antibiotics. The time for assay is less than 4 hr. It can be performed in a clinical isotope laboratory not designed solely for assay of serum folate.

The values obtained in serial blood samples when assayed twice at intervals of 2 wk by the isotope method were reproducible within a 10% variation. When serum is to be kept at room temperature in excess of 48 hr prior to assay, folate content must be protected against oxidative destruction by a reducing agent such as 2-mercaptoethanol. Microbiologic assay has less reproducibility. The radioisotope and microbiologic assays separate serum folate levels into closely similar recognized diagnostic groups.

It is important to note that in the current studies the nonradioactive methyl-folate was a racemic mixture and the radioactive methyl-folate was only the active stereoisomer. In view of the evidence, in this study and that of Ford et al.,⁵ that only the active isomer binds to milk, true serum folate values (assuming only the active isomer is present in serum) by the isotope method reported here may be only half the calculated values, and these may more closely superimpose on the *L. casei* values, rather than being higher.

Milk contains other specific binders in addition to the milk folate binder, which could be useful in developing radioisotope assays of a wide range of vitamins or minerals. Examples are the lactoferrin¹⁴ which avidly binds iron, and the milk B₁₂-binding protein.¹⁵

**ADDENDUM**

The only commercially available labeled methyl THFA is the material from Amersham/Searle. This material is of inadequate specific activity for this assay. For this reason, we have developed a modification utilizing commercially available ³HPGA of suitable specific activity.

In this modification, ³HPGA (specific activity 33 Ci/mM; Amersham/Searle) can be used instead of ³H methyl THFA to assay serum folate. The protocol is the same as described in this report for ³H methyl THFA; however, a two step or sequential assay is used. The serum to be assayed is first incubated with the milk binder and, after 30 min, ³HPGA is added. Separation by charcoal is the same. The standard curve is constructed by
Table 2.—Measurement of Serum Folate Levels by Sequential $^3$HPGA Assay as Compared With L. casei Assay

<table>
<thead>
<tr>
<th>Serum</th>
<th>L. casei Assay</th>
<th>$^3$HPGA Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>16.3</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>11.0</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Range 7.5–16</td>
<td>11.1</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>20.6</td>
<td>9.8</td>
</tr>
<tr>
<td>Folate-deficient</td>
<td>&lt;2 &lt; 1.9</td>
<td>2.75 2.8</td>
</tr>
<tr>
<td></td>
<td>&lt;2 &lt; 4.6</td>
<td>0.75 7.0</td>
</tr>
<tr>
<td></td>
<td>&lt;2 &lt; 1.1</td>
<td>0.75 2.5</td>
</tr>
<tr>
<td></td>
<td>&lt;1 &lt; 1.0</td>
<td>0.38 0.30</td>
</tr>
<tr>
<td></td>
<td>&lt;1     &lt;1</td>
<td>0.0 0.0</td>
</tr>
</tbody>
</table>

addition of $d$, $l$-methyl THFA to milk binder, followed after 30 min by addition of $^3$HPGA. Table 2 shows preliminary correlation between serum folate values obtained by this method as compared with the L. casei method. The use of $^3$HPGA not only obviates the need for $^3$H methyl THFA but also offers a more stable label to work with. A full report of this method is in preparation.

ACKNOWLEDGMENT

We wish to thank Miss Leona Bandel and Mrs. Z. Bokslawski for measurement of serum folates by L. casei. We also thank Miss Le Teng Go and Miss Lois Brenner, who reproduced a number of the studies here reported.

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