Radiometric microbiological assay of B vitamins. Part 2: extraction methods

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Overview

An important aspect of any assay method for the analysis of B vitamins is the extraction of the vitamin from the biological medium in which it is contained. The accuracy of an assay method in measuring the vitamin content of biological samples will be greatly influenced by the degree of extraction of the vitamin from its biological medium. However, despite their importance, extraction methods are the least studied and least characterized component of vitamin assays.

Several important factors should be recognized in the selecton of a specific extraction procedure for B vitamins. First, B vitamins are present in biological samples as parts of larger molecules or coenzymes. For example, niacin or niacinamide is a small portion of its biologically active forms, the nicotinamide adenine dinucleotides (i.e., NAD and NADP). Second, B vitamins can be present in biological samples in a bound form and in more than one biologically active form of the vitamin. Thus, the extraction method should be able to extract equally all biologically active forms of the vitamin from larger molecules or from bound forms.

For the most part, extraction methods of B vitamins used in conjunction with microbiological assays are based on enzymatic treatment or acid hydrolysis of samples. Acid hydrolysis is generally used to denature proteins and sample matrix with subsequent removal of the vitamin as well as to release the vitamin from the coenzyme molecule. Enzymatic treatment is used to breakdown a parent molecule, in the case of B vitamins, the coenzyme form, and release the vitamin moeity. These procedures are generally compatible with microbiological assays. It should be noted that in the extraction of some vitamins from biological samples, such as blood and human milk, the vitamin can be easily extracted in a simple heating or autoclaving step. For example, studies in this laboratory have shown that niacin in whole blood is easily extracted in the autoclaving step used to sterilize the test vial.[1] Therefore, not all samples require rigorous extraction treatments.

An important consideration with any extraction method is the efficiency of extraction. This is usually difficult to determine, since the "true" endogenous level of the vitamin is not known. However, extraction efficiency can be assessed by adding a known amount of the vitamin, in its biologically active form, to the sample. The complete recovery of the added

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Table 1 Percent recovery of B vitamins in biological samples using the radiometric-microbiological assay

Vitamin	Sample	Percent Recovery
Pantothenic acid	Whole blood	98.5 ± 6.5
Thiamine	Human milk	103.9 ± 7.3
Biotin	Plasma	92.8 ± 7.4
Niacin	Food	99.8 ± 7.1

vitamin will provide a semiquantitative measure of extraction efficiency. *Table 1* provides values for percent recovery of niacin, biotin, pantothenic acid, and thiamine in a variety of biological samples using the radiometric-microbiological assay (RMA) and extraction procedures described in this paper. True extraction efficiency can be measured only by incorporating an isotopically labeled form of the vitamin into the sample. This is a time-consuming procedure and has been applied only to studies of animal tissues and in a few cases to plant products.[2]

Extraction procedures used for the analysis of niacin, biotin, pantothenic acid, and biotin with RMA

Niacin

(a). *Whole blood.* Whole blood is diluted 1:200, 1:300, and 1:400 with deionized water, and 0.5 ml is added to test vials for assay. Red blood cells contain greater than 95% of all the vitamin with very small amounts present in plasma or serum. However, if plasma niacin levels are desirable, the plasma is diluted 1:2 and 1:4 with deionized water. Again, 0.5 ml of diluted plasma is added to test vials for analysis. It should be noted that precipitated protein or red cell debris or food debris does not interfere with the accurate measurement of the $^{14}CO_2$ released from the bacterial metabolism of L-1-^{14}C-valine, the basis of the RMA. Thus, the ability to add the diluted blood or plasma sample directly to the test vial without removal of precipitated debris significantly simplifies sample preparation.

(b). *Food.* One or two grams of ground (e.g., breakfast cereals are ground to a fine powder using a food processor) or homogenized food sample is added to 100 ml of 1 N HCl in 250 ml Erlenmeyer flasks and mixed. The solution is autoclaved for 90 min at 121° C. After cooling, the pH of the food solution is adjusted to 4.5 using 6 N NaOH and the total volume adjusted to a known level, usually 150 ml, with deionized water. Removal of precipitated food debris is not needed since it does not interfere with the RMA.[1,3] Aliquots of the acid-hydrolyzed food sample are stored at −70° C until assay. On the day of the assay, the samples are thawed and diluted 1:100, 1:200, and 1:500 with deionized water; 0.5 ml is added to the test vials for analysis using the RMA as previously described.[3]

Biotin

(a). *Plasma samples.* Plasma is obtained by centrifugation of whole blood at 900*g* for 10 min and stored at −70° C until assay. On the day of the assay, the plasma sample is thawed and diluted 1:2.5, 1:5, and 1:10 with deionized water; 0.5 ml is added to test vials for assay.

(b). *Food.* One or two grams of food or 5 ml of juices are mixed with 40 ml of 2 N H_2SO_4 in 200 ml Erlenmeyer flasks and mixed. Food samples are autoclaved for 90 min at 121° C, cooled, and pH adjusted to 4.5 with 6 N NaOH. The final volume is made up to 100 ml with deionized water and aliquots are stored at −70° C until assay. On the day of the assay, the samples are thawed and diluted in a 1:2 to 1:20 range with deionized water; 0.5 ml is added to the test vials for analysis using the RMA as previously described.[3]

Pantothenic acid

The procedure for extraction of pantothenic acid in whole blood and food is essentially the same. This procedure requires the preparation of two enzymes that are commercially available.

(a). *Alkaline phosphatase solution.* Sigma Type I-S from calf intestine (Sigma Chemical Co., St. Louis, MO, USA) is diluted with 20 mM $KHCO_3$ to make an 8 U/ml solution.

(b). *Pantetheinase enzyme preparation.* Pigeon liver acetone powder is obtained from Sigma (#L8376). This preparation contains the enzyme pantetheinase as well as high endogenous levels of pantothenic acid that must be removed prior to use. Four grams of pigeon liver acetone powder are diluted in 80 ml of 20 mM $KHCO_3$ buffer; the solution is mixed and centrifuged at 12,000*g* for 10 min at 4° C. The resulting supernatant is then purified from endogenous levels of pantothenic acid by passing the solution several times through an AG1-X8 Anion Exchange Resin (HCL form, 200–400 mesh) column (3 × 25 cm of resin) chased with 200 ml of 20 mM $KHCO_3$ buffer. Following each pass, the level of endogenous pantothenic acid is measured to determine the amount of contamination. In a typical preparation, the degree of pantothenic acid contamination is in the order of 2–3 μg/ml enzyme preparation. Following resin treatment, the pantothenic acid contamination can be reduced to approximately 10–15 ng/ml enzyme preparation. Pantetheinase enzyme activity can be determined by the method of Wittwer et al.[4] Enzyme solution is aliquoted and stored frozen at −70° C until used for extraction of whole blood or food samples.

Whole blood extraction procedure. Whole blood is diluted 1:1 with deionized water, and 0.25 ml of hemolyzed blood is added to a test tube containing 0.25 ml of alkaline phosphatase solution and 0.25 ml of purified pantetheinase enzyme solution. The total volume is brought to 1 ml with 20 mM $KHCO_3$ buffer. The contents of the test vial are mixed and incubated at 37° C for 18 h. Following the incubation period, the whole blood–enzyme solution is diluted with deionized water to give a final volume of 1:40, 1:80, and 1:160; 0.5 ml of these dilutions is used for assay.

Food extraction procedure. Ten to 20 grams of food sample are homogenized in approximately 80 ml of water using a blender and the pH adjusted to 6.5–7.0. Ten grams of this pH adjusted food homogenate is autoclaved at 121° C for 10 min. One ml of the autoclaved food samples is added to a test tube containing 0.4 ml of the alkaline phosphatase solution and 0.4 ml of the pantetheinase enzyme preparation. Total volume is made to 2 ml with 20 mM $KHCO_3$ buffer. This food-enzyme solution is incubated at 37° C for 18 h. An enzyme blank is also included. Following incubation, the food-enzyme solution is diluted to three different concentrations ranging from 1:100 to 1:400 with deionized water according to the sample to be analyzed. From these dilutions 0.5 ml is used for assay.

Thiamine

Our experience with the analysis of thiamine has been primarily in human milk samples. Based on initial studies, it appears that thiamine is freely available in the milk, since thiamine levels of milk samples analyzed without any extraction treatment did not differ when the same sample was treated with acid phosphatase, an enzyme used for the extraction of the vitamin in biological samples.[5] The procedure for thiamine analysis in milk samples is as follows: milk samples are diluted 1:25 or 1:50 with deionized water and 0.5 ml is used for assay.

Discussion

The extraction of B vitamins from biological samples is one of the most important steps in any analytical method. Differences in extraction procedures probably introduce the greatest degree of variability when comparing samples using different analytical methods. The literature is filled with

extraction procedures for water soluble vitamins, which have been validated to various degrees. It is imperative that the implementation of any extraction method be validated in each laboratory, and the procedure must be compatible with the analytical method to be used. The extraction procedures described above have been validated in this laboratory for their use with the RMA. Validation studies have not been described, since they are beyond the scope of this paper.

Although the extraction procedures described above are similar to those described in the literature for other microbiological assays of these B vitamins, they differ in that precipitated proteins or food debris is not removed prior to analysis. Furthermore, colored samples do not interfere with the RMA, and no further sample treatment is required. The deletion of these steps, which is required with other analytical methods, significantly simplifies sample preparation.

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References

1 Guilarte, T.R. and Pravlik, K. (1983). Radiometric-microbiological assay of niacin using *Kloeckera brevis:* analysis of human blood and food. *J. Nutr.* **113,** 2587–2594

2 Gregory III, J.F. (1988). Methods for determination of vitamin B6 in foods and other biological materials: a critical review. *J. Food Comp. Anal.* **1,** 105–123

3 Guilarte, T.R. (1991). Radiometric microbiological assay of B vitamins. Part 1: assay procedure. *J. Nutr. Biochem.* **2,** 334–338

4 Wittwer, C., Wyse, B., and Gaurth Hansen, R. (1982). Assay of the enzymatic hydrolysis of pantetheine. *Anal. Biochem.* **122,** 213–222

5 Defibaugh, P.W. (1987). Evaluation of selected enzymes for thiamine determination. *J. Assoc. Off. Anal. Chem.* **70,** 514–517