Radioimmunoassay that measures serum vitamin B12

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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 cyanocobalamin coupled to human serum albumin. Antibody titres and affinities were determined and the antiserum 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 6.7×10^{10} litres/mol. For maximum sensitivity the highest K_a (titre 1/66 000) was chosen. A tracer concentration of 22 pmol/l, an antiserum dilution of 1/100 000, and a sample volume of 0.1 ml were used. At an antiserum dilution of 1 in 100 000 the cyanocobalamin 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 cobinamide and hydroxycobalamin 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.¹⁻¹⁰ 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.¹¹ 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.¹²⁻²¹

Comparisons of RIDAs with microbiological assays have shown discrepancies attributable to biologically inactive cobalamin analogues.²²⁻²⁴ 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.^{25 26}

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 methods²⁷⁻³¹ 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 cyanocobalamin 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 (2.5×56 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.³² 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°C . Vitamin B12-HSA conjugate, 1.5 mg in 1.0 ml of deionised water,

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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 grade 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 × 12 mm Sarstedt) with 0.05 ml PBS and 0.1 ml 44 pmol/l cyano (Co^{57}) cobalamin (CT2, high specific activity, 370 KBq/ml; Amersham International Ltd.) and incubated at 20°C for 16 hours. Magnetisable particles (0.5 ml, 2 g/l),* freshly 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 magnetisable 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 magnetisable 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 cyano (Co^{57}) cobalamin tracer added.

The rabbit antisera 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. Cyano(Co^{57})cobalamin 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, K_a , litres/mols.

Rabbit 4 antiserum (0.35 ml) diluted 1/100, 1/1000, 1/5000, 1/10 000, 1/50 000, 1/100 000

*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. These particles were stored at 4°C, at about 50 g/l. For use in the assay, they were diluted to about 2 g/l in saline containing hydroxypropylmethyl cellulose (viscosity of 2% solution 4000cP; Sigma) at a concentration of 3 g/l.

and 1/500 000 in PBS was pipetted into tubes. Cyano(Co^{57})cobalamin 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 (Cytamen 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 cyano(Co^{57})cobalamin (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), cyano(Co^{57})cobalamin (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 $1750 \times g$ 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 hydroxycobalamin 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 cyano(Co^{57})cobalamin (table 1). Competition between Tween-20 and antisera 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 6.7×10^{10} l/mol for R4 to 2.6×10^{10} 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

Table 1 Assessment of serum albumin preparations for their capacity to bind cyano(Co^{57})cobalamin

PBS buffer	Percentage non-specific binding	
	With Tween-20	Without Tween-20
No albumin	48.2	0.4
BSA (i)	52.3	3.0
BSA (ii)	55.6	52.0
BSA (iii)	38.7	10.7
HPPF (iv)	47.9	3.2

- (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

Antiserum	Antibody affinity constant (K_a) $L/N \times 10^{10}$	Antibody titre per ml $\times 10^{-3}$
Rabbit 1	4.2	45 000
Rabbit 2	4.5	188 000
Rabbit 3	2.6	20 000
Rabbit 4	6.7	66 000
Rabbit 5	2.6	27 000

of Ahrenstedt and Thorell,³² but higher than those of van de Wiel *et al.*²⁸ The antibody binding affinity constants determined by Scatchard analyses showed that antibody affinities did not correlate with antibody titres. R4 antiserum did not have the highest antibody titre but it had the greatest binding affinity for cyanocobalamin (table 2).

R4 antiserum (1/100 000) and a tracer concentration of 22 pmol/l giving a B_0 value of 43% were used in the development of the assay protocol.

A typical standard curve is presented in fig 1. The results of the endogenous vitamin B12 binding capacity of normal human serum and non-immune rabbit serum (table 3) show that the B12 binding capacity of the human serum and the non-immune rabbit serum were no longer evident at dilutions of 1/1000 and 1/10 000, respectively. The non-specific binding value was 1.4%. As the R4 antiserum was diluted 1 in 100 000 for the assay, no interference from endogenous B12 binding proteins could occur. Human sera (vitamin B12 normal range 111–738 pmol/l) cannot be diluted 1 in 1000 because the concentration of vitamin B12 present in normal serum samples would be below 15 pmol/l, the limit of sensitivity (fig 1). Therefore, the endogenous vitamin B12 binding proteins have to be removed before assay.

Figure 1 Standard curve: percentage B/ B_0 against cyanocobalamin concentration (pmol/l). Abscissa: Cyanocobalamin concentration (pmol/l); antiserum dilution 1/100 000; tracer concentration 22 pmol/l; NSB = 3.0%; B_0 = 42.7%. Ordinate: % B/ B_0 .

Table 3 Determination of endogenous vitamin B12 binding capacity of normal human serum and non-immune rabbit serum dilutions prepared in buffer using no Tween-20

Dilution	Percentage cyano(Co^{57})cobalamin bound	
	Normal human serum	Non-immune rabbit serum
1/10	46.4	47.9
1/100	6.4	46.8
1/1000	1.8	16.5
1/10 000	1.4	2.7

NSB = 1.4%.

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% B_0 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 al.*²³ 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

Figure 2 Correlation of vitamin B12 concentrations assayed by RIA and *L. leichmannii*. Abscissa: B12 bioassay (pmol/l). Ordinate: B12 RIA (pmol/l).

Table 4 Intra and Interbatch precision of serum vitamin B12 assay

	Number	RIA range (pmol/l)	Mean (pmol/l)	CV (%)	Bioassay value (pmol/l)
Intrabatch:					
Serum 1	3	93–142	120	20.6	148
Serum 2	3	111–142	128	12.4	96
Serum 3	3	310–465	384	20.2	487
Interbatch:					
Serum 1	3	16–49	29	58.2	66
2	3	89–137	112	21.7	74
3	3	129–199	168	21.3	199
4	3	177–229	207	12.8	288
5	3	192–288	249	20.2	162
6	3	288–325	311	6.5	369
7	3	317–421	356	15.7	502
8	3	325–487	406	20.0	>922
9	3	413–553	485	14.5	>922
10	3	775–1151	922	20.6	>922

Table 5 Recovery of exogenous vitamin B12 from serum

Cyanocobalamin added (pmol/l)	RIA B12 (pmol/l)	% Recovery
0	251	
74	325	100
148	413	109
295	561	105
590	856	103

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 cobalamins. Other cobalamins and structurally related molecules have yet to be assessed for cross-reactivity, although all the cobalamins in serum should be converted to cyanocobalamin during the acetate-cyanide 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 antiserum 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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