Assessment of a radioisotopic assay for vitamin B₁₂ using an intrinsic factor preparation with R proteins blocked by vitamin B₁₂ analogues

BARBARA BAIN, GN BROOM, JACKIE WOODSIDE, RA LITWINCZUK, SN WICKRAMASINGHE

From the Department of Haematology, St Mary's Hospital and Medical School, Praed St, London W2 1PG

SUMMARY A competitive protein binding radioassay kit for serum vitamin B₁₂ has been assessed. Precision, linearity, sensitivity, and specificity have been found to be satisfactory. Falsely-normal assay results in patients with vitamin B₁₂ deficiency have not been observed.

Radioisotopic assays of vitamin B₁₂ are being increasingly introduced into haematological practice because of the considerable saving of technical time and the lesser likelihood of assay failure in comparison with microbiological assays. A serious inadequacy of many radioisotopic assays for vitamin B₁₂ was suggested when Kolhouse *et al*¹ observed that many commercial kits used "intrinsic factor" containing large amounts of R proteins; they suggested that the binding of cobalamin analogues in serum to the R proteins lead to a loss of specificity of the assay and a failure to detect vitamin B₁₂ deficiency in some patients. Although others have been unable to demonstrate such cobalamin analogues in serum^{2,3} manufacturers have nevertheless modified assay kits either by purifying intrinsic factor or by blocking R proteins thereby leaving intrinsic factor as the effective binding protein. We have assessed a commercial kit based on the latter principle, in which the contaminating R proteins are blocked by vitamin B₁₂ analogues which lack the nucleotide of cobalamin and thus combine with R proteins but not with intrinsic factor.

Material and methods

Microbiological assay of vitamin B₁₂ was carried out with *Lactobacillus leichmannii* and a competitive protein binding radioassay was carried out using Becton Dickinson B₁₂ ⁵⁷Co Radioassay Kit, in which the vitamin B₁₂-binding protein is porcine intrinsic factor and the contaminating R proteins are blocked by analogues, prepared by the hydrolysis of vitamin

B₁₂. A deoxyuridine suppression test was carried out on bone marrow aspirates from a number of patients using the method of Wickramasinghe and Saunders.⁴ Vitamin B₁₂ absorption was investigated using Dicopac (Amersham International). Red cell folate concentrations were measured using Becton Dickinson Folate Radioassay Kit (reference range in our laboratory 120-675 µg/l).

Microbiological and radioassay of vitamin B₁₂ was carried out in parallel on samples from 100 normal volunteers 55 of whom were women. Their age range was from 17 to 55 yr. Mean values and 95% reference ranges were derived.

Both types of vitamin B₁₂ assay were performed in parallel on 284 consecutive patient samples on which a serum vitamin B₁₂ assay had been requested by clinical staff; in the majority, an assay of red cell folate was carried out on a sample obtained simultaneously.

Reproducibility was studied for both microbiological and radioassay using a single large serum sample from a normal volunteer which was assayed five times in one batch and four times in another batch by both methods. Between-batch reproducibility of the radioassay for patient samples was also assessed by repeating assays on 20 consecutive samples from a single batch in another batch. Linearity was studied for both methods by serial dilutions of a sample of normal serum, of known vitamin B₁₂ concentration, in a pool of sera with low vitamin B₁₂ concentrations.

After introduction of the radioassay of serum vitamin B₁₂ into routine use the assay results were critically assessed to establish whether any normal assay results were obtained on patients who were

vitamin B₁₂-deficient, or whether falsely-low assay results were obtained on patients with normal vitamin B₁₂ status. Over an 18-month period all samples from patients with megaloblastic haemopoiesis or a strong clinical suspicion of vitamin B₁₂ deficiency were assayed both by radioassay and microbiological assay, and in such patients a vitamin B₁₂ absorption study was recommended irrespective of the serum vitamin B₁₂ concentration. During the first year of use of the radioassay a microbiological assay was done on all samples with a radioassay value of less than 200 ng/l, and to detect falsely-low radioassay results the clinical and laboratory investigations of patients with a radioassay result of less than 100 ng/l were reviewed.

Results

Serum vitamin B₁₂ concentrations on the 100 healthy subjects had a log-normal distribution both by microbiological assay and radioassay; logarithmic transformation was therefore used to derive means and normal (reference) ranges (Table) and to assess the correlation between the two methods. The difference between mean values for men and women was statistically significant only for the microbiological assay ($p < 0.05$) and was not of such a magnitude as to be of practical importance. There was a good correlation between the microbiological and radioassay results of the normal subjects ($r = 0.87$) and no significant difference between the mean values ($p > 0.05$). The 284 consecutive patient samples showed a somewhat worse correlation ($r = 0.78$, or $r = 0.80$ if grossly discrepant results from a single patient on antituberculous chemotherapy were excluded).

Reproducibility of the radioassay was better than reproducibility of the microbiological assay (coefficients of variation 7.0% and 10.7% respectively); this was attributable to greater between-batch variation with microbiological assay. Between-batch reproducibility studies for the radioassay on 20 consecutive patient samples gave a coefficient of variation of 9.9% and a correlation coefficient of 0.98.

Linearity of both assay methods was excellent and correlation between the results obtained from the

serial dilutions for both methods was also excellent ($r = 0.99$).

During the present investigation 20 patients were found who had been adequately investigated and were vitamin B₁₂-deficient. In two patients with a neurological presentation of pernicious anaemia and one patient with carcinoma of the stomach the finding of a low serum vitamin B₁₂ concentration on radioassay led to further investigation including a bone marrow aspiration. In the other 17 patients the diagnosis of vitamin B₁₂ deficiency was made independently of knowledge of a serum vitamin B₁₂ concentration, being based on the clinical history, bone marrow findings, deoxyuridine suppression test and studies of vitamin B₁₂ absorption. Serum B₁₂ assay results by the two methods on 24 samples from the 20 patients are shown in the Figure, together with results by the two methods on the 100 normal volunteers. In only one of the 20 patients with vitamin B₁₂ deficiency was a normal level (180 ng/l) found by radioassay; a repeat radioassay on the same sample was 62 ng/l, and radioassays on two other serum samples from the same patient were similarly low. This single normal value is therefore interpreted as being due to a technical error at a time when the assay had been recently introduced and this result has therefore not been plotted in the Figure. Apart from this single assay result the range of vitamin B₁₂ concentrations on 29 samples from the 20 deficient patients was from undetectable to 132 ng/l; the range of vitamin B₁₂ concentrations by microbiological assay on 24 samples was from undetectable to 175 ng/l, none falling within the normal range. During the period of the study all patients with megaloblastic haemopoiesis and a normal serum vitamin B₁₂ concentration were found to have another explanation for megaloblastosis, either folate deficiency, cytotoxic therapy or leukaemia. Microbiological assay of samples with a radioassay value of less than 200 ng/l did not reveal any unexpected vitamin B₁₂ deficiency.

During the period of the study we received serum samples from two patients with proven pernicious anaemia which had given misleadingly high results on another radioassay kit in which R proteins were not blocked; our radioassay results on these samples were 70 and 35 ng/l. Similarly one of our 20 vitamin B₁₂

Mean serum vitamin B₁₂ concentration (ng/l) (and 95% reference ranges) in 100 normal subjects

	Men (n = 45)	Women (n = 55)	Men plus women (n = 100)
Serum B ₁₂ (ng/l) by microbiological assay	401 (154–831)*	343 (178–660)*	368 (181–746)
Serum B ₁₂ (ng/l) by radioassay	359 (183–703)	318 (154–659)	336 (165–684)

*Significance of difference between values of men and women by microbiological assay, $p < 0.05$.

Serum vitamin B₁₂ concentrations by competitive protein binding radioassay and microbiological assay in 100 normal volunteers (○) and in 20 vitamin B₁₂-deficient patients (●). The lower 95% confidence limits for competitive protein binding radioassay (— · — · —) and for microbiological assay (— — —) are shown.

deficient patients had previously had two normal results with a different unblocked commercial kit. After the conclusion of the study we were referred a fourth patient with macrocytosis and peripheral neuropathy whom we found to have pernicious anaemia with serum vitamin B₁₂ concentrations of 115 and 105 ng/l on two occasions; this diagnosis had previously been considered to have been excluded on the basis of a normal serum vitamin B₁₂ with the same unblocked radioassay kit as had been used on our patient.

During the first year of use of the radioassay 1063 patient samples were assayed. Of these, 161 samples (15%) from 127 patients gave assay results below the reference range. Seventy-one samples (6.6%) from 54 patients gave assay results of less than 100 ng/l; further assessment of these 54 patients was made. Twenty-one patients had proven, or strongly suspected vitamin B₁₂ deficiency, four were vegetarians, and a further 20 patients had low concentrations by both microbiological and radioassay. In only nine patients did it appear that radioassay results were falsely low. Investigations of the 73 patients with serum vitamin B₁₂ concentrations between 100 and 164 ng/l were found to have been much less detailed but as would be expected, the proportion of patients with proven B₁₂ deficiency was lower in this group, while the proportion of unexplained low serum B₁₂ concentration was higher.

Discussion

We have assessed the Becton Dickinson Vitamin B₁₂ ⁵⁷Co Radioassay Kit, a competitive protein binding

radioassay. Linearity was found to be excellent, and reproducibility was satisfactory. The reference range was found to be similar to that for microbiological assay using *L. leichmannii*. The lower serum vitamin B₁₂ concentrations we have observed in women (significant only for the microbiological assay) is of interest. Since serum vitamin B₁₂ falls during pregnancy there may be a hormonal influence in non-pregnant women causing a somewhat lower concentration of serum vitamin B₁₂ than is found in men.

It has frequently been observed that some commercial kits used in some laboratories give misleadingly normal serum vitamin B₁₂ assay results in a significant proportion of patients with pernicious anaemia.^{1-5,6} This observation has been attributed to the presence in "intrinsic factor" preparations of R proteins which bind cobalamin analogues as well as cobalamin (vitamin B₁₂) leading to a reduction of specificity of the assay. The problem could have been aggravated by a low pH in most assay systems which reduced the binding of vitamin B₁₂ by intrinsic factor but not its binding by R proteins.¹ Some doubt has been cast on this explanation by the failure of others to find any evidence of such cobalamin analogues in serum.^{2,3} It is also difficult to reconcile a loss of assay specificity attributable to R proteins with the observations of others that an assay using an R protein as a binder, rather than intrinsic factor, may give an excellent separation of patients with pernicious anaemia from normal.^{7,8} Despite this uncertainty, assay methods have been modified either by purifying intrinsic factor or by blocking R proteins (but not intrinsic factor) with cobinamide, or other vitamin B₁₂ analogues. The commercial kit which we have assessed is based on the latter principle. We found the sensitivity of the assay to deficiency of vitamin B₁₂ to be comparable to that of a microbiological assay using *L. leichmannii*. Furthermore this kit was found to be capable of detecting low serum vitamin B₁₂ concentrations in four patients whose sera had given misleadingly normal values with two other commercial kits in which R proteins had not been blocked or removed. Since commercial kits have been modified by the purification of intrinsic factor, or by blocking of R proteins erroneously high B₁₂ values have been much less reported, and an improvement of performance of Becton Dickinson kits in quality control surveys was noted following such kit modification.⁹ Our findings in four patients whose sera had been assayed in other laboratories using unmodified kits support the need for modification.

Erroneously low results with radioassays have also been reported in prototype commercial kits,^{10,11} this being attributed to high non-specific binding in the supernatant, and inadequate removal of free vitamin

B₁₂ by the absorbent. It is also theoretically possible for erroneously low assay results to be produced by contamination of the patient's serum or counting vials by radioactive isotopes. With the kit assessed we found the incidence of falsely low assay results to be acceptably low. It is not uncommon to find that elderly patients, vegetarians and post-gastrectomy patients have low concentrations of serum vitamin B₁₂ without other features of B₁₂ deficiency. Low concentrations in such patients were found with the radioassay and must be regarded as valid results rather than indicating any lack of specificity of the assay. Since microbiological assays were not performed on all samples submitted, we cannot assess the frequency of falsely-low concentrations with a microbiological assay.

Because of the grave consequences of failure to recognise vitamin B₁₂ deficiency, an assay with a significant number of falsely-normal concentrations is unacceptable. The competitive protein binding radioassay assessed in this paper is acceptable in this regard, and also as regards linearity, precision, and specificity. Nevertheless in interpreting patient data it should be remembered that any test is subject to random errors of various types, and if any laboratory result conflicts with clinical judgement then further assessment of the patient is essential.

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Requests for reprints to: Dr BJ Bain, Department of Haematology, St Mary's Hospital Medical School, London W2 1PG.