Radioassay for Cobalamin (Vitamin B₁₂) Requiring No Pretreatment of Serum

Thomas M. Houts¹ and James A. Carney²

We describe a radioassay for cobalamin (vitamin B₁₂) in human serum or plasma that requires no boiling or other pretreatment of the sample. Normal chicken serum covalently coupled to magnetizable particles is used as the binding agent. The assay is performed at pH 12.9, at which pH all cobalamin in human serum is released from its binding proteins, whereas the binding agent maintains a high affinity for cobalamin (Kₐ 1.7 × 10¹⁰ L/mol). Under these assay conditions the binding protein shows a specificity for cobalamin similar to that of purified intrinsic factor. The assay is simple, rapid, and precise, and results correlate well with those of the Euglena gracilis microbiological assay and an intrinsic-factor binding assay.

Additional Keyphrases: magnetizable solid phase · reference interval · microbiological assay and intrinsic factor assay compared

Adequate stores of cobalamin (vitamin B₁₂) are essential for normal erythropoiesis, and normal neurological function. Cobalamin deficiency leads to megaloblastic anemia and characteristic neurological disorders. Particularly important are the facts that the neurological changes associated with cobalamin deficiency are often irreversible and may occur before significant abnormalities appear in the peripheral blood. Because concentrations of cobalamin decrease before clinical manifestations appear (1), it is essential to have routine, sensitive methods for determining these concentrations.

In human serum, virtually all the circulating cobalamin is bound to specific binding proteins, the transcobalamins. In the long-established microbiological methods for determination of serum cobalamin, the sample is diluted with an acidic buffer, then boiled to denature the transcobalamins and release the cobalamin. The assays depend on the growth of Lactobacillus leichmannii or Euglena gracilis, microorganisms for which cobalamin is a necessary growth factor. These microbiological methods, the accepted reference methods for cobalamin (2), are time consuming, often imprecise, and require training and technical expertise; moreover, L. leichmannii is subject to interference from a wide range of drugs, including antibiotics and tranquillizers (3).

Many laboratories now measure serum cobalamin by the simpler radioassay methods. In these assays the sample is boiled in the presence of cyanide, to denature the transcobalamins and convert all the circulating cobalamin to the more chemically stable cyanocobalamin form, before binding protein is added. Various binding proteins have been used, including intrinsic factor, saliva, human serum, and toadfish, trout, and chicken sera (4, 5). The most widely used of these are purified or crude intrinsic factor preparations, but many problems are associated with their use (6), including difficulty of purification, instability on storage, and variation in binding capacity with changes in serum protein and cobalamin concentrations.

Dangers arising from the clinical use of some radioassays for cobalamin have been reported, in which cobalamin-deficient patients had apparently normal serum values as determined by radioassay (7). Kolhouse et al. (8) have suggested that this was due to the presence of "cobalamin analogues" in human serum, which are measured by radioassays involving R protein, but measured to a much smaller extent by the microbiological assays and by radioassays in which purified intrinsic factor is used. Because intrinsic factor is the most specific of cobalamin-binding proteins (9), Kolhouse et al. suggest that only assays with the specificity of intrinsic factor should be used for the clinical determination of cobalamin in serum.

The purpose of the present study was to develop a radioassay as specific for cobalamin as those based on use of intrinsic factor, that would correlate with the reference microbiological assays and would avoid the need to boil the serum, thereby facilitating its use for screening purposes. Carrying out the assay at very high pH ensured that all the cobalamin in human serum was released from the transcobalamins and was available for assay (10). Chicken serum, however, retains its ability to bind cobalamin at this pH (11). Factors affecting the assay and data relating to its accuracy, precision, sensitivity, specificity, and correlation with other assays are presented.

Materials and Methods

Materials

Sodium hydroxide, sodium azide, sodium chloride, dibasic sodium phosphate, monobasic sodium phosphate, potassium chloride, potassium cyanide, and Triton X-100 were obtained from BDH Poole, Dorset, U.K., and were of "AnalaR" grade. [⁵⁷Co]Cyanocobalamin, 220 Ci/g, was obtained from The Radiochemical Centre, Amersham, Bucks, U.K. Spectrophotometrically calibrated cyanocobalamin solution ("Cytamen") was provided by Glaxo Labs, Greenford, Middlesex, U.K., and human serum albumin from Sigma Chemicals Ltd., Poole, Dorset, U.K.

Normal chicken serum was obtained as a 4-L pooled specimen from Cunningham and Greenbury, Dorcas Farm, Stoke Hammond, Bletchley, Bucks, U.K.

An analog-blocked intrinsic factor radioassay kit was obtained from Diagnostic Products Corp., Los Angeles, CA 90064. In this method the R protein present in the binder is

¹Technia Diagnostics Ltd., 283 City Rd., London ECIV 1JX, U.K.
²Present address: Department of Chemical Pathology, St. Bartholomew’s Hospital, London EC1A 7BE, U.K.

Received Aug. 4, 1980; accepted Nov. 12, 1980.

³The term "R protein" was originally devised to denote a cobalamin binding protein in human gastric juice that was devoid of intrinsic factor activity and showed rapid mobility on electrophoresis. Immunologically related proteins have been found in various human tissues and body fluids and have also been referred to as "R" proteins.
saturated with a cobalamin analog that has a high affinity for R protein but low affinity for intrinsic factor, and that selectively inactivates the R protein.

A gastric R protein radioassay kit, the “Quanta Count I,” was obtained from Bio-Rad Labs., Watford, Herts, U.K. The assay is performed at a pH of 2, thereby selectively inactivating the intrinsic factor present in the binder.

Reagent Preparation

*Bi*-releasing buffer: Per liter, 2.0 mol of KCl, 264 mmol of NaOH, 1 g of NaN₃, and 40 mg of KCN.

Solid-phase diluent: Per liter, 2.5 mol of KCl, 10 g of Triton X-100, and 1 g of NaN₃.

Cyanocobalamin standards: Prepare these by diluting the contents of a calibrated Cytochrome ampoule in a diluent containing, per liter, 56 mmol of Na₂HPO₄, 14 mmol of NaH₂PO₄, 60 mmol of NaCl, 1 g of NaN₃, and 40 g of human serum albumin.

[³⁷Co]Cyanocobalamin tracer: Prepare for use by diluting a 100 ng/L diluent containing 2.5 mol of KCl, 10 g of Triton X-100, and 1 g of NaN₃ per liter.

*Immobilized (solid-phase) chicken serum: Magneticizable cellulose/iron oxide particles were prepared, activated with CNBr, and coupled to chicken serum as described by Kamel et al. (12) at a ratio of 1 mL of chicken serum to 1 mL of solid-phase particles. After the coupling and washing, the particles were washed three times with the solid-phase diluent and resuspended in the same diluent at a concentration of 3.6 g of solid phase per liter, such that 100 μL bound 45–60% of 45 pg of [³⁷Co]cyanocobalamin in the absence of unlabeled cobalamin. The solid-phase-coupled chicken serum is stable for at least six months at 4 °C.

Assay Protocol

 Pipet 250 μL of patient’s serum or cobalamin standard into disposable polystyrene tubes, then add 250 μL of *Bi*-releasing buffer, 100 μL of tracer, and 100 μL of solid-phase chicken serum suspension. Incubate for 1 h, vortex-mixing the tubes at 15-min intervals. Sediment the solid-phase by standing the tubes for 5 min on a 15 × 20 cm multipoles magnet (Magnet Applications Ltd., London, U.K.) or, alternatively, by centrifugation at 1000 × g for 1 min. Aspirate and discard the supernates and measure the radioactivity in the tubes containing the solid-phase (bound fraction) with a gamma counter with a ⁵⁷Co channel.

To construct a standard curve, plot B/B₀ vs concentration of the cobalamin standards on logit-log paper, and determine the cobalamin concentration of serum samples by interpolation (Figure 1).

**Results**

**Assay Optimization**

*Effect of pH:* There was no suitable buffering ion, so we used NaOH/KCl, varying the concentration of NaOH over the range of 0.1–0.4 mmol/L, which provided an incubation pH of 12.1–13.2. From 0.2 to 0.4 mmol/L, we observed no effects on either the standard curve or the values obtained for serum samples. At a NaOH concentration of 0.1 mmol/L, binding in the standard curve increased, but serum samples had lower apparent cobalamin concentrations (Table 1). NaOH at 264 mmol/L (initial concentration) provides an incubation pH of 12.9 and is on the plateau for both binding and apparent serum cobalamin concentrations.

*Effect of ionic strength:* Increasing the KCl concentration in the releasing buffer and diluents from 50 mmol/L to saturated caused an increase in specific binding. The concentrations we use in the reagents is approximately half-saturated, which prevents crystallization of the salt during storage at low temperatures.

**Effect of KCN concentration:** We investigated the effect of KCN in the system and found no significant effect on either the standard curve or apparent cobalamin concentrations in serum samples over the range of 5–15 μg of KCN per assay tube. However, omitting cyanide led to significantly lower apparent cobalamin concentration in serum samples (Table 1).

**Dilution for standards:** Because the *Bi*-releasing buffer

**Table 1. Effect of NaOH and of KCN Concentration in the *Bi*-Releasing Buffer on Zero Standard Binding (B₀) and the Apparent Cobalamin Concentration of a Human Serum Pool**

<table>
<thead>
<tr>
<th>Conc in buffer, mmol/L</th>
<th>% bound for zero standard</th>
<th>Apparent cobalamin concn in human serum pool, ng/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>55.8</td>
<td>290</td>
</tr>
<tr>
<td>200</td>
<td>48.3</td>
<td>535</td>
</tr>
<tr>
<td>264</td>
<td>48.7</td>
<td>550</td>
</tr>
<tr>
<td>300</td>
<td>48.9</td>
<td>550</td>
</tr>
<tr>
<td>400</td>
<td>47.8</td>
<td>540</td>
</tr>
<tr>
<td>KCN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>47.9</td>
<td>340</td>
</tr>
<tr>
<td>0.3</td>
<td>48.2</td>
<td>480</td>
</tr>
<tr>
<td>0.6</td>
<td>46.1</td>
<td>495</td>
</tr>
<tr>
<td>0.9</td>
<td>45.3</td>
<td>475</td>
</tr>
</tbody>
</table>

![Fig. 1. Standard curve, showing mean and (bars) ±2 SD (n = 10)](Fig_1)
Table 2. Precision Studies (Three Pooled Sera)  

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD), ng/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-assay</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>103 (4.0)</td>
<td>3.9</td>
</tr>
<tr>
<td>B</td>
<td>469 (17)</td>
<td>3.6</td>
</tr>
<tr>
<td>C</td>
<td>1045 (25)</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Between-assay</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>108 (9.5)</td>
<td>8.8</td>
</tr>
<tr>
<td>E</td>
<td>522 (30)</td>
<td>4.7</td>
</tr>
<tr>
<td>F</td>
<td>1096 (55)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Within-assay variation was determined by assaying 20 replicates each of three human serum pools. Between-assay variation was determined by assaying each pool in duplicate on 10 consecutive days.

Assay Performance  

Precision: Within-assay variation was determined by assaying 20 replicates each of three human serum pools. Between-assay variation was determined by assaying three human serum pools in duplicate on 10 consecutive days. The results are shown in Table 2.

Analytical recovery: Accuracy as reflected in analytical recovery was tested by assaying two sera individually or mixed together in equal volumes, and by adding known amounts of cyanocobalamin to aliquots of sera sufficient to increase the concentration by 400 ng/L. Analytical recovery ranged from 101 to 110% (Table 3).

Sensitivity: The definition of sensitivity chosen is that of Rodbard (13) for the minimum detectable concentration. We measured the variation in response at zero dose for 20 replicates and used a one-sided t-statistic at the 95% confidence level. For standards and samples assayed in duplicate, the minimum detectable concentration is 21 ng/L.

Assay Validation  

Normal range: In 20 samples from healthy volunteers cobalamin concentration ranged from 196 to 630 ng/L. It has been suggested (8) that, to compensate for the skewness of the raw data, the normal range should be calculated as the mean plus or minus 2 SD after log transformation. The normal range calculated in this manner is 187-696 ng/L.

Correlation studies: Assay results were compared with those of other well-defined methodologies, as follows:

1. Samples (n = 83) covering the range of the method were assayed by the E. gracilis method at St. Bartholomew's Hospital and by the present method. The correlation coefficient (r) was 0.98, with a slope of 1.00 and a y-intercept of 30 (S_y/x = 49).

2. Samples (n = 39) in the low and normal range were assayed by the E. gracilis method, the analog-blocked intrinsic factor (IF) method, and the non-boiling method. The results are summarized as follows:

   Non-boiling method = 0.70(IF method) + 70; r = 0.95
   E. gracilis method = 0.79(IF method) + 44; r = 0.99

3. Two methods known to produce higher results than the microbiological assay were also tested. One is a gastric R protein radioassay, and the other is a chicken serum radioassay with incubation at pH 9.3, described by Green et al. (14). We analyzed 28 samples by both the non-boiling assay and the R protein assay, and 38 samples by both the non-boiling assay and method of Green et al. The results are summarized as follows:

   R protein method = 1.13(non boiling method) + 84; r = 0.91
   Method of Green et al. = 1.40(non-boiling method) + 129; r = 0.94

Binding Characteristics  

Affinity constant: The affinity constant for the binding of cobalamin to the solid phase/chicken serum under the conditions of the assay was estimated from a Scatchard plot (15) constructed from a standard curve (Figure 2). The negative slope of the line gives an affinity constant (K_a) of 1.7 X 10^10 L/mol.

Specificity: Recent publications (8, 20) have presented evidence that human serum contains several unidentified
Table 4. Specificity of the Non-Bolling Assay for True Cobalamin in Human Serum*  

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Specific cobalamin content (ng/fraction)</th>
<th>Non-specific cobalamin content (ng/fraction)</th>
<th>E. gracilis assay</th>
<th>Non- bolling assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>11.6 (3.2)</td>
<td>41.4 (11.0)</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>15</td>
<td>446.4 (37.1)</td>
<td>537.6 (67.0)</td>
<td>460</td>
<td>445</td>
</tr>
<tr>
<td>17</td>
<td>29.4 (8.4)</td>
<td>93.8 (29.9)</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>20</td>
<td>8.3 (2.8)</td>
<td>41.4 (13.0)</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>26</td>
<td>0.3 (0.8)</td>
<td>7.2 (5.9)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Data in columns 2 and 3 are compiled from an International Patent Application (20). Data in columns 4 and 5 are from R. H. Allen (personal communication).

cobalamin analogs that can mask cobalamin deficiency if measured by some radioassay systems. Although these analogs have not been identified, Kolhouse et al. (8) have achieved some separation by paper chromatography. To assess the cross-reactivity of these cobalamin analogs, Allen (20) used the present method to assay fractions from his paper chromatogram that had been assayed previously by methods specific for cobalamin and by methods that also measure the analogs. The fractions chosen for assay were those in which there was a marked discrepancy in apparent cobalamin content by the specific and nonspecific assays. The results for four methods are shown in Table 4.

Discussion

Assays for cobalamin in serum have invariably required pretreatment to release the vitamin from its transport proteins. This most commonly achieved by heating a diluted sample at 100 °C for 15–30 min. This is time consuming, inconvenient, and can lead to a loss of precision, indicating the need for a simpler procedure for releasing the vitamin. Use of high pH to dissociate cobalamin from human transcobalamin was demonstrated by Graebeck et al. (10), who observed complete dissociation at pH 12.9 in the presence of cyanide. Studies in this laboratory confirmed their observation that after a temporary increase of pH to 12.9, human serum regains a significant portion of its cobalamin-binding capacity on return to neutral pH. We, therefore, decided to investigate whether other cobalamin-binding proteins could still operate at very alkaline pH.

Kidroni and Grossowicz (11) had previously reported that chicken serum maintains its ability to bind cobalamin at very high pH, and that the binding is dependent on ionic strength (16). The present data confirm these observations and extend them to higher ionic strength and pH. Under the conditions of the assay, variations in the ionic strength of patient's samples were negligible.

Preliminary investigations of a non-boiling assay involving chicken serum at pH 12.9 were carried out using a liquid-phase binder and coated charcoal separation. Feasibility was demonstrated, but batch-to-batch variations in the efficiency of charcoal preparations to adsorb cobalamin, as well as a significant effect of pH on the separation of bound and free cobalamin, led to the choice of a solid-phase system.

The advantages of a magnetizable solid-phase separation system have been outlined before (12) and include low nonspecific binding (<2% in this assay), avoidance of the need for centrifugation, and absence of time-dependence in the separation step. The only substantial disadvantage is a loss of binding affinity, with that for the solid-phase chicken serum (1.7 × 10^12 L/mol) being lower than the figure of 1.1 × 10^12 L/mol reported by Newmark et al. (16). This may be partly due to the solid-phase technology, and partly to the higher pH of incubation. The affinity constant with the solid-phase system is similar to the value of 1.5 × 10^10 L/mol reported for purified porcine intrinsic factor (17) and is sufficiently high to enable a precise and sensitive assay.

Potassium cyanide is used in virtually all radioassays for cobalamin to convert the circulating forms of cobalamin (hydroxo-, methyl-, and adenosylcobalamin) to the more stable cyanocobalamin form (18). It also inhibits nonspecific binding of hydroxocobalamin to the amino groups of serum proteins (19). The amounts used have varied over the wide range of 10 to 400 µg per assay tube and we found that a small amount (10 µg per assay tube) suffices to produce reliable values for serum samples; a 50% variation in the amount of cyanide causes no significant changes in the assay (Table 1). Omission of cyanide resulted in significantly lower values, indicating that some is required.

It has recently been reported that human serum contains "cobalamin analogues" that interfere in some radioassays for cobalamin (8,20). The existence of these analogs provides a plausible explanation for the observed differences between some radioassays and the reference microbiological assays (14,21). It also provides an explanation for the failure of some radioassays to diagnose cobalamin-deficient patients (7). These analogs have not yet been identified, but can be partly separated from each other and from true cobalamin by paper chromatography (8,20). They appear to be different from the known cobamides produced by bacteria (22).

To help determine whether the present assay, like those using pure intrinsic factor, is specific for cobalamin or gives a measure of "total corins" as do R protein assays, we sent reagents to Dr. Allen. Fractions of an extract of normal human serum eluted from his paper chromatogram were assayed for apparent cobalamin content by the present method, intrinsic factor radioassays, nonspecific R protein assays, and the E. gracilis microbiological assay. The results (Table 4), demonstrate that the solid-phase chicken serum incubated at pH 12.9 shows a specificity for true cobalamin in human serum resembling that of intrinsic factor.

Comparison of the normal ranges found with a radioassay involving a purified porcine intrinsic factor and by the present technique is also indicative of the latter's specificity: 187 to 715 ng/L (8) and 187 to 696 ng/L, respectively.

Results with the non-boiling assay correlated more closely with the E. gracilis method than with either a gastric R protein radioassay or the chicken serum radioassay of Green et al. (14). The observed slopes and intercepts further indicate the existence of the analogs described by Kolhouse et al. (8), which are measured to a much smaller extent in the non-boiling radioassay and the E. gracilis assay than in the other two. The correlation of the non-boiling and E. gracilis assays against the analog-blocked intrinsic factor assay shows that although the slopes and intercepts of the regression lines varied slightly from the ideal, results from the three methods are similar.

The precision and sensitivity of the present method compare well with other methods available for the measurement
of cobalamin in human serum. Tests of "accuracy" as described here (Table 3) and in most radioassay systems do not measure accuracy in the statistical sense of the word. The purpose of such tests is to determine if there is a systematic bias owing to differences in behavior between the standards and patients' samples. Such a bias has been observed in radioassays based on intrinsic factor and in microbiological assays for cobalamin when serum proteins are not included in the aqueous standards (3,6). No such effects were observed in the present assay.

We find this assay to be simple, rapid, precise, sensitive, and specific for true cobalamin in human serum. The magnetizable solid-phase separation system, combined with the lack of a pre-treatment of the sample, opens the possibility of a fully automated continuous-flow radioassay system. A full clinical trial is required to confirm the diagnostic potential suggested by this data.

We thank Professor David L. Mollin for his helpful advice, Dr. Nefertiti Sourial for allowing us to use her data on the method of Green et al. (14), and Dr. Robert H. Allen for the specificity studies. We acknowledge Dr. Adrienne R. McGregor for her work in the preliminary studies.

References