Comparison of serum vitamin B₁₂ estimation by saturation analysis with intrinsic factor and with R-protein as binding agents

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SUMMARY It has been reported that serum vitamin B₁₂ levels assayed by saturation analysis methods may give misleadingly high results, so much so that the diagnosis of vitamin B₁₂ deficiency may be obscured. This defect was ascribed largely to assays using a vitamin B₁₂ binder other than pure intrinsic factor. To test out this hypothesis two assays were set up, one using saliva (non-intrinsic factor R-binder) and the other using human gastric juice (intrinsic factor) as B₁₂-binding agents. Both assays were able to differentiate sera from patients with pernicious anaemia from those from control subjects.

Published results accumulated over the past 10 years indicate that properly designed and performed saturation analysis vitamin B₁₂ assays are as reliable as microbiological assay methods for detecting low serum B₁₂ levels. The failure of some methods to do so does not appear to be due to the nature of the B₁₂-binding agent.

Routine assay of the serum vitamin B₁₂ (cobalamin) level is usually carried out by saturation analysis. Many variations of technique have been described, and published series have shown an excellent differentiation between samples from patients with clinical pernicious anaemia and from control groups. It is less certain that equally good results are obtained by others without a special interest in the test.

Doubt concerning the reliability of some of the tests has been expressed by Cooper and Whitehead,¹ who reported that the diagnosis in one-third of patients with pernicious anaemia was missed because a serum B₁₂ level within the normal range was obtained with saturation analysis. Kolhouse *et al.*² ascribed this to the use of B₁₂-binding agents in the test procedure other than intrinsic factor. Two claims were made. Firstly, it was reported, on the basis of chromatography of fractions assayed for B₁₂ activity by saturation analysis, that not only were cobalamins present in plasma (methylcobalamin, adenosylcobalamin, hydroxocobalamin, and sometimes traces of cyanocobalamin) but other cobamides not detected by microbiological assay with *Euglena gracilis* or *Lactobacillus leichmannii*. Only cobalamin was physiologically important, and if the method

measured those non-B₁₂ compounds a misleadingly high result was obtained. Secondly, it was claimed that when intrinsic factor was used as the binding agent only cobalamins were measured, and the result was valid in pernicious anaemia. If, however, a non-intrinsic factor binding agent was used, such as serum or saliva, the non-cobalamin factors were measured as well as the cobalamins and the result could then be misleadingly high.

To test this hypothesis two saturation analysis methods were set up based on that described by Carmel and Coltman³ and Hall.⁴ Both were identical but one used a human gastric juice in which 99% of the B₁₂ binding capacity was due to intrinsic factor and the other saliva in which all the B₁₂ binding was due to R-protein. We report our results with sera from patients with untreated pernicious anaemia and from control groups.

Material and methods

The method was essentially that described by Carmel and Coltman³ and Hall,⁴ modified by adding 1 ml B₁₂-free serum extract to all tubes in the standard curve. All dilutions were made in acetate cyanide buffer pH 4.8. Sera for assay were de-proteinised as described below.

SALIVA

Pooled human saliva from laboratory staff was centrifuged to remove debris and stored at -20°C in 1.2 ml aliquots. For use 1 ml was diluted in approximately 25 ml acetate-cyanide solution so that 0.1 ml of the diluted saliva bound 60-70% of 100 pg $^{57}\text{Co-B}_{12}$.

GASTRIC JUICE

Human gastric juice was brought to pH 10 to inactivate pepsinogen, and after 20 minutes the pH was adjusted to 7.0. Intrinsic factor concentration and total vitamin B_{12} binding capacity was measured.⁵ A gastric juice pool containing 99% intrinsic factor and virtually no R-binder was selected for this study. It was stored at -20°C and diluted in the same way as the saliva.

ACETATE-CYANIDE BUFFER pH 4.8

3.0 mol sodium acetate (56.6 ml) is mixed with 3.0 mol acetic acid (43.3 ml), and 20 mg sodium cyanide is added to each litre buffer. This pH gave optimal and complete recovery of B_{12} in the assay system.

 B_{12} -DEFICIENT SERUM EXTRACT

One part pooled serum is added to 3 parts acetate-cyanide buffer and heated in a boiling water bath for 20 minutes. The supernatant is retained, and B_{12} is removed by adsorption of 25 ml supernatant with 100 mg serum-coated charcoal. Charcoal is removed first by centrifugation and finally by passing the adsorbed supernatant through a millipore filter (Millex 0.45 μm). It is stored at -20°C .

SERUM-COATED CHARCOAL

Charcoal (Norit GSX) was activated at 160°C for 2 hours. One gram was left at room temperature with 10 ml pooled serum for 10 minutes, and the charcoal was washed with 10 ml acetate-cyanide buffer.⁶

EXTRACTION OF SERA FOR ASSAY

One millilitre serum is mixed with 3 ml acetate-cyanide buffer and heated in a boiling water bath for 20 minutes. The extracts are cooled and the supernatants retained for assay.

THE TEST

Each test consisted of 1 ml serum extract, 1 ml acetate cyanide buffer, and 100 pg $^{57}\text{Co-B}_{12}$ in 1 ml buffer. These were mixed, and 0.1 ml binding agent (the amount binding 60-70 pg of 100 pg B_{12}) was added. After 1 hour at room temperature serum-coated charcoal suspended in 0.5 ml saline was added. The standard curve was set up in a similar manner, 1 ml B_{12} -free serum extract replacing the

test serum and 1 ml acetate-cyanide buffer containing the serially diluted unlabelled B_{12} . Standards for $^{57}\text{Co-B}_{12}$, binder, and charcoal controls are included.

Patients

PERNICIOUS ANAEMIA

Twenty-five sera were available before treatment from 21 patients with pernicious anaemia. All these patients had a macrocytic anaemia with megaloblastic marrow changes, achlorhydria with gastric atrophy, impaired vitamin B_{12} absorption improved with additional intrinsic factor, and a low serum B_{12} level by microbiological assay with *Lactobacillus leichmannii*. All responded to treatment with B_{12} alone.

Blood was obtained from 52 normal subjects, who were members of the hospital and laboratory staff and in normal health.

Results and discussion

The purpose of the study was to compare intrinsic factor and R-binders when used for saturation analysis assay of serum B_{12} . The results in Table 1 and the Figure show that an assay using an R-binder

Table 1 Vitamin B_{12} estimation by saturation analysis (pg/ml) (mean $\pm$ SE)

	Salivary binder	Gastric juice binder
Controls	224-1000 m 548 ± 187 (48)* 28-216	152-1000 m 425 ± 146 (52) 0-200
Pernicious anaemia	m 140 ± 55 (25)	m 88 ± 50 (25)

*No. of sera.

was entirely satisfactory in the detection of sera with low levels of B_{12} , and all of 25 sera from patients with untreated pernicious anaemia were below the levels obtained with control samples. Taking 200 pg/ml as the lower limit of a normal range, we interpret the data with gastric juice as showing that two 'normal' samples are low (Figure) rather than that two pernicious anaemia sera were missed, but we would not argue with anyone who wishes to vary this interpretation. But the differentiation was not as satisfactory with gastric juice as with saliva.

Our data do not support the contention that saturation analysis using an R-binder may miss a substantial number of patients with pernicious anaemia, nor does it support the hypothesis that an R-binder is unsatisfactory as a binding agent because non-cobalamins blur the distinction between sera containing low levels of B_{12} and those with normal levels.

Serum B₁₂ levels in patients with untreated pernicious anaemia (○) and controls (●) by saturation analysis with both gastric juice and saliva as binding agent.

However, we are impressed by the ease with which an assay using charcoal and free B₁₂ binding protein can go wrong and give misleading results in uncritical hands. Reassay of the quantity of saliva required should be done at regular intervals.

Results were generally higher with a salivary binder than with an intrinsic factor binder. However, this did not influence the ability of the test to differentiate sera from pernicious anaemia patients from control sera. The higher levels with an R-binder may be related to the manner in which B₁₂ dissociates from the binder. The B₁₂-R-binder complex is relatively stable whereas B₁₂ readily dissociates from intrinsic factor.^{7,8}

Our data are in conformity with published results on B₁₂ assay using a variety of B₁₂ binding reagents. Some of these data are summarised in Table 2. Six studies in which sera from 210 patients with pernicious anaemia were assayed using R-binders showed only three patients overlapping the normal range. One pernicious anaemia sample out of 117 was also just normal when gastric juice was used. These studies assayed over 1000 normal samples. These data do not support the view that one-third of pernicious anaemia cases are missed or that circulating non-cobalamins invalidate the results of some saturation

Table 2 Serum samples assayed for vitamin B₁₂ content by saturation analysis

Binder	Number of sera from		
	Untreated PA found to be low	Normal subjects above PA range	PA in normal range
R-binder ⁹⁻¹³	210	658	3
Gastric juice ¹⁴⁻¹⁶ or hog stomach	117	365	1

PA = pernicious anaemia.

analysis assays. Finally, the diagnosis of pernicious anaemia should be based on all the features of the case, and interpretation of B₁₂ levels must take into account the possibility that an erroneously raised level may sometimes emerge from the laboratory.

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