Comparison of Radioassay and Microbiological Assay for Serum Folate, with Clinical Assessment of Discrepant Results

Lynn Baril and Ralph Carmel

Folate assays by use of radiolabeled folate provide obvious practical advantages over the standard microbiological assay, but remain incompletely tested. We therefore compared results for 415 sera with a kit involving $^3$H-labeled folate and the Lactobacillus casei microbiological method. We examined the patients' data when there were discrepancies between the two methods. Although the correlation overall was satisfactory, results were discrepant in 25% of cases. In 74% of the latter, the radioassay result appeared to be the correct one, primarily because L. casei results were suppressed by antibiotics being taken by the patient. The radioassay occasionally gave falsely high values for patients with liver disease and falsely low ones for patients who had received isotopes for scanning purposes. Several assay kits that make use of $^{125}$I- or $^{75}$Se-labeled folate were also tested. Although these results correlated with the results of $^3$H-labeled folate assay, various problems appeared, including the possible need for serum-supernate control tubes in one kit. Answers to these and other questions and careful clinical correlation of results are needed for any folate radioassays before their adoption for routine clinical use.

Additional Keyphrases: “kit” methods · variation, source of · liver disease · ascorbate as stabilizer

For obvious practical reasons, a reliable radioisotopic assay for serum folate would be a highly desirable substitute for the microbiological assays that have long been the standard. These reasons include the difficulty of working with microbiological methods, which limits the number of laboratories that can use them, the several days required before results are available, and interference with growth of the test microorganism by antibiotics and other drugs the patient may be receiving. However, an ideal radioassay has not yet been established. The many methodologic problems and still unresolved questions have been reviewed elsewhere (1). A further cautionary note may be provided by the recent report (2) that vitamin B$_{12}$ radioassay fails to identify a significant proportion of vitamin B$_{12}$-deficient sera that have demonstrably low concentrations by microbiological assay. Yet radioassay has long ago supplanted the cumbersome microbiological assay for that vitamin in most laboratories.

An unfortunate development in the face of the above problems has been the commercial introduction of a prodigious variety of radioassay kits for folate, and the widespread adoption of them by clinical laboratories. In fact, many of these kits have not been adequately tested, often do not perform up to manufacturers’ claims (3, 4), and furthermore are often modified by individual clinical laboratories without re-evaluation (1).

Proper testing of radioassay methods requires, among other things, careful comparison of results with those by the older method (Lactobacillus casei assay) and correlation with clinical and hematological data. Most published reports have provided comparison with microbiological assay. However, the number and selection of samples have not always been optimal, and in many instances (3–10) the correlation demonstrated between the assays has been inexact. Furthermore, even in the presence of an acceptable correlation coefficient, direct examination of the paired data often revealed a striking number of discrepancies (5, 11–14) which could not be attributed solely to the presence of antibiotics in the sera. Sometimes the authors thereupon only claimed that their radioassay was capable of differentiating between low and normal concentrations. Results were seldom examined for a wide variety of patients and clinical and hematological evaluation was rarely done in those cases where the two methodologies gave dissimilar results, aside from identifying instances where antibiotic use was responsible.

Therefore, we present our results, where patients' data were retrospectively evaluated in an attempt to identify sources of discrepancies. The radioassay kit selected for the main part of our study was one that we and others (3) have found to be among the most reliable of the many now commercially available.

Methods and Materials

Without conscious bias, we selected 415 serum samples submitted to our laboratory for folic acid determinations for use in the comparison. The blood samples had been centrifuged within 5 to 10 min of receipt; however, the interval from blood drawing to receipt varied. Sera were stored at −20 °C until assayed; repeated thawing and freezing was avoided. Because the sera were kept frozen and were also to be assayed for vitamin B$_{12}$, ascorbate was not added. Others have shown that serum stability is acceptable without added ascorbate (13, 15), although the point remains controversial (7). We routinely use the L. casei microbiological assay (15). Results were compared to those obtained with a $[^3]$Hpteroylglutamic acid ($^3$H-PGA) kit (Diagnostic Products Corp., Los Angeles, Calif. 90064), the radioassay being done exactly as specified by the manufacturer, except that buffer pH was adjusted to 9.5 instead of 9.2, as recommended (16, 17) for better equivalence of binding of PGA and 5-methyltetrahydrofolinic acid by milk binders; in the kit, β-lactoglobulin—or, more precisely, an
unidentified contaminant of it (18)—is used as the binder. Sample radioactivity was counted in 10 ml of Phase Combining Solubilizer (Amersham Searle Corp., Arlington Heights, Ill. 60005), in a liquid scintillation counter. Quench correction was done by internal standardization, according to the manufacturer’s (Diagnostic Products Corp.) directions.

Sera were considered to give discrepant results if the values by the two methods fell into different diagnostic categories (i.e., high, normal, indeterminate, or low) according to the manufacturers’ ranges for results with the kits and the range obtained by Herbert (15) for the L. casei assay. The charts of 85 of the 103 such patients encountered were available for review. The following data at the time of blood drawing were sought: hemoglobin concentration; mean corpuscular volume; leukocyte count; description of blood morphology; serum vitamin B₁₂ concentration, lactate dehydrogenase activity, and bilirubin concentration; medications taken; and clinical diagnoses. With this information in hand, we attempted to assess which assay result was more compatible with the patient’s clinical picture and what factors might account for aberrant results. For example, folate deficiency was diagnosed and the lower of the two folate results was accepted whenever unequivocal megaloblastic anemia (either based on bone-marrow morphology or on finding hypersegmented neutrophil nuclei) was accompanied by normal serum vitamin B₁₂ concentrations. We also accepted folate deficiency as probable if, in the absence of vitamin B₁₂ deficiency, macrocytic anemia was associated with at least one of the following: response to subsequent folate therapy; co-existing disorders commonly associated with folate deficiency, such as sprue, anticonvulsant use, or pregnancy; a striking history of poor dietary intake; or

<table>
<thead>
<tr>
<th>Method</th>
<th>Isotopic label</th>
<th>Folate standard</th>
<th>Folate binder</th>
<th>Stated normal range µg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnostic Products (Los Angeles, Calif. 90064)</td>
<td>³H-PGA</td>
<td>methyltetrahydropfolate</td>
<td>β-lactoglobulin</td>
<td>3-21</td>
</tr>
<tr>
<td>Schwarz/Mann (Orangeburg, N.Y. 10962)</td>
<td>¹²⁵I-PGA</td>
<td>PGA</td>
<td>β-lactoglobulin</td>
<td>4-20</td>
</tr>
<tr>
<td>Bio-Rad Laboratories (Richmond, Calif. 94804)</td>
<td>¹²⁵I-PGA</td>
<td>PGA</td>
<td>β-lactoglobulin</td>
<td>1.9-14.0</td>
</tr>
<tr>
<td>New England Nuclear (North Billerica, Mass. 01862)</td>
<td>¹²⁵I-histamine PGA</td>
<td>PGA</td>
<td>β-lactoglobulin</td>
<td>2.3-15.2</td>
</tr>
<tr>
<td>Amersham/Searle (Arlington Heights, Ill. 60005)</td>
<td>⁷⁷Se-&quot;seleno-folate&quot; methyltetrahydropfolate</td>
<td>porcine serum</td>
<td>&gt;4.0</td>
<td></td>
</tr>
<tr>
<td>L. casei (ATCC 7469, American Type Culture Collection, Rockville, MD 20852)</td>
<td>PGA</td>
<td>- - - -</td>
<td>5-25</td>
<td></td>
</tr>
</tbody>
</table>

(3-5 indeterminate)

Fig. 1. Comparison of ³H-PGA radioassay and L. casei assay folate results for 415 sera (r = 0.87, slope = 0.752, intercept = 2.279)

Not shown are 11 sera for which points on the graph are superimposed on existing points and 26 sera for which radioassay results exceeded 24 µg/liter. L. casei assay gave a value of 5.1 µg/liter for one of the latter, a serum which was contaminated by antibiotics (folate value was 37.2 µg/liter by radioassay); two others also showed discrepancies, albeit lesser ones (13.6 and 17.2 µg/liter by L. casei assay vs. 30.0 and 31.0 µg/liter by radioassay)
an otherwise unexplained increased activity of serum lactate dehydrogenase (EC 1.1.1.27). On the other hand, a low folate concentration was deemed incorrect and the higher value was accepted if the patient was taking folic acid supplements, or if he was taking antibiotics and had no stigmata of folate deficiency but had low folate values by microbiological assay, or if the patient very clearly had no evidence of megaloblastic anemia and also had no obvious common causes of low serum folate concentrations such as poor diet or recent consumption of alcohol.

We also assayed selected serum samples with some other radioassay kits (Table 1), following the manufacturers’ instructions in each instance and using their normal ranges. In one set of assays for each of two kits, serum-supernate control tubes were incorporated for each serum assayed. Such tubes were identical to the usual serum tubes except that an equivalent volume of buffer was substituted for the folate binder. The radioactivity in these tubes was subtracted from that of the serum tubes. We compared the resulting folate values to those obtained without subtracting the supernate control.

Results

Comparison of 3H-PGA Assay with L. casei Assay

Several sera were assayed repeatedly during several months, to determine inter-assay variation. The 3H-PGA assay gave a mean of 6.7 ± 1.7 (1 SD) µg/liter with a standard error of 0.33 for one normal serum in 26 observations, and a mean of 5.3 ± 0.5 µg/liter with a standard error of 0.16 for a second in nine observations. Respective L. casei assay means for the same two sera were 8.6 ± 1.9 and 4.3 ± 0.3 µg/liter, with standard errors of 0.24 and 0.02 in 60 and 108 observations, respectively. Omission of ascorbate can also be seen to have had no effect on folate stability. Similar evaluation of eight low-folate sera was done with L. casei assay. Mean values ranged from 1.2 ± 0.1 to 2.8 ± 0.4 µg/liter in eight to 15 observations; standard errors ranged from 0.03 to 0.11.

Comparison of results for the 415 sera by the two methods gave a correlation coefficient of 0.87, but Figure 1 demonstrates the wide scatter in many cases. Analysis by chart review was possible in 85 cases where there were discrepant results, as defined in the previous section (Table 2). The following specific points can be made: (a) isotopic contamination of serum, usually from gallium or technetium given the patient for scans (19), falsely lowered radioassay results in four cases, in two of which the values thereupon fell into a different diagnostic category; (b) 25 of the 45 falsely low L. casei assay results could be attributed to antibiotics given the patient, and in only 11 of those 25 sera did we suspect this problem during the assay by noting decreasing microbial growth with increasing volume of serum; and (c) 14 patients with megaloblastic anemia not caused by vitamin B12 deficiency, and thus apparently attributable to folate deficiency, gave falsely high results for 3H-PGA assay (3.2–11.2 µg/liter, as compared with <1.0–4.1 µg/liter by L. casei assay). Interestingly, 13 of those 14 patients had alcoholic liver disease, and the other had chronic renal failure. The 3H-PGA assay requires scintillation counting, and so we at first suspected quenching by hyperbilirubinemia of causing the falsely high radioassay results above. However, correcting for quench did not alter the values significantly.

Evaluation of 125I-labeled PGA and 75Se-labeled Folate Kits

We selected 34 sera for assay with one or another of the 125I-labeled PGA kits and 33 for assay with the 75Se-labeled folate kit. Because the numbers involved were small and many sera were selected specifically because of discrepancy between results by 3H-PGA and L. casei assay, only limited conclusions are possible. Nevertheless, in sera with discrepant results all the radioassay methods tended to agree with each other more often than with L. casei assay (Table 3). This was particularly true in sera from patients with liver disease, although in two such sera 125I-PGA assay results agreed with L. casei rather than with 3H-PGA assay results. 125I-PGA and 3H-PGA assay results correlated well (r = 0.91 for all 34 sera). However, the New England Nuclear kit, and to a much lesser extent the other two 125I-PGA kits, produced consistently lower results than did the 3H-PGA kit. The 75Se folate assay results correlated slightly less well with 3H-PGA assay results (r = 0.82) than did the 125I-PGA kits.

Evaluation of Serum-Supernate Controls

Residual binding for folate by serum was negligible with the 3H-PGA kit. Radioactivity in the serum-supernate control tube barely exceeded background activity and did not vary

<table>
<thead>
<tr>
<th>Discrepancy</th>
<th>L. casei correct</th>
<th>3H-PGA correct</th>
<th>No conclusion possible</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. casei higher than 3H-PGA (5 sera)</td>
<td>2*</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3H-PGA higher than L. casei (80 sera)</td>
<td>14</td>
<td>45</td>
<td>21</td>
</tr>
<tr>
<td>Total (85 sera)</td>
<td>16</td>
<td>45</td>
<td>24</td>
</tr>
</tbody>
</table>

* We encountered four such sera in which isotopic contamination had caused falsely lowered radioassay folate values, but in the two the discrepancy was not great enough to cause results to fall into a different diagnostic category.

Table 2. Clinical Analysis of Discrepancies Between Values for Folate by L. casei and 3H-PGA Assay

<table>
<thead>
<tr>
<th>Radioassay</th>
<th>No. sera</th>
<th>3H-PGA &amp; L. casei</th>
<th>3H-PGA only</th>
<th>L. casei only</th>
<th>Neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-PGA</td>
<td>34</td>
<td>13</td>
<td>13 (3)*</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Schwartz/Mann</td>
<td>11</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bio-Rad</td>
<td>12</td>
<td>5</td>
<td>5 (2)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>New England Nuclear</td>
<td>11</td>
<td>5</td>
<td>2 (1)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>[75Se] folate</td>
<td>33</td>
<td>18</td>
<td>8 (3)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>31</td>
<td>21 (8)</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate number of sera where low L. casei assay result was caused by antibiotic administration.

Table 3. Comparison of 125I-PGA and 75Se-Folate Radioassay Results with Those Obtained by 3H-PGA and L. casei Assays

Evaluation of Serum-Supernate Controls

Residual binding for folate by serum was negligible with the 3H-PGA kit. Radioactivity in the serum-supernate control tube barely exceeded background activity and did not vary

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among the 18 specimens tested, which included sera from patients with liver disease, renal failure, or leukemia. In contrast, supernatant counts with the [75Se]folate kit were sizeable in the 13 sera tested. Equally importantly, these counts varied among the different sera, ranging from 10 to 65% of the [75Se]folate counts bound by the folate binder for that particular serum (mean of 16% ± 24). Table 4 illustrates their effect on results for serum folate.

### Discussion

Comparisons of folate methodologies in which clinical correlation has been attempted have heretofore been rare. Although ours was not a prospective study and in several cases presence or absence of folate deficiency could not be established definitively, several conclusions are possible.

First, despite a respectable correlation coefficient overall, fully 25% of our sera fell in different diagnostic categories when tested by microbiological assay and by Diagnostic Products' 3H-PGA radioassay. The wide scatter in Figure 1 is striking.

Second, in those 61 cases where a reasonable conclusion could be drawn about which of the two assay results was correct, the radioisotopic assay was correct 74% of the time. This was so chiefly because of the many microbiological assay results suppressed by the antibiotics being given the patient. If we omit antibiotic-containing sera from analysis, then the distribution of incorrect results is similar between the two methods. Apparently, therefore, the chief advantage in reliability for the radioassay is that it is better if one often deals with sera from patients for receiving antibiotics. The two chief identifiable disadvantages of the 3H-PGA radioassay are that it gives falsely high results in patients with liver disease and, as described previously (19), falsely low ones when the patient has received injections of isotopes. The explanation for the apparent radioassay artifact in some patients with liver disease is not apparent. Quenching in the 3H-PGA assay did not appear to be responsible. We found the artifact to be the opposite of that described by Kamen and Caston (8).

Third, in a limited series, folate radioassays using gamma-emitting isotopes gave results generally corresponding with those of the 3H-PGA radioassay. However, several instances of variance were noted, particularly with the New England Nuclear kit, as noted by others (14). Kubasik et al. (3) have found low results with several other 125I-PGA kits that they tested. More extensive studies correlating results of 125I-PGA methods with clinical data are needed before any definite conclusions are drawn.

Fourth, we incorporated serum-supernate controls (i.e., tubes identical to the serum assay tubes except that the step of adding folate binder was omitted) into the H-PGA and the [75Se]folate radioassays. Shaw et al. (6) state that radioassays must incorporate such control tubes. Our results indicate that such supernate controls are unnecessary for the H-PGA assay but may be necessary with the [75Se]folate kit. Clearly, this question must be resolved for each radioassay before it is used.

In summary, our findings indicate that none of the assay methodologies is consistently reliable. Although significant discrepancies in results between microbiological and radioisotopic folate assays are frequent, the latter, at least in the form of Diagnostic Products' 3H-PGA kit, may be the most practical of those we have tested. Given the frequency of antibiotic contamination that we encountered, the latter method may actually be preferable to the microbiological assay because results with it are more often correct. However, sera from patients with liver disease often gave falsely high results by radioassay, and little is known about the other causes of discrepant results. Studies to elucidate the cause of the above artifact of the radioassay would be important. Obviously, too, all new methods must be subjected to prospective analyses with careful clinical correlation before they can be adopted for routine use.

We thank the manufacturers of the five radioassay kits listed in Table 1, each of whom donated one or two kits to us. (If more than two kits were required from a manufacturer, we purchased them).

### References


Microchromatographic Methods for Hemoglobin A₂ Quantitation Compared

Effie M. Brosious, Jane M. Wright, Rosalie M. Baine, and Robert M. Schmidt

On 20 consecutive work days during four weeks, one technologist performed 24 microchromatographic determinations of hemoglobin A₂ (Hb A₂) by each of four methods: the Efremov procedure requiring Tris/HCl buffer, the original Huisman technique with use of glycine developer, and two commercial test kits in which a modified glycine developer is used. The blood samples tested were obtained from 12 adults with no hematological abnormality and from 12 β-thalassemia carriers previously diagnosed by familial and hematologic studies. Results by the first method and the two commercial kits (one from Helena Laboratories and one from Isolab, Inc.) did not differ significantly in precision for either the normal or β-thalassemia trait samples. For both sample types, the second method yielded larger coefficients of variation than those obtained with the other methods. Moreover, the second method was the only one with which values overlapped for normal samples and samples with above-normal Hb A₂ concentrations.

Hemoglobin A₂ (Hb A₂) is increased in individuals with β-thalassemia trait. Methods available for quantitating Hb A₂ include column chromatography and electrophoresis followed by elution of hemoglobin bands. Densitometry was shown to be unreliable and inaccurate in a previous study (1). However, column chromatography adequately differentiated the samples from normal individuals and the samples from persons with β-thalassemia trait. The microchromatographic techniques (2, 3) and the newly marketed commercial-kit columns (Helena Laboratories, Inc., Beaumont, TX 77704; and Isolab, Inc., Akron, OH 44321) should provide a fast, simple column-chromatographic method for quantitation of Hb A₂, but the accuracy and precision of these methods must be confirmed.

We have compared these popular microchromatographic methods with respect to precision and normal ranges.

Materials and Methods

Whole-blood specimens were collected with disodium ethylenediaminetetraacetate as anticoagulant. Donors included 12 hematologically normal adults and 12 individuals confirmed by clinical and family studies to have β-thalassemia trait. Hemolysates were made from whole-blood samples by washing the erythrocytes four times with 8.5 g/liter NaCl and then lysing them with carbon tetrachloride and water as described elsewhere (4). The hemoglobin solutions were centrifuged for 15 min at 3000 rpm, and the clear supernate was removed as the test hemolysate. Hemolysates were stored at 4 °C during the 20-day study period. On each test day, the hemolysates were coded to eliminate operator bias and Hb A₂ was measured with the microchromatographic procedures described by Efremov et al. (2) and Huisman et al. (3) and also with the "Hb A₂ Quik Column" (Helena Laboratories, lot 1075335) and with "Quik-SEP" (Isolab Inc., lot 704051).

In the Tris method of Efremov et al. (2),¹ diethylaminoethylcellulose and hemolysates were equilibrated with

¹ We initially refer to each method by the primary author of the original paper describing the procedure. The methods are thereafter referred to by the developer/buffer used in the elution process.