Analytical Evaluation of a Folate Radioassay

Application to Serum and Erythrocyte Measurements

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ABSTRACT

Many commercial folate radioassay kits have proven to be unsatisfactory in the past. It has been found that the Schwarz/Mann Iodine-125 folate radioassay kit permits a rapid measurement of folic acid requiring only a single incubation at pH 9.3. At this pH, the relative affinity of pteroylglutamic acid (FA) and N-5-methyltetrahydrofolic acid (MTFA) for the milk binder is the same. Additionally, the radioassay was compared to the L. casei microbiological assay. Regression analysis of 42 patients' sera, folate concentration 0.9 to 14 µg per liter, gave a correlation coefficient of 0.963. At folate concentration greater than 14 µg per liter, results with the radioassay in many instances were lower than those with the microbiological assay. In some cases the discrepancy was as much as 50 percent. However, the recovery of pure FA, MTFA and mixtures of the two at concentrations of 20 to 40 µg per liter was satisfactory by radioassay. This would indicate that the microbiological assay is inaccurate at higher folate concentrations. It has been shown that the Schwarz/Mann folate kit is accurate, precise, unaffected by icterus and lipemia. It is, therefore, suitable for both serum and red blood cell folate assay. A discrepancy has been observed between serum and red cell folate concentration; consequently, a simultaneously assay of both folates is suggested.

Introduction

Until recently, folate has been measured by the cumbersome microbiological assay which is difficult to set up in a hospital laboratory. However, commercial vendors of kits have endeavored to make reagents for folate radioassay easily accessible and to render the assay relatively facile, rapid and accurate. The multiplicity of kits and procedures is very often confusing. In many instances, the microbiological reference procedure for comparison to the folate radioassay is not available in most laboratories.

Generally, problems associated with radioimmunoassay (RIA) commercial reagents arise from a variety of normal ranges quoted by various kit manufacturers, the plethora of analytical variations, and the near absence of a mandatory national quality control program. In many
cases, the misinformation and the lack of detailed information on the evaluation of the commercial kits, which are being increasingly used in the hospital laboratories, obligates the user to scrutinize critically these kits prior to their routine use. Owing to these reasons, the World Health Congress of W.H.O. has recognized the need for international standardization for *in vitro* diagnostic kits and reagents.4

Kubasik et al have recently documented problems associated with six commercial folate radioassay kits.9 In search of a suitable method for folate determination, an 125I-labeled kit for both the serum and red blood cell (RBC) folate assay,* was evaluated by us and compared to the *L. casei* microbiological or bioassay. Previously, this kit was investigated briefly by us and was found to be reliable for the folate assay.1

A detailed analytical evaluation of this kit and its comparison to the microbiological assay is presented. Additionally, data on the significance of simultaneous assay of serum and red blood cell folate and vitamin B12 determination are reported.

**Materials and Methods**

**Apparatus**

All reagents and patients’ sera were dispensed with semiautomatic ‘Lancer’ pipets with disposable tips.† Polypropylene 12 × 75 mm tubes with caps ‡ served both as the reaction and counting vials. A RC-3 refrigerated centrifuge with an HL-2 tube rack rotor§ was used to separate bound and free fractions. Sample counting and data reduction were done with an 1185 automatic gamma counter and interfacing pds/3 computer system.¶

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* Marketed by Schwarz/Mann, Orangeburg, NY 11982.
† Sherwood Medical Industries, St. Louis, MO 63103.
‡ Falcon Co., Oxnard, CA 93030.
§ Sorvall, Newtown, CT 06470.
¶ Searle Analytic Inc., Des Plaines, IL 60018.

**Reagents**

Pteroylglutamic acid or folate acid (FA) and N-5-methyltetrahydrofolic acid (MTFA) were used in recovery studies.11 Radioassay control sera “I” and “II”** were used for assay quality control. These lyophilized control sera were reconstituted with distilled water, stored frozen at −30° in 50 μl aliquots and thawed immediately before use.

All reagents for folate assay were supplied by the manufacturer and were reconstituted with distilled water. They were stored at 2 to 8° and brought to room temperature before use.

The folate assay kit contains reagents for a total of 200 determinations. The lyophilized reagents include: five individual FA standards (1.5, 3.0, 5.0, 10.0 and 20 μg per liter) in a lysine-gelatin-dextran buffer at pH 9.3, four vials each containing 1.2 μCi of 125I-labeled FA, and folate binder isolated from bovine milk. Additionally, a 40 μg per liter standard was included in all assays. The buffer used in the assay is provided as a dry powder containing lysine, lysine hydrochloride, gelatin and sodium ascorbate. It was prepared fresh daily and the pH adjusted to 9.3 ± 0.1 prior to use. A vial of ascorbic acid powder for the preparation of RBC hemolysate is provided. This lysing solution was prepared fresh daily. A dextran-charcoal suspension at 4° was used to bind the free fraction.

**Procedure**

Two separate blood specimens were drawn from patients after an overnight fast. One blood specimen (5 to 10 ml) was allowed to clot at room temperature for 30 min. The serum was then separated and divided into two equal proportions. One portion was shipped frozen for mic-
robiological assay elsewhere,* the other was frozen at \(-30^\circ\) for radioassay in our laboratory.

The hematocrit of the second blood specimen (2 to 5 ml), collected in ethylenediamine tetraacetic acid was determined by a Model Ssr Coulter Counter.† A 100 \(\mu\)l aliquot of the well suspended whole blood was added to 2 ml of the 0.2 percent aqueous ascorbic acid solution.

The resulting hemolysate (final dilution, 1:21) was allowed to stand at 20 to 25\(^\circ\) for 90 min for complete hydrolysis of folic acid polyglutamates. The hemolysate was then stored at \(-30^\circ\) until assayed for red cell folate.

A 50 \(\mu\)l aliquot of serum or hemolysate was added to the appropriately labeled tubes containing 900 \(\mu\)l of the buffer. The tubes were loosely capped, gently mixed and heated in the dark at 100\(^\circ\) for 15 min to free the folate from endogenous binders. After being cooled to 20 to 25\(^\circ\), 100 \(\mu\)l of \(^{125}\)I-labeled FA were added, followed by 100 \(\mu\)l of milk binder. After being mixed, the tubes were allowed to stand at room temperature in the dark for 30 min. A 0.4 ml suspension of cold dextran-charcoal was then squirted into each tube. The tubes were allowed to incubate for 5 min at room temperature and then centrifuged for 15 min at 1,800 \(\times\) g at 4\(^\circ\). The supernatant containing the bound fraction was decanted and counted. Standards and control sera were similarly treated. Nonspecific binding (NSB) for the assay was determined by adding 100 \(\mu\)l of \(^{125}\)I-labeled FA to 1 ml of the buffer without the addition of the folate binder. All assays were done in duplicate.

All tubes were counted for the time necessary to obtain 10,000 counts and the serum folate concentration was directly computed by the interfacing computer system. The RIA computer program used automatically corrected for NSB and interpreted the folate concentration from log-logit transformation of the experimental data.

\[
\text{Logit (y)} = \text{Log}_e \frac{y}{1-y}
\]

Where \(y = \frac{B}{B_o}\), \(B\) represents the counts per min (cpm) of the sample, and \(B_o\) the cpm of the zero standard, — the maximum binding obtained in the assay.

![Figure 1. Variability of the Schwarz/Mann folate standard curve.](image)
Red cell folate was calculated with the following formula using the assay value obtained directly by the computer:

\[
\text{RBC folate (µg/liter)} = \frac{2100 \times \text{folate assay value (µg/liter)}}{\text{hematocrit}}
\]

Results

It is not uncommon to observe batch to batch variation in commercial reagents. The variability of the folate standard curve with eight different lots of reagents was investigated by the present authors over a period of four months (figure 1). Each point on the curve represents a mean of 30 individual determinations. The \( B_0 \) for the freshly manufactured kits was 49 to 57 percent, decreasing progressively with the age of the kit. This, however, did not affect the results. The linearity of the relationship between sample concentration and binding obtained from log-logit transformation of the experimental data is shown in figure 2. Folate concentrations as high as 80 µg per liter could be accurately measured.

The effect of specimen blanks owing to the presence of endogenous folate binders has been documented. The effect of heat treatment on serum and hemolysate blanks was examined by us. This was done by omitting the folate milk binder from the assay system. The results showed a binding equal to ± 1 percent of NSB. It is, therefore, evident that the effect of specimen blank was eliminated by thermal destruction of the endogenous folate binders.

Assay sensitivity was experimentally determined to be 0.7 µg per liter (table I). Assay accuracy and the milk binder affinity for FA and MTFA was determined by the addition of FA, MTFA and their mixtures (table II) to patients' sera at concentrations of 20 to 40 µg per liter. Additionally, recovery of 1:1 mixtures of FA and MTFA at concentrations of 1.5, 3.0, 5.0 and 10.0 µg per liter from red blood cell hemolysates was carried out. The analytical recovery from serum was found to be 90 to 107 percent for FA, 90 to 103 percent for MTFA, and 90 to 102 percent for the mixtures (table II). The recovery of mixtures for FA and MTFA from hemolysates was 96 to 101 percent. These data indicate that the milk binder has equal affinity for both FA and MTFA at pH 9.3.

Assay quality control is shown by intra-assay and interassay variance (table III). These are comparable to the target values reported by the manufacturer, although the manufacturer did not adhere to the accepted methodology for determining the target values.

The effect of abnormal concentrations of bilirubin (220 mg per liter) and triglycerides (25,000 mg per liter) was investigated on folate recovery. The results were not significantly affected in either

\[
\text{TABLE II}
\]

Analytical Recovery of Folic Acid and N-5-Methyltetrahydrofolic Acid Added to Patient's Sera

<table>
<thead>
<tr>
<th>FA/MTFA Added µg/liter</th>
<th>FA/MTFA Expected µg/liter</th>
<th>FA/MTFA Recovered µg/liter</th>
<th>Percent of Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>20/20</td>
<td>48.6</td>
<td>49.0</td>
<td>100.8</td>
</tr>
<tr>
<td>40/20</td>
<td>63.5</td>
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<td>101.7</td>
</tr>
<tr>
<td>40/20</td>
<td>66.6</td>
<td>67.3</td>
<td>98.1</td>
</tr>
<tr>
<td>20/40</td>
<td>63.5</td>
<td>63.4</td>
<td>99.8</td>
</tr>
<tr>
<td>20/40</td>
<td>68.6</td>
<td>69.5</td>
<td>101.3</td>
</tr>
</tbody>
</table>

\[ \text{Absorbance vs. Folate (µg/liter)} \]
case. However, the lipemic sera showed 
a slight negative bias.

Regression analysis of 42 patients’ sera 
having folate concentration less than 14 
μg per liter assayed by radioassay and 
microbiological assay is shown in figure 
3. At folate concentrations greater than 14 
μg per liter, results with radioassay in 
some instances were lower than those ob­
tained with the microbiological assay (table 
IV). In some cases the discrepancy 
was as much as 50 percent. Since recov­
er with the radioassay at concentrations 
higher than 14 μg per liter was found to 
be quantitative, the microbiological pro­
cedure appears to be in error at higher 
folate concentrations.

In order to determine the significance 
of simultaneous serum and RBC folate 
assay, these were measured in serum and 
hemolysate. Additionally, vitamin B₁₂ 
was assayed in serum with the 
Schwarz/Mann radioassay kit. Eleven 
patients from a total of 52 showed a dis­
crepancy between the serum and RBC fo­
late concentration (table V). Ten patients 
were found to have normal RBC folate 
concentration but a subnormal folate 
concentration (normal range: serum, 5 to 
25 μg per liter; RBC, 175 to 900 μg per 
liter). However, one patient had subnor­
mal RBC folate and vitamin B₁₂ concen­
tration, but a normal serum concentra­
tion. It is possible this patient was not 
fasting, thus showing a normal serum fo­
late concentration, yet was folate and/or 
vitamin B₁₂ deficient.

Discussion

In contrast to six other folate radioassay 
kits examined by Kubasik et al.,⁹ the 
Schwarz/Mann folate kit was found by 
the present authors to be suitable for 
serum and RBC folate assay. This assay 
required a single incubation at pH 9.3. 
Givas and Gutcho have shown that at this 
 pH both FA and MTFA have an equal 
affinity for the milk binder, thus eliminat-
The data in table V show that a discrepancy between serum and RBC folate can occur. This would seem to suggest a need for simultaneous serum and RBC folate measurement. Recently, Morse and Maxwell have suggested concomitant determination of tissue (RBC) and serum folate since serum folate concentration fluctuates more readily than the tissue folate. Additionally, vitamin $B_{12}$ concentration should be measured if folate concentration is found to be subnormal, since subnormal vitamin $B_{12}$ concentration can be responsible for folate deficiency.

It has been concluded by the present authors that the Schwarz/Mann folate radioassay kit is accurate, precise, unaffected by icterus and lipemia and suitable for both serum and RBC folate assay. Our data are in excellent agreement with a recent report in which the folate assay system was assembled from individual components.7

### References


### Table IV

<table>
<thead>
<tr>
<th>Folate Bioassay (ug per liter)</th>
<th>Folate Radioassay (ug per liter)</th>
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<tbody>
<tr>
<td>13</td>
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<td>15</td>
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<td>30</td>
<td>9.0</td>
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### Table V

<table>
<thead>
<tr>
<th>Serum Folate (ug per liter)</th>
<th>RBC Folate (ug per liter)</th>
<th>Vitamin $B_{12}$ (ng per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>246.8</td>
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<td>2.9</td>
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<td>675.4</td>
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</tr>
<tr>
<td>9.3</td>
<td>167.1</td>
<td>105.4</td>
</tr>
</tbody>
</table>

Normal Ranges: 5 - 25

Serum Folate: 175 - 900

RBC Folate: 300 - 1000