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Analytical Methods Section

Production and purification of an R-protein–enzyme conjugate for use in a microtitration plate protein-binding assay for vitamin B_{12} in fortified food

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A protein-binding assay for the determination of vitamin B_{12}, based on the use of an R-protein–enzyme conjugate and the microtitration plate format, has been developed and applied to fortified foods. The assay limit of detection was 9 pg per well and the assay sensitivity 0·09 μg per 100 g of food. The immobilized phase of the assay, a B_{12}-keyhole limpet haemocyanin passively adsorbed to the plate wells, was synthesized by procedures giving significant advantages over previously reported approaches. The assay was simple to perform.

INTRODUCTION

Vitamin B_{12} (cyanocobalamin) is a member of a group of compounds collectively known as cobalamins, which are cobalt-containing corrinoids. Cyanocobalamin is actually an artifact of extraction processes where cyanide is added to stabilize the cobalamins (Chin, 1985). Vitamin B_{12} is essential for all cells in the body participating in the conversion of methylmalonyl CoA to succinyl CoA, and the methylation of homocysteine to methionine (Lee & Griffiths, 1985). It is found in a variety of forms in foods of animal origin (Paul & Southgate, 1978), predominantly as B_{12} and hydroxycobalamin. Products such as breakfast cereals are frequently fortified by the addition of cyanocobalamin.

The traditional methods of vitamin B_{12} analysis in food are microbiological in nature, usually based on the organisms *Lactobacillus leichmannii* or *Ochromonas malhamensis* (Chin, 1985). Of the two, the latter has greater specificity for cobalamins which more closely approximates the biological activity. In assays using *L. leichmannii* as much as 20% of total vitamin B_{12} activity in some samples has been reported to be due to non-cobalamins (Chin, 1985). In a direct comparison of analyses performed with the two organisms, significantly higher results were observed with *O. malhamensis* over a range of vitamin B_{12} values in food (Anon., 1984). Microbiological analysis of vitamins can be time-consuming (*O. malhamensis* requires 3–4 days for growth; *L. leichmannii* requires 1–2 days) and non-specific interferences can cause problems of interpretation even to experienced analysts.

In clinical environments, vitamin B_{12} is usually measured using radio-assays based on the competition of added ^{57}Co-labelled cyanocobalamin and sample cyanocobalamin for a limited number of binding sites provided by natural B_{12}-binding proteins such as intrinsic factor and R-protein (Herbert & Colman, 1985). The radio-assays are simpler and quicker to perform than the microbiological assays, although they require special facilities for the handling and disposal of the radioactive tracers. The use of radioisotopes is not generally perceived as desirable in a food context. However, several workers have examined the application of commercial radio-assay kits for vitamin B_{12} in food (Osterdahl & Johannsson, 1988).

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More recently, assays using enzyme-labelled B_{12}-binding proteins have been described (Tsalta & Meyerhoff, 1987). In the present paper the authors advance this approach and report a microtitration plate, enzyme-linked protein-binding assay for B_{12} in fortified foods which is rapid but simple enough for routine use in unspecialized laboratories.

MATERIALS AND METHODS

Materials

R-protein (from porcine mucosa), cyanocobalamin, horseradish peroxidase (HRP), keyhole limpet haemocyanin (KLH) and bromoacetyl bromide were all obtained from Sigma, Poole, UK; peroxidase substrate based on 3,3',5,5'-tetramethylbenzidine was obtained from Cambridge Veterinary Sciences, Cambridge, UK, and Nunc microtitration plates (Immunoplate I) were obtained from Gibco Europe Ltd, Uxbridge, UK. All other reagents were of the highest standard available. Three types of breakfast cereals, fortified and unfortified, were supplied by Kelloggs, Manchester, UK.

Production and purification of R-protein–HRP conjugate

The method used for conjugation of HRP with R-protein was adapted from that of Wilson and Nakane (1979). Sodium periodate (0·1 M, 0·05 ml) was added to an aqueous solution of HRP (1 mg in 250 ml) and left to react for 20 min before being dialysed against 1 mM acetate buffer (pH 4·4) for 2 h.

The dialysate was added to R-protein (10 000 units in 1 ml of 0·02 M carbonate/bicarbonate buffer, pH 9·2), and left to react for 2 h. Aqueous sodium borohydride (0·2 ml of a 1 mg ml^{-1} solution) was added to the derivatized enzyme and the mixture stirred for 1 h at room temperature before being dialysed overnight against phosphate-buffered saline (PBS) at 4°C.

The enzyme-linked R-protein was purified by FPLC. Aliquots (200 μl) were injected on to a Superose 6 column and eluted with PBS, flow rate 0·4 ml min^{-1}. Using a 5% cut off, two peaks were observed. Only the first showed both B_{12}-binding and HRP activity and was subsequently stored in glycerol/water (1 : 1, v/v) at −20°C until required for use in the B_{12} assay.

Production of B_{12}–KLH conjugate

The solid phase of the assay was provided by means of a B_{12}–KLH conjugate immobilized to the well surfaces of microtitration plates. The conjugate was produced by adding DL-dithiothreitol (15 mg) to a solution of KLH (21·7 mg in 8M urea, 1 mM EDTA and 50 mM phosphate buffer, pH 8·0, 20 ml) and leaving to react for 45 min. Meanwhile, bromoacetyl bromide (3·26 μl) was added to cyanocobalamin (25·5 mg) in dry acetyl formamide (5 ml) and allowed to incubate for 3 min before the reaction was stopped by the addition of 0·5 M phosphate buffer (pH 7·8, 2 ml). The activated cyanocobalamin was added to the reduced, denatured KLH and left stirring in the dark for 18 h at room temperature before dialysing against water. The product was lyophilized and stored at 4°C until required.

Preparation of microtitration plates coated with B_{12}–KLH

Each well of a microtitration plate was passively coated with the B_{12}–KLH conjugate (300 μl of a 5 μg ml^{-1} solution in 0·05 M carbonate/bicarbonate buffer, pH 9·6). After incubation at 4°C for 16 h the plates were washed three times with water, air-dried and stored in the dark at room temperature until required. Plate washing procedures in this and subsequent stages were performed using a Titertek Microplate Washer 120 (Flow Labs. Ltd, Rickmansworth, Herts, UK).

Sample preparation

The following extraction procedure was developed to be compatible with the maintenance of protein-binding activity. The extraction buffer was prepared by dissolving anhydrous disodium hydrogen phosphate (6·5 g) and sodium citrate (6 g) in 1 litre of water/methanol (1 : 1, v/v). A finely ground sample (10 g) was weighed into a centrifuge tube. Extraction buffer (40 ml) was added and the mixture was shaken for 20 min before being centrifuged at 5000 rpm for 10 min. The pellet was twice further extracted with 25 ml and then 15 ml of buffer using the same procedure.

The supernatants of each sample were pooled and, after adjusting the pH to 7·0, made up to 100 ml with extraction buffer (pH 7·0).

Assay protocol

Vitamin B_{12} standards were prepared by serial dilution of cyanocobalamin in an appropriate blank food matrix. Standards or sample extracts (100 μl) in triplicate and R-protein–HRP (100 μl of a 1 : 40, v/v, dilution in PBST) were placed in appropriate wells of a coated plate and incubated overnight at 4°C. The plate was then washed five times with PBST, and peroxidase substrate (200 μl) added to each well. After 30 min the reaction in each well was stopped by the addition of 2 M sulphuric acid (50 μl) and the optical density of each well determined at 450 nm using a Titertek Multiskan MCC plate reader (Flow Labs. Ltd, Rickmansworth, Herts, UK). The level of B_{12} in the sample was quantified by reference to the B_{12} standard curve performed on each plate.

RESULTS AND DISCUSSION

The objective of the present work is the production of technically simple yet rapid methods of analysis for application to food samples. The microtitration plate format seems eminently suitable for such a purpose, being readily applicable to large or small sample numbers and able to be subjected to either fully automated procedures or manual operation as required. The use of enzyme labels and colorimetric assay end-points leads to a requirement for instrumentation of comparatively low cost. An additional benefit is the possibility of applying common technology to the determination of multiple analytes. To this end the authors have previously described enzyme-labelled, microtitration plate procedures for the determination of pantothenic acid (Morris *et al.* 1988; Finglas *et al.*, 1988), biotin (Finglas *et al.*, 1986), the folate group (Finglas *et al.*, 1988*b*,*c*) and pyridoxamine (Alcock *et al.* 1990). Such procedures have been based on enzyme-linked immunosorbent assays (ELISA tests) or, as in the present report, protein-binding assays (PBAs).

Accordingly, the authors have examined the use of R-protein in a PBA for vitamin B_{12} suitable for food application. R-protein recognizes all corrinoids (cobalamins and non-active analogues) (Herbert & Colman, 1985) whereas intrinsic factor (used in some radioassays for clinical application) recognizes only cobalamins. In application to fortified food where a single vitamin form predominates, then the use of R-protein might be preferred because of its lower relative cost. Preparation of the binding protein–enzyme conjugate followed standard conjugation procedures; no problems with conjugate stability were encountered.

For use in the immobilized phase of the assay, B_{12}–KLH was synthesized by the bromoacetyl bromide method, in contrast to previous work on B_{12} which employed conjugates synthesized using the carboxylamide groups on the corrin ring (Olesen *et al.*, 1971; Van de Weil *et al.*, 1974). The authors observed that the bromoacetyl bromide conjugation procedure gave improved assay sensitivity as judged colorimetrically, and allowed the use of R-protein–HRP at a much higher dilution (Alcock, S. & Morgan, M. R. A., unpublished). These observations were made with B_{12}–KLH conjugates (synthesized by the bromoacetyl bromide method) having a lower B_{12}/KLH ratio than the conjugates synthesized using the carboxylamide groups, which might account for the improved assay sensitivities. The authors have previously utilized the bromoacetyl bromide procedure for conjugation of pantothenic acid to protein (Morris *et al.*, 1988) and believe it should receive greater prominence in the methodology available of protein–hapten conjugation.

Microtitration plates coated with B_{12}–KLH conjugate have been found to be stable for up to one year or more when stored at room temperature in the dark.

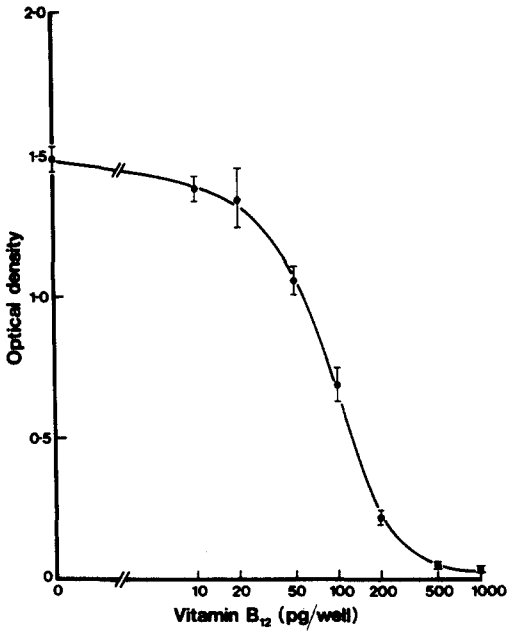

Fig. 1. Standard curve for vitamins B_{12}. Points are means of triplicates ± 1 SD.

Similarly, batches of R-protein–HRP conjugate can be kept at −20°C for several months without any appreciable loss of either enzyme activity or vitamin-binding capacity.

The results for the assay were obtained using the overnight incubation procedure. For a laboratory-based test, such a format is convenient. For wider application, shorter time periods may be of greater interest and it was observed (data not presented) that a 1 h incubation at 37°C gave apparently similar results to those obtained at 4°C with an overnight incubation. Such flexibility is an important facet of microtitration plate procedures.

The standard curve for B_{12} had a limit of detection (determined by subtracting two standard deviations from the mean zero value) of 9 pg per well and a very low background level of binding (Fig. 1).

Each batch of B_{12}–KLH conjugate is sufficient to coat 1000 microtitration plates at the 5 μg ml^{-1} level. The purified R-protein–HRP is used at a dilution of 1 : 40 in the assay and each purified batch is sufficient for 50 plates. The B_{12} standard curve (Fig. 1) is not only reproducible between assays using the same batches of plate-coating and enzyme-labelled R-protein conjugates, but also different batches of reagents. One of the major criticisms of the microbiological assay for vitamin B_{12} is the poor reproducibility found between assay runs performed on different days and also between different laboratories. The use of the PBA, therefore, can help to overcome this problem.

Extracts of non-fortified samples of different foodstuffs did not appear to interfere with the analysis as judged by comparison with behaviour of standards in assay buffer. Standard curves prepared in non-

Table 1. Recovery of vitamin B_{12} added to non-fortified cornflakes prior to extraction as measured by the protein-binding assay. Mean recovery = 82% (SD = 8·2%). Duplicate samples were extracted and measured at each level

Added B_{12} (ng per 100 g)	Recovered B_{12} (ng per 100 g)	% Recovery
50	39	78
100	83	83
150	108	71
200	185	93
400	340	85

fortified ordinary cornflakes and crunchy nut non-fortified cornflakes had correlation coefficients of 0·971 and 0·972, respectively. As well as demonstrating the lack of matrix interference, this also indicates that different types of cornflakes can be analyzed using the same procedures.

Table 1 shows the recovery of B_{12} added to unfortified cornflakes prior to extraction. Even though the analysis of fortified foods allows the use of a simplified extraction procedure compared to that normally used prior to microbiological assay for non-fortified foods, the recovery is less than quantitative, but is reproducible. Assuming that this is not a problem of fortification, then the results might suggest that it is the extraction procedure rather than the assay that contributes to the variability observed. This is borne out by measurement of intra- and inter-assay coefficients of variation on a homogeneous fortified cornflake sample. Values of 9·4% (n = 11, x = 1·44 μg per 100 g) and 9·7% (n = 7, x = 1·30 μg per 100 g), respectively, were obtained.

Table 2 shows the B_{12} content, as measured by the assay, of three retail samples of different fortified breakfast cereals and four non-fortified samples. As would be expected, the non-fortified samples were below the assay sensitivity of 0·09 μg per 100 g. The fortified samples gave values within the expected range provided by the manufacturers.

The protein-binding assay described in this work for the determination of vitamin B_{12} in fortified breakfast cereals can offer a number of advantages over the existing microbiological assay, notably improved reproducibility, easier to perform and much-reduced assay times. The PBA has the added advantage that it does not require radioisotopes as in the radioassay, and the reagents used are stable for up to 1 year.

Table 2. Analysis of vitamin B_{12} content (μg per 100 g) of retail samples of breakfast cereals, as measured by protein-binding assay, and label declarations (assay sensitivity = 0·09 μg per 100 g; each sample measured in duplicate)

Sample type	B_{12} content
Cornflakes	
fortified	1·3
non-fortified	<0·09
Crunchy nut cornflakes	
fortified	2·8
non-fortified	<0·09
Bran	
fortified	2·8
non-fortified	<0·09

Further work is needed to enable application of the assay to non-fortified foods. This could be achieved by improving assay sensitivity, by developing new extraction procedures of greater efficiency and with the possibility for analyte concentration.

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