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Analytical Methods Section

The appropriateness of using cyanocobalamin as calibration standards in competitive binding assays of vitamin B_{12}

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The forms of vitamin B_{12} that have been found in foods are cyanocobalamin, hydroxocobalamin, sulphitocobalamin, adenosylcobalamin and methylcobalamin. These cobalamins, with the exception of sulphitocobalamin, are also known to be present in serum and plasma. The addition of sodium cyanide, potassium cyanide, sodium metabisulphite or sodium nitrite in the extraction procedure employed for the determination of vitamin B_{12}, however, can convert these cobalamins into derivatives which include dicyanocobalamin and nitritocobalamin. It was observed that cyanocobalamin has a binding affinity for hog intrinsic factor equal to that of methylcobalamin, dicyanocobalamin and nitritocobalamin, but not to that of hydroxocobalamin, sulphitocobalamin and adenosylcobalamin. Thus, these latter three cobalamins cannot be measured accurately using a competitive binding assay which employs hog intrinsic factor as its binding protein and cyanocobalamin as the calibration standards unless they are converted to cyanocobalamin, methylcobalamin, dicyanocobalamin or nitritocobalamin before the assay.

INTRODUCTION

Competitive binding assay of vitamin B_{12} was first described by Herbert in 1958 (Herbert & Colman, 1988). This technique was subsequently employed for use in determining the amount of vitamin B_{12} in serum and plasma (Barakat & Ekins, 1961; Rothenberg, 1961) and was later applied to foods by a number of investigators (Richardson *et al.*, 1978; Beck, 1979; Marcus *et al.*, 1980; Casey *et al.*, 1982; Bennink & Ono, 1982; Osterdahl *et al.*, 1986).

The assay is carried out by the addition of a measured amount of radioactive vitamin B_{12} and a limited amount of vitamin B_{12} binding protein to the vitamin B_{12} in serum, plasma or food extracts. The free or unbound vitamin B_{12} is then separated from the protein-bound vitamin B_{12} and the radioactivity of either the former or the latter is measured. All of the binding protein will be bound by the radioactive and non-radioactive vitamin B_{12} present since the amount of radioactive vitamin B_{12} added is, by itself, sufficient to bind the small amount of binding protein. As both the radioactive and non-radioactive vitamin B_{12} compete to bind with the binding protein, the degree to which the radioactive count of the protein-bound vitamin B_{12} is inhibited is taken to be indicative of the amount of vitamin B_{12} present in the serum, plasma or food extracts. The actual amount of the vitamin is then determined by comparison with calibration standards.

Calibration standards employed in competitive binding assays are most often prepared from cyanocobalamin, whilst the radioactive vitamin B_{12} and the binding protein utilised are usually [^{57}Co]cyanocobalamin and hog intrinsic factor, respectively (Richardson *et al.*, 1978; Beck, 1979; Marcus *et al.*, 1980; Casey *et al.*, 1982; Nexo & Olesen, 1982).

The forms of vitamin B_{12} that have been identified in serum and plasma are hydroxocobalamin (OH-Cbl), cyanocobalamin (CN-Cbl), adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) (Nexo & Olesen, 1982). These four cobalamins, together with sulphitocobalamin (HSO_3-Cbl), have also been reported to occur in some foods (Farquharson & Adams, 1976).

Extraction of vitamin B_{12} from serum, plasma or food involves the addition of excess cyanide (sodium or potassium cyanide), sodium metabisulphite or sodium nitrite (Beck, 1979; Nexo & Olesen, 1982; AOAC, 1984). Preliminary observations made in the current

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Table 1. Conversion of five forms of vitamin B_{12} by excess potassium cyanide, sodium metabisulphite and sodium nitrite (in the dark)[a]

Before addition of compounds	After addition of compounds		
	+ KCN	+ $Na_2S_2O_5$	+ $NaNO_2$
OH-Cbl	diCN-Cbl	HSO_3-Cbl	NO_2-Cbl
HSO_3-Cbl	diCN-Cbl	HSO_3-Cbl	HSO_3-Cbl
CN-Cbl	diCN-Cbl	CN-Cbl	CN-Cbl
AdoCbl	diCN-Cbl	AdoCbl	AdoCbl
MeCbl	MeCbl	MeCbl	MeCbl

[a] Observations made based on absorption spectrophotometry of aqueous solutions. Spectra were determined 30 min after addition of compound. The ratio, in terms of number of moles, of KCN, $Na_2SO_2O_5$ and $NaNO_2$ to each form of vitamin B_{12} was 50:1, 1·5:1 and 15:1, respectively.

work showed that the presence of these compounds will result in the conversion of OH-Cbl, HSO_3-Cbl, CN-Cbl, AdoCbl and MeCbl into the various derivatives listed in Table 1, which include nitritocobalamin (NO_2-Cbl) and dicyanocobalamin (diCN-Cbl). This suggests that the vitamin B_{12} extracted from serum, plasma or foods (after addition of excess cyanide, sodium metabisulphite or sodium nitrite) could be in the form of HSO_3-Cbl, CN-Cbl, AdoCbl, MeCbl, diCN-Cbl and NO_2-Cbl (see Table 1), depending on the form of the endogenous vitamin B_{12}. As such, accurate determination of the extracted vitamin cannot be made using a competitive binding assay which employs hog intrinsic factor as its binding protein and CN-Cbl as the calibration standards unless the binding affinities of CN-Cbl and the forms extracted, for hog intrinsic factor, are similar.

A study was therefore undertaken to determine the relative binding affinity of OH-Cbl, HSO_3-Cbl, CN-Cbl, AdoCbl, MeCbl, diCN-Cbl and NO_2-Cbl for hog intrinsic factor. The relative binding affinity of these compounds for hog intrinsic factor was measured by their ability to compete with [^{57}Co]cyanocobalamin (CN[^{57}Co]Cbl) for hog intrinsic factor.

MATERIALS AND METHODS

Crystalline OH-Cbl, CN-Cbl, AdoCbl and MeCbl were purchased from Sigma Chemical Co. and dissolved in distilled, deionised water. An aqueous solution of HSO_3-Cbl was prepared according to Farquharson & Adams (1977) by addition of 0·1 mmol sodium metabisulphite to OH-Cbl (100 mg) and dissolving the mixture in distilled, deionised water (20·0 ml) with stirring for 30 min. Aqueous diCN-Cbl solution was prepared by addition of potassium cyanide (to a final concentration of 1 mM) to aqueous 16 μM OH-Cbl (Gimsing *et al.*, 1983) whilst aqueous NO_2-Cbl was prepared by addition of sodium nitrite (to a final concentration of 0·3 mM) to aqueous OH-Cbl of similar concentration (Kaczka *et al.*, 1951). The purity of these preparations was determined by thin-layer chromatography and, when other contaminating forms of cobalamin were detected, high-pressure liquid chromatography was used together with UV–VIS spectroscopy to purify the preparations.

A total of 174 10 mm × 75 mm polypropylene tubes were then labelled as follows: non-specific binding (3 tubes), total binding (3), OH-Cbl (24), HSO_3-Cbl (24), CN-Cbl (24), AdoCbl (24), MeCbl (24), diCN-Cbl (24), and NO_2-Cbl (24); then 0·05, 0·10, 0·15, 0·20, 0·25, 0·50, 0·75 and 1·00 ml of aqueous solutions of 322 pM OH-Cbl, 322 pM HSO_3-Cbl, 310 pM CN-Cbl, 334 pM AdoCbl, 328 pM MeCbl, 322 pM diCN-Cbl and 322 pM NO_2-Cbl were added to the tubes in triplicate. The volume of the solutions in the tubes was then adjusted to 1·0 ml with distilled water. The non-specific binding (NSB) and total binding (TB) tubes had distilled water (1·0 ml) added to them.

To each of the above tubes, aqueous CN[^{57}Co]Cbl (50 μl, 123 ng ml^{-1}) (specific activity: 100 kBq ng^{-1}; Diagnostic Products Corporation, Los Angeles, USA) was added. The tubes were vortexed and then incubated for 30 min at room temperature.

After incubation, a solution containing hog intrinsic factor bound to cellulose (1·0 ml), which was obtained from Diagnostic Products Corporation, was added to each of the tubes except for the NSB tubes. To each of the NSB tubes distilled water (1·0 ml) was added. All tubes were vortexed and then incubated for 60 min at room temperature.

Following incubation, the tubes were centrifuged at 3 000 × *g* for 30 min at 20°C in a Beckmann J-6B centrifuge with a JS-4·2 rotor. The supernatants were decanted and the residues were retained for counting in a Packard 5650 gamma counter for 1 min.

The amount of CN[^{57}Co]Cbl bound to the hog intrinsic factor in each tube was determined by calculating the counts per minute (c.p.m.) of the tube corrected for non-specific binding of CN[^{57}Co]Cbl to the tube:

$$\text{Net c.p.m.} = \text{c.p.m.} - \text{average c.p.m. of NSB tubes}$$

The amount of CN[^{57}Co]Cbl bound to the hog intrinsic factor in each tube was expressed as a percentage of the average amount of CN[^{57}Co]Cbl bound to the hog intrinsic factor in the TB tubes which had been corrected for non-specific binding of CN[^{57}Co]Cbl to the tubes:

$$\text{Bound CN[}^{57}\text{Co] Cbl (\%)} = \frac{\text{Net c.p.m.}}{\text{Average c.p.m. of TB tubes} - \text{average c.p.m. of NSB tubes}} \times 100$$

Statistical evaluation of the results was performed by the *U*-test of Wilcoxon, Mann & Whitney (Sachs, 1984).

RESULTS AND DISCUSSION

The relative binding affinities of OH-Cbl, HSO_3-Cbl, CN-Cbl, AdoCbl, MeCbl, diCN-Cbl and NO_2-Cbl for hog intrinsic factor were measured by their abilities to compete with CN[^{57}Co]Cbl for hog intrinsic factor. As

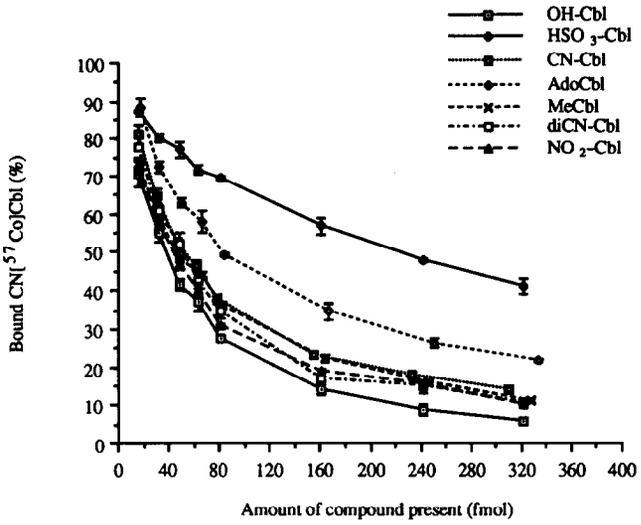

Fig. 1. The relative binding affinity of OH-Cbl, HSO_3-Cbl, CN-Cbl, AdoCbl, MeCbl, diCN-Cbl and NO_2-Cbl for hog intrinsic factor. Data are mean values and standard deviations from triplicate analyses.

is apparent from Fig. 1, the order of the compounds in terms of their binding affinity for hog intrinsic factor, from highest to lowest, was OH-Cbl, CN-Cbl, AdoCbl and HSO_3-Cbl. The difference in their binding affinities was statistically significant. Using the Wilcoxon, Mann & Whitney *U*-test, the *P* values obtained in the comparison of the binding affinity of OH-Cbl with CN-Cbl, CN-Cbl with AdoCbl, and AdoCbl with HSO_3-Cbl were $0{\cdot}05 < P < 0{\cdot}10$, $0{\cdot}025 < P < 0{\cdot}05$ and $0{\cdot}01 < P < 0{\cdot}025$, respectively. The relative binding affinity of MeCbl, diCN-Cbl and NO_2-Cbl for hog intrinsic factor was similar ($P > 0{\cdot}20$) and did not differ significantly from that of OH-Cbl or CN-Cbl ($P > 0{\cdot}20$).

The results obtained suggest that the measurement of vitamin B_{12} using a competitive binding assay which employs hog intrinsic factor as its binding protein and CN-Cbl as the calibration standards will only be accurate if the forms of cobalamin present in the serum, plasma or food extracts are CN-Cbl, MeCbl, diCN-Cbl or NO_2-Cbl. This is because MeCbl, diCN-Cbl and NO_2-Cbl have a similar binding affinity for hog intrinsic factor to that of CN-Cbl, whilst OH-Cbl, HSO_3-Cbl and AdoCbl do not.

As shown in Table 1, if vitamin B_{12} in serum, plasma or foods is extracted (1) in the presence of excess cyanide, OH-Cbl, HSO_3-Cbl, CN-Cbl and AdoCbl are converted to diCN-Cbl while MeCbl remains unchanged, (2) in the presence of sodium metabisulphite, OH-Cbl is converted to HSO_3-Cbl while HSO_3-Cbl, CN-Cbl, AdoCbl and MeCbl remain unchanged, or (3) in the presence of sodium nitrite, OH-Cbl is converted to NO_2-Cbl while the other four potential forms of vitamin B_{12} remain unchanged. Therefore, the presence of excess cyanide is essential for accurate assay results as OH-Cbl, HSO_3-Cbl and AdoCbl will be converted to diCN-Cbl.

CONCLUSION

The use of calibration standards prepared from CN-Cbl in the determination of vitamin B_{12} by competitive binding assay should be preceded by an extraction procedure which converts the potential forms of vitamin B_{12} into compounds which exhibit binding affinity, for the binding protein employed, equal to that of CN-Cbl.

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