Reversed-Phase HPLC Separation of Water-Soluble Vitamins on Agilent ZORBAX Eclipse Plus Columns

Application

Pharmaceutical, Food/Beverage

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Abstract

A mixture of six B vitamins and vitamin C were separated on the same Agilent ZORBAX Eclipse Plus C-18 stationary phase in three different particle sizes and column dimensions: 5 µm (4.6 × by 150 mm); Rapid Resolution (RR), 3.5 µm (4.6 × 100 mm), and RR High Throughput (RRHT), 1.8-µm (4.6 × 50 mm). A simple phosphate buffer-methanol mobile phase gradient was used. The method was designed to supplement United States Pharmacopeia (USP) methods in which these vitamins are separated singly on different columns by different methods, some involving complex ion-pair reagents. The shorter columns provided more rapid separations with little change in resolution. All compounds could be separated on the 1.8-µm column in 3.5 minutes. A gain in sensitivity was noted when the column length and particle size were reduced. The HPLC method was applied to the analysis of vitamins in a variety of commercial products, including multivitamins and various soft drinks. The columns chosen allowed for good separation, but the RRHT column also allowed quick method development without giving up the resolution and speed requirements for the method. The ZORBAX Eclipse Plus columns were shown to have high efficiency and superior performance characteristics with minimal tailing.

Introduction

Vitamins are essential nutrients for the proper functioning of the human body. The daily requirements are small, ranging from 2.4 µg to 90 mg [1]. Inadequate amounts of vitamins can cause numerous health problems, such as adrenal impairment (vitamin B) or scurvy or a compromised immune system (vitamin C). With the exception of pyridoxine and cyanocobalamin, water-soluble vitamins are not stored in the body [1]. Taking a multivitamin daily can ensure one is getting the proper amount of vitamins if diet alone cannot provide them.

Many vitamins easily degrade upon exposure to heat, light, and oxygen. Ascorbic acid is known to degrade quickly and easily in aqueous solutions. This degradation has been studied in solutions of different pH and in both aerobic and anaerobic conditions [2]. It has also been documented that the inside surface of glassware may contain materials that measurably degrade ascorbic acid within a short period of time. According to a study published by Margolis and Park, this is particularly true for autosampler vials used to hold samples for chromatography. Their paper gives several suggestions for preventing this decomposition [3]. In our study, vitamin samples were prepared every four

hours as needed. Although the percent relative standard deviation (%RSD) values given for ascorbic acid are very good, these injections were done immediately after the solution was made. Within a couple hours of preparing the solutions, we could see the peak areas for ascorbic acid decreasing with consecutive injections.

Currently, the United States Pharmacopeia (USP) has standard methods for the analysis of the vitamins used in our study; however, there is no USP method for the separation of all eight of the water-soluble vitamins in the mixture used. Some of the USP methods are complicated and involved the use of ion pair reagents (see Table 1). Ion pair reagents can often be used to improve resolution, but may result in irreversible changes in column performance [4]. The USP method for ascorbic acid is not currently a chromatographic method. Furthermore, these USP methods do not allow for the use of some newer column technologies, such as sub-2-micron particle sizes in shorter dimensions.

The main goal of the project was to create a single high-performance liquid chromatographic method that would allow separation of the following water-soluble vitamins: ascorbic acid, biotin, cyanocobolamin, niacinamide, panthothenic acid, pyridoxine, riboflavin, and thiamin. See Figure 1 for the structures of these vitamins. A secondary goal was to demonstrate the effect of decreasing particle size on resolution, separation time, and system pressure.

Experimental

Chromatographic experiments were conducted using an Agilent 1200 SL Rapid Resolution liquid chromatograph (LC) equipped with an autosampler and an 80-Hz diode array detector (Agilent Technologies, Inc., Santa Rosa, CA). Both isocratic and binary gradients were generated using this system. Columns used in the study were: 5-µm Agilent ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 150 mm (P/N: 959993-902), 3.5-m ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 100 mm (P/N: 959961-902), and 1.8-µm ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 50 mm (P/N: 959941-902) formats.

Vitamin standards were obtained from Sigma Aldrich (Milwaukee, WI). Identification of the vitamins in the standard mixture, supplements, and vitamin drinks was confirmed using the diode array detector with high-speed full-spectral UV-VIS detection.

Table 1. Reagents and Requirements for USP Methods (USP31-NF 26, May 2008. Ref. 5)

Vitamin	Reagents needed	Tailing Factor (TF)	%RSD t_R
Pyridoxine	Glacial acetic acid, sodium 1-hexanesulfonate, methanol, water	–	< 3%
Thiamine	Sodium 1-hexanesulfonate, phosphoric acid, hydrochloric acid, acetonitrile, water	< 2	< 3%
Cyanocobalamin	Methanol, water	–	–
Pantothenic acid	Monobasic potassium phosphate, phosphoric acid, methanol, water	–	< 2%
Biotin	Sodium perchlorate, phosphoric acid, dimethyl sulfoxide, acetonitrile, water	–	< 3%
Niacin	Sodium 1-hexanesulfonate, methanol, acetonitrile, glacial acetic acid, water	< 2	< 2%
Riboflavin	Glacial acetic acid, edetate disodium, sodium acetate, triethylamine, methanol, water	–	< 2%

Mobile phase channel A was 25 mM NaH_2PO_4 (pH = 2.5), mobile phase channel B was methanol. The flow rate was 1.0 mL/min. The complete chromatographic conditions are depicted in Table 2.

The mobile phase gradient was changed proportionally to match the column length to keep solvent-strength selectivity (k*) the same (see Table 3).

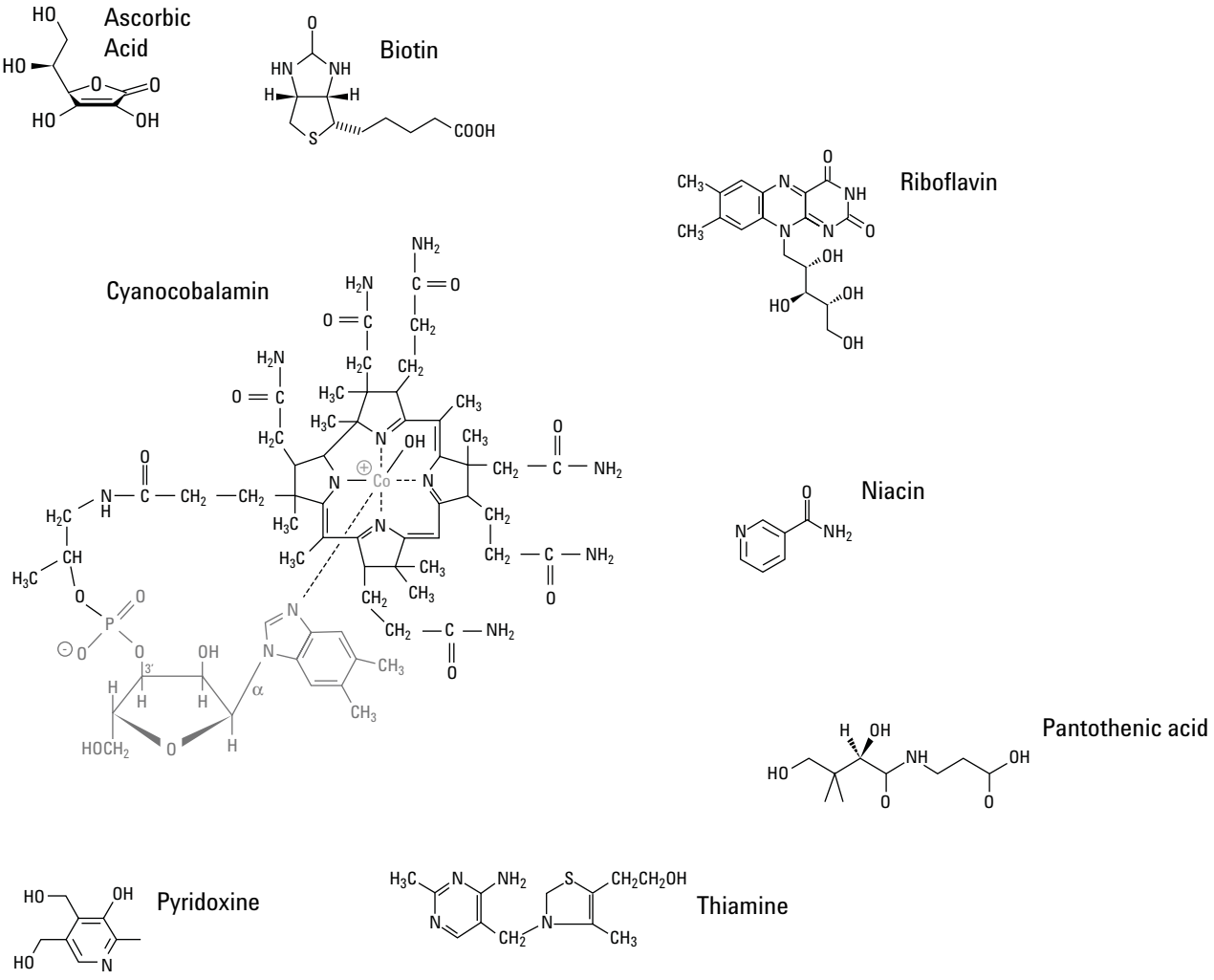

Figure 1. Structures of water-soluble vitamins used in this experiment.

Table 2. Chromatographic Conditions

LC	Agilent 1200 SL
Mobile phase A	25 mM NaH_2PO_4 pH = 2.5
Mobile phase B	Methanol
Flow rate	1.00 mL/min
Column compartment temperature	35 °C
Detection	220 nm, no Reference
Response time	0.05 s
Injection volume	Adjusted for column size: 5 µm, 5 µL 3.5 µm, 3.3 µL 1.8 µm, 1,7 µL
Detector flow cell	Micro flow cell (2 µL)

Table 3. Gradients for Equivalent k*

%B	5 µm	3.5 µm	1.8 µm
1	0.00 min →	0.00 min →	0.00 min
12	1.50 min	1.00 min	0.50 min
30	1.53 min	1.03 min	0.51 min

The individual standards and standard mixture were prepared by weighing out the appropriate masses of each vitamin using an analytical balance or microbalance, as needed, and dissolving in 10 mL of water. All samples that were analyzed for vitamins were purchased locally. The Vitaminwater (Glaceau, Flushing, NY) was injected without dilution. For sample preparation, the chewable vitamin tablets (Berkley & Jensen Children's Chewable Complete Multi Vitamins and Minerals Supplement, Natick, MA) were powdered and then dissolved in 100 mL of water. The adult tablet (Equate Adult Multivitamin, Walmart, Bentonville, AR) was treated in a similar manner. Table 4 outlines all of the final concentrations for standards and vitamin tablets. Prior to injection, all samples were filtered using Agilent syringe Econofilter, regenerated cellulose, 25-mm diameter, 0.20-µm porosity (P/N: 5185-5830).

Results and Discussion

Chromatographic Reproducibilty

The %RSDs for replicate injections of each vitamin are given in Table 5. These values were calculated based on seven replicate injections of each vitamin. In Table 5, the concentrations of each vitamin in the standard solution used to obtain reproducibility data on the 1.8-µm column is reported along with the %RSD values for retention time, area, and tailing factor (at 5 percent height). Some of the USP methods required that the %RSD for retention times be less than 2 or 3 percent (see Table 1); the method for cyanocobalamin had no such requirement. Table 5 shows that all the %RSD values were within the required range, and were actually much lower than the stated limits.

Table 4. Peak Identifications and Concentrations of Vitamins

Pk #	Vitamin	Conc. (g/L) in standard	Conc. (g/L) in soln. of adult vitamin tablet	Conc. (g/L) in soln. of chewable supplement
1	Ascorbic acid	8×10^{-2}	9×10^{-1}	6×10^{-1}
2	Niacin	4.5×10^{-2}	1.5×10^{-1}	2×10^{-1}
3	Pyridoxine	9×10^{-2}	5×10^{-2}	2×10^{-2}
4	Pantothenic acid	2.4×10^{-1}	1×10^{-1}	1×10^{-1}
5	Cyanocobalamin	9×10^{-2}	2×10^{-5}	6×10^{-5}
6	Biotin	Saturated	3×10^{-4}	4×10^{-4}
7	Riboflavin	1.5×10^{-1}	3.2×10^{-2}	1.7×10^{-2}

Table 5. %RSD on 1.8-µm Column

Vitamin	Conc. g/L	%RSD t_R	%RSD area	%RSD TF
Thiamine	1.20	0.7	0.3	1.3
Ascorbic acid	0.11	0.2	0.9	0.6
Niacin	0.15	0.5	0.3	0.9
Pyridoxine	0.09	0.3	1.8	1.9
Pantothenic acid	0.24	0.0	0.4	0.7
Cyanocobalamin	0.18	1.4	0.9	0.4
Biotin	Saturated	0.2	2.9	4.2
Riboflavin	0.15	0.2	2.3	0.9

Comparison of Tailing Factors at 5% Peak Height

Table 6 gives the retention times and tailing factors for each solute on the three ZORBAX Eclipse Plus columns used for these experiments. These columns have a very inert surface, which displays very little tailing for these water-soluble compounds. Note that the average tailing factors for all the vitamins studied were less than 1.2 on all the columns used. This is well within the range of acceptable tailing factors included in the USP methods (see Table 1).

Table 6. Comparison of Retention Times and Tailing Factors at 5% for Each Solute on the Three Agilent ZORBAX Eclipse Plus Columns

	5 µm		3.5 µm		1.8 µm	
Vitamin	Ret. time	Tailing factor	Ret. time	Tailing factor	Ret. time	Tailing factor
Ascorbic acid	2.10	1.1	1.41	1.2	0.76	1.2
Niacin	2.62	1.1	1.87	1.2	1.01	1.2
Pyridoxine	3.03	1.1	2.25	1.3	1.31	1.3
Pantothenic acid	4.02	1.1	2.95	1.1	1.68	1.2
Cyanocobalamin	4.47	1.1	3.33	1.1	1.98	1.2
Biotin	6.33	1.1	4.69	1.1	2.63	1.0
Riboflavin	7.24	1.1	5.40	1.1	3.04	1.1
Average (Tf)	–	1.1	–	1.2	–	1.2

Scalability from One Column Configuration to Another

The three column configurations gave a similar elution pattern as shown in Figure 2. The ZORBAX particles allowed for straightforward scalability from longer columns packed with 5-µm and 3.5-µm particles to the shortest column, packed with 1.8-µm particles. The same mixture was injected onto all three of the columns used. The gradient time was adjusted as described in Table 3 to give equivalent k* values.

Gain in Sensitivity When Comparing Three Different Column Configurations

Table 7 shows the gain in sensitivity for cyanocobalamin in the standard vitamin mix injected into the three different columns. Since the injection volume was scaled proportionally to column length, the peak heights cannot be compared directly. Instead, the amount injected is normalized, which allows the sensitivity to be compared. After the normalization, the ratio of the peak areas to injected amounts is the same. However, the data shows that there is a significant difference in the ratio of the heights and the injected amounts. There is a significant increase in the sensitivity of the 3.5-µm column compared to the 5-µm column, and another significant increase in the sensitivity of the 1.8-µm column. Table 7 shows the gain in sensitivity in comparison to the 5-µm column. The gain in sensitivity when the column is changed from a 5-µm column to a 3.5-µm column is 34 percent, while the gain in sensitivity when the column is changed from a 5-µm column to a 1.8-µm column is 52 percent. This gain in sensitivity is due to the higher efficiency (that is, lower dilution factors) of the smaller particles, not simply because the analytes spend less time in the column.

Table 7. Comparing the Sensitivity of Three Column Configurations (Cyanocobalamin)

Column	Normalized injection amount	Area	% area	Height	% height	% gain in sensitivity
5 µm	100	269	–	49	–	–
3.5 µm	66	171	64	48	98	34
1.8 µm	33	91	34	42	86	52

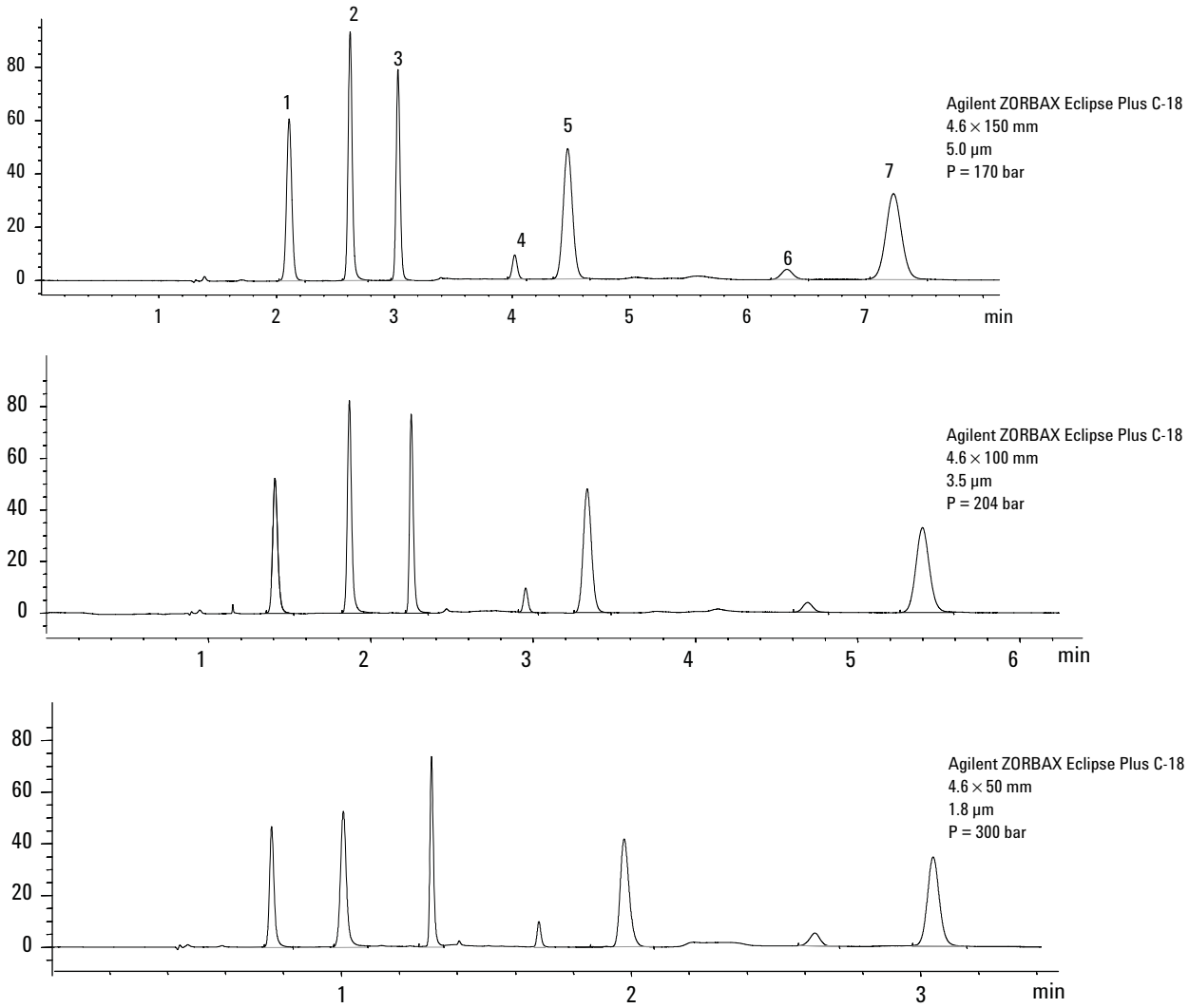

Figure 2. Scalability of Agilent ZORBAX Eclipse Plus C-18 column dimensions and the effect on analysis time and pressure (conditions shown in Table 2).

Comparing Speed and Pressure in Three Different Column Configurations

Figure 2 also shows the difference in separation time performed on the different columns. The separation was shortened from an 8-minute run time on the 4.6 x 150 mm, 5-µm column to a 3.5-minute run on the RRHT, 4.6 x 50 mm, 1.8-µm column. The time could have been shortened further by using a higher flow rate. With the good peak resolution shown in Figure 2 it would have been possible to increase the flow rate. However, when this was attempted, some of the peaks in the commercially available vitamins and drinks could not be identified due to the presence of other overlapping unidentified ingredients. As expected, the pressure increased when the particle size of the column packing was reduced. Figure 2 indicates the system pressures (P), in bar, for the three columns. The highest system pressure reached was 300 bar, when using the 1.8-µm column (RRHT). This is well within the operating range for both the Agilent 1100 (max. 400 bar) and the 1200 SL (max. 600 bar) systems. These results are summarized in Table 8.

Table 8. Gradient Separation Results of the Column Configurations, Holding k* Constant

Column Configuration			Separation Attributes	
Length (mm)	**Particle (m)**	**Flow (mL/min)**	**Gradient time (min)**	**Pressure (bar)**
150	5	1	8	170
100	3.5	1	6	204
50	1.8	1	3.5	300

Identification and Quantitation of Water-Soluble Vitamins in Vitamin Supplements

Figure 3 shows chromatograms of two different vitamin supplements. The upper chromatogram is for the Equate Adult Multivitamin, and the lower chromatogram is for the Berkley & Jensen Children's Chewable Multivitamin. The water-soluble vitamins that were positively identified in the sample are indicated on Figure 3 with the peak numbers assigned in Table 4. The primary identification was based on retention time, but a secondary positive identification was made for each vitamin using UV spectra. Although the standard mixture included seven of the water-soluble vitamins, an eighth water-soluble vitamin, thiamine, was added later to allow its identification and quantitation in the vitamin supplements.

In the adult vitamin, five of the seven vitamins that were in the stock solution were identified. Biotin was also identified, but neither an accurate retention time nor an area count could be recorded due to the very low concentration in the sample. In the children's chewable vitamin, six of the seven vitamins used in the stock solution were identified. In addition to these vitamins that were identified, in both the vitamins tested, the first peak that came out after the void was identified as thiamine. Each of the vitamins is needed in the body in different amounts. Due to the large range of concentrations in these samples, we were unable to get a chromatogram that showed good peaks for all the vitamins using UV-VIS detection. For example, ascorbic acid, niacin, and pyridoxine are needed in larger amounts and therefore are at higher concentrations in the vitamin tablets. Thus, the peaks are very large in the chromatograms. For the chromatograms depicted in Figure 3, the injection volume was changed to 15 µL as opposed to the 1.7 µL injection volume that was used for the standard mixture. These volumes were chosen in order to observe the peaks for the vitamins that were present in lower concentrations. Cyanocobalamin was not identified in any of the chromatograms of the vitamins, because it is only needed in very small amounts in the body, and only a few micrograms are present in the vitamin samples. Table 9 shows the quantitation data for thiamine, niacin, and pyridoxine in the adult and children's multivitamins. The measured values agree quite well with that expected from the label of the tablet bottles.

Some popular vitamin and energy drinks were also analyzed. These were sugar-free Red Bull (Red Bull, N.A., Inc., Santa Monica, CA) and strawberry-kiwi Vitaminwater. In these drinks, it was typically possible to identify ascorbic acid, niacin, and pyridoxine, but even when it was known to be added, a peak for cyanocobalamin did not appear in the chromatogram. This lack of signal was due to the low concentration of this vitamin in the drinks. Table 10 shows the quantitation data for niacin and pyridoxine in the sugar- free Red Bull and strawberry-kiwi Vitaminwater.

Table 9. Quantitation of Vitamins in Children's and Adult Multivitamins

Vitamin	Supplement	Expected value (mg/tab)	Measured value (mg/tab)
Thiamine	Adult multivitamin	1.5	–
Thiamine	Children's multivitamin	1.5	1.8
Niacin	Adult multivitamin	20	22.6
Niacin	Children's multivitamin	20	22.2
Pyridoxine	Adult multivitamin	2	2.0
Pyridoxine	Children's multivitamin	2	2.5

Table 10. Quantitation of Vitamins in Popular Drinks

Vitamin	Supplement	Expected value (mg)	Measured value (mg)
Niacin	Vitaminwater	12.5	12.1
Niacin	Red Bull, sugar free	21.2	30
Pyridoxine	Vitaminwater	5.0	5.8
Pyridoxine	Red Bull, sugar free	2.1	2.4

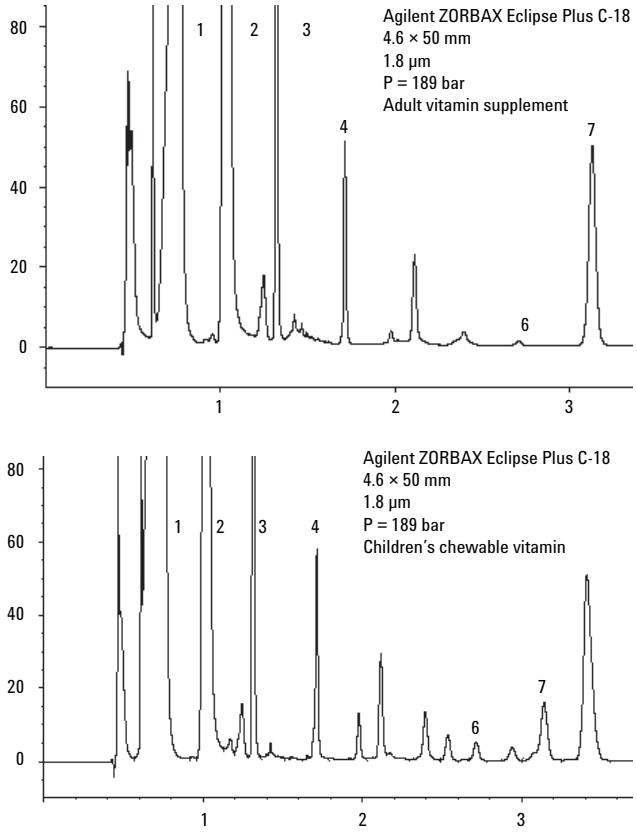

Figure 3. Chromatograms of solutions of an adult multivitamin supplement and a children's chewable vitamin tablet under the same conditions. Conditions are the same as in Figure 2 and positive identification of vitamins are as numbered in Table 4.

Conclusions

The chromatographic method allowed for the separation of water-soluble vitamins in a standard solution without the use of ion pair reagents. Six of the eight vitamins of interest were positively identified using retention times and UV spectra in an adult vitamin supplement. Seven of the eight vitamins of interest were positively identified by the same methods in a children's chewable multivitamin. Cyanocobalamin could not be identified in either tablet since it is found in very small amounts in these tablets, and the concentration in the vitamin drinks was below the limit of detection for this method. Quantitation of thiamine, niacin, and pyridoxine was performed for the two commercial vitamin tablets. Identification and quantitation of niacin and pyridoxine were also done for two popular energy/vitamin drinks. In this way, the method was proven suitable for the identification of vitamins in a variety of commercially available products.

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The method also showed that ZORBAX Eclipse Plus columns, which are available in a large variety of column dimensions and particle sizes, including RR formats, are useful for the separation and identification of water-soluble vitamins. These columns offer high efficiencies and display a very inert surface that gives superior performance with low tailing for these types of separations. The column dimensions chosen depend on the specific use objectives for the data being obtained. Shorter columns with smaller particles offer much shorter run times, which allow faster method development and higher throughput while still maintaining sufficient resolution.

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