Radioimmunoassay that measures serum vitamin B12

J J O’Sullivan, R J Leeming, S S Lynch, A Pollock

Abstract

Aims: To develop a specific radioimmunoassay for the routine determination of serum vitamin B12.

Methods: Antisera were raised in rabbits by immunisation with the monocarboxylic acid derivative of cyano-cobalamin coupled to human serum albumin. Antibody titres and affinities were determined and the antisera giving the highest binding affinity constant, $K_a$, was used to develop the assay protocol. Donkey-anti-rabbit gamma globulin-coated magnetisable particles were used to separate the bound from free vitamin B12. The considerable cobalamin binding capacity of human serum was destroyed by autoclaving in acetate-cyanide buffer. Sixty samples were assayed by the radioimmunoassay (RIA) and the Lactobacillus leichmannii assay. Recovery and cross-reactivity experiments were performed.

Results: Final rabbit antibody titres varied from 1/20 000 to 1/188 000. Scatchard plots did not correlate with the antibody titres. The $K_a$ values varied from 2.6 to $6.7 \times 10^{18}$ litres/mol. For maximum sensitivity the highest $K_a$ (titre 1/66 000) was chosen. A tracer concentration of 22 pmol/l, an antisera dilution of 1/100 000, and a sample volume of 0.1 ml were used. At an antisera dilution of 1 in 100 000 the cyano-cobalamin binding of the rabbit serum was diluted out. The assay showed excellent correlation with the microbiological assay, with 100% recovery of added vitamin B12. Levels of cross-reactivity for dicyanide cobamide and hydroxocobalamin were 9.8 and 8.1%, respectively.

Conclusions: The development of this immunoassay permits the measurement of serum vitamin B12 without important interference from cobalamin analogues, related corrinoids, and non-specific binders.

The first in vitro measurements of vitamin B12 in serum were by microbiological assays, developed in the 1950s and based on the requirements of certain organisms for corrinoids as growth factors. Although sensitive, cheap, and simple in concept, accurate and reproducible results require care and experience. The disadvantages include long incubation times, possible microbial contamination, and suppression of growth by antibiotics and cytotoxic drugs. Over the past 25 years microbiological assays have been replaced in most laboratories by radioisotope dilution assays (RIDA), using intrinsic factor (IF) and R-protein as binders. In the 1970s RIDAs achieved popularity because of claims that they were technically simpler, more accurate and reproducible than microbiological assays, free from microbial and iatrogenic interference, and commercially available as kits. Several vitamin B12 binders and separation systems have been used.

Comparisons of RIDAs with microbiological assays have shown discrepancies attributable to biologically inactive cobalamin analogues. High performance liquid chromatography provides a rapid and reproducible means of characterising physiological cobalamins but is not sufficiently sensitive to detect the concentrations found in human serum.

The availability of an immunoassay specific for vitamin B12 and free from interference by cobalamin analogues, related corrinoids, or non-specific binders would be of value for the routine measurement of serum vitamin B12. Although radioimmunoassay methods have been developed, they have not been widely used due to difficulties in preparing appropriate antibodies. We present here details of an immunoassay for the determination of serum vitamin B12 concentrations using antibodies raised in rabbits against the monocarboxylic acid derivative of cyano-cobalamin conjugated to human serum albumin.

Methods

Cyanocobalamin (crystalline, pure; Sigma) was subjected to mild acid hydrolysis with 0.4 M hydrochloric acid (BDH) and the monocarboxylic acid derivative purified on a column (2.5 x 56 cm) of QAE-Sephadex A-25 (Pharmacia LKB). The vitamin B12-human serum albumin (HSA, purified, lophilised; Behringwerke AG) conjugate was prepared by a modification of the method of Ahrenstedt and Thorell. The addition of the spacer molecule into the conjugate was omitted and TRIS buffer (AnalaR, BDH) was used instead of pyridine, followed by desalting on Sephadex G-15 (Pharmacia). The conjugate was then lyophilised, redissolved in deionised water to 1.5 mg/ml and stored at $-70^\circ$C. Vitamin B12-HSA conjugate, 1.5 mg in 1.0 ml of deionised water,
Radioimmunoassay for measurement of serum vitamin B12 was emulsified with 1·0 ml Freund's Complete Adjuvant and injected intradermally, at multiple sites, on the backs of five adult rabbits. Blood samples were collected monthly from the marginal ear vein and the antibody titres were determined. Injections were at monthly intervals over four months.

Four bovine serum albumin (BSA) preparations were evaluated for use in the assay diluent: (i) 98–99% BSA; (ii) RIA garde BSA; (iii) vitamin B12 and B12 binding factor deficient BSA (all from Sigma); and (iv) human plasma protein fraction (Blood Products Laboratory). Diluents contained 0·5% w/v BSA, with and without 0·2% v/v Tween-20.

Serial dilutions of antiserum were made in 0·05 M phosphate buffered saline (PBS) (pH 7·0) containing 0·5% BSA (98–99% albumin; Sigma) and 0·1% sodium azide. Each antiserum dilution (0·35 ml) was pipetted in duplicate into polystyrene assay tubes (75 x 12 mm Sarstedt) with 0·05 ml PBS and 0·1 ml 44 pmol/l cyanocobalamin (Co57) tracer, high specific activity, 370 KBq/ml, Amersham International Ltd.) and incubated at 20°C for 16 hours. Magneticisable particles (0·5 ml, 2 g/l) frequently resuspended and intermittently mixed to avoid settling out, was added to all tubes, except the "total activity" tubes. After mixing thoroughly the tubes were left standing, undisturbed, at 20°C for 30 minutes, then placed over a Magnetic Separator (Serono Diagnostics Ltd.) for five minutes. While standing on the magnets the supernatants were aspirated to waste using a Venturi water-jet pump. The pellets of magneticisable particles were resuspended in saline (1·5 ml) and the tubes were again left to stand on the magnets for five minutes. The supernatants were aspirated to waste and the tracer bound to the magneticisable particles was measured using an NE 1600 multiple well-type gamma counter. Included in each assay were two "total activity" tubes, containing only tracer and two "blank" or non-specific binding tubes, containing no antiserum. The titre of each antiserum was expressed as the antiserum dilution which bound 50% of the cyanocobalamin tracer added.

The rabbit antiserum were diluted in PBS as follows: R1 = 1/20 000; R2 = 1/95 000; R3 = 1/12 500; R4 = 1/30 000; and R5 = 1/17 500. Antiserum (0·35 ml) was pipetted into each tube. Cyanocobalamin was diluted in PBS to give final concentrations of 700, 350, 175, 88 and 44 pmol/l and 0·1 ml was pipetted into tubes to which PBS (0·05 ml) was added and mixed. After incubation at 20°C for 16 hours and separation as above Scatchard plots were prepared by plotting the bound/free ratio against total concentration of bound tracer. The slope gave the antibody affinity, Ka, litres/mols.

Rabbit 4 antiserum (0·35 ml) diluted 1/100, 1/1000, 1/5000, 1/10 000, 1/50 000, 1/100 000 and 1/500 000 in PBS was pipetted into tubes. Cyanocobalamin was diluted in PBS to give concentrations of 176, 88, 44, 22 and 11 pmol/l. Tracer (0·1 ml) and PBS (0·05 ml) were added. The tubes were mixed and incubated at 20°C for 16 hours before separation and antiserum dilution curves drawn.

Cyanocobalamin solution (Cytemen injection BP, 1000 mg/l; Glaxo) was diluted to give concentrations of 0, 15, 37, 74, 185, 369, 553 and 738 pmol/l and 0·1 ml pipetted into tubes followed by R4 antiserum (0·3 ml, 1/100 000). The tubes were mixed and incubated at 20°C in the dark for three hours. Then cyanocobalamin (Co57) tracer (0·1 ml, 22 pmol/l) was added. After mixing, the tubes were incubated at 20°C in the dark for 16 hours before separation. The percentage tracer bound was calculated as B/Bo, where B is the percentage tracer bound and Bo is the percentage of tracer bound by the zero standard.

Normal human serum and non-immune rabbit serum were diluted 1/10, 1/100, 1/1000 and 1/10 000 in PBS. Each dilution (0·35 ml), cyanocobalamin (0·1 ml, 22 pmol/l), and PBS (0·05 ml) were pipetted into tubes which were incubated at 20°C for 16 hours then separated with BSA-coated charcoal in PBS. To release vitamin B12 and to destroy vitamin B12 binders sera were diluted 1 in 3 for RIA and 1 in 10 for microbiological assay (Lactobacillus leichmannii) with 0·02 M acetate-cyanide buffer, pH 4·5. The tubes were sealed and autoclaved at 121°C for five minutes. After cooling and centrifuging at 1750 x g for 10 minutes supernatants (0·1 ml) were assayed.

For recovery of exogenous vitamin B12, cyanocobalamin (74, 148, 295 and 590 pmol/l) was added to human serum prior to the extraction procedure and the recovery determined by RIA.

Standard curves were prepared with cyanocobalamin, dicyanide cobinamide, and hydroxocobalamin and the cross-reactivity of each for the antibody binding sites was determined.

**Results**

**ASSESSMENT OF BSA PREPARATIONS USED IN ASSAY DILUENT**

Certain commercially available BSA preparations bound unacceptably high concentrations of vitamin B12 (table 1). Tween-20 is used in several assay diluents. We found Tween-20 in PBS bound cyanocobalamin (table 1). Competition between Tween-20 and antiserum decreased assay sensitivity and was omitted.

**ANTISERUM TITRATION AND AFFINITY CONSTANT DETERMINATION**

R2 produced the best response to the conjugate, giving a final antibody titre of 1/188 000 (table 2) and R3 the lowest, 1/20 000. Antibody affinities ranged from 6·7 x 1010 l/mol for R4 to 2·6 x 1010 l/mol for R3 and R5 (table 2), the affinities and titres not being in the same order. R4 with the highest binding affinity for cyanocobalamin was used for the assay of serum vitamin B12. The vitamin B12 conjugate antibody titres (table 2) were lower than those

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*The gamma globulin fraction of the serum of donkeys immunised against rabbit gamma globulins was coupled to particles of iron oxide (magnetic, precipitated) that had been coated with cellulose. The particles were made from sodium hydroxypoly(methyl cellulose viscosity of 2% solution 40000P; Sigma) at a concentration of 5 g/l.*
Table 1  Assessment of serum albumin preparations for their capacity to bind cyanocobalamin (Co⁵⁷)

<table>
<thead>
<tr>
<th>PBS buffer</th>
<th>Percentage non-specific binding With Tween-20</th>
<th>Without Tween-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>No albumin</td>
<td>48-2</td>
<td>4</td>
</tr>
<tr>
<td>BSA (i)</td>
<td>52-3</td>
<td>3-0</td>
</tr>
<tr>
<td>BSA (ii)</td>
<td>55-6</td>
<td>52-0</td>
</tr>
<tr>
<td>BSA (iii)</td>
<td>38-7</td>
<td>10-7</td>
</tr>
<tr>
<td>HPPF (iv)</td>
<td>47-9</td>
<td>3-2</td>
</tr>
</tbody>
</table>

(i) 98-99% bovine serum albumin.
(ii) RIA grade bovine serum albumin.
(iii) vitamin B12 and B12 binding factor deficient bovine serum albumin.
(iv) human plasma protein fraction.

Table 2  Antibody binding affinity constants and antibody titres for each of five rabbits

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antibody affinity constant (Kₐ) L/N × 10⁶</th>
<th>Antibody titre per ml × 10⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 1</td>
<td>4-2</td>
<td>45 000</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>4-5</td>
<td>188 000</td>
</tr>
<tr>
<td>Rabbit 3</td>
<td>2-6</td>
<td>20 000</td>
</tr>
<tr>
<td>Rabbit 4</td>
<td>6-7</td>
<td>16 000</td>
</tr>
<tr>
<td>Rabbit 5</td>
<td>2-6</td>
<td>27 000</td>
</tr>
</tbody>
</table>

Sixty sera were assayed by RIA and the L. leichmannii microbiological assay. The correlation coefficient was 0-85 (fig 2). The intrabatch coefficient of variation (CV) of three aliquots of the same serum varied from 12-4 to 20-6%. The interbatch CV was below 22% with the exception of one serum of very low vitamin B12 content, with a range of 16-49 pmol/l, where the CV was 58-2% (table 4).

Table 5 details the results of recovery of exogenous vitamin B12.

The dicyanide cobinamide and hydroxocobalamin curves were not parallel to the cyanocobalamin curve. The cross-reactivity calculated at 80% Bo gave 9-8 and 8-1% for dicyanide cobinamide and hydroxocobalamin, respectively.

Discussion
Serum vitamin B12 concentrations are currently determined either by RIDAs or microbiological assay. Results obtained using the first generation RIDAs led Kolhouse et al²³ and Cooper and Whitehead²⁴ to suggest that the non-purified vitamin B12 binding proteins used bound cobalamin analogues which resulted in normal vitamin B12 concentrations being reported for patients with clinical signs of vitamin B12 deficiency. Now, third generation RIDAs, utilising “purified intrinsic factor” or “blocked R-binders,” are being produced. Although several RIA methods for the measurement of vitamin B12 have been reported, none is in routine use.

Of the three BSA preparations evaluated, the least expensive gave the best results; the product description was misleading for our
Radioimmunoassay for measurement of serum vitamin B12

Table 4  Intra and Interbatch precision of serum vitamin B12 assay

<table>
<thead>
<tr>
<th>Number</th>
<th>RIA range (pmol/l)</th>
<th>Mean (pmol/l)</th>
<th>CV (%)</th>
<th>Bioassay value (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraibatch:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 1</td>
<td>93–142</td>
<td>120</td>
<td>20.6</td>
<td>148</td>
</tr>
<tr>
<td>Serum 2</td>
<td>111–142</td>
<td>128</td>
<td>12.4</td>
<td>96</td>
</tr>
<tr>
<td>Serum 3</td>
<td>310–465</td>
<td>384</td>
<td>20.2</td>
<td>487</td>
</tr>
<tr>
<td>Interbatch:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 3</td>
<td>16–49</td>
<td>29</td>
<td>58.2</td>
<td>66</td>
</tr>
<tr>
<td>Serum 2</td>
<td>89–137</td>
<td>112</td>
<td>21.7</td>
<td>74</td>
</tr>
<tr>
<td>Serum 1</td>
<td>129–199</td>
<td>166</td>
<td>21.3</td>
<td>99</td>
</tr>
<tr>
<td>Serum 4</td>
<td>177–229</td>
<td>207</td>
<td>12.8</td>
<td>298</td>
</tr>
<tr>
<td>Serum 5</td>
<td>192–288</td>
<td>249</td>
<td>20.2</td>
<td>162</td>
</tr>
<tr>
<td>Serum 6</td>
<td>288–325</td>
<td>311</td>
<td>6.5</td>
<td>369</td>
</tr>
<tr>
<td>Serum 7</td>
<td>317–421</td>
<td>356</td>
<td>15.7</td>
<td>502</td>
</tr>
<tr>
<td>Serum 8</td>
<td>325–487</td>
<td>406</td>
<td>20.0</td>
<td>&gt;922</td>
</tr>
<tr>
<td>Serum 9</td>
<td>413–593</td>
<td>485</td>
<td>14.5</td>
<td>&gt;922</td>
</tr>
<tr>
<td>Serum 10</td>
<td>775–1151</td>
<td>922</td>
<td>20.6</td>
<td>&gt;922</td>
</tr>
</tbody>
</table>

Table 5  Recovery of exogenous vitamin B12 from serum

<table>
<thead>
<tr>
<th>Cyanocobalamin added (pmol/l)</th>
<th>RIA B12 (pmol/l)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>251</td>
<td>100</td>
</tr>
<tr>
<td>74</td>
<td>132</td>
<td>100</td>
</tr>
<tr>
<td>148</td>
<td>413</td>
<td>109</td>
</tr>
<tr>
<td>295</td>
<td>561</td>
<td>105</td>
</tr>
<tr>
<td>590</td>
<td>856</td>
<td>103</td>
</tr>
</tbody>
</table>

NSB = 2.1%.

purposes. Sensitivity, intrabatch, and interbatch CVs in the RIA compared well with those obtained using the L leichmannii assay. The low levels of cross-reactivity indicate that the antibody binding sites recognise the differences in the conformation and electrostatic charges of these cobalamin analogs. Other cobalamin and structurally related molecules have yet to be assessed for cross-reactivity, although all the cobalamin in serum should be converted to cyanocobalamin during the acetate-cyanoide serum treatment. The RIA described can be routinely used for the measurement of vitamin B12 concentrations in serum and lends itself readily to automation, using robotic sampling techniques. Large numbers of assays can be performed per millilitre of antisera and the assay is much cheaper than commercial kits. There is potential for the development of a non-isotopic immunoassay, alleviating the problem of handling radioactive materials and for the production of monoclonal antibodies.

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11 Powell DEB, Thomas JH, Mandal AR, Dignam CT. Effect of drugs on vitamin B12 levels obtained using the Lactobacillus leichmannii method.
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