Radiometric microbiological assay of B vitamins. Part 1: assay procedure

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Overview

A variety of analytical methods have been described for the analysis of B vitamins. Among these, microbiological assays have been the most widely used and accepted method for the analysis of B vitamins in complex biological samples. Microbiological assays are based on the growth requirement of a test microorganism for a specific vitamin. The specific vitamin is exogenously added in suboptimal amounts so that microbial growth is limited by the vitamin content of the medium. The turbidity resulting from bacterial growth in a series of known vitamin standards is measured and used to develop a standard curve. The turbidity resulting from the growth of the microorganism in the unknown samples is then compared and the vitamin content of the sample determined. One difficulty encountered with conventional turbidimetric-microbiological assays is that they require extensive sample preparation and the procedure is tedious and time-consuming.

Radiometric-microbiological assays (RMAs) were developed to eliminate some of the technical problems associated with the conventional turbidimetric-microbiological method. The RMA is based on the measurement of $^{14}CO_2$ from the metabolism of a ^{14}C-labeled substrate by the test organism in the presence of the specific vitamin to be analyzed. In this laboratory, we have used the yeast *Kloeckera brevis*, which has an absolute requirement for niacin, biotin, thiamine, pantothenic acid, and vitamin B-6, as the test organism and L-1-^{14}C-valine as the radiolabeled substrate. The RMA method for these vitamins has been validated and applied to a variety of biological samples.[1-7] In this communication, we describe the RMA procedure for niacin, biotin, thiamine, and pantothenic acid.

Reagents

All solutions are prepared with distilled/deionized water.

Inoculum broth. Difco micro-inoculum broth (Difco Laboratories, Detroit, MI, USA) is used for the growth of the yeast *K. brevis*. The powder is rehydrated according to manufacturer's instructions and 10 ml aliquoted into 16 × 125 mm culture tubes (Fisher Scientific, Columbia, MD, USA). The test tubes are capped and autoclaved for 15 min at 121° C. The sterile test tubes are allowed to cool and kept refrigerated at 2–4° C for subsequent use.

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YM agar slants. Difco YM agar (Difco Labs) is rehydrated by resuspending 41 g in 1 L of water and heating to a boil to dissolve completely. The media is dispensed into culture test tubes in 8–10 ml aliquots, sterilized at 121° C for 15 min, and allowed to cool and hardened with the test tubes at a 5–10° angle from horizontal. Test tubes are stored at 2–4° C for subsequent use.

Vitamin solutions for preparation of assay growth media. Four different vitamin solutions are made containing all of the essential vitamins for the growth of the yeast *K. brevis* with the exception of the one vitamin to be assayed. For example, if the vitamin to be assayed is biotin, then the vitamin solution would contain all of the other vitamins except biotin. The vitamin concentrations in these solutions are: d-biotin, 0.2 μg/ml; d-calcium pantothenate, 40 μg/ml; folic acid, 0.2 μg/ml; inositol, 200 μg/ml; *p*-aminobenzoic acid, 20 μg/ml; riboflavin, 20 μg/ml; nicotinic acid, 40 μg/ml; thiamine HCl, 40 μg/ml; and pyridoxine HCl, 40 μg/ml. Each vitamin solution is stored at −70° C in 25 ml aliquots. A 25 ml aliquot is kept at 2–4° C for weekly use.

Vitamin standard solutions. Stock standard solutions of 100 μg/ml of nicotinic acid (niacin), thiamine, biotin, and pantothenic acid are prepared in deionized water and stored in 2 ml aliquots at −70° C. On the day of the assay, the stock solution for the vitamin to be assayed is thawed and diluted with water to the appropriate concentration to be added to the test vials for the development of a standard curve.

Nonradioactive valine solution. A 2.0 mg L valine/ml water solution is made and kept frozen at −70° C in 4 ml aliquots. This solution along with the vitamin solution is added to the basal medium to make the assay medium.

L-1-[^{14}C] valine solution: L-1-^{14}C-valine (specific activity 55–56 mCi/mmole) is obtained from Research Products International (Mount Prospect, IL, USA). A 10 μCi/ml L-1-^{14}C-valine stock solution is made in sterile deionized water and filtered through 0.22 μm filter into a sterile, rubber-capped serum vial. The stock solution is kept refrigerated. From this stock solution, a 0.25 μCi/0.1 ml working solution is prepared in a similar fashion, and 0.1 ml is used for inoculating each assay vial. The specific concentration of all solutions is determined using liquid scintillation spectrometry. It should be noted that L-1-^{14}C-methionine has also been used as the radioactive substrate.[3-6] However, we have found L-1-^{14}C-valine to be a better substrate with longer shelf-life.

Vitamin assay media preparation. The assay medium used is Vitamin Free Yeast Base Medium from Difco Laboratories. This base medium contains all of the essential nutrients for optimal yeast growth but lacks all of the essential vitamins. This allows the investigator control of the vitamin composition of the assay medium. By exogenously adding the appropriate concentration of vitamins needed for growth, with the exception of the one wanted to be assayed, the same basal medium can be used for the assay of niacin, biotin, thiamine, and pantothenic acid. The medium is prepared prior to assay as follows: Five ml of one of the four vitamin solutions, based on which vitamin is going to be assayed, and 3.13 ml of the nonradioactive 2 mg/ml L-valine solution are added to 8.35 g of vitamin free yeast base medium powder to make 100 ml of assay media with deionized water.

Microorganism maintenance and inoculum preparation

Kloeckera brevis (apiculata) ATCC 9774 (American Type Culture Collection, Rockville, MD, USA) is maintained on YM agar slants with three to four weekly subcultures. From a fresh agar slant incubated at 30° C overnight, one loop (1.5 mm diameter loop) full of *K. brevis* cells is aseptically transferred to a Micro-Inoculum broth test tube that had been warmed to room temperature. The cell suspension is vortexed to disperse the yeast cells. With the test tube cap slightly opened, the yeast suspension is incu-

bated in a horizontal position at a 5–10° angle for 20–22 hr at 30° C. Following incubation, the yeast suspension is centrifuged at 900*g* for 10 min to obtain a pellet of yeast cells. The supernatant is decanted and the pellet resuspended in 10 ml sterile saline. The pellet is vortexed, and the centrifugation step is repeated. The resulting pellet is resuspended in 10 ml sterile saline. A 1:50 or 1:100 dilution of the suspension is made, and 0.1 ml is used to inoculate each vial. It should be noted that the degree of dilution depends on how rapidly the yeast is growing. The routine maintenance of the test organism can be eliminated by lyophilizing the yeast cells and storing the lyophilized cultures at 2–4° C. Note: This yeast does not grow at 37° C.

Lyophilization of *Kloeckera brevis* cells

Yeast cells are prepared as for inoculum preparation with the exception that after the last centrifugation step the cells are resuspended in 1 ml of sterile saline. One tenth of a ml of this concentrated yeast suspension is placed into sterile lyophilization ampules and prefrozen in a dry ice–10% methanol solvent bath. Yeast cells are lyophilized by using an automated freeze drier (Virtis Company, Gardiner, NY, USA). Ampules are heat-sealed under vacuum while still connected to the drying chamber. Sealed ampules are then stored at 2–4° C until used.

Radiometric-microbiological assay procedure

To 20 ml serum vials (Arthur H. Thomas, Philadelphia, PA, USA), 2 ml of assay media preparation and vitamin standard solutions are added to make a 1–20 ng/vial standard curve for niacin, 1–15 ng/vial for thiamine and pantothenic acid, and a 10–250 pg/vial standard curve for biotin. Each standard curve usually has 8 vitamin levels, including a blank with no vitamin added. For analysis of biological samples, 0.5 ml of extracted, diluted sample is added to the vial. Two or three different dilutions of the unknown sample are generally used. Some extraction methods have been previously published, and specific details of these methods will appear in a future issue of this journal.[3-6] Total volume in standard curve and biological samples vials is made up to 5 ml with deionized water.

All test vials are sealed with aluminum seals (Arthur H. Thomas) fitted with rubber liners (West Company, Phoenixville, PA, USA) and autoclaved for 10 min at 121° C for the assay of niacin, biotin, and pantothenic acid, but only at 100° C for the thiamine assay since this vitamin is heat labile. Assay vials are allowed to cool and each aseptically injected with 0.25 μCi (0.1 ml) of L-1-^{14}C-valine and 0.1 ml of yeast inoculum preparation. Vials are vigorously shaken to mix contents and incubated at 30° C for 18–20 hours. The $^{14}CO_2$ produced from the metabolism of L-1-^{14}C-valine by *K. brevis* in the presence of the specific vitamin is measured using the Bactec 460 (Johnston Laboratories, Cockeysville, MD, USA), an automated gas flow ionization chamber system used in clinical microbiology laboratories. A ^{14}C radioactivity versus vitamin concentration standard curve is obtained and used for the determination of vitamin concentrations in biological samples by interpolation. *Figure 1* shows representative graphs of RMA dose-response curves for thiamine, niacin, pantothenic acid, and biotin.

Discussion

The RMA assay procedure described is essentially the same for the analysis of niacin, biotin, thiamine, and pantothenic acid. The RMA assay procedure for vitamin B-6 has been previously described in detail.[7] The difference among the RMA assay of these vitamins is the extraction procedures used prior to assay. Details of extraction procedures will be provided in a future issue of the *Journal of Nutritional Biochemistry*. For biological flu-

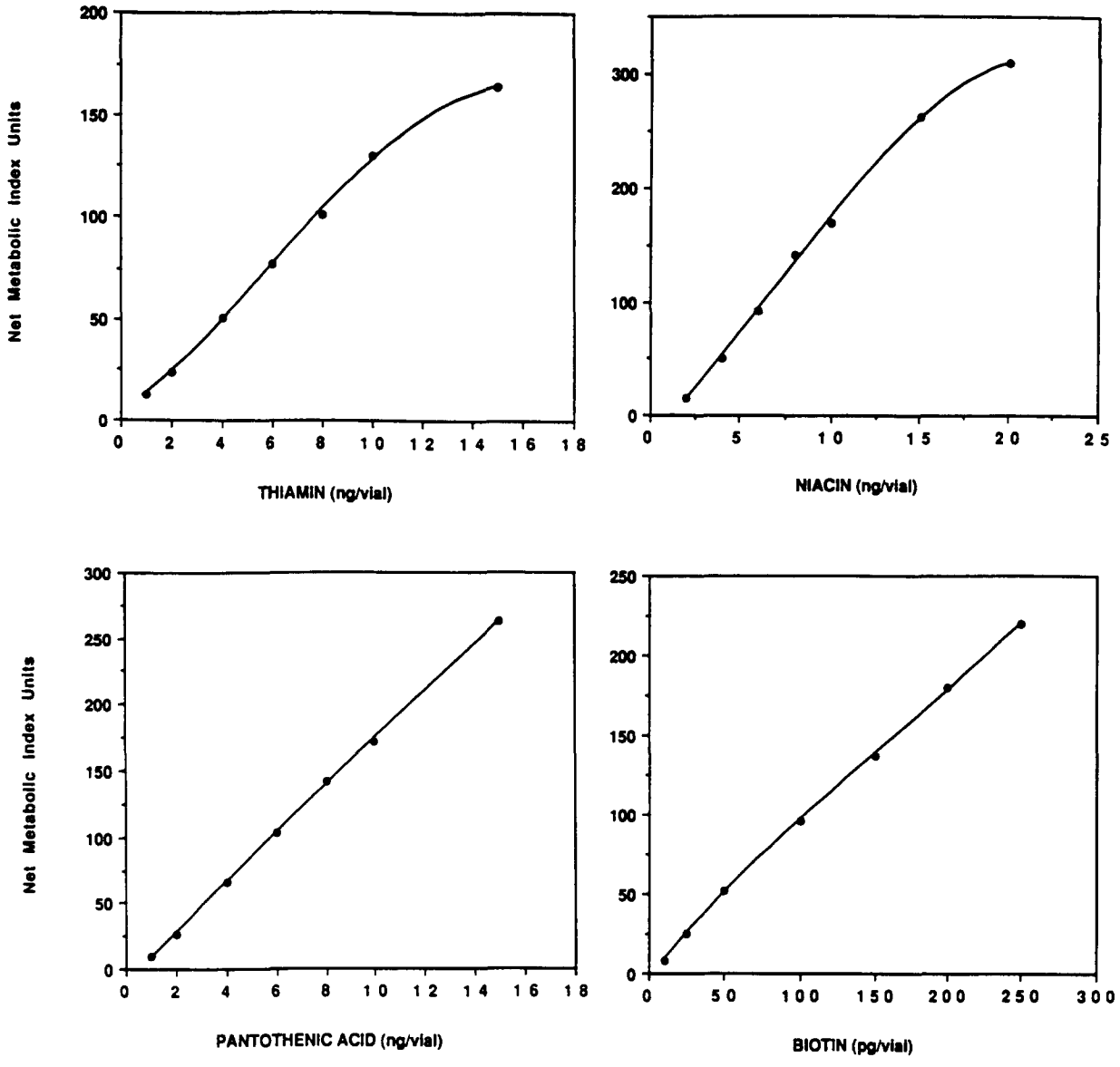

Figure 1 Representative graphs of radiometric-microbiological dose response curves for thiamine, niacin, pantothenic acid, and biotin. Net $^{14}CO_2$ production is represented as metabolic index units where 100 units equals 0.031 μCi of ^{14}C radioactivity.

ids such as human plasma or whole blood and milk, the extraction procedure for some of the vitamins, such as niacin and biotin, is simple and can be performed in a single autoclaving step. In the analysis of niacin and biotin in human plasma or whole blood, the sample is diluted with water, added to the test vial, and the autoclaving step (10 min at 121° C) used to sterilize the test vial is sufficient to extract the vitamin. In these cases, the extraction is simple because the vitamins are circulating freely or are easily released from their bound forms. For example, niacin in biological fluids is freely available as nicotinamide or as the coenzyme(s) nicotinamide adenine dinucleotides from which the nicotinamide moiety is readily released by heating.[3]

The extraction method for pantothenic acid is more complex since it requires a double enzyme extraction procedure to release the pantothenic acid moiety from its naturally occurring biologically active form Coenzyme A. This is required because the yeast does not grow in the presence of the intact coenzyme A molecule.

One of the advantages of the *K. brevis* RMA over the conventional turbidimetric-microbiological assays and other assays is that colored samples or precipitated whole blood, plasma, or food debris does not interfere with the accurate measurement of $^{14}CO_2$. With other methods, the samples have to be debris-free and colored samples may not be able to be analyzed. This advantage of the RMAs has significantly simplified sample preparation and assay procedure. The use of semi-automated instrumentation (i.e., Bactec 460) to measure the $^{14}CO_2$ allows for the analysis of large number of samples. These assays can be used in the analysis of samples where a total content of the vitamin is desired; they do not provide levels of specific vitamers unless they are previously separated by chromatographic methods. Nevertheless, the RMAs described can be used for the analysis of five different water-soluble vitamins in complex biological samples with essentially the same assay conditions. Recently, other methods have been developed for the analysis of these vitamins in biological samples.[8-14] These methods are primarily based on high pressure liquid chromatography or radioimmunoassay and radiodilution assay techniques. The selection of an assay method for the analysis of these vitamins should be based on the desired sensitivity, specificity, and applicability to the biological sample to be analyzed.

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