Simultaneous Determination of Niacin, Niacinamide, Pyridoxine, Thiamine, and Riboflavin in Multivitamin Blends by Ion-Pair High-Pressure Liquid Chromatography

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Abstract □ A high-pressure liquid chromatographic procedure for the simultaneous determination of niacin, niacinamide, pyridoxine, thiamine, and riboflavin was developed and applied to the analysis of multivitamin blends for these water-soluble vitamins. Reversed-phase ion-pair chromatography, using sodium hexanesulfonate as the counterion, was employed. Analysis time is shortened considerably and precision is improved by the application of this analytical technique as compared to the current official methods of analysis. Involved sample pretreatment is not required.

Keyphrases □ Vitamins, various—simultaneous high-pressure liquid chromatographic analyses in multivitamin preparations □ High-pressure liquid chromatography—simultaneous analyses of various vitamins in multivitamin preparations

Methods currently used for the analysis of niacin, niacinamide, pyridoxine, thiamine, and/or riboflavin in multivitamin preparations involve the application of derivatization and measurement of total UV absorption (1, 2), polarography (3, 4), GLC (5), electrophoretic analysis (6), TLC (7, 8), colorimetry (9, 10), or fluorometry (11). Several of these methods are accurate (1, 2, 10, 11); however, they are complicated, and strict attention must be paid to the preparation of reagents (because of their instability or caustic properties) to obtain meaningful analytical data. Two methods (1, 5) are not capable of distinguishing between niacin and niacinamide.

The specificity and quantitative accuracy of several methods with respect to the determination of water-soluble vitamins in multivitamin preparations are doubtful (7–10). None of the procedures referenced is amenable to the simultaneous determination of niacin, niacinamide, pyridoxine, thiamine, and riboflavin, and most are relatively time consuming. For these reasons, the feasibility of using high-pressure liquid chromatography (HPLC) was examined.

EXPERIMENTAL

Equipment—An HPLC pump[1] at a flow rate of 0.5 ml/min and a loop injector[2] (50-μl volume loop) were used. Eluates were monitored at 270 nm with a variable wavelength detector[3]. A 30-cm × 4-mm i.d. stainless steel column[4] was packed with porous silica particles averaging <10 μm in diameter chemically bonded to a monomolecular layer of octadecyltrichlorosilane.

Materials and Reagents—USP–NF reference standards were used for standard solutions of niacin, niacinamide, pyridoxine, thiamine, and riboflavin. Sodium hexanesulfonate[5] was obtained commercially and used without further purification.

Mobile Phase Preparation—The mobile phase was prepared by mixing 250 ml of reagent grade methanol with 750 ml of a 0.005 *M* solution of sodium hexanesulfonate containing 1% (v/v) acetic acid in a 1-liter erlenmeyer flask. Prior to use, the mobile phase was degassed by vacuum filtration through a 5-μm pore, 47-mm diameter, polytef filter[6].

Standard Curves—Mixed standard solutions were prepared in the aqueous portion of the mobile phase to contain 0.0125–0.110 mg of niacin and niacinamide/ml, 0.0033–0.026 mg of pyridoxine/ml, 0.0016–0.0110 mg of thiamine/ml, and 0.0020–0.019 mg of riboflavin/ml. A 50-μl aliquot of each standard mixture was injected, and observed peak heights at 270 nm were plotted *versus* concentration for each water-soluble vitamin.

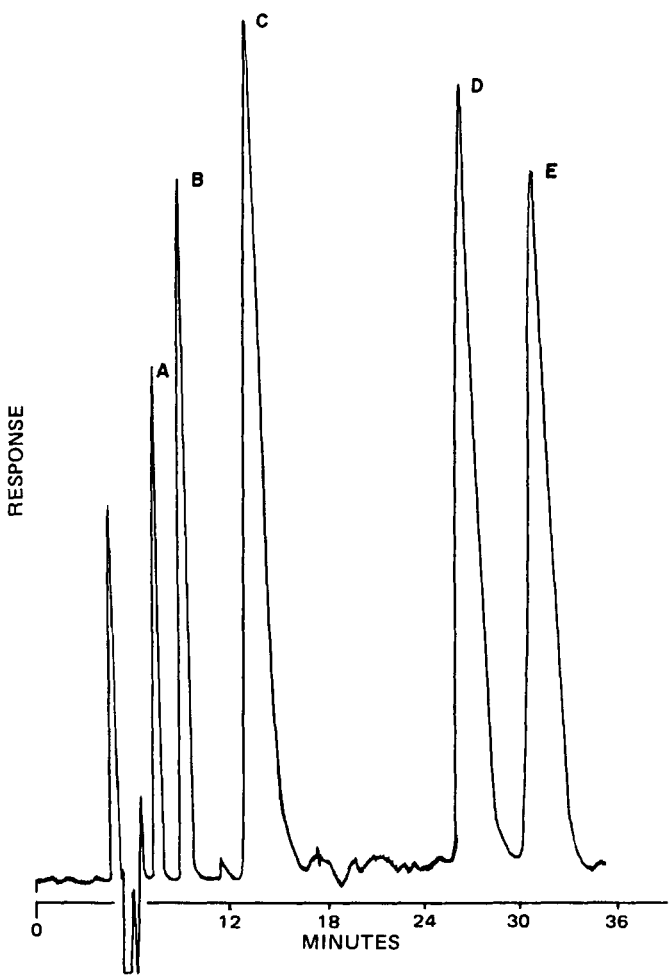

Figure 1—*Chromatographic trace of a standard mixture of niacin (A), niacinamide (B), pyridoxine (C), thiamine (D), and riboflavin (E). The conditions were: mobile phase, 25% methanol–75% 0.005 M sodium hexanesulfonate in 1% acetic acid; flow rate, 0.5 ml/min; detection, UV at 270 nm; and chart speed, 25.4 cm/hr.*

[1] Model 6000A, Waters Associates, Milford, Mass.
[2] Valco model CV-6-UHPa-C-20.
[3] Schoeffel model SF 770.
[4] μBondapak C_{18}, Waters Associates, Milford, Mass.
[5] Eastman-Kodak.
[6] Type LS, 47 mm, Millipore Corp.

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Table I—Precision of HPLC Method for the Determination of Niacin, Niacinamide, Pyridoxine, Thiamine, and/or Riboflavin in Five Multivitamin Blends

Multivitamin Blend	1 *RSD*, %				
	Niacin	Niacinamide	Pyridoxine	Thiamine	Riboflavin
1	—	1.0	—	1.3	1.0
2	—	1.0	0.9	2.6	1.2
3	2.8	—	—	3.0	2.8
4	1.0	—	—	0.5	—
5	—	2.4	0.9	—	1.0

Table II—Recovery of Niacin, Niacinamide, Pyridoxine, Thiamine, and/or Riboflavin from Five Multivitamin Blends

Multivitamin Blend	Recovery, %				
	Niacin	Niacinamide	Pyridoxine	Thiamine	Riboflavin
1	—	100	—	99	101
2	—	101	100	99	103
3	100	—	—	98	100
4	104	—	—	100	—
5	—	100	99	—	99

Sample Solution—No sample pretreatment was required for the multivitamin blends analyzed. Appropriate amounts of sample were dissolved directly in the aqueous portion of the mobile phase, placed in an ultrasonic bath for 10 min, diluted to volume, filtered if necessary, and chromatographed. Sample vitamin concentrations were determined *versus* single-point standards.

RESULTS AND DISCUSSION

Interest in the analysis of water-soluble vitamins in multivitamin preparations is immense. The complex matrixes of these preparations make chromatography an ideal choice for the determination, and the application of HPLC is particularly attractive due to its specificity, the lack of need for elaborate sample pretreatment schemes, and the physical properties of the vitamins themselves.

Recent literature reports indicated the successful application of reversed-phase ion-pair HPLC to the determination of niacin and niacinamide simultaneously (12, 13), riboflavin and thiamine simultaneously (13), and niacin, niacinamide, pyridoxine, and thiamine separately (13) in multivitamin preparations. A reversed-phase ion-pair HPLC technique for the simultaneous determination of niacinamide, pyridoxine, thiamine, and riboflavin analogous to the method described here was suggested previously (13); however, no reports of the application of this technique have appeared.

Liquid chromatographic methods involving ion exchange for the determination of niacinamide, niacin, pyridoxine, thiamine, and/or riboflavin were reported (14–17), but they are imprecise (18), result in limited column life (19), or are not amenable to the simultaneous determination of these vitamins. A normal phase chromatographic method for the determination of riboflavin in multivitamin preparations was reported (20), as was a reversed-phase system for folic acid, cyanocobalamin, and riboflavin (21). Relatively complicated sample pretreatment procedures are required for the application of many of these methods to the determination of water-soluble vitamins in multivitamin preparations.

In general, the complex matrixes of multivitamin preparations containing the water-soluble vitamins niacin, niacinamide, pyridoxine, thiamine, and riboflavin consist of polar diluents, fillers, oil-soluble vitamins, inorganic salts, dyes, preservatives, and other water-soluble vitamins such as folic acid and calcium pantothenate. Materials specifically tested for possible interference with the quantitation of niacin, niacinamide, pyridoxine, thiamine, or riboflavin and found to be either insoluble in the aqueous portion of the mobile phase or eluted at the solvent front include vitamins A, D, and E, butylated hydroxyanisole, butylated hydroxytoluene, calcium carbonate, elemental iron, yolk shade No. 2 dye, folic acid, and calcium pantothenate.

Cyanocobalamin was present in several multivitamin blend preparations but at levels not detectable (20 μg/g) by monitoring eluates at 270

Table III—Comparative Analyses between the HPLC Method, the Current Official Method, and the Amounts of Vitamin Added for Multivitamin Blend 1

Multivitamin Sample	Niacinamide, %			Thiamine, %			Riboflavin, %		
	HPLC	Official	Added	HPLC	Official	Added	HPLC	Official	Added
1-A	13.9 ± 0.3	14.5 ± 0.7	—[a]	2.8 ± 0.1	2.8 ± 0.1	—[a]	3.0 ± 0.1	2.8 ± 0.2	—[a]
1-B	13.9 ± 0.3	14.1 ± 0.7	—[a]	2.7 ± 0.1	2.7 ± 0.1	—[a]	3.0 ± 0.1	3.0 ± 0.2	—[a]
1-C	14.2 ± 0.3	14.5 ± 0.7	—[a]	2.7 ± 0.1	2.8 ± 0.1	—[a]	2.8 ± 0.1	2.8 ± 0.2	—[a]
1-D	13.9 ± 0.3	14.3 ± 0.7	14.5	1.9 ± 0.1	2.3 ± 0.1	2.1	2.6 ± 0.1	2.8 ± 0.2	2.8
1-E	14.2 ± 0.3	14.1 ± 0.7	14.5	2.0 ± 0.1	2.1 ± 0.1	2.1	2.5 ± 0.1	2.8 ± 0.2	2.8
1-F	13.4 ± 0.3	13.8 ± 0.7	14.5	2.0 ± 0.1	2.2 ± 0.1	2.1	2.8 ± 0.1	2.7 ± 0.2	2.8
1-G	13.3 ± 0.3	14.3 ± 0.7	14.5	2.2 ± 0.1	2.2 ± 0.1	2.1	2.9 ± 0.1	2.9 ± 0.2	2.8
1-H	14.0 ± 0.3	14.0 ± 0.7	14.5	2.2 ± 0.1	2.2 ± 0.1	2.1	2.9 ± 0.1	2.8 ± 0.2	2.8

[a] Values unavailable.

Table IV—Comparative Analyses between the HPLC Method, the Current Official Method, and the Amounts of Vitamin Added for Multivitamin Blend 2

Multivitamin Sample	Niacinamide, %			Pyridoxine, %			Thiamine, %			Riboflavin, %		
	HPLC	Official	Added	HPLC	Official	Added	HPLC	Official	Added	HPLC	Official	Added
2-A	3.7 ± 0.1	3.6 ± 0.2	3.8	0.46 ± 0.01	0.44 ± 0.03	0.45	0.58 ± 0.03	0.53 ± 0.03	0.52	0.34 ± 0.01	0.29 ± 0.02	0.30
2-B	3.7 ± 0.1	4.1 ± 0.2	3.9	0.44 ± 0.01	0.45 ± 0.03	0.46	0.54 ± 0.03	0.52 ± 0.03	0.53	0.33 ± 0.01	0.29 ± 0.02	0.31
2-C	3.6 ± 0.1	4.2 ± 0.2	3.8	0.43 ± 0.01	0.43 ± 0.03	0.45	0.53 ± 0.03	0.51 ± 0.03	0.52	0.42 ± 0.01	0.48 ± 0.03	—[a]
2-D	3.8 ± 0.1	4.1 ± 0.2	—[a]	0.42 ± 0.01	0.45 ± 0.03	—[a]	0.56 ± 0.03	0.53 ± 0.03	—[a]	0.34 ± 0.01	0.35 ± 0.02	—[a]
2-E	4.0 ± 0.1	4.1 ± 0.2	3.8	0.42 ± 0.01	0.45 ± 0.03	0.45	0.56 ± 0.03	0.53 ± 0.03	0.52	0.39 ± 0.01	0.35 ± 0.02	—[a]
2-F	3.7 ± 0.1	4.1 ± 0.2	—[a]	0.42 ± 0.01	0.45 ± 0.03	—[a]	0.55 ± 0.03	0.53 ± 0.03	—[a]	0.44 ± 0.01	0.35 ± 0.02	—[a]

[a] Values unavailable.

Table V—Comparative Analyses between the HPLC Method, the Current Official Method, and the Amounts of Vitamin Added for Multivitamin Blend 3

Multivitamin Sample	Niacin, %			Thiamine, %			Riboflavin, %		
	HPLC	Official	Added	HPLC	Official	Added	HPLC	Official	Added
3-A	13.6 ± 0.8	13.1 ± 0.7	—[a]	1.2 ± 0.1	0.9 ± 0.1	—[a]	1.1 ± 0.1	1.1 ± 0.1	—[a]
3-B	12.8 ± 0.7	12.9 ± 0.6	12.1	1.0 ± 0.1	0.9 ± 0.1	0.9	0.9 ± 0.1	1.0 ± 0.1	1.0
3-C	12.1 ± 0.7	13.1 ± 0.7	12.1	1.0 ± 0.1	0.9 ± 0.1	0.9	1.0 ± 0.1	1.0 ± 0.1	1.0
3-D	14.2 ± 0.8	12.8 ± 0.6	12.7	1.0 ± 0.1	1.0 ± 0.1	1.0	1.1 ± 0.1	1.0 ± 0.1	1.1
3-E	14.9 ± 0.8	13.5 ± 0.7	12.7	1.0 ± 0.1	1.0 ± 0.1	1.0	1.2 ± 0.1	1.1 ± 0.1	1.1

[a] Values unavailable.

Table VI—Comparative Analyses between the HPLC Method, the Current Official Method, and the Amounts of Vitamin Added for Multivitamin Blend 4

Multivitamin Sample	Niacin, %			Thiamine, %		
	HPLC	Official	Added	HPLC	Official	Added
4-A	9.8 ± 0.2	9.9 ± 0.5	10.0	0.95 ± 0.01	1.05 ± 0.05	1.00
4-B	10.6 ± 0.2	9.9 ± 0.5	—[a]	1.01 ± 0.01	1.06 ± 0.05	—[a]

[a] Values unavailable.

determining the vitamin content of the spiked samples *versus* single-point vitamin standards (Table II). Essentially quantitative recoveries were observed in all cases.

Comparative analytical data (between this HPLC procedure and the current official methods of analysis) are given in Tables III–VII for the five multivitamin blends. The average deviation of the HPLC method from the current official analytical procedures (percent relative to the current official analytical result) was 5.9% for niacin, 4.5% for niacinamide, 5.1% for pyridoxine, 6.4% for thiamine, and 4.5% for riboflavin. The HPLC assay (percent relative to the current official method analytical result) tended to give analytical values that were 3% high for niacin, 4% low for niacinamide, 2% low for pyridoxine, 0.5% low for thiamine, and

Table VII—Comparative Analyses between the HPLC Method, the Current Official Method, and the Amounts of Vitamin Added for Multivitamin Blend 5

Multivitamin Sample	Niacinamide, %			Pyridoxine, %			Riboflavin, %		
	HPLC	Official	Added	HPLC	Official	Added	HPLC	Official	Added
5-A	11.1 ± 0.5	11.4 ± 0.6	11.9	2.15 ± 0.04	2.3 ± 0.2	2.40	1.72 ± 0.03	1.7 ± 0.1	1.83
5-B	11.7 ± 0.6	11.8 ± 0.6	12.0	2.49 ± 0.04	2.3 ± 0.2	2.40	1.78 ± 0.04	1.7 ± 0.1	1.83

nm. Ascorbic acid, which elutes at the solvent front, tended to interfere with the quantitation of niacin when present in high concentrations; however, when the composition of the mobile phase was reduced from 25 to 22.5% methanol, the resolution of niacin from ascorbic acid was increased sufficiently to allow its accurate determination even in blends containing roughly 75% by weight of ascorbic acid. A small peak was noted just following the elution of pyridoxine due to the solubilization of small amounts of butylated hydroxytoluene, and a slight rise in the baseline of the chromatographic trace of multivitamin blends containing yolk shade No. 2 dye was noted; however, no interference with the quantitation of the water-soluble vitamins present was found.

All or portions of several multivitamin blend components are likely, if solubilized, to be retained on the column. A change in column performance was noted only after several days, however, during which time up to 20 samples containing varying amounts of the possible interferences were chromatographed. An overnight washing of the column with methanol regenerated it for further use.

The chromatographic trace of a standard mixture (50 μl injected) containing niacin (0.11 mg/ml, 0.1 aufs), niacinamide (0.11 mg/ml, 0.4 aufs), pyridoxine (0.026 mg/ml, 0.04 aufs), thiamine (0.011 mg/ml, 0.02 aufs), and riboflavin (0.019 mg/ml, 0.1 aufs) is presented in Fig. 1. The complete elution of all five water-soluble vitamins was accomplished within 35 min from injection. Recorder response *versus* vitamin concentration was linear for all five water-soluble vitamins over the concentration ranges described under *Experimental*. Correlation coefficients for standard curves were 0.9998, 0.9997, 0.9999, 0.9993, and 0.9998 for niacin, niacinamide, pyridoxine, thiamine, and riboflavin, respectively.

The mean y value and the y intercept were 2.52 and 0.12 for niacin, 3.47 and 0.22 for niacinamide, 3.85 and 0.17 for pyridoxine, 3.77 and 0.01 for thiamine, and 3.31 and 0.002 for riboflavin, respectively. The reproducibility of standard mixture injections as determined on 2 days with two separate standard mixtures was, at the worst, ±1.2% (2 *SD* relative to the average) for the five vitamins. Thus, single-point standards are amenable to the determination of these water-soluble vitamins by this procedure.

The precision of this method, as determined on four separate weighings of single samples of five different multivitamin blends, is presented in Table I. Although the water-soluble vitamin content of the five blends in solution varied widely (0.026–0.8 mg/ml for niacin, 0.032–0.076 mg/ml for niacinamide, 0.0036–0.016 mg/ml for pyridoxine, 0.002–0.008 mg/ml for thiamine, and 0.002–0.012 mg/ml for riboflavin), reproducibility was good in all cases. Decreases in precision noted for a particular water-soluble vitamin present in more than one sample matrix were directly proportional to the amount of vitamin present and the ease of dissolution of the sample matrix being assayed.

Recovery of the water-soluble vitamins present in each of the five multivitamin blend preparations was tested by adding the appropriate vitamins in amounts equal to those already present in the sample and

1% high for riboflavin. These deviations reflect the degree of imprecision and nonspecificity encountered using the current official methods of analysis.

The described HPLC method should be applicable to a variety of multivitamin preparations with little or no modification. Its flexibility with respect to sample concentration and composition and the lack of required elaborate sample pretreatment enhance accuracy and reduce analysis time greatly.

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