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Semiautomated, Simultaneous Assay of Thiamine, Riboflavin, Pyridoxine, and Niacinamide in Multivitamin Preparations

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Abstract □ A semiautomated analytical system was developed for the simultaneous determination of thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), and niacinamide in multivitamin preparations. The automated analytical methods are similar in principle to the USP manual procedures.

Keyphrases □ Multivitamin preparations—semiautomated, simultaneous analysis □ Vitamin analysis—thiamine, riboflavin, pyridoxine, and niacinamide in multivitamin preparations

A semiautomated method was developed for the simultaneous analysis of four vitamins (niacinamide, pyridoxine, thiamine, and riboflavin) in multivitamin formulations; it is similar in principle to the USP (1) manual procedures. The method is a significant extension of the work by Khoury (2, 3) and Albright and Degner (4), who developed methods for simultaneous determination of two vitamins in multivitamin formulations.

EXPERIMENTAL

Equipment—The analytical train consisted of the following Technicon[1] modules: Liquid Sampler II, three proportioning pumps (model I), a dialyzer (model I), two colorimeters (model I) with 15-mm. flow cells, two Fluorometer I units, two double-pen recorders[2], and an autoclave[3].

Reagents—*Niacinamide Buffer (pH 6.8)*—Prepare by dissolving 206.5 g. sodium phosphate dibasic (A.R.) and 57.3 g. citric acid (A.R.) in 2 l. of distilled water. Before use, dilute 200 ml. buffer and 2 ml. polysorbate 80[4] to 1 l.

Pyridoxine Buffer—Prepare a 20% (w/v) sodium acetate (A.R.) solution (Solution A) and a second solution (Solution B) consisting of 470 g. ammonium chloride (A.R.) and 470 ml. ammonium hydroxide (A.R.) (58%) dissolved in water and diluted to 2 l. with water. Mix equal portions of Solutions A and B plus 4 ml. of polysorbate 80/l. before use.

Buffer (pH 4.0)—Prepare by dissolving 219 g. sodium phosphate dibasic (A.R.) and 258 g. citric acid (A.R.) in water and diluting to 2 l. with water. Before use, dilute 200 ml. buffer plus 10 g. potassium chloride (A.R.) to 1 l. with water.

Cyanogen Bromide[5]—Use 7% (w/v) in distilled water.

Sulfanilic Acid—Disperse 50 g. of sulfanilic acid[5] in approximately 800 ml. water. Add ammonium hydroxide (58%) slowly until dissolution of the sulfanilic acid is achieved. Adjust to pH 4.5 with concentrated hydrochloric acid (A.R.), and dilute to 1 l. with water.

N, *2,6-Trichloro-p-benzoquinoneimine Solution*—Dissolve 200 mg. of *N*,2,6-trichloro-*p*-benzoquinoneimine[5] in 500 ml. isopropyl alcohol.

Table I—Relative Standard Deviations of Three Types of Vitamin Preparations[a]

	Thiamine		Riboflavin		Pyridoxine		Niacinamide	
	Automated	Manual	Automated	Manual	Automated	Manual	Automated	Manual
Decavitamin drops	1.2	2.7	1.9	3.5	1.7	1.9	3.2	3.8
Vitamin premix for nutritional products	2.1	0.8	1.1	9.1	2.1	3.2	3.4	5.2
Decavitamin tablet granulations	2.1	1.2	2.0	3.4	0.6	4.9	4.2	5.1

[a] Values are given as percentages.

[1] AutoAnalyzer, Technicon, Tarrytown, NY 10591

[2] Model 67A-(TC)-2PHPH570-00.
[3] American Sterilizer Co., model 57CR.
[4] Atlas Chemical.
[5] Eastman Kodak.

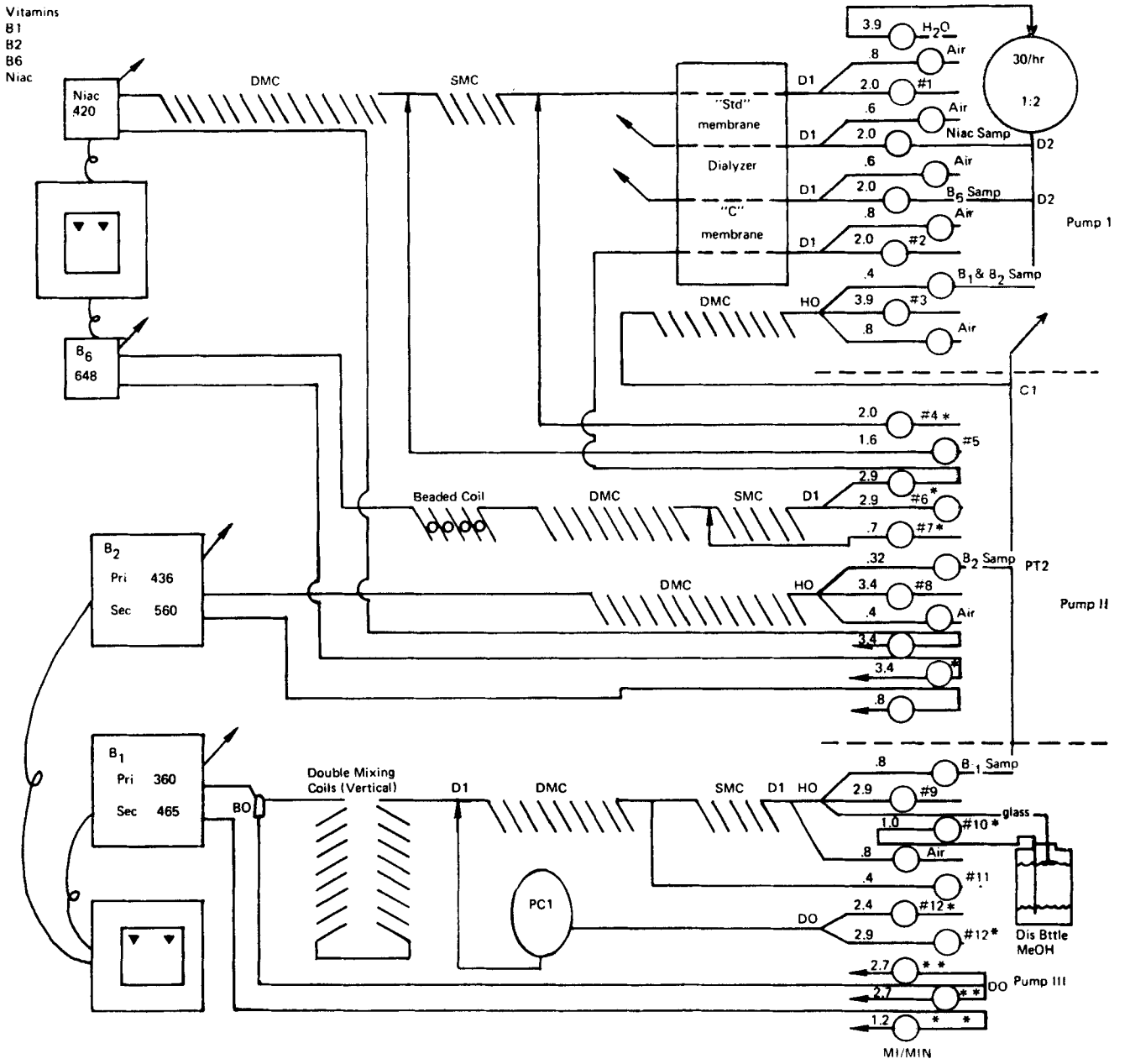

Figure 1—*Schematic diagram of automated system. Key: 1, niacin buffer; 2, pyridoxine buffer; 3, pH 4.0 buffer; 4, 7% cyanogen bromide; 5, 5% sulfanilic acid; 6, isopropyl alcohol; 7,* N,2,6-trichloro-p-benzoquinoneimine *solution; 8, pH 4.0 buffer; 9, 1% potassium chloride; 10, heavy mineral oil; 11, oxidizing reagent; 12, isobutyl alcohol; *, solvaflex pump tubing; and **, acidflex pump tubing. All others are standard pump tubing.*

Potassium Ferricyanide Oxidizing Reagent for Vitamin B_1—Prepare fresh daily 0.3% (w/v) solution of potassium ferricyanide in 10% (w/v) sodium hydroxide.

Isobutyl Alcohol (A.R.)—Saturate with water.

Methanol (A.R.), isopropyl alcohol (A.R.), and heavy mineral oil were also used.

Standards—A stock standard solution containing 0.200 mg./ml. each of thiamine USP, riboflavin USP, and pyridoxine USP and 2.000 mg./ml. of niacinamide USP was prepared by autoclaving the riboflavin in 0.02 *N* acetic acid, cooling, and adding the remaining vitamins, with subsequent dilution to volume with 0.02 *N* acetic acid. Autoclaving was necessary to solubilize riboflavin. This solution was stable for 4 weeks when stored at 5°.

A working standard was prepared daily by adding a 10.0-ml. aliquot of stock standard solution to a 200-ml. volumetric flask and autoclaving with 20 ml. of 0.1 *N* hydrochloric acid for 5 min. at 121°. The flask was subsequently cooled and diluted to volume with distilled water. Four additional levels of standard concentrations were prepared by diluting 10, 20, 30, and 40 ml. of working standard to 50 ml. with distilled water.

These resultant standard solutions were equivalent to 2, 4, 6, 8, and 10 mcg./ml. of vitamins B_1, B_2, and B_6 and 20, 40, 60, 80, and 100 mcg./ml. of niacinamide.

Sample Preparation—An accurately measured quantity of sample, equivalent to 1–5 mg. of vitamins B_1, B_2, and B_6 and 10–50 mg. of niacinamide, was placed in a 500-ml. volumetric flask with 50 ml. of 0.1 *N* hydrochloric acid, autoclaved for 5 min. at 121°, cooled, and diluted to volume with distilled water.

Procedure—A schematic diagram of the automated system is shown in Fig. 1. An aliquot of each sample was divided among the four subsystems for analysis of appropriate vitamins where respective transmittance or fluorescent intensities were recorded (Figs. 2 and 3).

DISCUSSION

Interference from dyes in the sample with both pyridoxine and niacinamide determinations necessitated the use of a dialyzer.

Table II—Comparison of Manual and Automated Analysis Results

Sample	Thiamine		Riboflavin	
	Automated	Manual	Automated	Manual
Vitamin premix				
A	7.50 mg./g.	7.33 mg./g.	3.70 mg./g.	3.75 mg./g.
B	3.22 mg./g.	3.13 mg./g.	4.79 mg./g.	4.77 mg./g.
C	3.66 mg./g.	3.58 mg./g.	6.56 mg./g.	6.50 mg./g.
Decavitamin granulation				
A	1.52 mg./470 mg.	1.54 mg./470 mg.	1.75 mg./470 mg.	1.63 mg./470 mg.
B	1.51 mg./470 mg.	1.55 mg./470 mg.	1.74 mg./470 mg.	1.70 mg./470 mg.
C	1.05 mg./500 mg.	1.03 mg./500 mg.	1.38 mg./500 mg.	1.50 mg./500 mg.
Decavitamin syrup	0.23 mg./ml.	0.22 mg./ml.	0.33 mg./ml.	0.33 mg./ml.
Decavitamin drop				
A	1.26 mg./0.6 ml.	1.23 mg./0.6 ml.	1.42 mg./0.6 ml.	1.43 mg./0.6 ml.
B	1.26 mg./0.6 ml.	1.27 mg./0.6 ml.	1.47 mg./0.6 ml.	1.45 mg./0.6 ml.
	Pyridoxine		Niacinamide	
	Automated	Manual	Automated	Manual
Vitamin premix				
A	7.44 mg./g.	7.46 mg./g.	85.7 mg./g.	89.2 mg./g.
B	1.50 mg./g.	1.53 mg./g.	29.1 mg./g.	28.3 mg./g.
C	1.72 mg./g.	1.77 mg./g.	40.2 mg./g.	41.4 mg./g.
Decavitamin granulation				
A	1.34 mg./470 mg.	1.35 mg./470 mg.	17.3 mg./470 mg.	15.9 mg./470 mg.
B	1.34 mg./470 mg.	1.31 mg./470 mg.	17.7 mg./470 mg.	15.6 mg./470 mg.
C	1.11 mg./500 mg.	1.12 mg./500 mg.	15.6 mg./500 mg.	15.7 mg./500 mg.
Decavitamin syrup	0.23 mg./ml.	0.22 mg./ml.	2.14 mg./ml.	2.07 mg./ml.
Decavitamin drop				
A	1.23 mg./0.6 ml.	1.19 mg./0.6 ml.	8.67 mg./0.6 ml.	8.52 mg./0.6 ml.
B	1.24 mg./0.6 ml.	1.21 mg./0.6 ml.	8.70 mg./0.6 ml.	8.76 mg./0.06 ml.

During sample preparation, niacinamide is partially hydrolyzed to niacin; but by treating standard and sample in a like manner, the degree of hydrolysis was found to be the same for standard and sample. The niacinamide buffer must be below pH 7 if ferrous ion is present to prevent ferrous hydroxide formation, which decreases the efficiency of the dialysis membrane.

The thiamine method of Khoury (3) was used with slight modification. Methanol was found to improve the partitioning of thiochrome from the aqueous phase to the isobutyl alcohol. Methanol, after contact with standard, solvaflex, or acidflex tubing, caused quenching of fluorescence. This necessitated addition of methanol by displacement with mineral oil.

Removal and inversion of the fluorometer door to remove water from the cell were used in lieu of flushing the lines with methanol as in Khoury's method (3). Addition of water to the isobutyl alcohol reagent prevented precipitation of potassium chloride at the point of introduction of isobutyl alcohol in the analytical train.

The natural fluorescence in a sample, which might interfere in riboflavin determination, can be measured by the addition of sodium

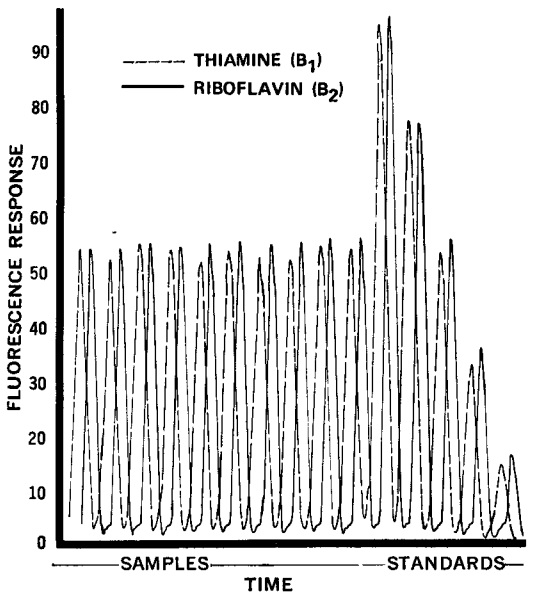

Figure 2—*Typical curves for vitamin B_1 and B_2 standards and samples.*

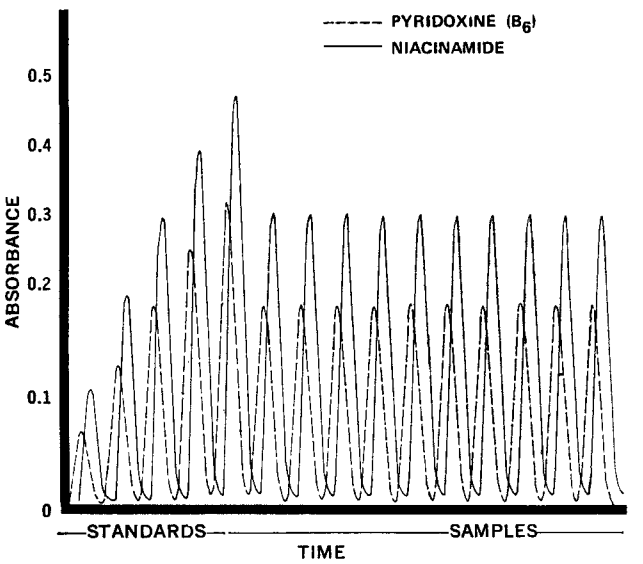

Figure 3—*Typical curves for vitamin B_6 and niacinamide standards and samples.*

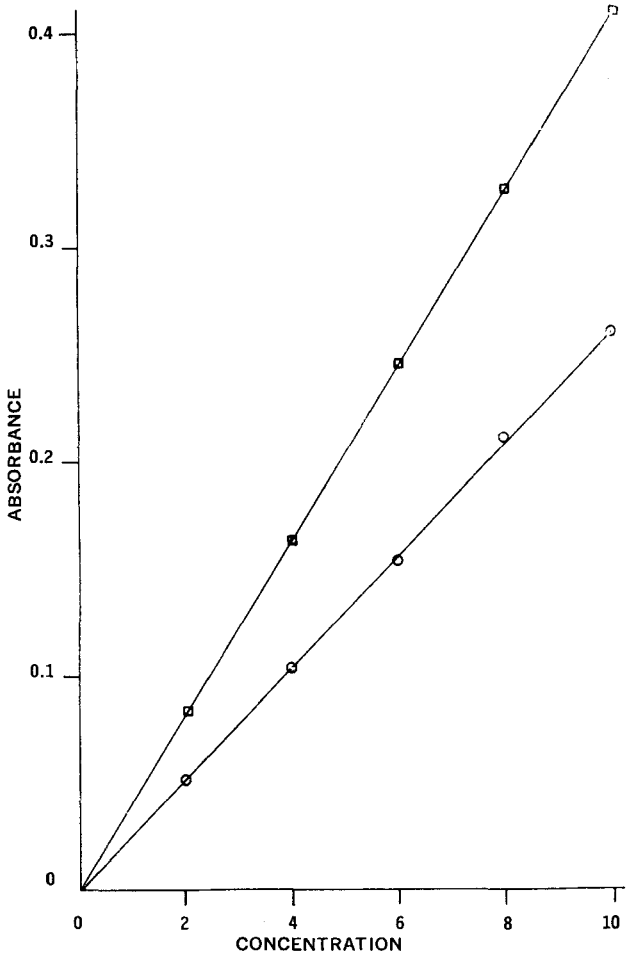

Figure 4—*Standard curves for pyridoxine and niacinamide. Key:* O, *pyridoxine (mcg./ml.); and* □, *niacinamide (mcg./ml. × 10).*

hydrosulfite to the sample after analysis. The sodium hydrosulfite reduces riboflavin to nonfluorescent leucoflavin. No measurable background fluorescence was observed for any of the samples assayed routinely.

The color reaction for pyridoxine is for *para*-unsubstituted phenols. A correction can be made by adding boric acid to complex selectively pyridoxine and reassaying the samples for interfering phenols. No measurable amount of interference was observed for any samples assayed routinely. If ascorbic acid is present, addition of copper chloride to the pyridoxine buffer is necessary to oxidize interfering ascorbic acid.

RESULTS

Standard Curves—The average response (transmittance or fluorescence) for each vitamin at five concentrations was determined. A plot of the five values for each vitamin gave a straight line (Figs. 4 and 5).

Reproducibility of Standards—The reproducibility of the method was checked by the analysis of 10 standards. The coefficient of variations observed were: 1.77% for thiamine, 1.17% for riboflavin, 0.52% for pyridoxine, and 0.85% for niacinamide.

Reproducibility of Sample Preparations—A decavitamin drop, a decavitamin granulation, and a vitamin powder mixture were assayed each day for 10 consecutive days by both the automated procedure and the manual USP XVII procedures. A comparison of the standard deviations calculated for both automated and manual assay results is shown in Table I. A further comparison of the automated and manual procedures is given in Table II for a variety of samples.

SUMMARY

An automated method for the simultaneous assay of four vitamins (thiamine, riboflavin, pyridoxine, and niacinamide) in multivitamin preparations was described. The automated system proved reliable and provided an approximate fivefold increase in assay capability.

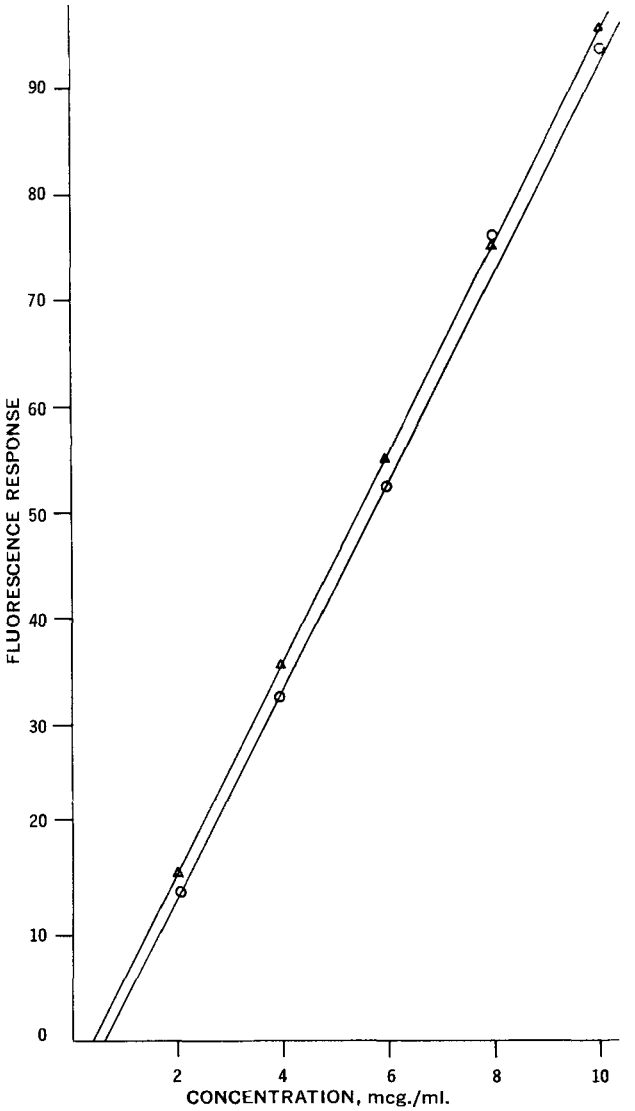

Figure 5—*Standard curves for vitamin* B_1 (O) *and vitamin* B_2 (Δ).

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