Automated Assay of Vitamin B-12 by the Abbott IMx® Analyzer

Steven C. Kuemmerle, Gary L. Boeltinghouse, Suzanne M. Delby, Teri L. Lane, and Royce P. Simonsen

A nonisotopic assay of vitamin B-12 in human serum or plasma is described, performed with the Abbott IMx® analyzer. The sample is first treated at pH >12.5 to release bound vitamin B-12 and to convert all forms to cyanocobalamin. Next, the analyte is bound, at lower pH, by vitamin B-12-specific binding protein, immobilized to a solid phase of polymeric microspheres. Detection involves monitoring the activity of the tracer enzyme (alkaline phosphatase) coupled to a derivative of cyanocobalamin. Total assay precision is 7.9% for vitamin B-12 at 200 ng/L, 6.6% at 400 ng/L, and 6.7% at 800 ng/L. Assay sensitivity, calculated as 2 SD from the zero calibrator, is 37 (±9) ng/L. The dynamic range extends to 2000 ng/L.

Analytical recovery of 300 and 600 ng/L additions of vitamin B-12 to sera with basal concentrations of 30–400 ng/L was 102.5%. Results of the assay correlated well with those of commercially available radioisotope assays. No interference was observed in specimens from patients with pernicious anemia, chronic or acute myelogenous leukemia, or renal failure. Cross-reactivity with cobinamide (1 g/L) was <0.00003%. Vitamin B-12 measurements for blood specimens drawn into serum, EDTA, or heparinized plasma-collection tubes agreed within 3%.

Additional Keyphrases: enzymatic methods · cyanocobalamin

Vitamin B-12, a member of the corrin family, is a cofactor for the conversion of methylmalonyl coenzyme-A (CoA) to succinyl CoA. Vitamin B-12 is also a cofactor in the synthesis of methionine from homocysteine and is implicated in the metabolism of folic acid and, along with folate, is required for DNA synthesis (1, 2). Anemia is the major disorder related to low serum concentrations of vitamin B-12. Megaloblastic anemia, characterized by an increase in mean corpuscular volume, results from vitamin B-12 deficiency (3); a major cause of the deficiency is pernicious anemia, the main feature of which is the impeded uptake of vitamin B-12. However, several other conditions manifest themselves as low serum vitamin B-12 content, including iron deficiency, normal near-term pregnancy, vegetarianism, partial gastrectomy/ileal damage, oral contraception, parasitic competition, pancreatic deficiency, treated epilepsy, and advancing age (2, 4-6). Disorders associated with above-normal concentrations of serum vitamin B-12 include liver diseases and myeloproliferative diseases (4).

We have developed a rapid, nonisotopic assay of vitamin B-12 in serum and plasma for use with the Abbott IMx® (Abbott Labs, Abbott Park, IL) automated clinical analyzer. The assay requires no manual pretreatment, is simple to set up and perform, and reduces laboratory costs associated with disposal of the radioimmunoassay components used in most current clinical assays of vitamin B-12.

Materials and Methods

Reagents

Porcine intrinsic factor (IF) was purified from adult hog duodenum by vitamin B-12–agarose (Sigma Chemical Co., St. Louis, MO) affinity chromatography based on the method of Allen and Majerus (7), modified as described.1 Approximately 40 pig duodena were harvested and kept on ice until the steps described were carried out. All liquids used to clean and prepare the duodena were kept ice-cold (temperature ~8 °C) as much as possible. The duodena were cut, cleaned, and placed in an ice-cold isotonic saline solution (NaCl 9.0 g/L). The strips were then minced and blended in cold phosphate-buffered saline (PBS; per liter, 14.6 g of K2HPO4, 2.18 g of KH2PO4, and 9.0 g of NaCl). The contents were blended at low speed for 1 min, transferred to a large container, and diluted to 3 L with ice-cold de-ionized water. The contents of the larger container were stirred for ~90 min. Enough 600 g/L perchloric acid reagent was added to keep the pH <2.0. The material was then centrifuged at 2700 x g until the solids were pelleted (~30 min). The supernatant liquid was decanted and then was adjusted to 50 mg/L in K2HPO4 buffer (pH 6.5–7.5). We let the solution stand for ~16 h at 4 °C, by which time a precipitate had formed. The supernate was decanted from the solids and then centrifuged for 45 min at 2700 x g. Again, the supernate was decanted from the solids and was filtered through Celite to remove any colloids. We then added ~50 mL of vitamin B-12–agarose affinity resin and stirred the solution overnight at 2–8 °C. After discontinuing the stirring, we filtered the crude mixture through a coarse sintered-glass funnel and loaded the remaining gel, containing bound IF, into a glass chromatographic column. The column was washed with successive 500-mL volumes each of GSS reagent (7.5 g of glycine, 34 g of sucrose, and 59 g of NaCl per liter), PBS, and 4 mol/L NaCl, and finally with 1 L of PBS.

The IF was eluted with guanidine·HCl, 3.8 mol/L. The initial IF fractions eluted from the column, assayed as described below, contained the IF selected for use in this assay. Later fractions yielded material with lesser performance. We tested the IF in these early fractions

1 Nonstandard abbreviations: IF, intrinsic factor; PBS, phosphate-buffered saline; and BSA, bovine serum albumin.
for the presence of R-proteins, which bind many porphyrim-ring-containing compounds (i.e., cobinamides), by radioassay with $^{57}$CoB-12. The IF tested, which cross-reacted $<0.004\%$ with cobinamides, was then exhaustively dialyzed, with several changes of de-ionized water.

IF binding activity and purity were determined in duplicate samples with use of a $^{57}$CoB-12 (Amersham, Inc., Arlington Heights, IL) charcoal-binding assay.

The following IF preparations were made—undiluted, and diluted 2-, 4-, 8-, 16-, 32-, and 128-fold—in a buffer containing, per liter, 6.8 g of KH$_2$PO$_4$ and 0.5 g of bovine serum albumin (BSA), adjusted to pH 7.5 with KOH, 5 mol/L (PO$_4$/BSA). To each glass culture tube we added the following: 1 mL of PO$_4$/BSA, 100 µL of IF dilution, and 100 µL of tracer (100 µg/L cyanocobalamin USP plus $^{57}$CoB-12 added to yield 12 000–15 000 counts/min, in PO$_4$/BSA); blank tubes (B$_0$) contained 1.1 mL of PO$_4$/BSA and 100 µL of tracer. After vortex-mixing the samples, we incubated them for 1 h at room temperature. We then added 1 mL of well-mixed charcoal solution (3.0 g of DARCO 100 mesh G-60 (Fisher Chemical, Pittsburgh, PA; no. D127-500) in a diluent of 38.1 g/L borate and 1.5 g/L BSA, pH 8.5), and incubated the solution for 30 min at room temperature. The charcoal was then pelleted by centrifugation, and the radioactivity remaining in the aspirated supernate was determined by counting the $\gamma$ emission of $^{57}$Co.

We calculated the B-12 binding capacity as follows:

$$\text{Fraction bound (F)} = 1 - \frac{(B-B_0)}{B}$$

where B is counts/min in tubes containing IF, and B$_0$ is counts/min in blank tubes.

Vitamin B-12 binding, µg/L of IF preparation =

$$\frac{10.0}{\text{ng of vitamin B-12 per tube}} \times DF$$

where DF is the dilution factor for each IF dilution assayed.

Typical yields for vitamin B-12 binding in the IF preparations used in this study ranged from 2000 to 5000 µg/L.

The purity of the IF preparations was determined by cobinamide competition radioassay. Eight solutions were prepared, containing cobinamide at 200 000, 20 000, 2000, 1000, 200, 100, 50, and 25 g/L. Each of these was combined with an equal volume of IF preparation sample diluted to a vitamin B-12 binding capacity of $\sim50$ mg/L, determined as described above. The eight resulting solutions were assayed for the amount of vitamin B-12 binding capacity remaining, by using the charcoal assay described above (dilution factor = 1). All IF preparations used in this study were insensitive ($<5\%$ loss of vitamin B-12 binding capacity) to the presence of cobinamide at concentrations of $\geq$100 mg/L for IF samples containing 50 µg/L vitamin B-12 binding capacity. Samples prepared to contain R-protein (see below) at a vitamin B-12 binding capacity of 59 µg/L lost $\sim10\%$ of their binding capacity in the presence of cobinamide at 12.5 µg/L and 38% of the binding capacity with cobinamide at 25 µg/L.

Purified IF was stored at $-20^\circ$C until covalently linked to 0.485-µm-diameter aminomethacrylate micro-particles (Seradyn, Indianapolis, IN) via a heterobifunctional linking molecule (Abbott Labs) active for a primary amine functional group on the microparticle and for an R-SH functional group on the IF protein. The non-IF vitamin B-12 binding protein (R-protein) was obtained from Sigma Chemical Co. and had a label activity of 10–25 mega-units/g of protein. One unit of R-protein will bind 1.0 ng of vitamin B-12 at pH 7.5 and 25 °C (8).

Enzyme tracer was constructed by covalently linking cyanocobalamin, modified to possess a primary amine functional group (Abbott Labs), to alkaline phosphatase (EC 3.1.3.1; Boehringer-Mannheim Biochemicals, Indianapolis, IN) via a homobifunctional linking molecule (Abbott Labs) active for primary amine functional groups.

Cyanocobalamin USP Reference Standard (7.68 mg/g) was used to formulate the calibrator stock solution. The concentration of this stock was verified spectrophotometrically [$A(1\%, 1$ cm) = 204 at 361 nm (9)], and calibrators were prepared from it by volumetric dilution.

**Apparatus**

The IMx automated clinical analyzer instrument system was used in this study and has been described in detail elsewhere (10).

**Assay Protocol**

The IMx B-12 assay is a two-step competitive assay requiring a minimum of three reaction cells per run. Approximately 12 drops (~480 µL) of extractant 1, consisting of an excess of the physiologically inactive vitamin B-12 analog cobinamide, albumin, and buffer, was added into the predilution well of reaction cell 1. The same amount of extractant 2, composed of $\alpha$-monothioglycerol in dilute acetic acid, was added dropwise into the predilution well of reaction cell 2. Both of these first two reaction cells serve double duty as reagent containers and as cells for assay of samples, allowing a full 24 sample run with this configuration. Starting with reaction cell 1, we added $\sim100$ µL of serum or plasma, calibrator, or control to each sample well. The sample carousel and IMx vitamin B-12 reagent pack—consisting of sodium hydroxide (1 mol/L)/potassium cyanide (50 mg/L) denaturant, microparticle-immobilized IF binder, alkaline phosphatase-conjugated cyanocobalamin enzyme tracer, and 4-methylumbelliferyl phosphate substrate (1.2 mmol/L)—were placed in the instrument and the assay sequence was initiated.

Forty microliters of sample (or calibrator), 40 µL of denaturant, and the 15 µL of extractant are combined by the IMx and incubated at 34 °C for ~8 min. This frees vitamin B-12 from serum binding proteins and converts
all physiological forms to cyanocobalamin. This use of high alkalinity, in combination with a thiol reagent, was suggested previously by Allen and Majerus (11). After 155 μL of solid-phase vitamin B-12 binder (IF) reagent is added to lower the incubation pH (to 9.8–10.2) and to bind free cyanocobalamin from the sample, the samples are incubated for an additional 25 min. An aliquot of the incubation solution is deposited onto the reaction cell matrix, where the vitamin B-12-bound microparticles are captured by the fibers of the matrix. After washing, 75 μL of enzyme-conjugated tracer reagent is applied to the matrix and allowed to incubate for ~4 min, after which 60 μL of the substrate solution is added in excess. Methylumbelliferone is generated in inverse proportion to the amount of vitamin B-12 in the original sample. The vitamin B-12 in the sample is quantified by using a calibration curve, constructed electronically by the IMx from the methylumbelliferone fluorescence rates obtained for six cyanocobalamin calibrators, ranging from 0 to 2000 ng/L. The assay time required for six results is 54 min; 61 min is required to assay 24 samples.

Comparison Assays

The Quantaphase B12/Folate Assay Kit (Bio-Rad Labs, Richmond, CA) was used in our laboratory, according to manufacturer's instructions, as vitamin B-12 comparison assay 1. In addition, vitamin B-12 values for pernicious anemia serum samples were provided by the laboratory of John Lindenbaum, Department of Medicine, Columbia University, New York, NY (comparison assay 2; a vitamin B-12 radioimmunoassay standardized against the Lactobacillus leichmannii microbiological assay).

Results and Discussion

IMx B-12 standard curve. Figure 1 shows a standard curve generated from cyanocobalamin calibrators at 0, 100, 250, 500, 1000, and 2000 ng/L. As shown by the relationship of rate vs concentration, the dynamic range extends from 0 to 2000 ng/L.

Assay precision. Assay precision was determined by replicate assays of control samples over a 19-day period, performed with 10 IMx instruments (9 run-days per instrument). Each of three concentrations of control was assayed four times per run, with two runs per day, for each instrument. The total number of assays was 180 per concentration of control; total n = 720 per concentration of control. Control values were calculated by using a calibration curve generated and stored on the first day of the study. Within-run, between-run, and total precision were determined for each instrument according to Krouwer and Rabinowitz (12). The precision data given in Table 1 were derived from the pooled variance and control values over all instruments (13). Total CVs considered over the 10 instruments ranged from 6.7% to 9.8% for the low-concentration control, from 5.5% to 9.1% for the medium control, and from 6.0% to 8.3% for the high control.

Accuracy and detection limit. To eight patients' samples we added 300 or 600 pg of cyanocobalamin per milliliter. Vitamin B-12 concentrations were determined with the IMx B-12 by assaying both vitamin-supplemented and unsupplemented samples. The analytical recovery of added B-12 was determined by subtracting the result for the unsupplemented sample from the value for the supplemented sample for each patient and dividing by the amount of vitamin B-12 added. As shown in Table 2, average recovery for the 300 ng/L addition was 100% (range after adding vitamin B-12, 921–735 ng/L) and for the 600 ng/L addition was

<table>
<thead>
<tr>
<th>Table 1. IMx B-12 Assay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean concentration, ng/L</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>198</td>
</tr>
<tr>
<td>400</td>
</tr>
<tr>
<td>785</td>
</tr>
</tbody>
</table>

n = 720 per concentration (see text).

<table>
<thead>
<tr>
<th>Table 2. Analytical Recovery of Vitamin B-12 Added to Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial vitamin B-12 concentration, ng/L</td>
</tr>
<tr>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>300 ng/L</td>
</tr>
<tr>
<td>273</td>
</tr>
<tr>
<td>35</td>
</tr>
<tr>
<td>258</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>294</td>
</tr>
<tr>
<td>151</td>
</tr>
<tr>
<td>435</td>
</tr>
<tr>
<td>411</td>
</tr>
<tr>
<td>Average</td>
</tr>
</tbody>
</table>

* For samples of normal human serum into which cyanocobalamin was added.
105% (range after adding vitamin B-12, 621–1035 ng/L). The data presented in Table 3 demonstrate the recovery of cyanocobalamin from serum samples upon their dilution through the normal range of the assay (see below).

Samples from 30 individuals (vitamin B-12 range 212–1191 ng/L) were collected into each of serum (no anticoagulant), heparin, or EDTA collection tubes. Vitamin B-12 values for the heparin-treated samples averaged 99.4 (SD 10.4)% of the serum values, and EDTA results were 98.1 (SD 9.3)% of the serum vitamin B-12 concentrations. From these results we conclude that the IMx B-12 assay is appropriate for determinations of either serum or plasma vitamin B-12.

The detection limit was defined as the mean of the zero rate plus 2 SD (replicates of 10 per run). For the IMx B-12 assay, the detection limit was determined with each of 24 separate instruments (two runs each) and was found to be 37 (SD 9) ng/L (n = 48; range 22–55 ng/L).

Assay interferences. Vitamin B-12 assays involving purified IF as analyte binder must be insensitive to interference from antibodies to IF present in sera of patients with pernicious anemia. These antibodies bind IF and disrupt binding of the tracer molecule, which can lead to an erroneously high result (14). We obtained from the laboratory of John Lindenbaum (Columbia University) 36 serum samples from patients showing a positive titer for anti-IF antibody. These samples were assayed in our laboratory by both the IMx B-12 and comparison assay 1. Our results, along with vitamin B-12 values determined with comparison assay 2, are presented in Figure 2, which shows good agreement among the assays.

Vitamin B-12 assays involving the use of R-protein as an analyte binder, or IF contaminated with R-protein, may overestimate serum vitamin B-12 values, because of the presence of nonfunctional vitamin B-12 analogs in human serum that bind R-protein (15). Cobinamide, a physiologically nonfunctional analog of cyanocobalamin that readily binds to R-protein but not to IF, can be used as a prototype to check for potential assay cross-reactivity with nonfunctional vitamin B-12 analogs in human serum (16). As shown in Figure 3, the IMx B-12 assay was insensitive to cobinamide added to human serum to a final concentration of \(1 \times 10^6\) ng/L (1 mg/L). These results demonstrate that the accuracy of the IMx B-12 assay is not affected by the presence of nonfunctional vitamin B-12 analogs in human serum.
The effects of several other potentially interfering substances were examined as presented in Table 4. The IMx B-12 assay was not sensitive to broad variations in the concentrations of endogenous total protein or triglycerides. Human serum samples fortified with either bilirubin or hemoglobin, through the indicated ranges, gave results in the IMx B-12 assay that were equivalent to those by comparison assay 1. Assay of samples from patients with chronic myelogenous leukemia (n = 19) or renal failure (n = 11) gave equivalent results for the IMx B-12 assay and comparison assay 1: 248–1705 and 291–1786 ng/L, respectively, by the IMx (99% of the reference assay results). For samples from hematocarcinoma patients (n = 31), the IMx B-12 assay gave values (234–1609 ng/L) averaging 4% lower than those by comparison assay 1.

Samples of pooled normal human serum were supplemented with cyanocobalamin to ~35 000 ng/L. To these samples we added 0, 50, or 100 units of R-protein per milliliter and assayed the samples either undiluted or diluted 100-fold with either IMx System MEIA diluent or IMx B-12 zero calibrator. The samples with excess R-protein, when assayed undiluted, were expected to give vitamin B-12 values >2000 ng/L. Instead, they gave values lower than the control (no R-protein), and within the dynamic range of the assay, as shown by the data presented in Table 5. Upon dilution, the samples with added R-protein gave values comparable with that for the control in the range of 35 000 ng/L.

**Correlation and normal range.** Comparison of results of the IMx B-12 assay vs those of comparison assay 1 for 159 patients' serum specimens with vitamin B-12 concentrations ranging from ~0 to 2000 ng/L, gave the following: r = 0.98, slope = 1.02 (±0.02), and intercept = −19 ng/L. A second trial with an additional 124 patients' specimens in the same concentration range gave r = 0.98, slope = 1.007 (±0.02), and intercept = −28 ng/L.

Serum samples from 297 persons, clinically evaluated as normal by their mean cell volume and hematocrit, were analyzed for vitamin B-12 in our laboratory by the IMx. Their vitamin B-12 concentrations ranged from 110 to 1381 ng/L; the mean was 425 ng/L, and the 95% confidence interval for the log-normal distribution was 156–946 ng/L.

In conclusion, as demonstrated by the results presented, the Abbott IMx B-12 assay provides precise, sensitive, and accurate quantification of vitamin B-12 concentrations in serum or plasma, over an effective range up to 2000 ng/L. The assay is insensitive to interferences from high concentrations of vitamin B-12 analogs, bilirubin, triglycerides, and hemoglobin. In addition, the assay provides reliable quantification of vitamin B-12 in samples from patients with myelogenous leukemia, liver cancer, renal failure, and pernicious anemia. Assay automation on the IMx provides for rapid results, in a nonradioisotopic format, with minimum operator intervention.

We thank Lynn Codacovi, Greg Mattingly, and Chris Bieniarz of Abbott Laboratories for their synthesis and contribution of the linking reagents and cyanocobalamin derivatives used in this work.

**References**