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A Rapid HPLC Method for Multivitamin Analysis

This article describes validated methods for the analysis of multivitamins in tablets, animal feed premixes, and beverage samples. The authors present extensive validation and method performance data from high performance liquid chromatography analyses of water- and fat-soluble vitamins. They obtained limits of detection of 0.6–1.2 ng with greater than 90% recovery for spiked samples of water-soluble vitamins and 1–10 ng with 87–98% recovery for fat-soluble vitamins.

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Vitamins are vital substances for many organisms to ensure healthy growth and development. Because vitamins generally are not biosynthesized within the human body, they must be obtained from food. Therefore, their determination is important in the food and pharmaceutical industries. Common vitamins are classified in two categories: water-soluble vitamins, which include ascorbic acid (vitamin C), niacin, niacinamide, pyridoxine (vitamin B_6), thiamine (vitamin B_1), folic acid, riboflavin (vitamin B_2), pantothenic acid, cyanocobalamin (vitamin B_{12}), and biotin (vitamin H); and fat-soluble vitamins, which include vitamins A, D, E, and K. These vitamins are common ingredients in multivitamin formulations, processed food, and fortified beverage products (1).

Documented in several reference sources (2–4), official analytical methods for vitamins often involve extraction and chemical reactions followed by titration, spectrophotometry, fluorometry, or high performance liquid chromatography (HPLC). Analysts can use these procedures to assay individual vitamins separately. However, a single-extraction procedure followed by simultaneous analysis of the entire water- or fat-soluble vitamin group is more cost-effective, so many analytical chemists have pursued the simultaneous analysis approach (5–12).

In an earlier study, our laboratory examined systematically controlling the column and mobile-phase variables that affect the ion-pair reversed-phase separation of common water-soluble vitamins (13). We found that these measures yielded improved separations.

In this article, we describe how we applied the method to analyze multivitamin tablets, fortified drinks, and feed premixes and then compiled extensive method performance parameters. We also developed a parallel method that uses reversed-phase separation and programmable UV detection for fat-soluble vitamins. Both methods are rapid — providing completed extraction and analysis in 1 h — and can yield quantitative data for diverse samples.

EXPERIMENTAL

Apparatus: We used an HPLC system that comprised a series 200 binary LC pump, a series 200 autosampler, an LC-101 column oven, and an LC-235C diode-array UV detector (all from Perkin-Elmer Corp., Norwalk, Connecticut). Spectral data from the diode-array detector aided in peak identification during method development and peak-purity assessment during sample analyses. We used wavelength programming to optimize the detection of diverse vitamins. A model 1022 integrator or a Turbochrom workstation (both from Perkin-Elmer) was used for data handling, system control, and spectral archiving. We also used an ultrasonic heated bath (Fisher Scientific, Fairlawn, New Jersey) set at greater than 200 W for sample extraction.

We used an 8.3 cm × 4.6 mm, 3-µm d_p, reduced-activity 3x8 C8 column (Perkin-Elmer) for water-soluble vitamin analysis. The bonded phase had 5% carbon loading, 200-m^2/g surface area, and 80-Å pore diameter. This column was selected because of its efficiency, batch-to-batch consistency, and low residual silanol activity. We used a similar C18 column for fat-soluble vitamin analysis. We also used a 10 mm × 2.1 mm, 10-µm d_p C18 guard column and a 3.2 cm × 4.6 mm, 10-µm d_p scavenger column (14).

We obtained vitamin standards and chemical reagents of the highest purity grade available from various suppliers, including Aldrich (Milwaukee, Wisconsin); Fisher Scientific; Mallinckrodt (St. Louis, Missouri); and Sigma (St. Louis, Missouri). We purchased HPLC-grade 1-hexanesulfonic acid (sodium salt) from Eastman-Kodak (Rochester, New York). All organic solvents and water were of HPLC-grade and from J.T. Baker (Phillipsburg, New Jersey). The vitamin tablets and food products were purchased on the open market in Connecticut.

HPLC conditions for vitamin analysis: *For water-soluble vitamins:* Our mobile phase was 15:85 (v/v) methanol–10 mM hexanesulfonate, 1% acetic acid, and 0.13% triethylamine in water. The flow rate was 1.5 mL/min at 35 °C at a pressure of 340 kPa (2400 psi). We performed UV-absorbance detection at 275 nm (295 nm from 0–0.9 min for samples containing high concentrations of Vitamin C).

For fat-soluble vitamins: We used a mobile phase of 92:8 methanol–water. The flow rate was 1.5 mL/min at 35 °C at a pressure of 260 kPa (1800 psi). We performed UV-absorbance detection at 265 nm for 8.0 min and 285 nm thereafter.

Standard and sample preparation procedures: *Preparation of calibration standards:* Folic acid solution (1.00 mg/mL) was prepared in 0.1 M sodium bicarbonate and adjusted to pH 7.0 with phosphoric acid. We prepared riboflavin (0.100 mg/mL) and all other water-soluble vitamins (1.00 mg/mL) in water. Fresh standard solutions for each water-soluble vitamin were prepared weekly and kept refrigerated until used. We prepared mixed water-soluble vitamin standard solutions from the individual water-soluble vitamin standard solutions. The mixed water-soluble vitamin calibration solution was transferred into capped, amber autosampler vials and used immediately. Under

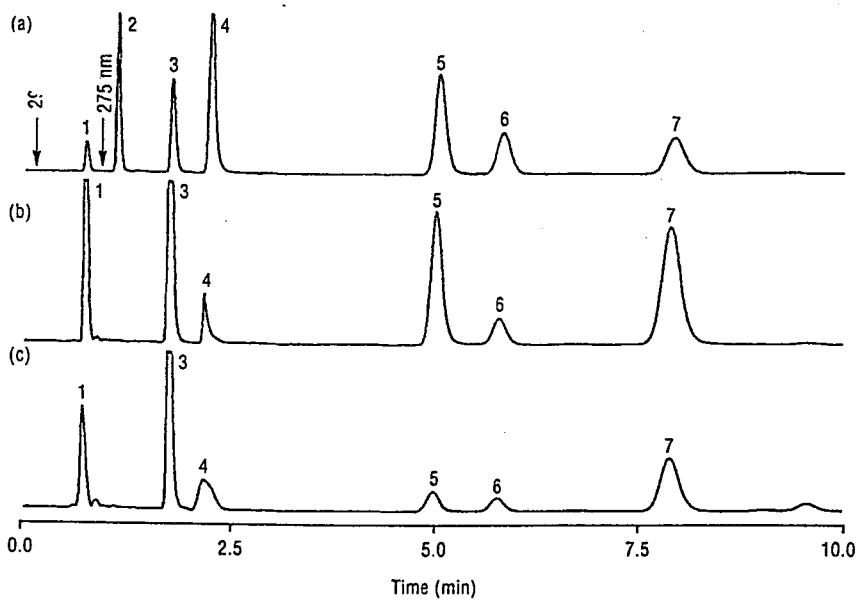

FIGURE 1: HPLC chromatograms of water-soluble vitamins in (a) a calibration standard mixture, (b) a multivitamin capsule, and (c) a multivitamin tablet. Conditions are described in the text. Peaks: 1 = vitamin C, 2 = niacin, 3 = niacinamide, 4 = pyridoxine, 5 = thiamine, 6 = folic acid, 7 = riboflavin.

hese conditions, most water-soluble vitamins ·e 1 stable except ascorbic acid, which ... discernible degradation in several ıours. We prepared fat-soluble vitamin stanlards in acetonitrile and kept them stable in :apped amber vials under refrigeration.

Sample preparation: For water-soluble vitanins in multivitamin tablets or feed premixes, ve followed a four-step procedure. First, we ground one multivitamin tablet or 1 g of feed ıremix with a mortar and pestle and transferred he ground powder into a 125-mL Erlenmeyer lask. Second, we poured exactly 10 mL of 1% ımmonia in dimethyl sulfoxide into the ground ıowder and sonicated it in an ultrasonic bath for ? min. Afterward, we added exactly 90 mL of ?% acetic acid in water to the mixture and ·tirred with a magnetic stir bar for 1 min. Third, ve ultrasonicated the mixture at approximately 10 °C for 5 min. Fourth, we filtered the extract hrough a 0.45-µm membrane filter into amber ·ials while it was still warm. After this four-part ırocedure, we analyzed samples immediately ıy injecting 5–10 µL of the sample solution nto the high performance liquid chromato·raph.

Water-soluble vitamins in beverage samples: ·or powdered-drink mix, we dissolved 1 g of ıowder in 20 mL of water, filtered the mixture hrough a 0.45-µm membrane filter into an am·er sample vial, and immediately injected the ar· For baby formula, we mixed the ready-o· baby formula or concentrate 1:1 with .% acetic acid in water to precipitate the pro·eins. Next, we filtered the mixture through a ·.45-µm membrane filter into an amber sample ial and then injected the sample immediately.

Fat-soluble vitamins in multivitamin tablets r feed premixes: We also followed a four-step rocedure for preparing these samples. First, we ground one multivitamin tablet with mortar and pestle and transferred the tablet powder into a capped 15-mL test tube. Second, we sequentially poured exactly 8 mL of extracting solution (1% citric acid in 80% ethanol–water) and 4 mL of hexane into the test tube. We recapped the test tube, shook it, and vortexed the mixture for 1 min. Third, we ultrasonicated the mixture in an ultrasonic bath (>200 W) at approximately 40 °C for 5 min, centrifuged the mixture for 1 min to separate layers, and transferred the hexane layer into a 10-mL volumetric flask. Fourth, we loosened the tablet powder at the bottom of the tube with a spatula. We repeated the hexane extraction at least once to obtain exactly 10 mL of hexane extract or dilute to volume with hexane if necessary. We immediately analyzed the samples by injecting 5 µL of the extract into the high performance liquid chromatograph.

RESULTS AND DISCUSSION

HPLC separation and analysis of water-soluble vitamins in multivitamin tablets: Figure 1 shows the chromatograms of the calibration standards and two multivitamin formulations. The C8 column separated all seven common water-soluble vitamins in less than 10 min with excellent resolution and peak symmetry. The mobile phase was optimized to maximize resolution (13). As described earlier, we chose methanol instead of acetonitrile or tetrahydrofuran for better resolution of the peaks eluted early in the run. We adjusted the levels of the ion-pair reagent and triethylamine to control the retention of thiamine and pyridoxine (13). We used 275 nm as a primary detection wavelength for water-soluble vitamins; however, we found that switching the wavelength to 295 nm (0–0.9 min) was useful to prevent detector saturation

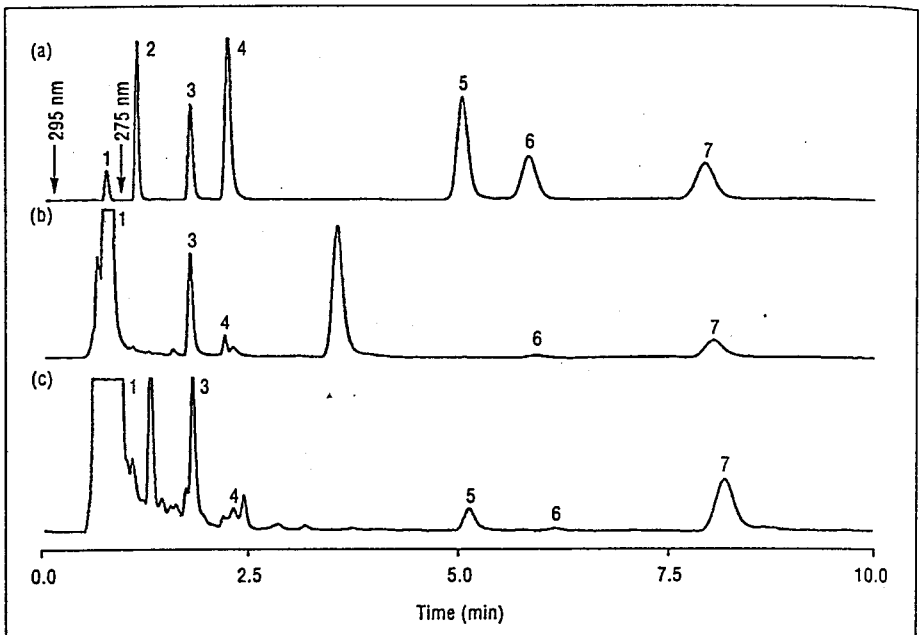

FIGURE 2: HPLC chromatograms of water-soluble vitamins in (a) a standard mixture, (b) a fortified drink, and (c) baby formula. Conditions are described in the text. Peaks: 1 = vitamin C, 2 = niacin, 3 = niacinamide, 4 = pyridoxine, 5 = thiamine, 6 = folic acid, 7 = riboflavin.

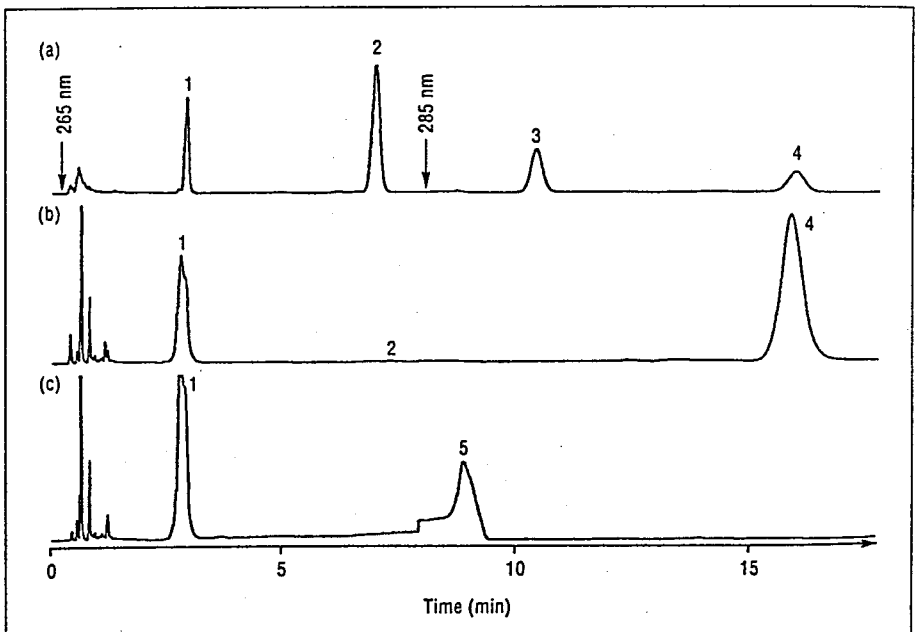

FIGURE 3: HPLC chromatograms of fat-soluble vitamins in (a) a calibration standard mixture, (b) a multivitamin tablet, and (c) a multivitamin capsule. Conditions are described in the text. Peaks: 1 = vitamin A acetate, 2 = vitamin D, 3 = vitamin E, 4 = vitamin E acetate, 5 = vitamin E succinate.

for many formulations containing high levels of vitamin C.

We found six water-soluble vitamin peaks in the chromatograms of the multivitamin tablet and capsule (Figures 1b and 1c). Spectral data from the diode-array detector confirmed peak identity (using the wavelength of maximum absorbance [λ_{max}]) and peak-purity index (PI). The pyridoxine peak in some samples sometimes was broadened or split into peaks with similar spectra indicating the presence of pyridoxine vitamers (5,6). Quantitative and validation data are presented later.

Figure 2 shows chromatograms of water-soluble vitamins in a fortified drink and a ready-to-serve baby formula sample. Data indicated many water-soluble vitamins are eluted as distinctive peaks and can be analyzed effectively from these samples with minimal sample preparation.

Separation and analysis of vitamins A, D, and E in multivitamin tablets: Figure 3 shows

the HPLC chromatograms of fat-soluble vitamins in calibration standards and in two multivitamin preparations using the reduced-activity C18 column and programmed-wavelength detection. Peaks for vitamin A were broadened in the samples (with PI less than 1.2), indicating the presence of isomers. Separation of fat-soluble vitamins using the water-soluble vitamin C8 column yielded broadened peaks and low efficiency. We switched the detection wavelength from 265 nm to 285 nm after the elution of vitamin D to enhance the detection of vitamin E. Injection volumes of more than 5 µL caused peak distortion in the hexane extract and must be avoided.

Analysis of water-soluble vitamins and fat-soluble vitamins in animal feed premix: Figure 4 shows the chromatograms of water-soluble vitamins and fat-soluble vitamins extracted from a sample of animal feed premix. The HPLC chromatograms of the extract yielded distinctive vitamin peaks and useful data for quality-control purposes.

Method performance parameters: Table I lists the analytical method performance parameters of precision, linearity, range, and sensitivities for both the water- and fat-soluble vitamin analyses (2,15). For most vitamins, we achieved precision levels of less than 0.2% relative standard deviation (RSD) for retention time and less than 1% RSD for peak area. Using a column oven is important to minimize retention shifts caused by temperature changes (16).

The poorer peak-area precision of vitamin C was caused by its instability in aqueous solution. We observed excellent linearities (linear coefficients of correlation [r] greater than 0.999) throughout a wide concentration range (more than 10^3) for most vitamins. We found detection limits for water-soluble vitamins to be approximately 0.4–1.2 ng for water-soluble vitamins at 275 nm and for vitamins A and D at 265 nm and less than 10 ng for vitamin E at 285 nm.

Table II lists the λ_{max}, PI, and other system-suitability parameters of the calibration solutions. Both λ_{max} and PI are determined by the diode-array detector and can be printed in the quantification report. We determined the peak-purity indices using a numerical algorithm that compared the upslope and downslope spectra (17). System-suitability parameters (column plate count [n], tailing factor [A_s], resolution [R], and retention factor [k]) were calculated automatically using the data system's system-suitability software.

We determined plate count and tailing factor according to the U.S. Pharmacopeial regulations (2). Column efficiency was more than 10,000 plates using a test probe (*tert*-butylbenzene); however, we obtained lower efficiency values of 6000–8000 plates for vitamins. The resolutions were greater than 3 for all vitamins, indicating a robust separation. The retention factor for vitamin C was 0.2, which is too low for reliable analysis in many food products.

Analysis data of vitamins in tablet, beverage, and feed premix samples: Table III lists the method precision and accuracy data for a multivitamin tablet sample. We obtained an assay precision of 2–3% RSD for water-soluble vitamins and 2–4% RSD for fat-soluble vitamins. Recoveries of spiked standards typically were greater than 90% for most vitamins. We found that our assay data typically were within 10% of the product labels' claims.

Table IV compares the assay data of a multivitamin capsule, an animal feed premix, a fortified beverage, and a baby formula with their respective label claims.

Summary of analytical method performance and methodology guidelines: Table V summa-

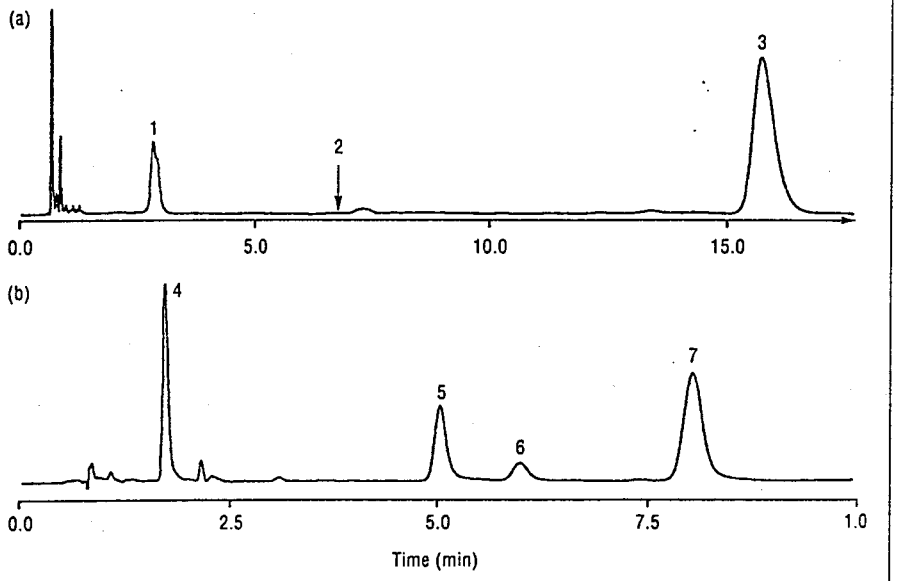

FIGURE 4: HPLC chromatograms of (a) water- and (b) fat-soluble vitamins in an animal feed premix sample. Conditions are described in the text. Peaks: 1 = vitamin A acetate, 2 = vitamin D_3, 3 = vitamin E acetate, 4 = niacinamide, 5 = thiamine, 6 = folic acid, 7 = riboflavin.

TABLE I: HPLC Analytical Method Performance Parameters for Water- and Fat-Soluble Vitamin Analysis

	Retention (min)	Retention (RSD%)	Peak Area (RSD%)	Linearity (r)*	Range (ng)	Detection Limit (ng)†
Water-Soluble Vitamins						
Vitamin C	0.726	0.220	1.50	0.9973	1–8000	0.6
Niacin	1.086	0.148	0.48	0.9999	1–4000	0.6
Niacinamide	1.731	0.081	0.50	0.9999	1–4000	1.0
Pyridoxine	2.185	0.107	0.48	0.9995	1–4000	0.5
Thiamine	4.989	0.080	0.33	0.9994	1–4000	1.2
Folic acid	5.771	0.166	0.35	0.9993	1–800	0.6
Riboflavin	7.880	0.084	0.88	0.9995	1–800	0.6
Fat-Soluble Vitamins						
Vitamin A acetate	3.00	0.12	1.07	0.9990	1–9000	0.90
Vitamin D_2	7.13	0.13	0.81	0.9993	1–4000	0.35
Vitamin E	10.63	0.09	0.89	0.9990	5–18,000	4.6
Vitamin E acetate	16.34	0.10	0.89	0.9989	5–18,000	8.7

* Coefficient of linear correlation.
† Detection limit measured at signal-to-noise ratio (S/N) = 3.

TABLE II: Typical λ_{max}, Peak Purity Indices, and System Suitability Peak Parameters of Water- and Fat-Soluble Vitamins

	λ_{max} (nm)	PI	*n* (Plate Number)	A_s	*R*	*k*
Water-Soluble Vitamins						
Vitamin C	242	1.2	—	1.2	—	0.3
Niacin	260	1.0	—	1.3	4	0.9
Niacinamide	260	1.0	—	1.5	7	2.0
Pyridoxine	291	1.0	—	1.6	4	2.8
Thiamine	245	1.0	—	1.4	14	6.7
Folic acid	282	1.0	—	1.2	3	9.0
Riboflavin	267	1.1	>6000	1.2	5	12.8
Fat-Soluble Vitamins						
Vitamin A acetate	326	1.0	6000	1.1	—	4.6
Vitamin D_2	265	1.1	7000	1.1	17	12.5
Vitamin E	291	1.0	7000	1.2	8	19.3
Vitamin E acetate	284	1.0	8000	1.0	9	30.0

TABLE III: Analysis Data of Water- and Fat-Soluble Vitamins in a Multivitamin Tablet*

	Tablet 1 (mg)	Tablet 2 (mg)	Tablet 3 (mg)	Average (mg)	Label (mg)	Precision (RSD %)	Recovery of Spikes (%)
Water-Soluble Vitamins							
Vitamin C	62.1	74.0	70.2	68.8	60	8.8	101
Niacin	0	0	0	0	0	0	96
Niacinamide	19.2	18.50	18.1	18.6	20	2.8	93
Pyridoxine	2.52	2.51	2.41	2.48	2.0	2.6	98
Thiamine	1.51	1.45	1.45	1.47	1.5	2.3	95
Folic acid	0.416	0.422	0.416	0.418	0.40	0.8	95
Riboflavin	1.67	1.62	1.70	1.66	1.7	2.5	92
Fat-Soluble Vitamins							
Vitamin A acetate	4.60	4.45	4.40	4.48	†	2.3	91
Vitamin D_2	0.013	0.014	0.014	0.014	0.010	4.2	94
Vitamin E acetate	32.4	31.5	32.8	32.2	30.0	2.0	87

* In recovery experiments, the spike levels were 150 mg for vitamin C, 2–20 mg for the other water-soluble vitamins, 5.0 mg of vitamin A acetate, 0.5 mg of vitamin D_2, and 25 mg of vitamin E acetate.
† Tablet contains 500 IU of vitamin A as vitamin A acetate and β-carotene.

TABLE IV: Analysis Data of Water-Soluble Vitamins in Fortified Drinks, Baby Formula, Animal Feed Premix, and a Multivitamin Capsule

	Tang (mg/6 oz Serving)		Baby Formula (mg/5 oz Serving)		Feed Premix (mg/g)		Capsule (mg)	
	Label	Found	Label	Found	Label	Found	Label	Found
Water-Soluble Vitamins								
Vitamin C	60	78	9.0	14.5*	—	—	150	176
Niacinamide	1.8	2.5	1.050	0.97	9.87	7.18	25	26
Pyridoxine	0.22	0.29	0.060	0.088	—	—	2.0	2.23
Thiamine	—	—	0.100	0.175	2.43	2.26	10	11.3
Folic acid	0.080	0.039	0.015	0.015	0.49	0.28	1.0	1.06
Riboflavin	0.16	0.25	0.150	0.175	3.37	1.66	5.0	5.38
Fat-Soluble Vitamins								
Vitamin A acetate	—	—	—	—	3.1	4.5	2.75	3.95
Vitamin D_2	—	—	—	—	—	—	—	—
Vitamin D_3	—	—	—	—	0.14	0.10	—	—
Vitamin E acetate	—	—	—	—	75	65.2	†	—

* Impurity peak in the sample.
† Capsule contains vitamin E succinate.

rizes the analytical performance parameters for the HPLC method developed for water and fat-soluble vitamins — including precision, accuracy, limit of detection, selectivity, linearity and range, and ruggedness. We also will discuss guidelines for achieving better accuracy and robustness.

The method precision was excellent for all water-soluble vitamins except vitamin C because of its instability problems (5,6). Degradation of several labile water-soluble vitamins (for example, folic acid and niacin) in aqueous solution also can affect method accuracy. In our study, we prepared calibration standards for water-soluble vitamin analysis daily, and analyzed the sample extracts immediately afterward. Using Peltier-cooler autosampler trays can reduce sample degradation during analysis.

The diverse structures and stability problems further hampered the development of a single extraction procedure for the entire vitamin group. Our procedure for water-soluble vitamins uses a two-step sonication with dimethyl sulfoxide followed by an acidic extracting solution to facilitate recoveries of both polar and hydrophobic vitamins. Our optimization study indicated that a 5-min ultrasonic extraction provided good recovery of water-soluble vitamins in the test samples. We were able to facilitate the extraction of fat-soluble vitamins using a two-phase solvent system (ethanol–water and hexane). We increased the method specificity by using a high-efficiency column and peak confirmation with automated spectral analysis. We tested method ruggedness by using several columns from different batches and by testing the mobile phase on a variety of columns including those from different manufacturers (13). We evaluated column life by continuous

TABLE V: Summary of Analytical Method Performance Parameters for Vitamin Analysis

Performance Parameter	Water-Soluble Vitamins	Fat-Soluble Vitamins
Retention time precision (%)	<0.3	<0.15
Peak area precision (%)	<1.0*	<1.1
Tablet assay precision (%)	<3†	<3
Spike recovery (%)	90–100*	98 (vitamin A) 87 (vitamin E)
Limit of detection (ng, $S/N = 3$)	0.6–1.2	<1 (vitamins A and E) <10 (vitamin E)
Selectivity	PI < 1.1 $R > 3$ $n > 6000$ (Confirmation via spectral annotations of λ_{max} and purity index)	PI < 1.1 $R > 5$ $n > 8000$
Range (ng)	1–>1000	1–>10,000 (vitamin A) 5–>20,000 (vitamin E)
Linearity (r)	>0.9995	>0.999
Mobile-phase preparation ruggedness (%)	±5	±3
Column lifetime (injections)	>1000	>1000
Compatibility with other columns	Fair	Excellent
Temperature variation ruggedness	Sensitive	20–45 °C

* Except for vitamin C, which is unstable in a water solution.
† 10% for vitamin C.

injection of the standard solution. Both ns survived more than 1000 injections of idard sample with little efficiency degradation.

Method limitations and improvements: The accurate determination of vitamins in pharmaceutical and food products poses a difficult challenge for analytical chemists. Extraction efficiency, interferences from excipients, potential complications from isomerizations, and analyte degradation all are critical concerns. The requirements for a single extraction–analysis scheme of the entire vitamin group further complicates these difficulties. Although we applied the methods described in this article to many pharmaceutical preparations and fortified products, other samples with lower analyte concentrations (for example, natural food samples and sera) might require additional sample preparation or more-selective detection (5–7).

Several water-soluble vitamins — pantothenic acid, vitamin B_{12}, and biotin — were excluded from our study because of their lower sample concentration (B_{12}) or low UV absorptivity (biotin and pantothenic acid). Users might need to perform gradient analysis for these vitamins (9).

The instability of vitamin C due to oxidation in aqueous solution can be reduced by adding an antioxidant such as monothioglycerol to the extracting solution (8). The early n of vitamin C reduces the method use- for this analyte in many food samples.

The quantitative extraction of multivitamin tablets containing multiminerals — especially iron, calcium, and magnesium — might require additional sample preparation procedures or specialized treatment to reduce possible interferences (8). Therefore, the extraction scheme detailed in this article should be viewed as a starting point for further improvisation and validation for specific samples.

SUMMARY

We developed rapid HPLC methods for both water- and fat-soluble vitamin groups for survey or quantitative analysis. The improved analysis yielded excellent method performance for many pharmaceutical and fortified food samples.

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