

Microbiological assay for vitamin B₁₂ performed in 96-well microtitre plates

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Abstract

A simplified microbiological assay for vitamin B₁₂ estimation, completed on microtitre plates using a colistin sulphate resistant strain of *Lactobacillus leichmannii* (NCIB 12519), and cryopreserved cultures is described. The new assay correlated well with a more conventional "tube" assay and was not influenced by the presence of antibiotics in serum. Evaluation of assay performance showed excellent interassay and intra-assay precision with quantitative recovery of added cyanocobalamin over a wide range of additions (94.9%–102.1%). The advantages of short incubation time, easy reading, and minimal reagent costs make this assay an attractive option in the routine clinical laboratory and in research.

The clinical value of the serum cobalamin concentration as a highly sensitive indicator of deficiency was originally established in the 1950s using microbiological assay with either *Euglena gracilis*¹ or *Lactobacillus leichmannii*^{2,3} as the test organism. Traditional vitamin B₁₂ microbiological assays, however, are cumbersome and time-consuming, demanding specialised technical input. Another disadvantage of these assays is that the presence of antibiotics in patient sera can inhibit the growth of the assay organism giving falsely low results.⁴ Most clinical laboratories therefore use competitive protein binding assays for the routine estimation of serum vitamin B₁₂. These assays are commercially available in kit form.

In 1987 the introduction of a colistin sulfate resistant strain of *L. leichmannii* meant that assays could be completed openly without aseptic precautions⁵ and the subsequent development of a test organism cryopreservation technique further shortened the assay protocol and improved overall assay performance.⁶ We describe here a simple microbiological assay for vitamin B₁₂ which takes advantage of these recent developments and is performed in 96-well microtitre plates. The assay also exploits the fact that antibiotic interference can virtually be eliminated by treating affected samples with an inactivating enzyme.⁷

Methods

L. leichmannii (NCIB 12519, ATCC 43787) was obtained from Torry Research Station, Aberdeen, Scotland. Vitamin B₁₂ (*Lactobacillus*)

assay broth base was obtained from E Merck, Darmstadt, Germany. Flat-bottomed 96-well microtitre plates (400 µl/well capacity) were from A/S Nunc, Roskilde, Denmark, and mylar plate-sealers from Dynatech Laboratories, Chantilly, Virginia, USA. Colistin sulphate was supplied by Pharmax Ltd, Bexley, Kent, and colistimethate sodium was from Warner Lambert, Ann Arbor, Michigan, USA. Beta lactamase (EC 3.5.2.6) ex *Bacillus cereus* was from Koch Light Ltd, Suffolk, and polypropylene tubes (4.5 ml) for serum extraction, and microtubes (2 ml) for storage of organism at -70°C, were from Sarstedt, Wexford, Ireland. Cyanocobalamin, of verified concentration, was from Duncan Flockhart, Middlesex. Tween-80 was from Sigma Chemicals. Additions of serum extract and buffer were made using a Stepper automatic pipette from Socorex, Renens, Switzerland. Inoculated assay medium was dispensed using an eight channel Titertek dispenser, and plates were read on a Titertek Multiskan Plus Mark 2 plate reader, both from Flow Laboratories, Ayrshire, Scotland.

Results were calculated either manually or using a data reduction programme (Softmax, Molecular Devices Inc). All other chemicals used were of reagent grade.

SERUM EXTRACTION

The procedure for extracting vitamin B₁₂ from serum was as described previously.⁵ Briefly, serum was diluted 1 in 10 with a buffer containing sodium hydroxide (8.3 mmol/l), acetic acid (20.7 mmol/l), and sodium cyanide (0.45 mmol/l), pH 4.5. After mixing, autoclaving (115°C/10 minutes), and centrifugation (1000 × g/10 minutes) the supernatant extracts were decanted. As a reduced volume of extract supernatant (300 µl) is needed to complete the microtitre plate assay it was necessary to determine if extraction by autoclaving of a lesser volume of dilute serum in a smaller tube would give a similar recovery of vitamin B₁₂. A comparison of the results obtained on 98 sera using a small extraction volume (serum:buffer 200 µl:1800 µl) with the larger extraction volume (serum:buffer 0.5ml:4.5 ml) gave virtually identical results (*t*-test; *p* < 0.001).

PREPARATION OF CRYOPRESERVED ORGANISM

Culture broth was prepared by dissolving 83 g/l vitamin B₁₂ assay broth base, 2.0 ml/l Tween 80, and 250 ng/l cyanocobalamin in water. After boiling and cooling, 500 mg/l colistin sulphate (or 2 g/l colistimethate sodium) was added. Broth was dispensed in 20 ml aliquots into glass screw-capped containers and stored

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Accepted for publication
7 February 1991

at -20°C . A culture of *L. leichmannii* was started by resuspension of freeze dried organism (or cryopreserved culture) in one of these broths and incubation overnight (37°C). This growth was subcultured for two successive days by adding $10\ \mu\text{l}$ to a second broth ($20\ \text{ml}$) each morning and incubating overnight. This procedure was essential to ensure that the culture was well established. The percentage survival of viable organisms, and their subsequent response under assay conditions, depends on the growth phase at the time of freezing. Cells in the log phase give the best response.⁶ Cultures are prepared as follows. On the morning after the third overnight incubation $2\ \text{ml}$ of culture was added to each of two fresh broths. These broths were incubated in parallel, and turbidity in one (at $595\ \text{nm}$) was monitored hourly in a Beckman model 35 spectrophotometer (Beckman, High Wycombe, Buckinghamshire). When growth reached the log phase (after about eight hours) an equal volume of sterile glycerol ($800\ \text{ml/l}$) was then added to the other culture. It was mixed thoroughly and dispensed in $1\ \text{ml}$ aliquots into microtubes. These were placed in a polystyrene box with walls $1\ \text{cm}$ thick, frozen down, and stored at -70°C . These cultures are stable for many months.⁶

The organism may also be maintained by serial subculture and prepared as described previously.⁵

ASSAY PROCEDURE

Extracts of patient serum (see above) and control sera were dispensed ($2 \times 100\ \mu\text{l}$ and $2 \times 50\ \mu\text{l}$) into microtitre plate wells using a "Stepper" automatic pipette. Compensating volumes ($50\ \mu\text{l}$) of extraction buffer were added to the wells containing $50\ \mu\text{l}$ of extract. Thus each serum extract is assayed at two dilutions in duplicate. A standard curve was prepared in quadruplicate; it comprised a range of cyanocobalamin concentrations from $0-5\ \text{pg/well}$. An additional four wells were set aside at the zero point as an uninoculated blank. Compensating volumes of extraction buffer were added as appropriate to bring all well volumes to $100\ \mu\text{l}$. Assay medium was prepared by dissolving $6.2\ \text{g}$ of dried vitamin B₁₂ assay broth base and $150\ \mu\text{l}$ of Tween 80 in each $100\ \text{ml}$ of water. After the medium was boiled and cooled colistin sulphate ($11\ \text{mg}/100\ \text{ml}$) was added (or colistimethate sodium $44\ \text{mg}/100\ \text{ml}$). A phial of cryopreserved organism was rapidly thawed (37°C) and added to the cooled medium ($200\ \mu\text{l}/100\ \text{ml}$ of medium). After thorough mixing on a magnetic stirrer $200\ \mu\text{l}$ of inoculated medium was added to every well, using an eight-channel pipette. Blanks to zero the plate reader were created by adding $10\ \mu\text{l}$ of a 4% solution of Stericol disinfectant to the extra set of wells at the zero point of the standard curve. The addition of disinfectant had the effect of preventing organism growth without contributing to the optical density of the blank. Each plate was covered with a plate sealer, mixed by inversion, and incubated at 37°C . After 20 hours the contents were resuspended by inversion, the plate sealers removed, and each plate was read

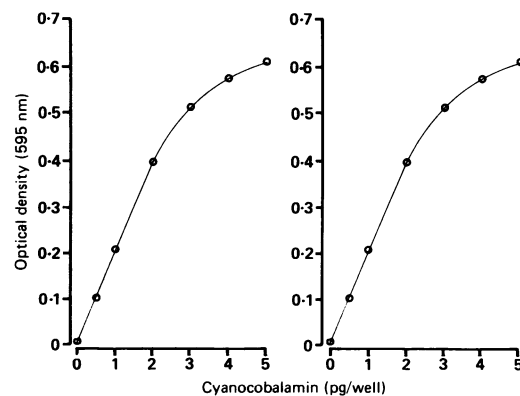


Figure 1 Response of *L. leichmannii* to cyanocobalamin when grown on microtitre plates.

at $595\ \text{nm}$ on the plate reader. A standard curve was constructed (fig 1) and the vitamin B₁₂ concentrations of the unknowns calculated.

COMPARISON WITH TUBE ASSAY

The assay as performed on microtitre plates was compared with a more traditional "tube" assay. The "tube" assay was based on that of Kelleher *et al*⁵ and incorporated the use of the cryopreserved organism.⁶ The results of 580 serum samples were compared.

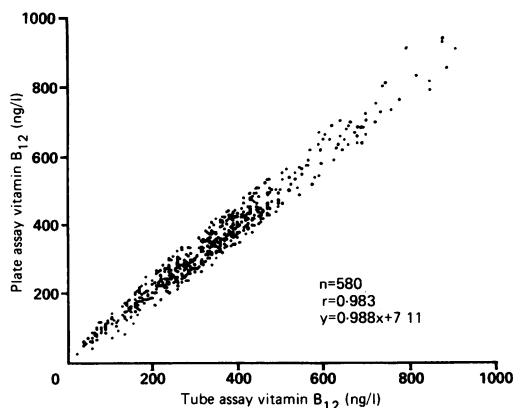
ASSAY PERFORMANCE

Control sera were used to determine the reproducibility of the new assay. These five sera were assayed 10 times in one assay and once in 10 different assays to determine within- and between-assay precision. The analytical recovery of added cyanocobalamin was also determined after additions of $100\ \text{ng/l}$, $200\ \text{ng/l}$, $300\ \text{ng/l}$, $400\ \text{ng/l}$ and $500\ \text{ng/l}$ of cyanocobalamin ($n = 10$ in each case) to a serum with a low vitamin B₁₂ concentration.

SAMPLES CONTAINING ANTIBIOTIC

An analysis of 3291 vitamin B₁₂ microbiological assays using the tube assay⁷ had shown that those antibiotics which caused interference were almost exclusively of the β -lactam type (penicillins or cephalosporins). The study showed that antibiotic interference could be virtually eliminated by pre-treating sera with a β -lactamase preparation. As the incidence of antibiotic interference in this plate assay was similar (9%) it was important to evaluate the effectiveness of such treatment. Sera containing interfering antibiotics were identified by the fact that either no growth occurred in the microtitre plate wells at all, or that the higher dilution of serum extract gave the higher result (because it contained less antibiotic). These sera were treated with β -lactamase. Each phial of enzyme ($700\ \text{units/phial}$) was reconstituted with $5\ \text{ml}$ of distilled water. Although stable for only seven days at 4°C , reconstituted enzyme may be stored in aliquots at -20°C for many weeks. The effectiveness of β -lactamase in treating samples affected by antibiotics was evaluated by measuring the recovery of a "spike" of cyanocobalamin added to each

Figure 2 Regression analysis of serum vitamin B₁₂ values obtained in a direct comparison of assays completed in glass tubes (13 × 100 mm) and in microtitre plates.



sample. Each antibiotic inhibited sample from the diagnostic service was reassayed in triplicate as previously described.⁷ One tube (A) contained only serum (200 μ l). Another tube (B) contained serum (200 μ l) and β -lactamase (20 μ l), and a third tube (C) contained serum (200 μ l), enzyme (20 μ l), and cyanocobalamin (20 μ l), equivalent to a "spike" of 100 ng/l. The samples were mixed and incubated at 37°C for 30 minutes, made up to 2 ml with buffer, and then extracted and assayed as normal. The assay result of tube A will confirm the antibiotic interference. If tube B gives the correct serum vitamin B₁₂ result then this is verified by the recovery of 100% of the cyanocobalamin "spike" added to tube C.

Results

A comparison of the results of 580 serum samples assayed by the "tube" assay and by the microtitre plate assay are expressed in fig 2. The correlation was highly significant ($r = 0.983$, $p < 0.001$); the line of best fit is given by the equation $Y = 0.99X + 7.1$.

The performance data for inter- and intra-assay variations are shown in table 1. The CV% values range from 1.2 to 3.5% for the interassay variation, and from 1.9 to 4.3% for the intra-assay series. Analytical recovery of cyanocobalamin using this assay was excellent (table 2), with recovery values ranging from 94.85%–102.1% of added cyanocobalamin. The validity of the serum vitamin B₁₂ results for the antibiotic-containing samples after treatment with β -lactamase was verified by determining the percentage recovery of a cyanocobalamin spike (tube C). The mean recovery from 84

Table 1 Reproducibility of control serum values as estimated using the microtitre plate assay

Mean (SD) (ng/l)	CV%
<i>Interassay:</i>	
106 (3.7)	3.5
152 (4.9)	3.2
243 (5.0)	2.1
283 (6.8)	2.4
379 (4.4)	1.2
<i>Intra-assay:</i>	
105 (4.5)	4.3
151 (4.1)	2.7
238 (5.2)	2.2
281 (6.7)	2.4
376 (7.0)	1.9

Table 2 Analytical recovery of added cyanocobalamin from serum

Added (ng/l)	Measured (ng/l)	Recovery %
100	102.1	102.1
200	189.7	94.85
300	295.7	98.65
400	402.5	100.63
500	484.5	96.9

samples was 100.18% (CV = 13.95%). The success of the enzyme treatment prompted another modification of the assay technique. Because β -lactamase has no effect on normal serum,⁷ all sera are now pretreated with the enzyme, incubated for 30 minutes at 37°C, and extracted as normal. This avoids the delay occasioned by the repeat assay of affected samples. Of 2000 samples assayed using this modification, none has shown evidence of suppressed organism growth.

Discussion

This simple plate assay for vitamin B₁₂ makes use of some recent developments in vitamin B₁₂ microbiological assay procedure⁵⁻⁷ and takes advantage of advances in microtitre plate reader technology. Inoculation with a colistin sulphate resistant organism allows the assay to be completed openly on the general laboratory bench without aseptic precautions. The use of cryopreserved cultures eliminates serial subculturing and has been shown to add consistency to between assay precision.⁶ The considerable time saving with this assay is primarily due to the speed of absorbance reading offered by modern automated microtitre plate readers when compared with the tedium of reading each tube of a "tube" assay on a spectrophotometer.

The assay performs well in the laboratory. A comparison with the previous "tube" assay showed good correlation ($r = 0.98$, $p < 0.001$). Cyanocobalamin can be quantitatively recovered (table 2), and the precision, both between and within assay is excellent (table 1). The limit of detectability of the assay is below 20 ng/l, and adaptations can easily be made to provide accurate results in duplicate on very small serum volumes (< 50 μ l). A previous study in this laboratory had shown that pretreatment of sera with a β -lactamase preparation was effective in eliminating the interference of penicillins, cephalosporins, and Augmentin before serum vitamin B₁₂ assay.⁷ We included a similar step for antibiotic-containing samples in this assay and this also proved effective, with a valid result obtained on all 84 routine such samples tested. This result was verified by the recovery of 100.18% (CV 13.95%) of a cyanocobalamin "spike" added to the samples. We now find it cost effective to pretreat all assay samples with β -lactamase and have noted no interference in over 2000 patient sera studied.

Microbiological assays have been the reference methods for the estimation of vitamin B₁₂ for over 30 years. This simple and inexpen-

sive "plate" assay using *L leichmannii* retains the specificity of traditional assays and can be used in the general laboratory. Accurate results may be obtained from very small sample volumes and the assay is not affected by the often cited^{4,8} problem of antibiotic interference. The assay has very low operating costs and is particularly well suited to handling large volumes of samples.

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