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Analytical Methods Section

The appropriateness of using cyanocobalamin as calibration standards in *Lactobacillus leichmannii* A.T.C.C. 7830 assay of vitamin B_{12}

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The appropriateness of using solutions of cyanocobalamin as the calibration standards in *Lactobacillus leichmannii* A.T.C.C. 7830 assay of total vitamin B_{12} in foods, plasma and serum was examined. This is because the forms of the vitamins that may be present after the addition of sodium cyanide, potassium cyanide, sodium metabisulphite or sodium nitrite in the sample extraction procedure are hydroxocobalamin, sulphitocobalamin, cyanocobalamin, adenosylcobalamin, methylcobalamin, dicyanocobalamin and nitritocobalamin, depending on the form of endogenous vitamin B_{12}. Therefore, if *Lactobacillus leichmannii* A.T.C.C. 7830 has a higher or lower growth response to cyanocobalamin than to the other potential forms of cobalamin, the amount of vitamin B_{12} determined by comparisons with calibration standards prepared from cyanocobalamin will be an under- or over-estimation. The results of this study showed that the growth response of *Lactobacillus leichmannii* A.T.C.C. 7830 to cyanocobalamin was similar to its growth responses to hydroxocobalamin, sulphitocobalamin, dicyanocobalamin and nitritocobalamin but lower than that to adenosylcobalamin and higher than that to methylcobalamin. Thus, total vitamin B_{12} cannot be measured accurately using *Lactobacillus leichmannii* A.T.C.C. 7830 assay which employs cyanocobalamin as its calibration standards if adenosylcobalamin or methylcobalamin is present.

INTRODUCTION

Microbiological assays of vitamin B_{12} in foods, serum and plasma are based on the observation that certain microorganisms can reproduce only in the presence of the vitamin. The assays require a medium deficient in vitamin B_{12} but otherwise able to support dense growth of the test organism. When vitamin B_{12} in sample extracts is added to the assay medium followed by inoculation with the test organism, the organism grows in proportion to the dose of the vitamin. This growth is measured in terms of the turbidity produced or by monitoring a chemical by-product of growth such as acidity. Over a certain vitamin B_{12} concentration range, the measured growth response will be directly proportional to the amount of vitamin B_{12} present, and, within this range, the sample extracts and calibration standards can be compared.

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Extraction of vitamin B_{12} from foods, serum or plasma involves the addition of excess cyanide (sodium or potassium cyanide), sodium metabisulphite or sodium nitrite (Beck, 1979; Nexo & Olesen, 1982; AOAC, 1984). These compounds are known to have a stabilizing effect on hydroxocobalamin. Besides hydroxocobalamin (OH-Cbl), the forms of vitamin B_{12} that have been identified in foods are cyanocobalamin (CN-Cbl), sulphitocobalamin (HSO_3-Cbl), adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) (Farquharson & Adams, 1976). These cobalamins, with the exception of HSO_3-Cbl, were also found in serum and plasma (Nexo & Olesen, 1982).

Calibration standards that are used in the microbiological assay of vitamin B_{12} in foods, serum and plasma are usually prepared from CN-Cbl (Nexo & Olesen, 1982; AOAC, 1984). The vitamin B_{12} extracted from the aforementioned samples (after addition of sodium cyanide, potassium cyanide, sodium metabisulphite or sodium nitrite), however, could be in the form of HSO_3-Cbl, CN-Cbl, AdoCbl, MeCb, dicyanocobalamin (diCN-Cbl) and nitritocobalamin (NO_2-Cbl), depending on the form of the endogenous vitamin B_{12} (Muhammad, 1990). Thus, the accuracy of a microbiological assay

will vary according to the forms of the extracted vitamin B_{12}, unless the organism employed exhibits equal growth responses to HSO_3-Cbl, AdoCbl, MeCbl, diCN-Cbl, NO_2-Cbl and CN-Cbl.

A study was therefore undertaken to determine the growth responses of *Lactobacillus leichmannii* A.T.C.C. 7830 to OH-Cbl, HSO_3-Cbl, CN-Cbl, AdoCbl, MeCbl, diCN-Cbl and NO_2-Cbl. *Lactobacillus leichmannii* A.T.C.C. 7830 is the most widely used organism in the microbiological determination of vitamin B_{12} (Schneider & Stroinski, 1987).

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 of 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 16 μM aqueous 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 McCartney bottles were labelled as follows: uninoculated blank (3 bottles), inoculated blank (3), OH-Cbl (24), HSO_3-Cbl (24), CN-Cbl (24), AdoCbl (24), MeCbl (24), diCN-Cbl (24) and NO_2-Cbl (24); then 0·25, 0·50, 0·75, 1·00, 1·25, 2·50, 3·75 and 5·00 ml of aqueous solutions of 64·4 pM OH-Cbl, 64·4 pM HSO_3-Cbl, 62·0 pM CN-Cbl, 66·8 pM AdoCbl, 65·6 pM MeCbl, 64·4 pM diCN-Cbl and 64·4 pM NO_2-Cbl were added to the appropriate bottles in triplicate. The volume of the solution in each bottle was then adjusted to 5·0 ml with distilled water. The uninoculated and inoculated blanks each had 5·0 ml of distilled water added to them.

Lots of Bacto B-12 assay medium USP (5·0 ml) (Difco Laboratories) were then dispensed into all the McCartney bottles and the bottles 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).

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 Biochrom 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 was 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.

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

RESULTS AND DISCUSSION

Figure 1 shows that *Lactobacillus leichmannii* A.T.C.C. 7830 exhibits a similar growth response to OH-Cbl, HSO_3-Cbl, CN-Cbl, diCN-Cbl and NO_2-Cbl ($P > 0·20$) but this growth response is less than that to AdoCbl ($0·05 < P < 0·10$) and more than that to MeCbl ($0·01 < P < 0·02$).

The findings indicate that accurate determinations of (1) OH-Cbl, HSO_3-Cbl, CN-Cbl and AdoCbl extracted from foods, serum or plasma in the presence of excess cyanide, and (2) OH-Cbl, HSO_3-Cbl and CN-Cbl extracted in the presence of sodium metabisulphite or

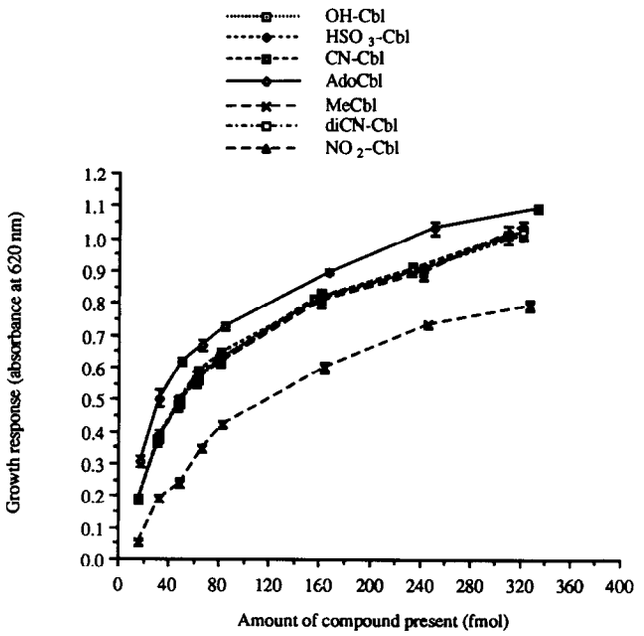

Fig. 1. Growth responses of *Lactobacillus leichmannii* A.T.C.C. 7830 to OH-Cbl, HSO_3-Cbl, CN-Cbl, AdoCbl, MeCbl, diCN-Cbl and NO_2-Cbl. Data are mean values and standard deviations from triplicate analyses.

sodium nitrite, can be made with the *Lactobacillus leichmannii* A.T.C.C. 7830 assay if the calibration standards employed are prepared using CN-Cbl. The amount of AdoCbl extracted in the presence of sodium metabisulphite or sodium nitrite, however, will be overestimated and the amount of MeCbl extracted in the presence of cyanide, sodium metabisulphite or sodium nitrite will be underestimated. This is because OH-Cbl, HSO_3-Cbl, CN-Cbl and AdoCbl are converted to diCN-Cbl in the presence of excess cyanide while MeCbl remains unchanged. In the presence of sodium metabisulphite and sodium nitrite, OH-Cbl is converted to HSO_3-Cbl and NO_2-Cbl, respectively, while HSO_3-Cbl, CN-Cbl, AdoCbl and MeCbl remain unchanged (Muhammad, 1990).

The major forms of vitamin B_{12} in serum and plasma are AdoCbl and MeCbl (Nexo & Olesen, 1982) while AdoCbl and OH-Cbl are the predominant forms in foods in terms of frequency of occurrence and of quantity (Farquharson & Adams, 1976). AdoCbl and MeCbl, however, can be converted to OH-Cbl in the presence of light. Under aerobic conditions, the times taken for complete conversion of AdoCbl and MeCbl into OH-Cbl after exposure to light from a 15 W Osram white bulb at a distance of 20 cm are 20 and 15 min, respectively (Farquharson & Adams, 1977). The time taken for complete conversion of AdoCbl and MeCbl into OH-Cbl under the fluorescent light in our laboratory was less than 1 h.

CONCLUSION

Accurate measurement of vitamin B_{12} in foods, serum and plasma can be made using *Lactobacillus leichmannii* A.T.C.C. 7830 assay and CN-Cbl as the calibration standards if the sample extracts are exposed to light before their analyses.

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