# Comparison of Radiometric Microbiologic Assay and Competitive Protein Binding Radioassay for Plasma and Red Blood Cell Folate Levels

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*We compared the radiometric microbiologic assay with a commercially available competitive protein binding radioassay kit for plasma and red blood cell folate. The correlation coefficient between these two assays was r = 0.78 for plasma folate and r = 0.87 for red blood cell folate. The sensitivity of the radiometric microbiologic assay was 96% for red blood cell folate.*

Folate deficiency occurs in a wide variety of conditions including alcoholism, malnutrition, and malabsorption. Patients undergoing renal dialysis, receiving anticonvulsants, or taking oral contraceptives may also develop folate deficiency. In addition, when increased use of folate occurs, as in pregnancy, hemolytic anemia, and malignancy, folate deficiency may arise (1–5). However, many people with biochemical evidence of folate deficiency are asymptomatic and have no hematological abnormalities. It is only when the synthesis of thymidylate is impaired beyond a critical level that the clinical features of folate deficiency become apparent (3).

There are three methods of determining folate levels: microbiologic assay, competitive protein binding radioassay, and radiometric microbiologic assay. The microbiologic assay for folate determination has been used as the standard method for many years. This assay is both highly sensitive and specific, but is very tedious and time-consuming, which prevents its widespread use. Microbiologic methods allow an accurate determination of the folate content of biological extracts, provided the folates are first hydrolyzed to the monoglutamate derivatives (6–9).

Several competitive protein binding radioassay procedures for the determination of serum, plasma, and red blood cell (RBC) folate have been described (10, 11). This assay is widely used and easy to perform, but does not provide folate levels in agreement with the microbiologic assay for RBC folate. One advantage of the competitive protein binding radioassay is that it is not influenced by drugs such as folate antag-

onists or antibodies, which might affect the microbiologic assay (11–15).

The radiometric microbiologic assay was developed by Chen and coworkers in 1978. They showed an excellent correlation with the microbiologic assay with a correlation coefficient of r = 0.96 for plasma folate and r = 0.98 for RBC folate. Good specificity and sensitivity of measurement were also established (6).

We compared the radiometric microbiologic assay with a competitive protein binding radioassay in the determination of plasma and RBC folate to see which method more accurately detects folate deficiency.

## Materials and Methods

We studied a total of 74 subjects. All had plasma folate determination by both methods and all had RBC folate determination by radiometric microbiologic assay. Only 47 subjects had RBC folate determination by the competitive protein binding radioassay. Subjects were evaluated by history, physical examination, and hematological indices by a staff physician prior to folate determination.

**Radiometric Microbiologic Method:** Venous blood from fasting subjects was drawn into heparinized tubes. A small aliquot was set aside to determine the hematocrit using a microhematocrit method.

To determine the RBC folate concentration, 1 ml of whole blood was diluted with 9 ml of deionized distilled water, vortexed for 2 min to ensure complete hemolysis of red blood cells, and then further diluted with 10 ml of 0.05 M phosphate buffer containing 150 mg% of freshly added ascorbic acid (pH 6.1). The resulting hemolysate was incubated at 37 °C for 20 min, aliquoted into 0.5 ml samples, and stored at −70 °C. On the day of the assay, the hemolysate was thawed and further diluted 1:5 with 0.05 M phosphate buffer containing 150 mg% of freshly added ascorbic acid. For the assay, 0.25 ml and 0.5 ml of the diluted hemolysate were added to duplicate 20 ml vials (Wheaton, Millville, NJ) containing 5 ml of Folic Acid Casei Medium (Difco Laboratories, Detroit, MI). The volume was brought to 9.8 ml with 0.05 M phosphate buffer.

For plasma folate measurement, the remaining blood was centrifuged at 400 g for 10 min. The plasma was separated, aliquoted, and stored at $-70\,°C$. On the day of the assay, a plasma aliquot was thawed and diluted 1:20 with a phosphate-ascorbate buffer. Other dilutions (1:10, 1:40) were also used, depending on the results obtained with the 1:20 dilution.

*Lactobacillus casei* (American Type Culture Collection, 7469a, Rockville, MD) was then transferred from agar slants (Lactobacilli Agar AOAC, Difco Laboratories) and incubated for 16–18 hr. On the morning of the assay, 0.5 ml of the suspension was transferred to another 10 ml of the same broth and incubated at 37 °C for 6–7 hr. The bacteria were centrifuged, washed three times with 10 ml of sterile Folic Acid Casei Medium, and resuspended in 10 ml of this medium. The suspension was further diluted 1:100 and 0.1 ml was used to inoculate each 20-ml vial using a tuberculin syringe.

Twenty milligrams of dried pteroylmonoglutamic acid (PGA) were suspended in 100 ml of a 10% ethanol solution. The pH was adjusted to 10.0 with 0.1 N sodium hydroxide to dissolve the folic acid, and then brought to pH 7.0 with 0.05 N hydrochloric acid. The stock solution was stored frozen in aliquots at $-70\,°C$. The working pteroylmonoglutamic acid standard, prepared on the day of the assay, was diluted in 0.05 M phosphate buffer to contain 1 ng of pteroylmonoglutamic acid/ml. For each assay, six different concentrations of the working pteroylmonoglutamic acid standard (0, and 0.1, 0.2, 0.4, 0.6, and 0.8 ng) were prepared and added to duplicate 20-ml vials containing 5 ml of Folic Acid Casei Medium with 1 mg ascorbic acid/ml. The volume was brought to 9.8 ml with the 0.05 M phosphate buffer.

All the vials were then autoclaved for 5 min at 15 psi. After cooling, 1.0 $\mu$Ci (0.1 ml) of [1 - $^{14}$C] gluconic acid and 0.1 ml of the bacterial suspension were added aseptically.

All the vials were simultaneously incubated at 37 °C for 18–19 hr. Bacterial growth was measured by quantifying the amount of $^{14}CO_2$ evolved using a Bactec 460. The radiometric microbiologic assay is based on the principle that bacterial metabolism can be detected by measuring radioactive $CO_2$ produced through the bacterial action of the C-14-labeled substrate. The radioactive $CO_2$ ionizes the air in the ionization chamber producing an electrical current, which is measured by the amplifier, converted to the digital form, and printed out as the "growth index." The rate of growth is proportional to the folate concentration. The radioactive $CO_2$ produced is then automatically trapped by an absorbent-filled chamber for safe and easy disposal. (The Bactec is also commonly used in the United States for the detection of bacterial growth in blood cultures [16].)

The plasma folate and whole blood folate levels for unknowns were interpolated from the standard curve. Results for RBCs were expressed in ng/ml of packed red cells using the following equation:

$$RBC\ folate = \frac{whole\ blood\ folate - plasma\ folate}{\frac{\%\ hematocrit}{100}}\left(1 - \frac{hematocrit}{100}\right).$$

**Radioassay:** The Simultrac kit (Becton Dickinson Immuno-

diagnostics, Orangeburg, NY) for simultaneous measurement of folate and vitamin $B_{12}$ was used. It is a competitive protein binding radioassay.

Heparinized venous blood was taken from fasting subjects and the hematocrit was determined using a microhematocrit method. The blood was prepared for RBC folate measurement by radioassay in a manner identical to that used for the radiometric microbiologic assay. This was a 1:20 dilution. The remaining blood was centrifuged and the plasma collected. The plasma and hemolyzate aliquots were then frozen at $-70\,°C$.

Duplicate tubes for each standard, plasma, and RBC sample were prepared and folate levels measured according to the manufacturer's instructions. A standard curve was plotted and plasma and hemolysate concentrations were interpolated and expressed in ng/ml folate. The hemolysate result is multiplied by 20 to give the whole blood folate concentration in ng/ml folate. The RBC folate is expressed in ng/ml of packed red cells, using the following equation:

$$RBC\ folate = \frac{whole\ blood\ folate}{\frac{\%\ hematocrit}{100}} \qquad (17).$$

## Results

The normal ranges for plasma and RBC folate by radiometric microbiologic assay were 4–20 ng/ml and > 140 ng/ml, respectively. These were determined by assaying 57 normal volunteers not on vitamin supplement. The competitive protein binding radioassay normals were determined by 50 normal volunteers and were 1.9–9.8 ng/ml for plasma folate and > 125 ng/ml for RBC folate.

The radiometric microbiologic assay identified 28 subjects with low plasma folate while the competitive protein binding radioassay identified 7 subjects with low plasma folate. The radiometric microbiologic assay identified 33 subjects with low RBC folate while the competitive protein binding radioassay failed to identify any subjects as having low RBC folate (Table 1). All the subjects who were taking vitamin supplements showed normal-to-high plasma and RBC cell folate values by both methods.

Figures 1 and 2 compare plasma and RBC folate results by the radiometric microbiologic assay and competitive protein binding radioassay. The correlation coefficients were r = 0.78 for plasma and r = 0.87 for RBCs.

The sensitivity and specificity of the radiometric microbiologic assay and competitive protein binding radioassay for plasma and RBC folate are listed in Table 2. Sensitivity is defined as the probability of being able to identify correctly those who do have a particular disease while specificity is the proba-

**TABLE 1. Subjects with Low Folate**

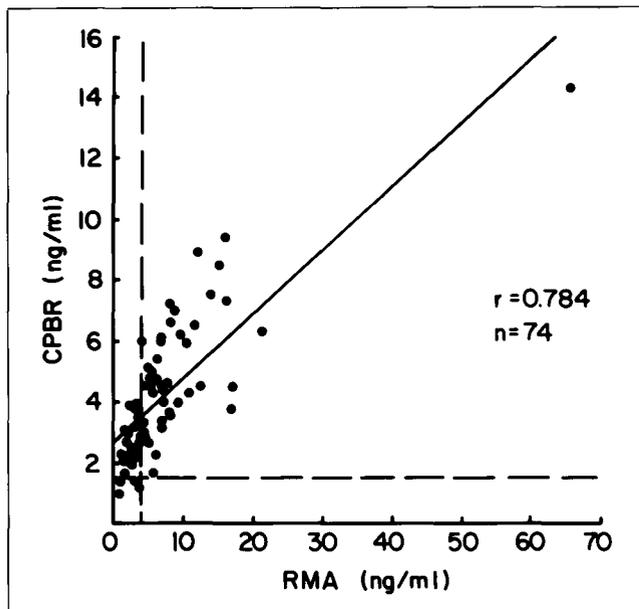|  | Plasma | Red Blood Cell |
| --- | --- | --- |
| Radiometric microbiologic assay | 28/74 | 33/74 |
| Competitive protein binding radioassay | 7/74 | 0/47 |

**FIG. 1.** Comparison of radiometric microbiologic assay and competitive protein binding radioassay for plasma folate measurements. Dotted lines represent lower limit of normal ranges.
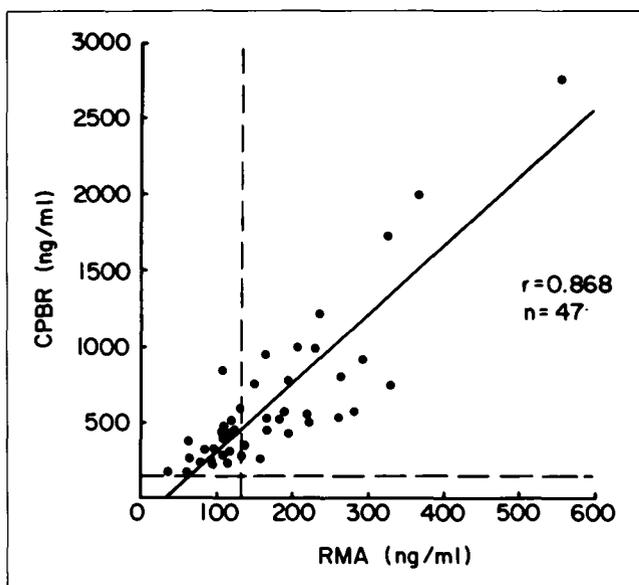


**FIG. 2.** Comparison of radiometric microbiologic assay and competitive protein binding radioassay for red blood cell folate measurements. Dotted lines represent lower limit of normal ranges.

bility of being able to identify correctly those who do not have the disease (*18*).

## Conclusion

Our results showed a poor correlation between the radiometric microbiologic assay and the competitive protein binding radioassay for plasma folate levels. We found a better correlation between the two methods for RBC folate levels, but there was large variation among the individual results. The variable response of polyglutamates for the binding proteins probably gave falsely high RBC folate levels using competitive protein

### TABLE 2. Sensitivity and Specificity

|  | Sensitivity | Specificity |
|---|---|---|
| Radiometric microbiologic assay— | | |
| Plasma folate | 64% | 61% |
| RBC folate | 96% | 68% |
| Competitive protein binding radioassay— | | |
| plasma folate | 36% | 89% |
| RBC folate | 24% | 100% |

binding radioassay (*9*). In contrast, the radiometric microbiologic assay allows a more accurate determination of all folates, provided that they are converted to the monoglutamate forms (*19,20*). The variable responses of different monoglutamate derivatives also make the radioassay procedures unsuitable for the determination of a mixture of monoglutamate derivatives (*11*). We also note that only when a low RBC folate value is present can the folate deficiency be identified using other hematological indices (*5*).

We have demonstrated that the measurement of RBC folate levels by the radiometric microbiologic assay is probably related to the fact that some patients with biochemical evidence of folate deficiency may not show clinical and hematological manifestations (*3*). We believe, therefore, that the radiometric microbiologic assay is most suited for routine clinical use.

## Acknowledgment

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