Experiences with dual protein bound aqueous vitamin B₁₂ absorption test in subjects with low serum vitamin B₁₂ concentrations

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SUMMARY A dual isotope vitamin B₁₂ absorption test in which vitamin B₁₂ is given both in aqueous solution and bound to protein (chicken serum), was evaluated in 26 controls and 68 patients with subnormal serum vitamin B₁₂ concentrations (19 with pernicious anaemia, 13 with iron deficiency, seven after partial gastrectomy, seven with malabsorptive states, five with folate deficiency, four with chronic alcoholism and 13 in whom no cause was apparent). In control patients protein bound absorption decreased with age; isotope excretion was 1.0% or over in those aged under 60 and 0.5% or over in those aged 60 and above. Malabsorption of protein bound vitamin B₁₂ with normal aqueous absorption occurred in five patients with iron deficiency, three with alcoholism, two after partial gastrectomy, two with folate deficiency and in one with a malabsorptive state. In alcoholics abstinence produced an improvement in protein bound absorption. All patients in the group for whom no cause could be found for the subnormal serum vitamin B₁₂ concentration had normal aqueous absorption but four had malabsorption of protein bound vitamin. Although the dual isotope test gave reproducible results and was consistent with the standard Schilling test some anomalies were detected; nine patients had reduced aqueous absorption with normal protein bound absorption. Despite this the dual test may prove useful in determining the importance of a subnormal vitamin B₁₂ concentration where the cause is not clinically apparent. Further development is needed before it can be considered for routine use.

The Schilling test is commonly used to measure absorption of vitamin B₁₂ in aqueous form. This correlates with the absorption of vitamin B₁₂ from food in healthy subjects.¹ There are, however, patients with vitamin B₁₂ deficiency whose Schilling test is normal but who show reduced absorption of the vitamin when it is bound in vitro to protein prior to ingestion.²⁻⁴ This combination of normal aqueous but decreased protein bound absorption is associated with achlorhydria and may be seen after partial gastrectomy⁵ or vagotomy^{5,6} in atrophic gastritis^{3,7} and during treatment with cimetidine.^{8,9} Patients with atrophic gastritis may have a subnormal serum vitamin B₁₂ concentration despite a normal Schilling test¹⁰ which, on occasion, has been associated with megaloblastic anaemia^{4,6,11} and neuropathy.¹¹ The failure of the Schilling test to detect malabsorption in

these patients may be because the gastric lesions are less severe than those typical of pernicious anaemia.

We have previously used vitamin B₁₂ bound to chicken serum for absorption studies⁴ and have now combined this with a test of aqueous vitamin B₁₂ absorption. We report the development and application of this combined test in the investigation of subnormal serum vitamin B₁₂ concentrations.

Material and methods

DEVELOPMENT OF THE TEST

The tests used during the development phase were carried out on volunteers. Approval was given by the district ethical committee. Our original test used 4 ml chicken serum and 1 µg B₁₂ labelled with cobalt 57(⁵⁷CoB₁₂). It was thought advisable, in a combined test, not to increase the total amount of vitamin B₁₂ because the percentage absorption of the vitamin is inversely related to the dose. The first dual test

devised, therefore, consisted of 0.5 µg $^{58}\text{CoB}_{12}$ in aqueous solution (CR3P, Amersham International), followed by 0.5 µg $^{57}\text{CoB}_{12}$ with 4 ml of chicken serum. Table 1 shows the results. The aqueous absorption seemed to be satisfactory, but the protein bound absorption was unexpectedly low. In two subjects our original test had given results of 0.4% and 1.1%, whereas in the combined test the excretions from the bound form were 0% and 0.3%, respectively. The decreased absorption may have been due to the proportion of chicken serum to B_{12} (4 ml to 0.5 µg) being higher than in the original single test.

A series of dual tests was therefore carried out using a 2 ml dose of chicken serum plus 1 µg $^{57}\text{CoB}_{12}$, a combination that almost completely saturated the binder in vitro. The protein bound results were again very low (table 1). Subsequent tests, on a different group of patients and controls, were performed in the reverse order using 1.0 µg $^{57}\text{CoB}_{12}$ with 2 ml chicken serum followed two hours later by aqueous $^{58}\text{CoB}_{12}$. Protein bound absorption was better with this order of administration (table 1). The test was subsequently used in this form.

THE DUAL TEST

After an overnight fast the subject drank 30 ml aqueous solution of 1 µg (0.037 MBq; 1 µCi) cyanocobalamin labelled with cobalt-57 to which had been added 2 ml sterile chicken serum within a few minutes of mixing. Two hours later a drink of 0.5 µg (0.019 MBq; 0.5 µCi) cyanocobalamin labelled with cobalt-58 was given, followed by a flushing dose of 1000 µg hydroxocobalamin. Resumption of normal eating was allowed after a further two hours. The excretion of labelled vitamin was measured in urine passed in the 26 hour period from the time of the first drink. A LKB Compugamma counter was used for measuring radioactivity. The counting rates for cobalt 57 at the 0.5% excretion level were between two and three times the background of 16 counts per minute, 95% confidence limits +0.02% to -0.02%.

SCHILLING TESTS

Eleven standard Schilling tests using 1 µg

cyanocobalamin were performed for comparison with the aqueous part of the dual test.

VITAMIN B_{12} ASSAYS

Serum cobalamin concentrations were determined by radioisotope saturation analysis with human intrinsic factor as the binder (reference range 170–900 ng/l).

CONTROL SUBJECTS AND PATIENTS

The control subjects comprised 26 volunteers, 15 men and 11 women, aged 21–85 years; 15 were under 60 years of age. Each had normal serum vitamin B_{12} and folate concentrations.

Nineteen patients had pernicious anaemia (five men and 14 women). Eleven had serum intrinsic factor binding antibodies. Seven patients had undergone partial gastrectomy. There were 40 other patients with subnormal serum vitamin B_{12} concentrations of whom 13 had iron deficiency, four were chronic alcohol abusers, five had folate deficiency, and seven had malabsorption syndromes. Thirteen patients in whom no reason for a low serum vitamin B_{12} concentration was apparent (idiopathic low B_{12} group) were also studied.

Ten subjects underwent a second dual test between one and 17 months after the first to show its reproducibility. Vitamin B_{12} was not given in the interval between the two tests.

Results

The figure shows the results of the absorption tests in all subjects.

In the control group, the mean excretion of isotope in the protein bound part of the test was 2.7% (SEM 0.4%). In those aged under 60 it was 3.6 (0.5)% (range 1.3–7.5) and in older controls it was 1.4 (0.2)% (range 0.6–3.6). We interpreted an excretion of less than 1.0% as abnormal in subjects under 60 years of age, and less than 0.5% as abnormal in older subjects.

In the patients with pernicious anaemia excretion of aqueous vitamin was abnormal in all cases. Protein bound vitamin B_{12} absorption was defective in 17, but normal in two elderly patients.

Table 1 Vitamin B_{12} absorption with various combinations of protein bound and aqueous vitamin B_{12}

No of subjects	Test dose (µg vitamin B_{12})		Urinary excretion (%)			
	First	Second	Aqueous test		Chicken serum test	
			Mean	Range	Mean	Range
17	0.5 µg Aqueous	0.5 µg + 4 ml CS*	18.4	1.2–41.8	0.1	0.0–0.5
8	0.5 µg Aqueous	1.0 µg + 2 ml CS*	23.5	6.8–39.4	0.1	0.0–0.3
8	1.0 µg + 2 ml CS*	0.5 µg Aqueous	16.1	8.8–23.3	0.9	0.1–2.8

*CS = $^{57}\text{B}_{12}$ bound to chicken serum.

Urinary excretion of protein bound and aqueous vitamin B₁₂ in all subjects. ○ = aged under 60; ● = aged 60 and over.

All seven patients who had had partial gastrectomy showed subnormal excretion in one or both parts of the dual test. In four both parts were normal, in two the protein bound part was low, and in one only the aqueous absorption was low.

Of the other patients with low serum vitamin B₁₂ and iron deficiency, five had a low protein bound absorption, three malabsorption of both forms, and two a low aqueous absorption. In three, both parts of the test were normal.

No consistent results were obtained in the heterogeneous group with malabsorptive states, but three

patients, one with bacterial contamination of the bowel, one with subtotal villous atrophy, and one receiving metformin, had normal protein bound B₁₂ absorption with abnormal aqueous absorption.

All four patients with alcohol abuse showed a subnormal protein bound vitamin B₁₂ absorption, and one patient also had a subnormal aqueous result. Two patients who abstained from alcohol for several months showed, on repeat testing, improved absorption of protein bound B₁₂ from 0% (aqueous 36%) to 7.2% (aqueous 32.1%) and 0.1% (aqueous 11%) to 1.3% (aqueous 18.3%).

Table 2 Reproducibility of dual tests

Age of patient (years)	Interval between tests (months)	Urinary excretion (%)			
		Protein bound test		Aqueous test	
		First	Repeat	First	Repeat
36	1	1.7	1.4	28.1	30.6
75	1	0.2	0.4	29.4	25.0
59	1	1.1	1.7	15.3	15.3
66	1	0	0.3	3.7	3.3
70	1	1.0	0.6	24.9	24.0
62	11	0.8	1.0	21.2	22.6
61	12	3.6	4.3	13.7	23.2
76	15	0.4	0	8.7	8.7
47	16	4.6	1.8	20.8	14.8
35	17	3.5	3.7	15.0	26.7

Two patients with folate deficiency had subnormal protein bound B₁₂ absorption and one patient had subnormal aqueous (7.5%) but normal protein bound absorption.

All patients in the idiopathic group had normal aqueous absorption and four had subnormal protein-bound B₁₂ absorption. Three of these four had parietal cell antibodies which were not present in the others. One with parietal cell antibodies also had intrinsic factor antibodies, and a year later had an abnormal Schilling test (7.1%).

In 11 subjects a comparison was made between the aqueous part of the dual test and a standard Schilling test performed separately. The mean excretion in the aqueous part was 7.8% compared with 8.0% in the Schilling test (paired $t = 0.172$, NS).

Table 2 shows that the reproducibility of the test was good.

Discussion

A combined test of aqueous and protein bound cyanocobalamin is more convenient for both patient and laboratory than two separate tests. Doscherholmen *et al*¹² described a combined test using egg yolk as a binder, but the preparation of the test doses is intricate. Our objective was to develop a test requiring only minimal preparation of the reagents and suitable for use by an isotope laboratory with appropriate counting equipment.

We chose a two hour interval between doses¹³ to lessen the risk of free ⁵⁸Co vitamin B₁₂ being taken up by unbound chicken serum. The order in which the aqueous and protein bound vitamin were given was important. Giving the aqueous dose first, seemed to reduce the absorption of the protein bound isotope. The reason for this is not clear because in other experiments, when two aqueous doses were given separately, little or no inhibition was noted. Results proved satisfactory when the protein bound dose was given first.

An important feature of this dual test is the comparability of the aqueous part with the standard Schilling test. The reproducibility of the aqueous part was as good as that of the Schilling test.^{14 15} The protein bound part of the test was also reproducible, repeat tests showing no change in clinical interpretation. The variability that was noted may reflect inadequate urine collection. As with any test entailing prolonged urine collection, the importance of collecting all urine has to be emphasised to the patient. We had to discard very few results because of obvious undercollection, but the omission of a small volume at the time of maximal isotope excretion would go undetected. With a well shielded sensitive gamma counter and prolonged counting times, the error from low counting rates is minimal and insignificant compared with that from incomplete urine collection. Another important consideration is the adequacy of binding of the cyanocobalamin to the chicken serum. Unbound vitamin B₁₂ must not be present, or this will greatly increase apparent absorption. It is advised that the binding capacity of each batch of chicken serum should be measured, although we found little variability.

The protein bound part of the test gave higher absorption than our original protein bound test,⁴ probably because of the reduced proportion of chicken serum to vitamin B₁₂. The decreased absorption in the elderly, however, in alcohol abusers, and in most patients with iron deficiency who had subnormal vitamin B₁₂ concentrations was again evident. This probably indicates hypochlorhydria.

In nine patients the aqueous absorption was reduced, but protein bound absorption was normal. The results in three of these patients, two with pernicious anaemia and one after partial gastrectomy, must be anomalous, probably due to the presence of free vitamin B₁₂ in the protein bound dose due to an error in measuring the volume of chicken serum or isotope. Similar discrepancies occurred in two patients with iron deficiency and one with folate

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deficiency, and may be due to the same cause; this emphasises the need for particular care in preparing the protein bound dose. The three other patients with the anomalous result had malabsorptive states, a high proportion of the group. This has previously been seen in two patients with gluten enteropathy investigated by our original protein bound test,⁴ and requires further study.

Further developments to eliminate anomalous results are necessary. The test would also be more effective if protein bound excretion in normal subjects could be enhanced. Although we have evaluated the test in a variety of conditions associated with low serum vitamin B₁₂ concentrations, we believe its real usefulness lies in the investigation of patients with low serum vitamin B₁₂ concentrations for which the cause is unknown. A dual test is appropriate in these circumstances because the Schilling test is usually normal in these patients. In the 13 patients we evaluated, four had subnormal protein bound absorption. Many years of observation may be required before it is known how many of these will develop typical pernicious anaemia, although one developed an abnormal Schilling test one year later.

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