An Improved Radioisotope Dilution Assay for Serum Vitamin B₁₂ Using Hemoglobin-coated Charcoal

By Yong K. Liu and Louis W. Sullivan

A radioisotope dilution assay for vitamin B₁₂, using hemoglobin-coated charcoal, was modified in several respects, the most prominent of which was the use of cyanide extraction of sera. With these modifications, the B₁₂ levels were significantly higher in sera from normal subjects and patients with folate deficiency, whereas sera from patients with pernicious anemia and other B₁₂-deficient states were only minimally affected. The use of cyanide extraction thus resulted in a clearer differentiation of B₁₂-deficient sera from other sera; all 23 patients with pernicious anemia had B₁₂ levels below 140 pg/ml, whereas all of 85 normal and 218 folate-deficient subjects had B₁₂ levels above 156 pg/ml. The microbiologic assay with Euglena gracilis was similarly affected by cyanide extraction of sera. Thus, it appears that the use of cyanide results in more complete extraction of B₁₂ from serum proteins.

The use of radioisotope dilution methods for the assay of vitamin B₁₂ in serum has been widely adopted in recent years. Such assays are based on the principle that after release from endogenous binding proteins, vitamin B₁₂ in serum can be measured by its competition with added radioactive cyanocobalamin for binding by intrinsic factor, serum, transcobalamin I, or saliva. Hall noted that the accuracy of such assays depends upon a similar affinity of intrinsic factor (or other binding protein) for the vitamin B₁₂ released from the serum and the added radioactive cyanocobalamin, and he suggested the use of cyanide in extracting B₁₂ from sera. It has been reported that a major fraction of the vitamin B₁₂ in serum is methylcobalamin, with lesser amounts of hydroxocobalamin and 5,6-dimethylbenzimidazole coenzyme B₁₂. The binding affinities of intrinsic factor, human serum, and of human gastric juice for a number of analogues of vitamin B₁₂ have been shown to differ considerably.

It has been shown that in the presence of cyanide ion, vitamin B₁₂ analogues in serum are converted to cyanocobalamin. The effect of cyanide extraction of sera in a radioisotope dilution assay for vitamin B₁₂ was thus investigated.
This simple modification improved the reliability of the assay by enhancing the differentiation of normal from vitamin B12-deficient sera.

**MATERIALS AND METHODS**

The vitamin B12 content of serum was measured by the radioisotope dilution method of Lau et al., using intrinsic factor as the B12 binding protein and hemoglobulin-coated charcoal to separate free B12 from B12 bound to intrinsic factor.4 Our protocol differs from that of Lau et al. in the following respects:

First, 0.1 ml of sodium cyanide or potassium cyanide (100 μg/ml) was added to assay tubes containing the unknown sera, prior to heating in the water bath.

Second, a lower concentration (400 pg/ml) of radioactive cyanocobalamin standard was used 57Co-B12, with a specific activity of 10–12 mCi/mg, (Rubratope-57, high specific activity, E. R. Squibb, New Brunswick, N. J.) was diluted to a concentration of 400 pg/ml with distilled water or normal saline.

Third, a proportional reduction in the concentration of intrinsic factor in the working solution to bind between 70–80% of 400 pg 57Co-B12/ml was made. Because National Formulary Intrinsic Factor Reference Standard (NFIF) is no longer available, we have used Intrinsic Factor 10X (IF) (Nutritional Biochemicals, Cleveland, Ohio). This intrinsic factor preparation has a potency ten times that of NFIF in the Schilling test and the guinea pig intestinal mucosa homogenate assay.19 The IF stock solution is prepared by dissolving 10 mg of IF in 500 ml of normal saline and is stored in 5-ml aliquots at −20°C until used. For use in the assay, an aliquot of the IF stock solution is thawed 5–10 min in a 22°C water bath and diluted with sufficient physiologic saline to give a concentration of IF that will bind 70–80% of the 400 pg of 57Co-B12, as determined from binding studies of each IF stock solution. The final concentration of IF in the working solution is usually 1.8–2.7 μg/ml.

Fourth, samples were centrifuged at 3000 rpm for 1 hr after the addition of hemoglobin-coated charcoal to ensure complete sedimentation of the charcoal.

Calculation of the vitamin B12 level (pg/ml) of the unknown serum sample was performed according to the following formula, modified from Lau et al.

\[
P_{\text{B12}} = \frac{400 \times \text{US-SC}}{\text{IF-SC}} - 1
\]

IF = cpm of intrinsic factor control; US = cpm of unknown serum; and SC = cpm of supernatant control.

The vitamin B12 content of a number of sera was also assayed with *Euglena gracilis*.20 Hematologic evaluations, including bone marrow aspirations, were performed in most patients with serum B12 levels of less than 200 pg/ml. The diagnosis of pernicious anemia was established either by radioactive vitamin B12 absorption tests or by in vitro assay of the intrinsic factor activity of the patient’s gastric juice obtained by augmented histamine stimulation.

**RESULTS**

The results of paired assays of 108 sera with and without cyanide extraction are given in Fig. 1. Without cyanide extraction, the vitamin B12 content of sera from 85 adult volunteers and patients without anemia ranged from 104 to 1364 pg/ml, with a mean of 453 pg/ml. Using cyanide extraction, higher values were obtained in 82 of the 85 sera, with a range of 156–1740 pg/ml and a mean of 665 pg/ml. Without cyanide extraction, B12 levels in 14 of these 85 sera were between 104 and 199 pg/ml, whereas with cyanide extraction B12 levels of these sera ranged from 156 to 360 pg/ml; 11 sera had B12 levels above 200 pg/ml. In 23 patients with untreated pernicious anemia, serum B12 levels
<table>
<thead>
<tr>
<th></th>
<th>Serum I Pernicious Anemia</th>
<th>Serum II Normal</th>
<th>Serum III Alcoholic Hepatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Cyanide</td>
<td>With Cyanide</td>
<td>Without Cyanide</td>
</tr>
<tr>
<td>28</td>
<td>56</td>
<td>344</td>
<td>459</td>
</tr>
<tr>
<td>30</td>
<td>64</td>
<td>335</td>
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<td>36</td>
<td>72</td>
<td>376</td>
<td>480</td>
</tr>
<tr>
<td>38</td>
<td>68</td>
<td>360</td>
<td>495</td>
</tr>
</tbody>
</table>

Mean ± SD
- Serum I: 35 ± 3, 64 ± 6
- Serum II: 350 ± 18, 470 ± 16
- Serum III: 2078 ± 52, 3092 ± 113

Range
- Serum I: 28–38, 56–72
- Serum II: 335–376, 459–495
- Serum III: 2024–2146, 2980–3240

Coefficient of variation (％)
- Serum I: 9.3, 8.6
- Serum II: 5.1, 3.4
- Serum III: 2.5, 3.7
Fig. 1. Effect of cyanide on serum vitamin B₁₂ in radiisotope dilution assay. Note higher B₁₂ levels obtained with cyanide extraction in majority of sera, as shown by increased slope of curve and displacement of curve to the left.

without cyanide extraction ranged from 0 to 123 pg/ml, with a mean of 60 pg/ml. After cyanide extraction the B₁₂ levels of these same sera ranged between 17 and 140 pg/ml, with a mean of 83 pg/ml.

Reproducibility

The B₁₂ level of five aliquots of each of three sera (low, normal, and high B₁₂ levels) was measured in the same assay with and without cyanide extraction (Table 1). The reproducibility obtained with all three sera with and without cyanide extraction was satisfactory. However, B₁₂ values were uniformly higher after cyanide extraction.

The variability between assays using cyanide extraction was assessed by measuring the B₁₂ content of two sera 12 times over an 8-mo period (Table 2). The B₁₂ level in serum from a patient with pernicious anemia ranged between 0–62 pg/ml, with a mean of 25 pg/ml and a median of 16.5 pg/ml. The B₁₂ level in a normal serum ranged from 185 to 323 pg/ml, with a mean of 260 pg/ml and a median of 272 pg/ml.

Effect of Cyanide Extraction of Denatured Sera

The endogenous B₁₂ content of six normal sera was destroyed by alkalinizing the sample with 1 N NaOH to a pH of 12 and then heating at 100°C for 30 min. No B₁₂ was detected in these sera with cyanide extraction.

Effect of Cyanide on Assay of Serum B₁₂ with Euglena gracilis

The B₁₂ content of 49 sera was determined in one assay with Euglena gracilis with and without cyanide extraction (Fig. 2). Higher B₁₂ levels were obtained after cyanide extraction in the sera from healthy adults and patients without anemia. In three of 13 sera from patients with untreated pernicious anemia, slightly higher B₁₂ levels were obtained after cyanide extraction.
Table 2. Variability Between Assays of Vitamin B\textsubscript{12} Content of Two Sera Using Cyanide Extraction

<table>
<thead>
<tr>
<th>Assay Number</th>
<th>Serum Pernicious (py/ml)</th>
<th>Serum Normal (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>192</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>204</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>185</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>249</td>
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<tr>
<td>5</td>
<td>1</td>
<td>301</td>
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<tr>
<td>6</td>
<td>62</td>
<td>272</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>283</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>309</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>323</td>
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<td>11</td>
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<td>12</td>
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</tr>
<tr>
<td>Mean</td>
<td>25</td>
<td>260</td>
</tr>
<tr>
<td>Median</td>
<td>16.5</td>
<td>272</td>
</tr>
<tr>
<td>Range</td>
<td>0–62</td>
<td>185–323</td>
</tr>
</tbody>
</table>

Clinical Application

With the modified radioisotope dilution method, the B\textsubscript{12} content of sera from 40 normal physicians, technicians, and medical students ranged between 158 and 1180 pg/ml, with a mean of 496 pg/ml. The serum B\textsubscript{12} level of 126 patients with addisonian pernicious anemia or B\textsubscript{12} deficiency after gastrectomy were all less than 150 pg/ml. The B\textsubscript{12} content in 111 of these 126 sera were below 125 pg/ml (Table 3). All of 218 patients with megaloblastic anemia due to folate deficiency had serum B\textsubscript{12} levels above 150 pg/ml.

DISCUSSION

The use of cyanide extraction in the hemoglobin-coated charcoal isotope dilution assay for vitamin B\textsubscript{12} gave higher values for sera from normal volunteers and from nonanemic hospitalized patients. The effect of cyanide extraction was greater in sera with higher B\textsubscript{12} levels. There was a minimal increase in the B\textsubscript{12} level of sera from patients with untreated pernicious anemia. Thus, cyanide extraction of sera resulted in clearer separation of normal from vitamin B\textsubscript{12}-deficient sera.

Table 3. Serum Vitamin B\textsubscript{12} Levels of 126 Patients With Addisonian Pernicious Anemia or B\textsubscript{12} Deficiency After Gastrectomy

<table>
<thead>
<tr>
<th>Vitamin B\textsubscript{12} (pg/ml)</th>
<th>Number</th>
<th>Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–25</td>
<td>13</td>
<td>10.3</td>
</tr>
<tr>
<td>26–75</td>
<td>34</td>
<td>27.0</td>
</tr>
<tr>
<td>76–125</td>
<td>64</td>
<td>50.8</td>
</tr>
<tr>
<td>126–146</td>
<td>15</td>
<td>11.9</td>
</tr>
<tr>
<td>Mean = 81</td>
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</tbody>
</table>
Fig. 2. Effect of cyanide on serum vitamin B\textsubscript{12} levels by microbiologic assay with *Euglena gracilis*. Cyanide extraction resulted in higher B\textsubscript{12} levels in most sera, with a resulting symmetrical shift of curve to the left.

The B\textsubscript{12} binding capacity of intrinsic factor in the working solution was stable and reproducible. Lower concentrations of intrinsic factor frequently exhibited variable B\textsubscript{12} binding capacity.

With our method, the variation of B\textsubscript{12} levels within one assay, or between different assays, was similar to that obtained with microbiologic assays\textsuperscript{25,26} or other radioisotope dilution methods.\textsuperscript{5,10} It has been reported\textsuperscript{4,5} that the hemoglobin-coated charcoal assay tends to give lower values for B\textsubscript{12} content of sera from vitamin B\textsubscript{12}-deficient patients, as compared to microbiologic assays using *Euglena gracilis* or *Lactobacillus leichmannii*. With cyanide extraction such a discrepancy was seldom found.

The structure of vitamin B\textsubscript{12} in serum has not been completely elucidated. However, current evidence suggests that a major component is methylcobalamin.\textsuperscript{14} This compound is unstable on exposure to light, converting to hydroxocobalamin.\textsuperscript{13} In the presence of cyanide, hydroxocobalamin is further converted to cyanocobalamin, the most stable of all known B\textsubscript{12} analogues.\textsuperscript{16} The complete release of vitamin B\textsubscript{12} from endogenous protein binders is essential for accurate determinations of serum B\textsubscript{12} content. Employing microbiologic methods in which serum proteins are removed by heat denaturation and precipitation, Killander\textsuperscript{25} and Mathews\textsuperscript{27} found lower B\textsubscript{12} levels in the serum extract when cyanide was not used. A significant amount of B\textsubscript{12} was recovered when the precipitate was again autoclaved in the presence of cyanide. These observations suggest that cyanide enhances the extraction (rather than prevents the destruction) of serum B\textsubscript{12} by heating. The actual chemical nature of this effect of cyanide remains unclear.

**ACKNOWLEDGMENT**

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**REFERENCES**

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An Improved Radioisotope Dilution Assay for Serum Vitamin B\textsubscript{12} Using Hemoglobin-coated Charcoal

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