PRACTICAL APPLICATION OF A SIMPLIFIED VITAMIN B_{12} RADIOASSAY

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A simplified radioassay for the measurement of serum vitamin B_{12} concentration was reassessed. Results of the radioassay were compared with results from the Euglena gracilis assay in 76 normal volunteers, 19 patients with Addisonian pernicious anemia, and 45 patients with gastric resection. Normal range of the radioassay was 200–900 pg/ml with a mean of 514 ± 156 pg/ml. Clear separation without overlap was possible by both methods between normal volunteers and vitamin B_{12}-deficient patients with pernicious anemia or gastric resection. The simplicity, economy, and reproducibility of the assay is stressed. Large numbers of sera can be assayed in a short period of time with very little expense and the test results made available within 24 hr. All equipment required can be found in the routine chemistry and nuclear medicine laboratories. The use of naturally occurring specific-binding substances with minimum preparation and cost seems an alternative answer to the rising expense of the prepackaged "kits."

Radioassays for the determination of serum vitamin B_{12} have been available for over a decade (*1–5*). Technical difficulties with the assay and discrepancies when the radioassay is compared with the microbiological assay have limited its use to only the larger institutions (*6–8*). Now with the appearance of the so-called prepackaged kits, assays are available to smaller hospitals which have limited nuclear medicine facilities. The prepackaged radioassay "kits" have been shown to be reliable and accurate but still they represent considerable expense when large numbers of studies are required (*2,5,7*). Tibbling described a method for determination of vitamin B_{12} in serum by radioassay, using naturally occurring binding substances and requiring only limited biochemical and nuclear medicine facilities (*4*). We have used this method to confirm its reliability and accuracy when compared with the microbiological Euglena gracilis assay. More importantly, our results emphasize how a simplified and economic method can be developed that requires only equipment found in most routine chemistry or nuclear medicine laboratories. The use of a naturally occurring specific-binding substance with minimum preparation and cost seems an alternative answer to the rising expense of the prepackaged "kits."

The radioassay for determining the vitamin B_{12} concentration in serum utilizes the principle of saturation analysis or "competitive protein binding." This depends upon measurement of the dilution of added radioactive vitamin B_{12} by unlabeled B_{12} present in serum. A carrier with vitamin B_{12} binding capacity (pooled human serum) is added to a mixture of radioactive and nonradioactive vitamin B_{12} that has previously been boiled to remove endogenous B_{12} binders. Isotopically labeled vitamin B_{12} then competes with the endogenous (cold) B_{12} for selective binding sites in the pooled serum. Once these binding sites are saturated, the free B_{12} is separated from the bound B_{12} by the addition of diethylaminoethyl (DEAE) cellulose. Centrifugation and counting of the precipitate permits calculation of the isotope dilution and an estimate of the serum vitamin B_{12} concentration.

MATERIALS

Acetate buffer. Forty-eight milliliters of 0.7 *M* acetic acid and 52 ml of 0.7 *M* sodium acetate are diluted with water to 1,000 ml and the pH is adjusted to 4.7.

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Potassium cyanide. Stock solution: 40 mg KCN and 25 ml of water may be stored up to 1 month at 4°C. Working solution: 0.5 ml of stock solution is diluted to 50 ml with water and prepared daily as used.

Tris buffer, 0.1 *M*. Tris-hydroxymethylaminomethane (12.1 gm) is dissolved in 1,000 ml of water and adjusted to pH 8.5.

DEAE cellulose powder. This is Whatman DE-11, 200 mg/assay.

^{57}Cobalt B_{12} solution. Cobalt-57-vitamin B_{12} (Rubritope^{57}, E. R. Squibb & Sons) has a specific activity of 12.5 μCi or more per microgram. One milliliter is diluted with 180 ml of physiological saline to make a final concentration of vitamin B_{12} of approximately 1,000 pg/ml. The solution is kept frozen in suitable portions and 2.5 ml are required to make working standards every 2 weeks.

Test serum binder solution. One hundred fifty milliliters of pooled human serum from healthy individuals with normal vitamin B_{12} concentration is diluted to 500 ml with physiological saline and kept frozen in suitable portions for daily assay. With each new batch of test binder serum used, the optimal amount per assay has to be re-evaluated. This is done by incubating 4 ml of extraction mixture (after boiling and centrifugation) with 0, 25, 50, 100, 150, and 200 μl of the test-binding serum solution. Good correlation is demonstrated between the amount of pooled serum added and the activity found in the DEAE cellulose.

Vitamin B_{12} standards. Known vitamin B_{12} solution (Merck Sharp & Dohme Research Laboratories, West Point, Pa., Cyanocobalamin, 20 μg/ml) is diluted with physiological saline to 0.1 μg/ml and kept frozen in individual vials. The number required with each individual assay is thawed, the remainder kept frozen and stored at —20°C. Working standards are diluted to contain 0–2,000 pg/ml (125, 200, 500, 1,000, and 2,000).

METHODS*

The specific tube contents and reagent dilutions required are listed in Table 1. Known standards, the unknown serum, the low serum control, and the standard pool are all set up according to this protocol. Each tube first receives 0.2 ml of serum, physiological saline, and known standards. One-half milliliter of KCN, 0.1 ml of thawed ^{57}Co-B_{12} solution, and 4 ml of 0.07 *M* acetate buffer are added to each tube. Individual tubes are capped, mixed on a Vortex mixer, and boiled at 100°C for 15 min. To the initial tube, which is not boiled, the ^{57}Co label is added directly in the counting vial. The remaining tubes, after 15 min of boiling, are cooled and then centrifuged. Four milliliters of filtrate from each of these tubes are transferred into the remaining counting vials, 0.1 ml of test serum binding solution is added to each vial, and they are left at room temperature for approximately 90 min. At the end of incubation, 6 ml of 0.1 *M* Tris buffer and 200 mg of DEAE cellulose powder are added to each tube. The tubes are sealed and the contents shaken approximately 100 times. The tubes are then centrifuged and the supernatant removed. The residual DEAE cellulose is washed

* According to the method of Tibbling (*4*).

TABLE 1. VITAMIN B_{12} RADIOASSAY

	Tube No.	Distilled H_2O (ml)	KCN (ml)	Serum for assay (ml)	Saline (ml)	Standard (ml)	^{57}Co B_{12} (ml)	Acetate buffer (ml)
100% Tag*	1	2.0	—	—	—	—	0.1	—
0% standard	2	—	0.5	—	0.2	—	0.1	4.0
125 pg/ml standard	3	—	0.5	—	—	0.2	0.1	4.0
250 pg/ml standard	4	—	0.5	—	—	0.2	0.1	4.0
500 pg/ml standard	5	—	0.5	—	—	0.2	0.1	4.0
1000 pg/ml standard	6	—	0.5	—	—	0.2	0.1	4.0
2000 pg/ml standard	7	—	0.5	—	—	0.2	0.1	4.0
Normal control	8	—	0.5	0.2	—	—	0.1	4.0
Low control	9	—	0.5	0.2	—	—	0.1	4.0
Patient serum	10	—	0.5	0.2	—	—	0.1	4.0
Patient serum	11	—	0.5	0.2	—	—	0.1	4.0
Patient serum	12	—	0.5	0.2	—	—	0.1	4.0

* Tube No. 1 is set up directly in a counting vial. Tube Nos. 2–12 are boiled, then centrifuged. Four milliliters of filtrate of each are placed in numbered counting vials; 0.1 ml of serum-binding solution is added to each. Tubes are incubated at room temperature for 90 min; 6 ml of Tris buffer and 200 mg DEAE cellulose are added to each, washed, and then precipitate is counted.

TABLE 2. COMPARATIVE RESULTS BETWEEN RADIOASSAY AND EUGLENA GRACILIS ASSAY OF VITAMIN B_{12}

		Radioassay			Euglena gracilis assay		
	No. of patients	Mean	s.d.	Range (pg/ml)	Mean	s.d.	Range (pg/ml)
Normal controls	76	514	±156	200–900	348	±148	185–825
Pernicious anemia	19	34	± 35	0–130	39	± 20	11–80
Gastric resection	45	280	±169	3–713	230	±240	35–760

twice with 10 ml of water by inverting the tube 50 times. Tubes are again centrifuged and the washings discarded. The remaining activity on the DEAE cellulose is counted on a Packard Model 5200 Autogamma scintillation counter for 10 min or until 10,000 counts. The radioactivity found in the tubes of known standard concentrations is used to construct a standard curve. The concentration in picograms per milliliter is plotted on the abscissa and counts per minute of the bound activity on the ordinate (Fig. 1). The concentration of vitamin B_{12} in the unknown serum can then be calculated from this standard curve.

The microbiological assay was performed using the standard Euglena gracilis assay of Ross (*9*).

RESULTS

The serum concentration of vitamin B_{12} was measured by both the radioassay method and by the Euglena gracilis assay method in 76 normal volunteers, 19 patients with known untreated Addisonian pernicious anemia, and 45 patients following gastric resection. Table 2 illustrates the comparative results between the two assay methods. In 76 normal volunteers, the mean concentration of vitamin B_{12} by the Euglena gracilis assay was 348 pg/ml and by the radioassay, the mean concentration was 514 pg/ml. In 19 patients with Addisonian pernicious anemia, the mean vitamin B_{12} concentration by the Euglena gracilis assay was 39 pg/ml and by the radioassay was 34 pg/ml. In 45 patients with gastric resections, the mean vitamin B_{12} concentration by the Euglena gracilis assay was 230 pg/ml and by the radioassay was 280 pg/ml. The range for normal volunteers by the microbiological method was 185–825 pg/ml and by the radioassay was 200–900 pg/ml. In those patients with pernicious anemia the range by the microbiological method was 11–80 pg/ml and by the radioassay, 0–130 pg/ml. There was no overlap found between the normal volunteers and the patients with Addisonian pernicious anemia. However, the range for gastric resections was 35–760 by the Euglena gracilis method and 3–713 pg/ml by the radioassay. No specific pattern was observed in this group, and values were observed that were both distinctly in the normal and the abnormal ranges. A comparison between the radioassay and the Euglena gracilis assay including all groups is plotted in Fig. 2. These findings show good correlation between the two methods with a correlation coefficient of 0.82. Figure 3 depicts the separation of these three groups when vitamin B_{12} levels determined by the radioassay are compared. No overlap is seen between normal volunteers and those patients with pernicious anemia. However, there is a broad range of values in those patients with gastric resections.

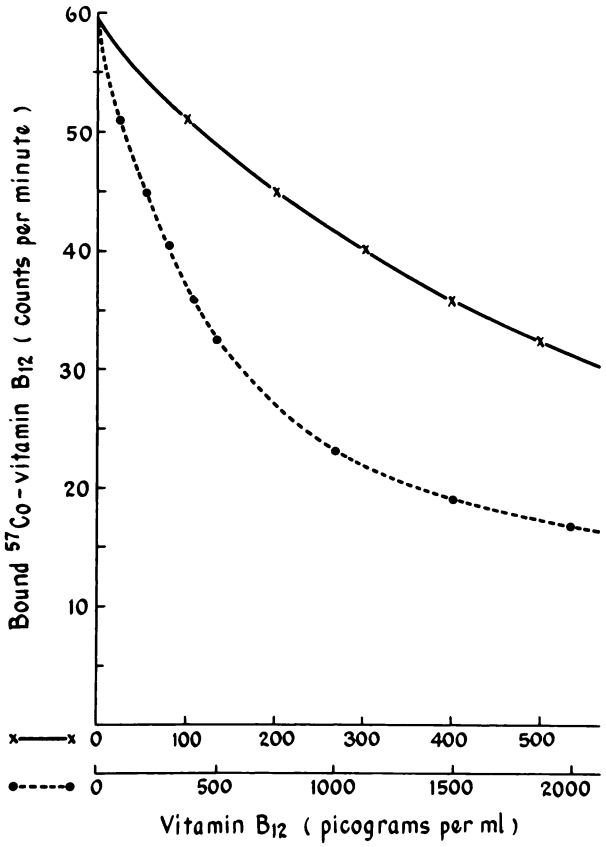

FIG. 1. Standard curve for determination of concentration of vitamin B_{12} in unknown serum. Concentration of vitamin B_{12} is plotted in picograms per milliliter on abscissa and counts per minute of bound activity on ordinate. Note use of dual scale to expand sensitivity of curve at low and high concentration levels.

DISCUSSION

Until recently, serum vitamin B_{12} concentrations were measured in this hospital by the standard Euglena gracilis assay. The basis for the bioassay depends upon the culture of the specific microorganisms (i.e., Euglena gracilis or Lactobacillus leishmanii) which require vitamin B_{12} for growth in a medium deficient in vitamin B_{12}. When unknown serum is added, the result in growth measured by optical density changes represents the amount of vitamin B_{12} present in unknown serum. These microbiological methods are laborious and time-consuming. They may not be entirely reliable for sera from patients receiving certain antibiotics or cytotoxic drugs. Other disadvantages include the need for maintenance of the culture of microorganisms, performance of the test under sterile conditions, the expense required in technical time, the inability to perform large numbers of tests at one time, and the long delay in returning results to the clinician.

The radioassay method as now available has none of the disadvantages of the bioassay. This method has the specific advantages of accuracy and sensitivity inherent to the use of radionuclides as well as the specificity inherent in using competitive protein-binding analysis. Moreover, the radioassay permits a large number of studies to be performed with the results reported the following day. Emergency measurements of serum vitamin B_{12} concentrations are now possible; results may take 2 weeks or longer using the Euglena gracilis assay. Reproducibility of the radioassay is at least equal to the biological assay. In comparing duplicate values in 12 normal volunteers, 45 patients with gastric resection, and 6 patients with pernicious anemia, the variation of values found was no greater than that of the Euglena gracilis assay. To test replication, a serum pool was performed in 24 separate assays and showed a range from 640–780 pg/ml of vitamin B_{12}. The recovery of added B_{12} was evaluated and found to be from 86–106%. Both the replication and recovery were also equally as good as the Euglena gracilis assay.

The appearance of the prepackaged "quick kit" for routine radioassays of hormones, vitamins, and drugs has been a distinct asset for the smaller laboratories but has placed a greater financial burden upon the department required to run large numbers of tests. It is primarily for economic reasons that we stress the simplified procedure described here. The specific-binding protein used is contained in normal human serum and requires no preprocessing. This obviates the need for purchasing commercial intrinsic factor or preprocessed specific-binding substances. Counting equipment no more elaborate than already exists in the routine nuclear medicine department is

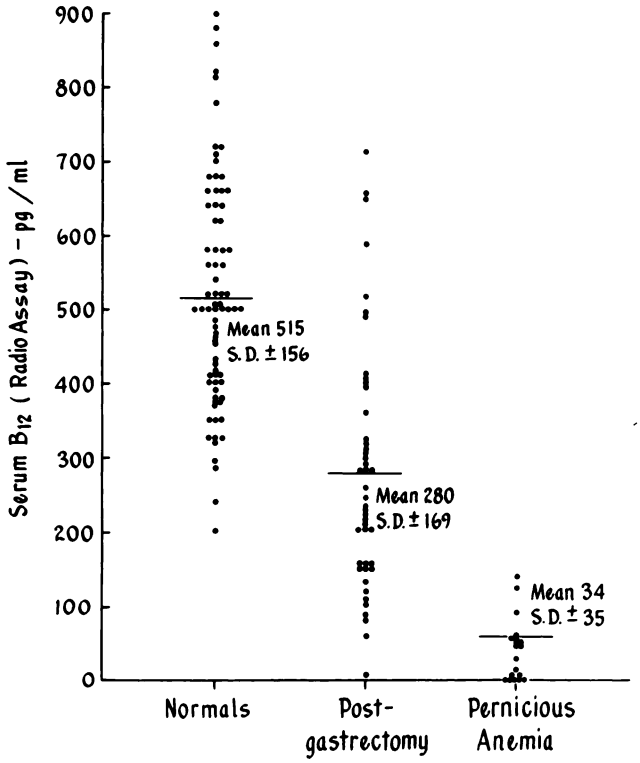

FIG. 3. Serum vitamin B_{12} levels by radioassay are shown for 76 normal controls, 45 patients with gastric resection, and 19 patients with pernicious anemia.

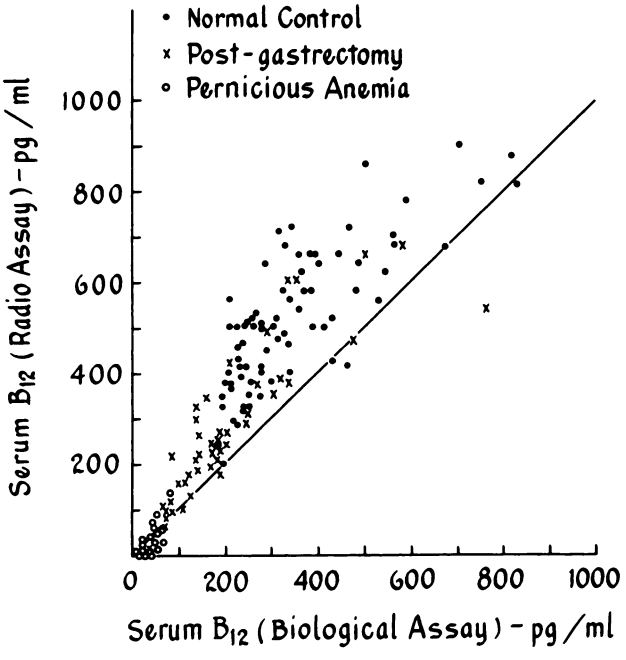

FIG. 2. Radioassay for serum vitamin B_{12} is compared with Euglena gracilis assay in 76 normal controls, 45 patients with gastric resection, and 19 patients with pernicious anemia.

required. A manual well-type scintillation counter or an automated gamma counter enables an individual technologist to conduct as high as 50 assays per day at a cost of less than ten cents per test.

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