Two Radioassays for Serum Vitamin B₁₂ and Folate Determination Compared in a Reference Interval Study

Robert H. Christenson,¹,² Georgette A. Dent,¹,² and Alexander Tukszynski¹

For 154 subjects, we verified that vitamin B₁₂ and folate status was normal, using as criteria the average polymorphonuclear lobe count, mean corpuscular volume, and hemoglobin concentration. We then used blood from these subjects to compare values obtained with two radioassay kits, each designed for simultaneous vitamin B₁₂ and folate determination. Although regression analysis showed reasonable correlation between the folate (r = 0.87) and vitamin B₁₂ (r = 0.94) kits, we observed significant differences in the overall mean values for vitamin B₁₂ (p < 0.01) and folate (p < 0.001) as measured with the kits in this population. Radioassay standard-curve data for the folate assays were similar, but these data indicated greater sensitivity to low concentrations for one vitamin B₁₂ kit than the other. Using reference intervals recommended in the kit inserts, we found that the vitamin B₁₂ status for 9% of these subjects would have been misclassified by one kit, 2% by the other.

Additional Keyphrases: megaloblastic anemia • vitamin deficiency

Improper diet, malabsorption, and alcoholism are three major causes of deficiency of vitamin B₁₂ or folate, or both, in humans (1). Because subnormal concentrations of vitamin B₁₂ and folate in blood often result in megaloblastic anemia (1), assay of these constituents in serum provide important etiological information that can be used for diagnosis and treatment of these disorders. Before radioassay techniques were developed, vitamin B₁₂ and folate were quantified by time-consuming and interference-prone microbiological assays (2, 3). Because the vitamin B₁₂ complex includes a Co atom, radioassays have been developed in which [¹⁵⁶Co]cyanocobalamin is used as tracer. Purified intrinsic factor is used in assays of competitive binding. For folate determination, radioassays have been developed involving folate binders for competitive binding and [¹²⁵I]labeled pteroylglutamic acid as tracer. Because [⁷⁷Co] and [¹²⁵I] have sufficiently different isotopic counting spectrums, the decay of these species can be separately monitored, thus providing a basis for simultaneous determination of vitamin B₁₂ and folate.

Herbert (4–6) has documented that the presence of nuclear hypersegmentation, as reflected by increased average polymorphonuclear leukocyte lobe counts in peripheral blood smears, is among the earliest signs for folate and vitamin B₁₂ deficiencies. Other well-known indices of anemia include mean corpuscular volume (MCV) and hemoglobin concentration.

We determined the average polymorphonuclear leukocyte lobe count, hemoglobin concentration, and MCV in blood samples from a group of ostensibly healthy blood donors, to verify the absence of vitamin B₁₂ and (or) folate deficiencies in these subjects. We then used concurrently-collected serum samples from these individuals in comparing two commercial kits for simultaneous determination of serum vitamin B₁₂ and folate. This study was intended to more firmly establish the normal reference interval.

Methods and Materials

Patients’ specimens. The blood specimens we studied were from 154 ostensibly healthy blood donors, 59 men and 95 women. From each of these 154 subjects we collected two 7-mL specimens of blood into Vacutainer Tubes (Becton Dickinson, Rutherford, NJ 07070) while the donor was supine. The first of these two tubes contained EDTA as anticoagulant (lavender-cap tube; BD cat. no. 02-685-2B) and the specimen was used within 1 h of collection to produce a peripheral blood smear to determine hemoglobin and MCV. The second tube contained no anticoagulant (red-top tube; BD cat. no. 02-083-02). After the clot had formed, this specimen was centrifuged (10 min, 1000 x g). The resulting serum was decanted into a separate plastic tube, capped, and stored at −10 °C until analysis (no longer than seven days).

Vitamin B₁₂ and folate assays. For all vitamin B₁₂ and folate assays, we used the “Quanaphase” (Bio-Rad Laboratories, Richmond, CA 94804) and “SimuITRAC-S” (Becton Dickinson Immunodiagnostics, Orangeburg, NY 10962) radioassay kits, carefully following the manufacturers’ directions. Because donor specimens contain proteins that interfere with the competitive binding of the folate binders and purified intrinsic factor used for folate and vitamin B₁₂ assay, both kits include a 100 °C heating step before testing, to denature interfering substances. In both kits, cyanocobalamin is used for standardization and its [⁷⁷Co]-labeled analog as tracer for vitamin B₁₂ radioassay. For folate determination, the pteroylglutamic acid form of folic acid is used for standardization and its [¹²⁵I]-labeled analog as tracer in both kits. For use in establishing a 100%-binding reference point, both kits include standards containing no vitamin B₁₂ or folate. In both kits, the highest-concentration standards for vitamin B₁₂ and folate determination contain 2000 ng and 20 μg of these respective constituents per liter.

We used the Multi-Prias™ Gamma Counting System (Packard Instrument Co., Downers Grove, IL 60515) for all gamma scintillation counting and data reduction done in this study.

Determination of MCV and hemoglobin. We determined the MCV and the concentration of hemoglobin of the specimens in the “Coulter S-plus” (Coulter Electronics Inc., Hialeah, FL 33010), carefully following the supplier’s protocol. Hemoglobin concentration is determined by measuring the absorbance of the lysed specimen at 525 nm after hemoglobin is reacted with cyanide to form cyanmethemoglobin; MCV is assessed from the average distribution of erythrocytes ranging in volume between 36 and 360 μm³.

Average polymorphonuclear leukocyte lobe count. To prepare peripheral blood smears we used an automated system (LARC; Corning Glass Works, Biomedical Technical Center, Raleigh, NC 27604) for the centrifuging and staining. The smears were examined and the polymorphonuclear leukocyte lobes counted in a LARC leukocyte analyzer (Corning Glass Works), set for semi-automatic operation. For calculation of average polymorphonuclear leukocyte lobe count, we
used the following formula: average polymorphonuclear leukocyte lobe count = (total number of lobes in 100 successive neutrophils)/100 (4–7).

Results

Table 1 summarizes the quality-control variables derived from logit-log linearization of standard curves for 10 vitamin B₁₂ and folate runs made with each of the two kits. In Table 1, we use maximum binding to represent the percentage of the added total ¹²⁵I-labeled pteroylglutamic acid or added (⁵⁷Co)cyanocobalamin tracer that is bound to folate binders or purified intrinsic factor in the zero standards for the assays, i.e., the nonspecific binding. To calculate the nonspecific binding values shown in Table 1, we used the expression: (100% × the radioisotopic counts per minute detected in "blank" tubes devoid of purified intrinsic factor or folate binders)/total radioactivity (in counts/min) of ¹²⁵I or ⁵⁷Co tracer added. We found the maximum binding and nonspecific binding characteristics for both kits to be within acceptable limits.

Constituent values corresponding to 80%, 50%, and 20% binding provide useful information about a radioassay’s useful range and analytical sensitivity; 100% binding represents the radioisotopic response of the zero standard on the ordinate, and 0% binding is extrapolated from the intercept of the standard curve on the abscissa. For use in Table 1, we calculated, after logit-log data reduction, the vitamin B₁₂ or folate concentration corresponding to 80%, 50%, and 20% binding relative to the assay’s zero standard from respective standard curves. For folate determination, concentrations were similar for the two at 50% and 80% binding. The slight dissimilarity in folate concentration for the kits at 20% binding (Table 1) does not represent a substantial difference, because the 20%-binding values observed for the two kits are well below the 20.0 ng/L (highest) standard used in each.

Overall, we found the useful range for both folate kits to be wide, and the day-to-day reproducibility and analytical sensitivity good.

Table 1 also lists calculated vitamin B₁₂ concentrations corresponding to 20%, 50% and 80% binding for these kits. For the Quantaphase vitamin B₁₂ assay, the 20%, 50%, and 80% binding concentrations were within the concentration values of standards provided with the kit. In contrast, we observed that, while the 50% and 80% binding concentrations for the SimulTRAC-S vitamin B₁₂ assay were within the standards used in the assay, these values were nearly twice those observed for 50% and 80% binding with the Quantaphase procedure. Further, we found the Simul-TRAC-S vitamin B₁₂ concentration corresponding to 20% binding to be 2974 (SD 674) ng/L, substantially exceeding the 2000 ng/L (highest) standard used in the kit.

Table 2 summarizes our data on hemoglobin concentration, MCV, and average polymorphonuclear lobe count for our subjects. The values for hemoglobin and MCV agreed well with the normal reference intervals defined for our hematology laboratory. The average polymorphonuclear lobe count data shown is consistent with values reported elsewhere (4–7).

Figure 1 illustrates the relation between vitamin B₁₂ as measured with the Quantaphase kit and with the Simul-TRAC-S kit; the mean values for the two kits differ significantly (p < 0.01). However, good proportionality between the two assays is demonstrated by the slope of 0.84, the y-intercept of −14 ng/L, and the correlation coefficient of 0.94 derived from linear-regression analysis of the data shown in Figure 1.

Figure 2 shows a plot of the folate data we obtained by use of the Quantaphase and SimulTRAC-S kits. Linear-regression analysis of these data resulted in a slope of 0.29, a y-intercept of 2.0 μg/L, and a correlation coefficient of 0.87.

<table>
<thead>
<tr>
<th>Vitamin B₁₂</th>
<th>Bio-Rad kit</th>
<th>BD kit</th>
<th>Bio-Rad kit</th>
<th>BD kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.8 ± 5.5</td>
<td>44.8 ± 2.3</td>
<td>46.6 ± 3.2</td>
<td>48.1 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>2.73 ± 1.17</td>
<td>1.97 ± 0.51</td>
<td>2.64 ± 1.35</td>
<td>1.97 ± 0.52</td>
<td></td>
</tr>
<tr>
<td>−1.02 ± 0.02</td>
<td>−0.968 ± 0.081</td>
<td>−0.98 ± 0.08</td>
<td>−0.951 ± 0.582</td>
<td></td>
</tr>
</tbody>
</table>

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Table 2. Data for Males, Females, and Combined Groups

<table>
<thead>
<tr>
<th>Test</th>
<th>Overall (Mean ± SD)</th>
<th>Women (Mean ± SD)</th>
<th>Men (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>136 ± 12 g/L (154)</td>
<td>1297.9 g/L (95)</td>
<td>147 ± 8.8 g/L (59)</td>
</tr>
<tr>
<td>Average</td>
<td>2.54 ± 0.14 (153)</td>
<td>2.53 ± 0.12 (94)</td>
<td>2.55 ± 0.15 (59)</td>
</tr>
<tr>
<td>polymorphonuclear leukocyte lobe count</td>
<td>90.2 ± 4.4 fL (154)</td>
<td>90.6 ± 4.6 fL (95)</td>
<td>89.6 ± 4.1 fL (59)</td>
</tr>
<tr>
<td>Mean corpuscular volume (MCV)</td>
<td>468.4 ± 236.3 g/L (151)</td>
<td>464.3 ± 263.4 g/L (94)</td>
<td>475.1 ± 185.4 g/L (57)</td>
</tr>
<tr>
<td>Vitamin B₁₂ (ng/L), Bio-Rad kit</td>
<td>383.5 ± 211.2 g/L (153)</td>
<td>378.1 ± 234.0 g/L (94)</td>
<td>392.1 ± 170.0 g/L (59)</td>
</tr>
<tr>
<td>Folate (μg/L), Bio-Rad kit</td>
<td>11.33 ± 10.33 (151)</td>
<td>12.2 ± 12.38 (94)</td>
<td>9.96 ± 5.36 (57)</td>
</tr>
<tr>
<td>Range: 2.6–71.0</td>
<td>Range: 2.6–71.0</td>
<td>Range: 2.6–25.7</td>
<td></td>
</tr>
<tr>
<td>Folate (μg/L), BD kit</td>
<td>5.31 ± 3.50 (153)</td>
<td>5.52 ± 4.15 (94)</td>
<td>4.97 ± 2.96 (59)</td>
</tr>
<tr>
<td>Range: 1.9–28.5</td>
<td>Range: 1.9–28.5</td>
<td>Range: 2.3–11.6</td>
<td></td>
</tr>
</tbody>
</table>
When the overall mean folate value for the kits was compared, we found that the values obtained with the Quantaphase kit were significantly \( p < 0.001 \) higher than those with the SimuITRAC-S kit.

In this study, neither kit showed a significant sex- or age-related within-assay difference in vitamin \( B_12 \) or folate concentrations.

For our study population the normal reference intervals (95% confidence limit) for vitamin \( B_12 \) were 116–817 ng/L for the SimuITRAC-S kit, 205–810 ng/L for the Quantaphase kit. For folate, the corresponding normal reference intervals (95% confidence limit) were 2.3–14.6 \( \mu g/L \) and 4.0–42.7 \( \mu g/L \), respectively.

**Discussion**

The mean values for polymorphonuclear lobe count, MCV, and hemoglobin concentration for these individuals correlate well with reported normal values. Because these laboratory tests are reliable indices of vitamin \( B_12 \) or folate status (4–6), they serve to verify the general good health of the subjects included in this study.

From our standard curve data, we conclude that the analytical performance of both of the folate kits was satisfactory in our hands.

Our results for vitamin \( B_12 \) concentrations corresponding to binding at 20, 50, and 80% (relative to the zero standard) indicate that the Quantaphase assay is more sensitive analytically than is the SimuITRAC-S kit in discriminating low values for vitamin \( B_12 \). Because the laboratory-assisted diagnosis of vitamin \( B_12 \) deficiency depends on reliable low-range determination of this constituent, it is especially important to have good analytical sensitivity at the low end of the standard curves used for vitamin \( B_12 \) assay.

Our mean folate value obtained with the Quantaphase kit significantly \( p < 0.001 \) exceeded that for the SimuITRAC-S kit. Although package inserts4,5 for the Quantaphase and SimuITRAC-S kits suggest normal reference ranges for folate (2.2–17.3 and >2.0 ng/L, respectively), in these same inserts it is recommended that each laboratory determine its own normal reference intervals. For the population included in this study, we determined normal reference intervals (95% confidence limit) of 4.0 to 42.7 \( \mu g/L \) for the Quantaphase folate kit, substantially greater than the 2.2–14.6 \( \mu g/L \) value recommended in the package insert for this assay. In contrast, the folate normal reference intervals (95% confidence limit) for folate, 2.3–14.6 \( \mu g/L \), we determined for use with the SimuITRAC-S kit agreed well with the >2.0 \( \mu g/L \) value listed in that kit’s package insert. In our study population, the normal reference intervals for folate observed with the SimuITRAC-S kit were more consistent with published data (6) than were those observed for the Quantaphase kit.

For our population, we found that the normal reference intervals (95% confidence limit) for vitamin \( B_12 \) with the Quantaphase kit significantly \( p < 0.01 \) exceeded those for the SimuITRAC-S kit. When we used the 180–960 ng/L normal range for vitamin \( B_12 \) listed in the SimuITRAC-S package insert4 to interpret the vitamin \( B_12 \) status of our study subjects, 9% of them were categorized as abnormal. The Quantaphase kit insert5 lists the normal value for vitamin \( B_12 \) as >300 ng/L; when the vitamin \( B_12 \) status of our subjects was so interpreted, 2% were classified as abnormal. Evidently the normal range for vitamin \( B_12 \) quoted for the SimuITRAC-S kit is not appropriately high.

Our study leads us to strongly concur with recommendations that normal-range studies be completed by each laboratory before assays are used in patient evaluation.

We greatly appreciate the cooperation of the Durham Red Cross Blood Center in procuring the specimens used in this study.

**References**