Comparison of Two Methods for Radioassay of Vitamin B\textsubscript{12} in Serum

Eugene P. Frenkel, Jerry D. White, Joan S. Reisch, and Richard G. Sheehan

A reference radioassay for vitamin B\textsubscript{12} in serum, in which human serum is used as binder and DEAE-cellulose is used to separate free and bound B\textsubscript{12}, was compared (194 sera) with a packaged kit ("Phadebas B\textsubscript{12} Test"), in which intrinsic factor is used as binder and polysaccharide for separation. The correlation coefficient was 0.949. With the kit, added B\textsubscript{12} was identifiable, proportional dilution characteristics were excellent, and serial reproducibility was good. The normal range for the reference assay was 200–1000 pg/ml; all B\textsubscript{12}-deficient patients had values of <165 pg/ml. In a separate study, 38 patients with documented (clinical and laboratory) B\textsubscript{12} deficiency had mean serum values of <150 pg/ml by the reference assay, 250 pg/ml by the kit. For the kit, the normal range was 250–1100 pg/ml. All patients with serum values of <150 pg/ml by kit assay were B\textsubscript{12} deficient; false positives were seen in the 150–250 pg/ml range.

Additional Keyphrases: normal range • commercial kit • “Sephadex” • “Phadebas B\textsubscript{12} Test” • anemia • diagnostic aids

The radioisotopic competitive inhibition assay for serum vitamin B\textsubscript{12} has been validated as a means of identifying B\textsubscript{12} deficiency in man (1–6). Characteristically, in such assays the endogenous B\textsubscript{12} is first extracted from the binding proteins in serum, then endogenous B\textsubscript{12} is admixed with exogenous isotopically-labeled B\textsubscript{12} in the presence of a standard binder, and the bound and unbound portions are then separated. Methodologic variations primarily involve the two latter steps (4, 5, 7).

The recent availability of one such modification in kit form has expanded the potential for more widespread use of the isotopic assay method. The present study compares our previously reported method (3, 5) (used in well over 10,000 assays) with this packaged kit ("Phadebas B\textsubscript{12} Test"; Pharmacia Lab., Inc., Piscataway, N. J. 08854). In our method human serum is added as the binder and diethylaminoethyl (DEAE) cellulose is used to separate free and bound B\textsubscript{12}, whereas in the packaged kit intrinsic factor is used as the binder and polysaccharide particles ("Sephadex") for the separation.

Methods

Assay Methods

Our assay (hereafter termed "reference" assay) was performed as previously reported (3, 5). The "Phadebas B\textsubscript{12} Test" was performed exactly as indicated in the package insert. In addition to the differences in standard binder and the techniques for separating the bound and unbound fractions, the two methods also differ in the following:

<table>
<thead>
<tr>
<th>Reference assay</th>
<th>Phadebas assay</th>
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</thead>
<tbody>
<tr>
<td>1. 2 ml of serum required</td>
<td>1. 0.5 ml of serum required</td>
</tr>
<tr>
<td>2. True duplicates are performed, on aliquots from the original sample</td>
<td>2. Replicates are studied; the &quot;duplicate&quot; portion is obtained from a dilution of the original serum</td>
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<tr>
<td>3. Recovery step permits estimation of nonspecific binding caused by imperfect extraction of B\textsubscript{12} from endogenous proteins</td>
<td>3. Not done</td>
</tr>
<tr>
<td>4. The amount of (radiolabeled) B\textsubscript{12} added is accounted for in calculating the final results</td>
<td>4. The added (radiolabeled) B\textsubscript{12} is ignored in the final calculation</td>
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<tr>
<td>5. Requires only selected standards for any laboratory that has previously prepared a standard curve</td>
<td>5. Requires a complete standard curve with each run</td>
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Statistical Analysis

The two methods were compared by fitting a least-squares regression line to the data (x, reference method; y, kit method) and comparing that line to the line of true identity. The ratio of the difference in the residual or error sums of squares (divided by its degrees of freedom, 2) divided by the residual sum of squares for the linear-regression model \( y = a + bx \) (divided by its degrees of freedom, \( n - 2 \)) provided the \( F \) statistic (8).

Results from multiple inter-laboratory evaluation of specimens were analyzed by determining the correlation coefficient (9).

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1 Phadebas B\textsubscript{12} Test kits were provided for the study by E. Lindgren, Pharmacia Laboratories, Inc., Piscataway, N.J. 08854.
Results
Comparative Assays

One hundred ninety-four specimens were assayed in duplicate by both methods. Figure 1 depicts the pattern of these results. The fitted regression line was $y = -27.01 + 1.26x$ (where $y$ was the kit method, $x$ our assay). The correlation coefficient was 0.949. The $F$ statistic, for comparison of the two models, was 108.20 with 2 and 192 degrees of freedom; this was significant at the 0.0005 level.

Delineation of Range of “Normal”

The primary use of the serum vitamin B$_{12}$ assay has been to identify the B$_{12}$-deficient state. The delineation of “normal” depends on the criteria used to denote B$_{12}$ depletion. In a separate study, we evaluated 60 sera in the low range by both methods. Of these sera, 38 were from patients with a classical clinical picture of B$_{12}$ deficiency (most had pernicious anemia) with evidence of macrocytic anemia and megaloblastic bone marrow and normal or high serum folate concentrations. All of the patients had had a complete hematologic recovery after therapy with physiologic doses of vitamin B$_{12}$, and the patients with pernicious anemia had evidenced histamine-fast achlorhydria and subsequently a characteristic Schilling test. The previously established normal range by our “standard” assay was 200–1000 pg/ml; however, to date, in about 400 documented B$_{12}$-deprived patients, all clinically B$_{12}$-deficient patients have had values of <165 pg/ml. In fact, virtually all have been <140 pg/ml. Figure 2 demonstrates the values obtained from these two assays. All 38 of the B$_{12}$-deficient patients had concentrations of <160 pg/ml by our “standard” assay, and no normal individual’s concentration was <170 pg/ml; 13 sera obtained from patients with no clinical or other laboratory evidence of vitamin B$_{12}$ deficiency had values in the range 170–200 pg/ml. Assay with the Phadebas kit demonstrated that all of the documented B$_{12}$-deficient patients had B$_{12}$ values of <250 pg/ml, and no serum from the other patients had a value of <140 pg/ml. In the range of 140–250 pg/ml, the Phadebas assay correctly identified all of the B$_{12}$-deficient patients, but also included 15 non-deficient patients.

The upper range of normal for the reference assay has been about 1000 pg/ml. Sharp delineation of the upper level is not possible because of the limited types of clinical conditions that are associated with high values. We derived the value for the upper limit of “normal” by contrasting serum values from patients with entities associated with high serum B$_{12}$ concentrations (e.g., chronic granulocytic leukemia, myelofibrosis, Di Guglielmo syndrome, and acute hepatic necrosis) with values obtained from normal persons. Sera from patients with these disorders, assayed by the kit method, had absolute values greater than 1100 pg/ml.

Experimental Variables

**Recovery of exogenous B$_{12}$.** Recovery of added crystalline B$_{12}$ was evaluated with the kit. Table 1 compares the ability of the two methods to account for B$_{12}$ added to a known serum sample. Except in the very low range, recovery of the added B$_{12}$ was >86% for the kit, a result reasonably comparable to the reference assay.

**Results after proportional dilution.** Progressive dilutions of serum showed a linear decrease in the measured concentration by the kit assay (Figure 3), as has been demonstrated for our assay. Such proportional dilution characteristics suggest specificity of the assay, because no substances appear to be in the serum that alter the binding kinetics.

**Serial reproducibility.** As one measure of repro-
Table 1. Recovery of Exogenous $B_{12}$ Added to Serum

<table>
<thead>
<tr>
<th>Endogenous</th>
<th>Added $B_{12}$ (cryst.)</th>
<th>Predicted total</th>
<th>Measured total pg/ml</th>
<th>Recovery, %</th>
<th>Kit</th>
<th>Measured total pg/ml</th>
<th>Recovery, %</th>
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</thead>
<tbody>
<tr>
<td>82</td>
<td>25</td>
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<td>882</td>
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</table>

Fig. 3. The effect of proportional dilution of serum on the determined $B_{12}$ values by the Phadebas kit
@ indicates the original serum value. The solid line (---) indicates perfect proportionality between volume and concentration. The dashed line (----) indicates the pattern obtained by the assay.

Reproducibility in our reference assay we used a "Moni-Trol" reference serum, including an aliquot in each day's run (Figure 4). Three separate pools were evaluated, and as noted, the values by the kit assay were about 50 pg/ml higher than by our reference assay. In addition, results with the kit method were more variable, as evidenced by the wider spread of duplicates on any given assay day and variation from week to week when compared to the reference assay.

Use of a standard curve. Previous reports from our laboratory have documented the need for and the value of a standard curve(s). We demonstrated that after establishing a standard curve for the laboratory, a 200-pg standard (plus the above reference standard) could serve as a safe and satisfactory criterion of reproducibility in any subsequent assay by the reference method.

A complete standard curve has been included in the package-insert directions for the Phadebas $B_{12}$ Test. Figure 5 illustrates a composite of 12 standard curves by the kit method, and shows the variation in percentage "bound" is such from curve to curve that the resulting values extend over a range of almost 100 pg/ml, making use of a single standard invalid.

Inter-laboratory reproducibility. Inter-laboratory reproducibility was evaluated for the Phadebas kit assay in a separate study on sera from 25 patients not included above. Each serum was divided into aliquots, which were distributed for evaluation by geographically separate laboratories. At least two laboratories ran each specimen. The correlation coefficient for the comparison of these laboratories was 0.918 (sample size, 25). This correlation was significant at the 0.005 level.

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2 Dade "Moni-Trol II," obtained from Scientific Products Division, American Hospital Supply Corp., Grand Prairie, Tex., and diluted fivefold in de-ionized water before use.
Discussion

Although the radioisotopic competitive inhibition assay for serum vitamin B₁₂ has been documented as a rapid and reliable means of identifying B₁₂ deficiency (1-6, 10), very few comparative methodologic studies have been performed. In most such studies, the radioisotopic assay has been compared to one or another form of more classical microbiologic assay (6, 10, 11); in only a few have different radioassay methods been compared (3, 10, 12). Because there are only small methodologic differences among the currently popular radioisotopic assays (10), one mode of evaluating a new assay procedure is by comparing it to one of these prior methods. The recent availability of a "packaged radioisotopic kit method for B₁₂" made such a comparative trial of interest, because such a kit would make the assay method useful to a much larger number of clinical laboratories.

Our study demonstrates that this kit (Phadebas) provides reasonable reproducibility and recovery of added crystalline B₁₂, two commonly used criteria for "validating" an assay procedure (12). An important mode of validation of any B₁₂ assay is the correct identification of clinically deficient patients. Our range of normal for the "standard" assay is 200 to 900 pg/ml. To date, all of the patients with clinically evident vitamin B₁₂ deficiency [e.g., megaloblastic anemia and (or) neurologic deficit responsive to repletion with physiologic quantities of B₁₂] have had serum B₁₂ concentrations of <160 pg/ml. Values of 160-200 pg/ml represent a "grey zone," into which fall values for patients with various degrees of gastric resection or who are more than 70 years old (3, 5). Some of these patients have a shift in the mean lobe average for their polymorphonuclear leukocytes, and some have occasional macro-ovalocytic change of their circulating erythrocytes, but none have evidence of marrow megaloblastosis. We are continuing to observe these patients, to help clarify this borderline range. To date, no patient with a serum vitamin B₁₂ value of <160 pg/ml by our method has failed to demonstrate tissue evidence of B₁₂ deficiency. As noted in Figure 2, the kit method did correctly identify all 38 of the documented cases of vitamin B₁₂ deficiency; all of these patients had B₁₂ values of <250 pg/ml. No patient with a value of <140 pg/ml failed to demonstrate histological evidence of vitamin B₁₂ deficiency. Some patients with no tissue manifestations of B₁₂ deficiency were seen who had values between 140 and 250 pg/ml. Because the B₁₂ assay is best used as a laboratory correlate in megaloblastic states, this "grey zone" is reasonably acceptable at the clinical level. The upper range of normal can reasonably be estimated to be 1000 pg/ml. From the data presented, the range of normal for the Phadebas kit is 250-1000 pg/ml.

References