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Analytical Methods Section

Comparison of a competitive binding assay with *Lactobacillus leichmannii* A.T.C.C. 7830 assay for the determination of vitamin B_{12} in foods

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The results obtained by a competitive binding method for the determination of vitamin B_{12} in food were compared with those obtained by a widely used microbiological assay with *Lactobacillus leichmannii* A.T.C.C. 7830. Both assays were performed on the same sample extract. The extraction was carried out at pH 4·5, 121°C for 10 min with 0·1 M sodium acetate–acetic acid buffer in the presence of potassium cyanide. A high correlation (r^2 = 0·841) between the results from the two methods was found. In the case of pork and yoghurt, the large differences observed could be due to the presence of substances in their extracts which interfere with the assays. The competitive binding assay can therefore be applied to the determination of vitamin B_{12} in some foods.

INTRODUCTION

The microbiological assay using *Lactobacillus leichmannii* A.T.C.C. 7830 is at present the most widely used method for determination for vitamin B_{12} in foods. This method is described by the Association of Official Analytical Chemists (AOAC, 1984). It is, however, time-consuming: it requires from two to three days to complete.

For many years, competitive binding assays employing specific vitamin B_{12} binding protein have been used by clinical laboratories to monitor serum levels of vitamin B_{12}. These assays are rapid and can be performed within 8 h. The basic principle of the assay is that by allowing a fixed amount of isotope-labelled vitamin B_{12} to react with a specific vitamin B_{12} binding protein of limited capacity, the labelled vitamin B_{12} saturates the binding protein and is thus partitioned into two moieties, free and bound. An unknown amount of unlabelled vitamin B_{12} may therefore be quantitated by comparing its distribution (into free and bound forms) with distributions yielded by a set of standards comprising known amounts of vitamin B_{12} introduced into the system.

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Owing to its speed and the ease in which the competitive binding assay can be performed, several investigators (Richardson *et al.*, 1978; Beck, 1979; Marcus *et al.*, 1980; Casey *et al.*, 1982; Bennink & Ono, 1982; Osterdahl *et al.*, 1986) have tried to apply it to selected foods. The vitamin B_{12} binding proteins used by these investigators were intrinsic factor and chicken serum binding proteins. The source of the intrinsic factor was not stated but most of the commercially available intrinsic factor is obtained from hog.

Comparison of the competitive binding assay with *Lactobacillus leichmannii* A.T.C.C. 7830 assay for the determination of vitamin B_{12} in foods has been performed (Richardson *et al.*, 1978; Casey *et al.*, 1982; Osterdahl *et al.*, 1986). Good correlation between the two assays was obtained in two out of the three studies reported. Richardson and his associates (1978) found that the agreement between the two methods was very good in some instances and poor in others. Wherever there was a difference between the results from the two methods, the competitive binding assay gave the lower figure. They attributed this to the extraction method not releasing all of the vitamin B_{12} in a form capable of binding to intrinsic factor, although it was all usable by the microorganism. It was also suggested that the samples may contain substances that are capable of stimulating the growth of the microorganism.

This study was therefore carried out in order to examine the comparability of the microbiological assay using *Lactobacillus leichmannii* A.T.C.C. 7830 and the

competitive binding assay which employs hog intrinsic factor as its binding protein.

MATERIALS AND METHODS

The procedures in this study were carried out under fluorescent light and the ten food samples analysed were chicken liver, lamb, egg, beef, cheese, flounder, pork, chicken, milk and yoghurt.

Extraction of food samples

About 1·0 g of each homogenised food sample was placed into a 50 ml Oak Ridge polypropylene centrifuge tube and a freshly prepared 0·1 M sodium acetate–acetic acid buffer (pH 4·5) which contained potassium cyanide (to final concentration of 1 mM) (30 ml) was added.

The contents of the tubes were sonicated while in an ice-bath for 30 s at full power using a Branson B-12 sonifier. The pH of the contents of all the tubes was 4·5. After sonication, the tubes were capped loosely and autoclaved at 121°C for 10 min.

The tubes were cooled and then centrifuged at 12 000 *g* for 15 min at 20°C in a Beckmann J-21B centrifuge with a JA-20 rotor. An aliquot of each extract (supernatant), as listed in Table 1, was taken and adjusted to about 40 ml with distilled water. The pH of the solution was adjusted to 6·0 with 0·1–1·0 M sodium hydroxide and distilled water was added to make the volume of the solution 100·0 ml (50·0 ml for yoghurt extract).

Lactobacillus leichmannii A.T.C.C. 7830 assay

Lots of the diluted food extracts (1·0 ml) were distributed, in triplicate, into McCartney bottles. In addition to this, McCartney bottles containing increasing amounts of cyanocobalamin (CN-Cbl) as the calibration standards were also prepared. To these bottles, in triplicate, 0·00 (for uninoculated blanks), 0·00 (for inoculated blanks), 0·25, 0·50, 0·75, 1·00, 1·25, 2·50, 3·75 and 5·00 ml of aqueous 63·6 pM CN-Cbl were added. The volume of the solution in each of the bottles was then made to 5·0 ml with distilled water.

Lots of Bacto B-12 assay medium USP (5·0 ml) (Difco Laboratories) were dispensed into the bottles and they were autoclaved at 121°C for 5 min. After cooling, each bottle, except for the uninoculated blanks, was inoculated aseptically with inoculum (50 μl) and the bottles were then incubated at 37°C for 48 h. The inoculum contained 0·1 mg dry cells of *Lactobacillus leichmannii* A.T.C.C. 7830 per 10·0 ml of suspension (Lichtenstein & Reynolds, 1957).

Table 1. Volumes of food extracts analysed

Food	Volume (ml)
Chicken liver	0·5
Lamb	5·0
Egg	5·0
Beef	5·0
Cheese	7·0
Flounder	7·0
Pork	20·0
Chicken	20·0
Milk	20·0
Yoghurt	25·0

Following incubation, the bottles were brought to room temperature and 1% Dow Corning antifoam C emulsion (50 μl) was added to each of them. With an uninoculated blank as reference, the absorbance of the inoculated blanks was determined in a calibrated LKB Biochem Novaspec 4049 spectrophotometer at 620 nm. The uninoculated blank used was the one which showed an absorbance, when read against distilled water, closest to the average absorbance of the three uninoculated blanks prepared. Calibration of the spectrophotometer was performed in accordance to the method prescribed by AOAC (1984). The results of the analysis were disregarded, and the analysis was repeated, if (1) any turbidity was present in the uninoculated blanks, or (2) the absorbance of the inoculated blanks corresponded to a dried cell weight of *Lactobacillus leichmannii* A.T.C.C. 7830 which was greater than 0·1 mg per bottle (AOAC, 1984). With absorbance reset at 0·000 using an inoculated blank that was representative of the rest of the inoculated blanks, the absorbance of the contents of the remaining bottles was measured.

A calibration curve was prepared by plotting absorbance readings for each level of CN-Cbl solution used against the amount of CN-Cbl in the respective bottles. The amount of vitamin B_{12} (expressed as CN-Cbl equivalents) in each diluted food extract used was then determined by interpolation from the calibration curve.

Competitive binding assay which employs hog intrinsic factor as its binding protein

The diluted food extracts (1·0 ml) were distributed, in triplicate, into 10 mm × 75 mm polypropylene tubes. In addition, tubes containing increasing amounts of CN-Cbl as the calibration standards were also prepared. To these tubes, in triplicate, 0·00 (for non-specific binding tubes), 0·00 (for total binding tubes), 0·05, 0·10, 0·15, 0·20, 0·25, 0·50, 0·75 and 1·00 ml of aqueous 318 pM CN-Cbl was added. The volume of the solution in each of the tubes was then adjusted to 1·0 ml with distilled water.

To each of the above tubes aqueous [^{57}Co]cyanocobalamin (50 μl, 123 ng ml^{-1}) (i.e. CN[^{57}Co]Cbl) (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) (Diagnostic Products Corporation) was added to each of the tubes except for

the non-specific binding (NSB) tubes. To 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 3000 *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 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:

Net c.p.m. = c.p.m. − average c.p.m. of NSB tubes

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

Bound CN [57Co]Cbl (%) =

$$\frac{\text{Net c.p.m.}}{\text{Average c.p.m. of TB tubes} - \text{average c.p.m. of NSB tubes}} \times 100$$

The calibration curve was prepared by plotting the percentages of bound CN[^{57}Co]Cbl for each level of CN-Cbl solution used against the amount of CN-Cbl in respective tubes. The amount of vitamin B_{12} (expressed as CN-Cbl equivalents) in each diluted food extract used was then determined by interpolation from the calibration curve.

RESULTS AND DISCUSSION

Figure 1 shows that there is a high correlation between the microbiological assay using *Lactobacillus leichmannii* A.T.C.C. 7830 and the competitive binding assay which employs hog intrinsic factor as its binding protein. The coefficient of determination (r^2) for nine of the samples using both methods was 0·841. There was also no significant difference (*t*-test, $P > 0{\cdot}50$) between the vitamin B_{12} content of chicken liver when determined by the two methods although the values were too high to be included in the correlation analysis.

Based on the *t*-test, however, the agreement between the two methods was very good in eight out of the ten foods analysed. In the case of pork, the competitive binding assay gave the higher figure, whilst the opposite was observed for yoghurt. The former finding may be attributed to the presence of substances in the pork extract which inhibit the growth of the *Lactobacillus leichmannii* A.T.C.C. 7830, whilst the latter could be due to substances in the yoghurt extract which stimulate the growth of the microorganism instead, thus leading to erroneous results. Alternatively, there may be substances in the yoghurt extract which can reduce the binding of vitamin B_{12} to hog intrinsic factor.

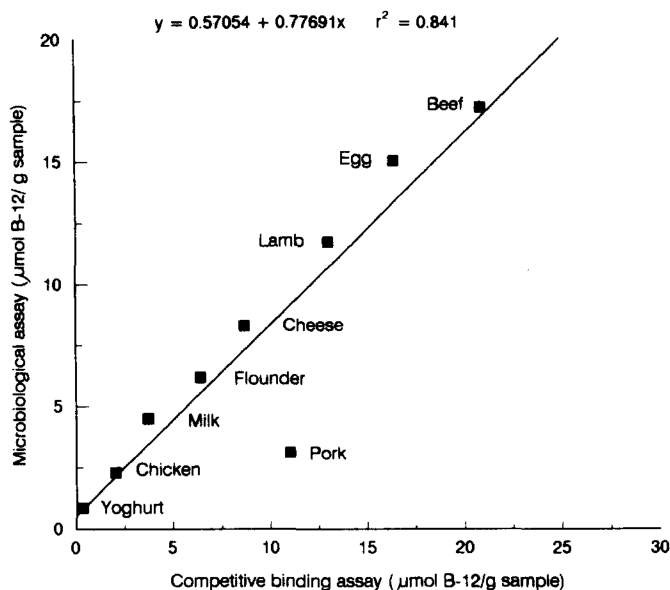

Fig. 1. Correlation between the microbiological method using *Lactobacillus leichmannii* A.T.C.C. 7830 and a competitive binding assay which employs hog intrinsic factor as its binding protein. The vitamin B_{12} content of the food samples was expressed as CN-Cbl equivalents. Both assays were performed on the same sample extract. Data are mean values from triplicate analyses of each sample. The extraction of 1·0 g of each sample was carried out at pH 4·5, 121°C for 10 min with 0·1 M sodium acetate—acetic acid buffer (30·0 ml) in the presence of potassium cyanide (30 μmol). Previous findings indicate that this procedure is efficient in extracting vitamin B_{12} from foods (Muhammad, 1990). The values obtained for chicken liver using the microbiological and competitive binding assays were 285 and 275 μmol g^{-1}, respectively.

Using completely different food samples, Richardson and his associates (1978) found that in all instances where there was a difference between the results from the competitive binding assay and the microbiological method, the former gave the lower figure. These investigators did not obtain instances where the competitive binding assay gave the higher figure.

The forms of vitamin B_{12} that have been identified in foods are hydroxocobalamin, cyanocobalamin, sulphitocobalamin, adenosylcobalamin and methylcobalamin (Farquharson & Adams, 1976). The addition of excess cyanide in the extraction procedure converts the first four of these cobalamins into dicyanocobalamin whilst methylcobalamin remains unchanged (Muhammad, 1990). The experimental procedure, however, was carried out under fluorescent light; this results in the conversion of methylcobalamin into hydroxocobalamin which is further converted into dicyanocobalamin in the presence of excess cyanide. Dicyanocobalamin was found to be stable under the conditions employed.

Solutions of cyanocobalamin were used as the calibration standards, as (1) the growth responses of *Lactobacillus leichmannii* A.T.C.C. 7830 to equal amounts of cyanocobalamin and dicyanocobalamin and (2) the binding affinity of cyanocobalamin and dicyanocobalamin for hog intrinsic factor were found to be similar (Muhammad, 1990). In addition, the results obtained by interpolation from a calibration curve prepared using

dicyanocobalamin instead of cyanocobalamin showed no significant difference (t-test, $P > 0.20$).

This study can only indicate whether the two assays achieve the same results, and does not establish whether one is more accurate than the other. *Lactobacillus leichmannii* A.T.C.C. 7830 is known not to be specific for vitamin B_{12} and responds to deoxyribosides and vitamin B_{12} analogues which exist in human serum (Kolhouse *et al.*, 1978), erythrocytes, liver and brain (Kanazawa & Herbert, 1983) but it has not been established whether these analogues are present in foods. The competitive binding assay which employs hog intrinsic factor as its binding protein is supposed to be more specific for vitamin B_{12}, as intrinsic factor binds with a very narrow spectrum of corrinoids (Lien *et al.*, 1973).

CONCLUSION

The competitive binding assay which employs hog intrinsic factor as its binding protein has potential for application to the analysis of vitamin B_{12} in foods. It has two advantages over the *Lactobacillus leichmannii* A.T.C.C. 7830 method. Firstly, it is quicker to perform and, secondly, it is more specific for vitamin B_{12}. Nevertheless, it is a more expensive method.

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