Commercially-Supplied Binders for Plasma Cobalamin (Vitamin B_{12}), Analysis—“Purified” Intrinsic Factor, “Cobinamide”-Blocked R-Protein Binder, and Non-purified Intrinsic Factor—R-Protein Binder—Compared to Microbiological Assay

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Recent evidence (Kolhouse et al., *N. Engl. J. Med.* 299: 785–792, 1978) demonstrates that commercial cobalamin (Vitamin B_{12}) radioassay kits contain nonspecific R-protein binding agents that can give falsely normal results in patients who are actually cobalamin deficient. We tested three kits: with “purified” intrinsic factor as the binder, with intrinsic factor and the nonspecific R-protein sites blocked with “cobinamide,” and non-purified intrinsic factor—R-protein binder. Results with use of the first two compared well with those by a microbiological assay (*Lactobacillus leichmannii*) and are in harmony with clinical impressions.

**Additional Keyphrases:** radio- and microbiological assay compared - reference intervals - variation, source of - liver disease - “kit” methods - cut-off values

Intrinsic factor can bind biologically active cobalamin (vitamin B_{12}); there is also a group of “R-proteins,” which can bind both biologically active cobalamin and non-biologically active cobalamin analogs (1). The R-proteins are cobalamin binders most often identified by their sources rather than by their functions, which mostly are unknown. These sources include saliva, tears, milk, plasma from fetuses or polycythemia vera patients, bile, gastric juice, cerebrospinal fluid, and leukocytes and erythrocytes. Gräseck (2) grouped all cobalamin binders other than intrinsic factor (IF) and transcobalamin II into a category called “R,” which initially referred to rapid electrophoretic mobility. Kolhouse et al. (1) state that the term R-protein was originally devised to denote a cobalamin-binding protein in human gastric juice that was devoid of IF activity.

Vitamin B_{12} radioassay kit manufacturers have used intrinsic factor concentrates as binders since the introduction of these kits. These concentrates contain both IF and R-proteins. Kolhouse et al. (1) showed that the amount of R-protein in these concentrates ranged from 51 to 85%. Serum and plasma contain significant amounts of non-biologically active cobalamin analogs, which are not measured with “benchmark” vitamin B_{12} microbiological assays or radioassays in which pure or “purified” IF is used as the binder. No clinical value can now be assigned to measuring the cobalamin analogs in plasma, but most of the binder in commercial radioassay kits obviously results in measurement both of these analogs and of active cobalamin. In 21 patients with clinical evidence of cobalamin deficiency, a radioassay with pure IF used as the binding agent correctly placed all patients well below the range for normals, and results correlated with those by microbiological assay. The same radioassay, but with R-protein as the binder, demonstrated significant overlap with the expected range for normal persons (7).

Since late 1978, there has been much activity by commercial producers of vitamin B_{12} radioassay kits to modify their binding agents to eliminate the nonspecific effects of R-proteins. There have been two approaches:

1. **Negate the R-protein binding sites:** This can be accomplished by flooding the IF—R-protein combination binder with an analog such as cobinamide. Cobinamide will not bind to IF, but will bind to R-proteins. If added in great excess (>100-fold) it will quench all the nonspecific cobalamin binding sites.

2. **Purify the IF:** IF concentrate can be purified by prolonged treatment with proteolytic enzymes such as trypsin (EC 3.4.21.4) or chymotrypsin (EC 3.4.21.1), or both, or by affinity chromatography.

Using commercially available radioassay kits, we have evaluated both of these approaches for measuring plasma cobalamins, and compared them with *Lactobacillus leichmannii* microbiological vitamin B_{12} assay.

**Methods and Materials**

Three vitamin B_{12} radioassay kits—“Dualcount,” “Dualcount with Cobinamide Blocking,” and “Dualcount with Purified B_{12} Binder”—were obtained from Diagnostic Products Corp., Los Angeles, Ca 90064. The vitamin B_{12} microbiological assay was done as described by Spray (3).

According to the manufacturer, the cobinamide preparation used in the blocked kit contained other analogs in unknown quantities, and had been established by the supplier to have a certain potency with R-protein and no effect with IF.

“Purified IF” in the commercial kits was prepared by the technique known as biospecific absorption or affinity chromatography, which depends on a reversible complex being formed between IF and a solid-phase-bound bioactive cobalamin. In this stage of purification the main impurities to be eliminated are the R-proteins. The specific retention and elution of IF on the affinity resin with the exclusion of R-protein is predicated on two observations. The first is that IF has a higher affinity for bioactive cobalamin than nonbioactive cobalamin analogs. This necessitates that the conjugation of the vitamin to the affinity resin results in a resin–vitamin complex in which the structure of the vitamin moiety is compatible with the binding site of IF. The second is that IF and R-proteins have different binding characteristics with respect to pH and ionic strength. That is to say, at the proper pH and ionic strength the affinity resin binds IF and excludes R-proteins. The resin-bound IF is eluted by reversible inactivation of IF: a mild protein denaturant is added to the column eluent and the pH and buffer components are changed. Subsequent removal of the denaturant results in a high yield of active IF.

Purity of the active IF was established in three ways:

1. Binding of bioactive cobalamin was completely inhibited when active IF was first incubated with antibody to IF.

2. IF purity was also assessed from binding properties. IF is active at basic pH but not at acidic pH; and its binding of
Fig. 1. Non-purified intrinsic factor and R-protein binder compared with "cobinamide"-blocked R-protein binder for radioassay of plasma B12

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B_{12} (\text{ng/L}) = \text{"Cobinamide" - Blocked Binder}
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n = 109. Mean of the non-purified IF and R-protein binder radioassay was 679 ng/L of the R-protein-blocked binder radioassay values, 516 ng/L. The regression equation is \( y = 0.94 x + 122 \). In Figs. 1-4, — is the theoretical perfect correlation line, for comparison.

Fig. 2. Results of Lactobacillus leichmannii microbiological assay and "cobinamide"-blocked R-protein binder radioassay for plasma B12 compared

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B_{12} (\text{ng/L}) = \text{"Cobinamide" - Blocked Binder}
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n = 127. Mean of the microbiological assay values was 425 ng/L; mean of the R-protein-blocked binder radioassay values was 395 ng/L. The regression equation is \( y = 1.01x + 36 \). In Figs. 1-4, — is the theoretical perfect correlation line, for comparison.

Radiolabeled bioactive cobalamin is little affected by the presence of cobalamin analogs. R-protein, in contrast, is active at both acidic and basic pHs and is inhibited by the presence of cobalamin analogs in a binding assay. Therefore, by comparing the cobalamin binders in assays performed at acidic and basic pH, and at basic pH in the presence of cobalamin analogs, the purity of IF preparations can be determined. A purified IF preparation will have very low cobalamin binding ability at acidic pH, and the binding at basic pH will be little affected by the presence of cobalamin analogs. In contrast, purified R-protein will have nearly equal binding capacity at both acidic and basic pHs with bioactive cobalamin, but binding will be substantially inhibited by the presence of cobalamin analogs.

3. The purified IF binder does not measure "cobinamide" in concentrations up to 2 mg/L.

We did not independently verify the purity of either the IF or the cobalamin preparations.

Heparin-treated plasma was used in all cases, and all assays were performed as duplicates. All isotope samples were counted on a Model 1185 Gamma Counter (Searle Analytic, Des Plaines, IL 60018) until a minimum of 5000 counts had accumulated, to make counting error negligible. Radioassay data were examined by use of a 9830 Hewlett-Packard calculator, with a statistical approach in which variance-weighted least-square regression analysis was performed on the logit transformation of the percent bound (4).

Patients were judged to be cobalamin deficient on the basis
of data on megaloblastic hematological data, clinical diagnosis and consultation, and, in some cases, abnormal results for Schilling tests and bone-marrow examinations. Patients were judged to have liver disease based on a variety of abnormal liver-evaluation data and clinical diagnosis.

**Results and Discussion**

Our first experience with the changes in cobalamin radioassay-kit binding proteins was with the introduction of a "cobinamide"-blocked binder. This kit contained the combination porcine IF and R-protein binding agent we had been routinely employing ("Dualcount") but "cobinamide" was now added (in excess of 2 ng/L) to block nonspecific R-protein binding sites. As shown in Figure 1, plasma cobalamin values for normal volunteers and for patients were shifted, lower results being obtained with use of the "cobinamide"-blocked binder.

We then tested and compared the radioassay kits, using "purified" IF as the binder, the "cobinamide"-blocked binder, and the *Lactobacillus leichmannii* microbiological assay. The microbiological assay and "cobinamide"-blocked radioassay showed good correlation (Figure 2) with about a 30 ng/L positive bias in favor of the microbiological assay. Similar results were observed when the radioassay kit with "purified" IF as the binder was compared to the microbiological assay (Figure 3). Again, we saw a positive bias (about 42 ng/L) in favor of the microbiological assay.

The comparison between samples assayed with use of the two binders, "purified" IF and "cobinamide"-blocked, was quite good (Figure 4). These data lead us to conclude that all three cobalamin assay procedures give essentially the same results.

Figure 5 described the results for normal individuals, cobalamin-deficient patients, and patients with liver disease when assayed by four methods. Three of the cobalamin-deficient samples were too small to be assayed with the non-purified binder. The assays involving the "cobinamide"-blocked and "purified" IF binders show an acceptable cutoff between cobalamin deficient patients and normal individuals, and also match the results by microbiological assay. As expected, the assay involving non-purified binder shows higher results. Our previous reference interval for this assay was 300–900 ng/L. We also recommend an indeterminate range for all assays, i.e., a range in which the cobalamin value alone may be suggestive but inconclusive in contributing to a differential diagnosis. As expected, patients with liver disease demonstrate higher values for plasma cobalamin than does the normal population.

Both Kolhouse et al. (1) and Cooper and Whitehead (5) have recently recommended the use of pure IF in radioassay kits or methods to provide a reliable result for cobalamin in plasma or serum. From our data presented here, it appears that although the "cobinamide"-blocked binder can be used, we agree with the above investigators that pure or "purified" IF would be the better choice of a binder, for analytical reasons. Any possible nonspecific effects due to other binders would be eliminated.

**References**


