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FACTORS AFFECTING THE ION-PAIR CHROMATOGRAPHY OF WATER-SOLUBLE VITAMINS

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SUMMARY

Factors controlling the separation of seven water-soluble vitamins on reversed-phase columns were systematically evaluated. Factors studied include both mobile phase constituents and column parameters. Data showed that a mobile phase containing hexanesulfonate (5 mM), methanol (15%), acetic acid (1%), and triethylamine (0.10–0.13%) yielded excellent separations with several C₈ and C₁₈ columns. Lowering the methanol concentration in the mobile phase enhanced the resolution of early eluting peaks, while the triethylamine level controlled the peak shape and retention of thiamine. The analytical precision, robustness, and sensitivity of the developed liquid chromatographic (LC) separation were evaluated. The stability of the LC separation was found to be satisfactory for over a 4-month period.

INTRODUCTION

Vitamins are vital substances for healthy growth and development of many organisms. Their determination is important in the food and pharmaceutical industries. Water-soluble vitamins (WSVs), include vitamin C, vitamins B₁, B₂, B₆, and B₁₂, and coenzymes niacin, biotin, folic acid, and pantothenic acid. They are common ingredients in multivitamin formulations. Official analytical methods are documented in the *United States Pharmacopeia/National Formulary* (USP XXI/NF XVI)¹. These assay procedures involve extractions and chemical reactions, followed by titration (ascorbic acid and pyridoxine), spectrophotometry (niacin), fluorimetry (thiamine and riboflavin), liquid chromatography (LC) (folic acid), or microbiological testing (cyanocobalamin and calcium pantothenate). These USP procedures are tedious and must be applied to each individual vitamin separately.

Recently, LC has been shown to be useful for the simultaneous determination of several WSVs. Separations based on ion-exchange^{2,3} and ion-pair chromatography on phenyl⁴, C₂ (ref. 5), C₈ (ref. 5), and C₁₈ columns⁶⁻⁸ were reported. The best separations were obtained with 1-hexanesulfonate as the ion-pairing reagent and

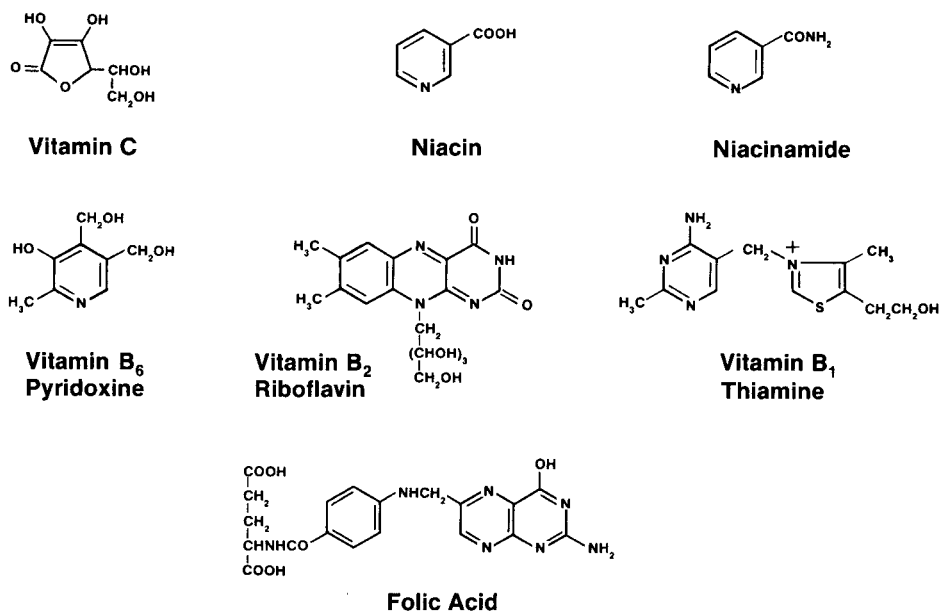


Fig. 1. Chemical structures of the seven water-soluble vitamins separated in this study.

Ion-pairing reagent

Although sodium 1-hexanesulfonate was most commonly used in the literature, the effect of C₅–C₁₀ alkylsulfonates was evaluated in this study to search for the optimum reagent. Retention data for 7 WSVs vs. alkylsulfonate concentration levels of 0–20 mM in the mobile phase are listed in Table I. Data for C₅, C₆, and C₇ alkylsulfonates are plotted in Fig. 2. Several significant trends with increasing concentration and alkyl chain length of the ion-pairing reagent are observed: (i) the retention times of highly water-soluble vitamins, ascorbic acid and niacin, (acidic solutes) remain unchanged, while that of niacinamide increases slightly; (ii) pyridoxine and thiamine, basic solutes that ion-pair with the sulfonates, show steep increase in retention times especially with long-chain alkylsulfonates; (iii) the retentions of hydrophobic solutes, folic acid and riboflavin, decrease slightly.

The optimum reagent appears to be hexanesulfonate (4–7 mM), confirming several reports using the same reagent at a 5 mM level^{7,8}. In this range, thiamine elutes between folic acid and riboflavin, yielding good separation of all seven WSVs in a reasonable analysis time. Pentanesulfonate is useful in the 7–15 mM range, although the resolution between niacinamide and pyridoxine is less satisfactory than that which is obtained with hexanesulfonate. The use of heptanesulfonate at the 0.5–2.5 mM level is problematic due to the very sharp increase of thiamine retention. Higher alkylsulfonates (C₈ and C₁₀) are unsatisfactory due to excessive retention (see Table I) and severe peak tailing of thiamine.

Mobile phase pH

The effect of pH in ion-pair chromatography is well understood¹². Fig. 3 shows the effect of mobile phase pH on WSV retention. The pH of the mobile phase containing 1% acetic acid and 5 mM sodium hexanesulfonate was adjusted from pH 2.4

TABLE I

RETENTION TIME OF WSVs vs. ALKYL SULFONATE

Column: Pecosphere-3CR C₈ at 2.0 ml/min; mobile phase: methanol-water (15:85) containing various concentrations of alkylsulfonate, 0.13% TEA, and 1% acetic acid.

Alkyl group	Sulfonate (mM)	Ascorbic acid	Niacin	Niacinamide	Pyridoxine	Folic acid	Thiamine	Riboflavin
C ₅	0	0.52	0.61	0.86	0.67	3.24	0.52	5.95
	1	0.51	0.65	0.84	0.70	2.67	0.65	5.19
	2	0.51	0.66	0.86	0.79	2.79	0.79	5.70
	5	0.51	0.66	0.87	0.97	2.72	1.25	5.59
	10	0.51	0.66	0.90	1.14	2.62	1.72	5.40
	20	0.51	0.66	0.91	1.25	2.14	2.25	4.17
C ₆	1	0.50	0.65	0.88	0.86	2.69	0.95	5.50
	2	0.51	0.66	0.89	1.07	2.58	1.47	5.40
	5	0.50	0.65	0.93	1.42	2.55	3.10	5.20
	10	0.50	0.65	0.95	1.81	2.48	5.30	4.69
	20	0.50	0.64	0.96	2.01	2.00	7.30	3.80
	C ₇	1	0.49	0.64	0.90	1.25	2.52	2.15
2		0.50	0.65	0.97	1.67	2.55	4.95	4.90
5		0.50	0.65	0.99	2.32	2.32	11.13	4.14
10		0.49	0.65	1.02	3.03	2.26	> 30	3.93
C ₈		1	0.51	0.66	0.91	1.36	2.43	5.55
C ₁₂	2	0.50	0.65	1.03	2.63	2.36	> 30	4.11
C ₁₈	1	0.51	0.65	0.94	1.96	2.32	> 40	4.26

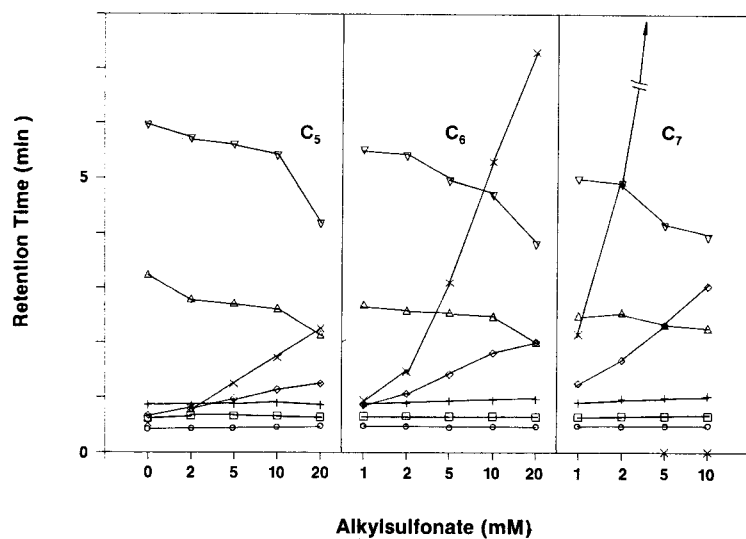


Fig. 2. The effect of alkylsulfonate on WSV retention. Column: Pecosphere-3CR C₈; mobile phase: methanol-water (15:85) containing various concentrations of alkylsulfonate, 1% acetic acid, and 0.13% TEA; flow-rate 2 ml/min. (O) Ascorbic acid; (□) niacin; (+) niacinamide; (◇) pyridoxine (Vit. B₆); (△) folic acid; (×) thiamine (Vit. B₁); (▽) riboflavin (Vit. B₂).

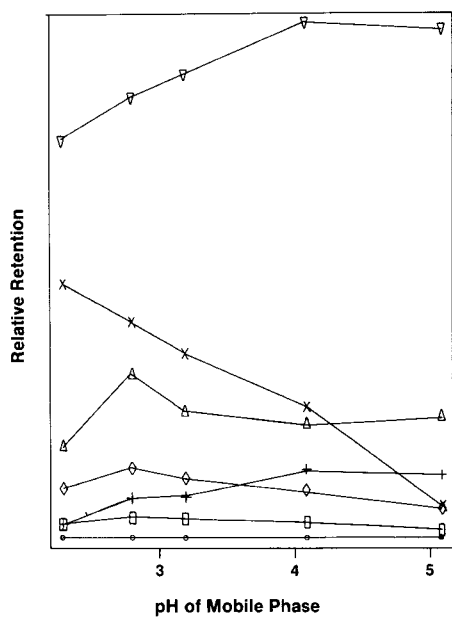


Fig. 3. The effect of pH on WSV retention. The aqueous portion of the mobile phase contains 5 mM hexanesulfonate and is adjusted to various pHs. Other mobile phase parameters and symbols are the same as in Fig. 2.

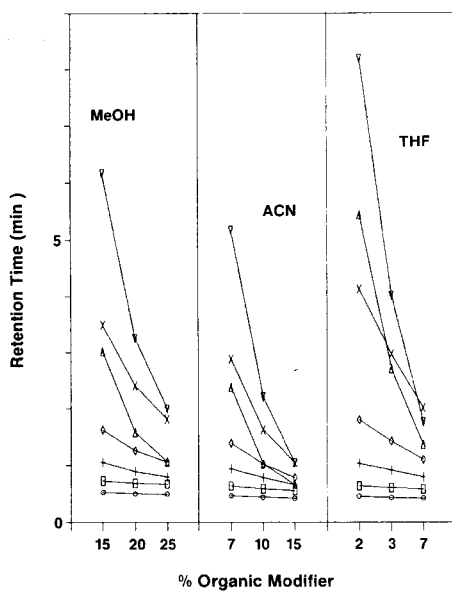


Fig. 4. The effect of organic modifier on WSV retention. Mobile phase contains various levels of organic modifier in 5 mM hexanesulfonate, 1% acetic acid, and 0.13% TEA. Symbols as in Fig. 2. MeOH = Methanol, ACN = acetonitrile, THF = tetrahydrofuran.

to 5.2 by the addition of sodium hydroxide or phosphoric acid. The most significant effect is the reduction of retention time of thiamine with increasing pH. Pyridoxine also shows some reduction though the effect is much less pronounced. These reductions can be attributed to less effective ion-pairing at the higher pH¹². The pH for optimum resolution under the experimental mobile phase conditions ranges from 2.8 to 3.2.

Organic modifiers

Fig. 4 shows the retention data vs. three concentration levels of organic modifiers: methanol, acetonitrile, and tetrahydrofuran (THF). The concentration levels were adjusted to compensate for the difference in elutropic strength of each solvent. As expected, the elutropic strengths rank in the order of THF > acetonitrile > methanol. Solute capacity factors (k') increase with lowered solvent strengths. The increase is steep for folic acid and riboflavin which are retained by solvophobic interactions. Changes of elution order between folic acid and thiamine were observed in acetonitrile and THF.

Fig. 5 shows the chromatograms obtained using 15% methanol, 7% acetonitrile and 3% THF respectively. Methanol gives the best overall resolution, especially for the early eluting WSVs. Acetonitrile appears to give slightly sharper peaks but

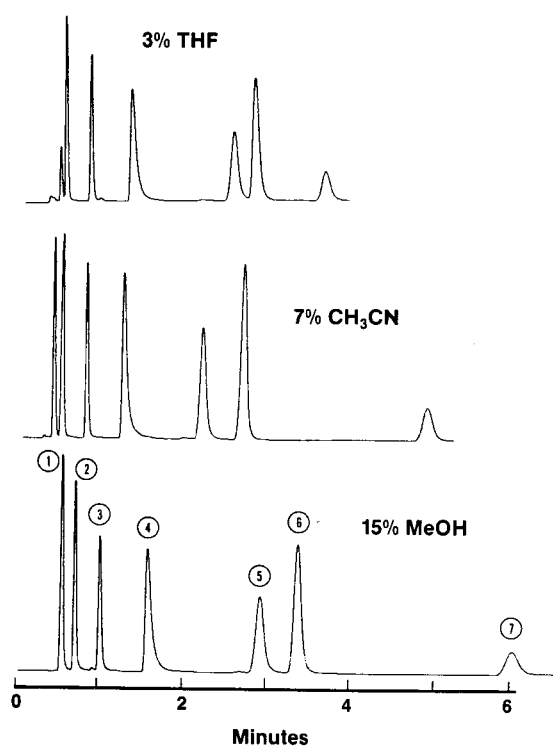


Fig. 5. Chromatograms showing the separation of WSVs using different organic modifiers. See the experimental section for column and mobile phase conditions. Peak identification: 1 = ascorbic acid; 2 = niacin; 3 = niacinamide; 4 = pyridoxine; 5 = folic acid; 6 = thiamine; 7 = riboflavin.

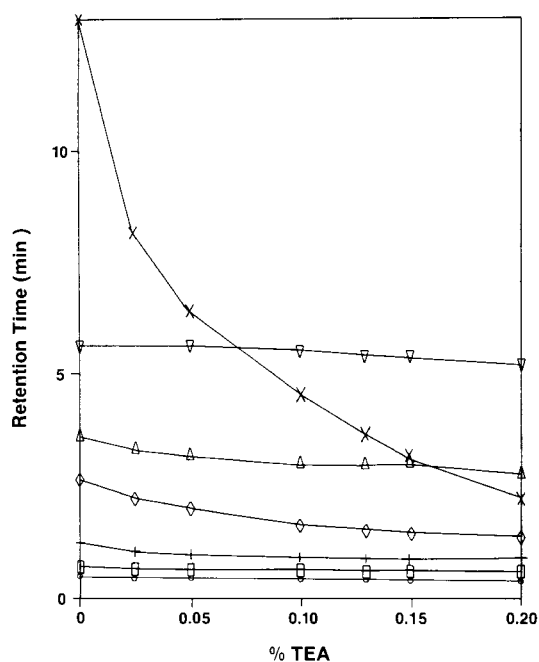


Fig. 6. The effect of TEA on WSV retention. The mobile phase conditions are listed in Table III except TEA% is varied. Symbols as in Fig. 2.

yields inadequate separation for ascorbic acid and niacin. THF produces very small peaks for ascorbic acid possibly caused by its reaction with impurities in THF.

Mobile phase additives

The effect of mobile phase additives including acetic acid, triethylamine (TEA), and ammonia was studied. Results are summarized below:

Acetic acid. Acetic acid was used to acidify the mobile phase to facilitate the ion-pairing of basic solutes with the alkylsulfonates. Presence in the mobile phase also improves the peak shape of folic acid. A 1% acetic acid level appears to be satisfactory.

Triethylamine. The use of triethylamine as a mobile phase additive to reduce peak tailing of basic solutes is well documented¹². Without TEA, thiamine showed poor peak shape on most of the columns evaluated. This phenomenon is caused by residual silanol groups or trace metals in some silica material as suggested by previous research^{13,14}. The effect of varying concentrations of TEA at methanol concentration of 15–25% was studied. The retention data at 15% methanol vs. % TEA are shown in Fig. 6. As expected, only the retention of the basic solutes (*i.e.*, thiamine and pyridoxine) is significantly affected by TEA. Thiamine shows a drastic reduction of retention time from 13 to under 3 min when the TEA concentration is increased from 0 to 0.2%. Under the experimental conditions, a TEA concentration of 0.10–0.13% gives symmetrical peaks and good separation of thiamine from riboflavin and folic acid. TEA levels below 0.03% yield severely tailing peaks for thiamine (with peak symmetry factors > 2)¹². Ammonia can be used at 0.1–0.4% levels instead of TEA, but is much less effective in improving the peak shape of thiamine.

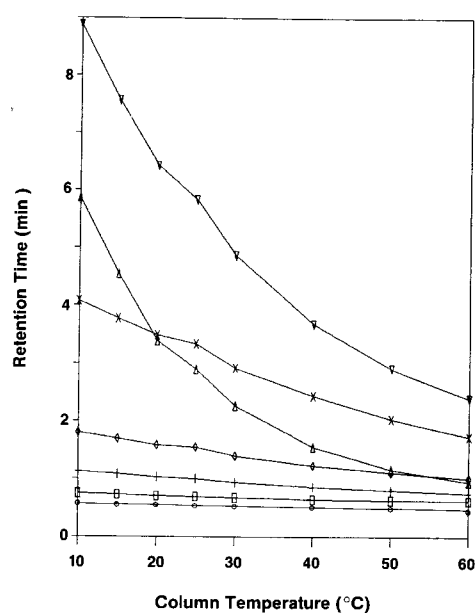


Fig. 7. The effect of column temperature on WSV retention. The mobile phase conditions are listed in Table III except column temperature is varied. Symbols as in Fig. 2.

Column temperature

All solutes show reduced retention times at elevated column temperatures as shown in Fig. 7. The temperature effects are: (i) slight for solutes with low k' such as ascorbic acid, niacin, and niacinamide; (ii) moderate for pyridoxine and thiamine whose retention mechanism is ion-pairing; (iii) strong for highly retained molecules such as folic acid and riboflavin whose retention mechanism is solvophobic effect.

Data show good band spacing between peaks and indicate that resolution of all WSVs is possible under the ambient temperature range of 22–28°C.

Summary of optimum mobile phase conditions

Table II summarizes the optimum ranges of mobile phase conditions leading to superior separation of seven WSVs. The criteria are resolution, peak shape, and

TABLE II
SUMMARY OF OPTIMUM CONDITIONS

<i>Factors</i>	<i>Optimum range</i>
Ion-pairing reagent	4–7 mM 1-hexanesulfonate
pH	2.8–3.2
Organic modifier	12.5–20% methanol
Additives	1% acetic acid 0.10–0.13% TEA
Column temperature	22–28°C

shows that smaller particles (*i.e.*, 3- μm) generate higher performance by delivering higher efficiency per unit time¹⁷. Pecosphere-3CR C₈ (83 mm long, 3- μm particles) yields similar resolution of the seven WSVs in 6 min similar to what Pecosphere-5CR C₈ (150 mm long, 5- μm particles) does in 12 min. If lower resolution is acceptable, separation of the seven WSVs can be performed in 0.5–2.0 min using a very short (33 mm long) 3- μm C₈ column^{15,17}. However, considering both resolution and performance, the best choice appears to be a longer 3- μm C₈ column.

Repeatability of retention time and peak area

Repeatability was evaluated by injecting a standard WSV solution using an autosampler either consecutively or over an 8-h period. The column was thermostatted to eliminate temperature effects. The coefficients of variation (C.V.) of retention time and peak area of each solute were calculated and are listed in Table III.

Data show excellent precision of retention time (< 0.01 min) and peak area (average C.V. of 0.39% for eight consecutive analyses and 0.6% for the 8-h analysis; averages excluding ascorbic acid). Ascorbic acid showed a peak area precision of 3.6–10% C.V., caused by a decrease in the peak area, attributable to the oxidation of ascorbic acid in aqueous solution¹⁸.

These excellent precision levels demonstrate that WSVs can be analyzed reproducibly under ion-pairing conditions using TEA as an additive. The accurate quantitation of ascorbic acid, however, requires special precautions to reduce oxidation of both the standard and extracted sample solutions.

Column lifetime

Column lifetime was evaluated by repetitive injections of a standard sample over a 9-day period. A total of 1700 injections was performed. No significant change in column performance was observed and the resolution of all seven WSVs was maintained. The pressure drop at 1.5 ml/min remained constant at 2800 p.s.i. and the column efficiency remained at 10 200–10 400 plates for the thiamine peak. The column was protected with a pellicular guard column and scavenger column as detailed in the experimental section. These data suggested that the LC separation employed is capable of long-term stability with reasonable operating care.

Sensitivity

WSVs are commonly detected by a variable-wavelength absorbance detector

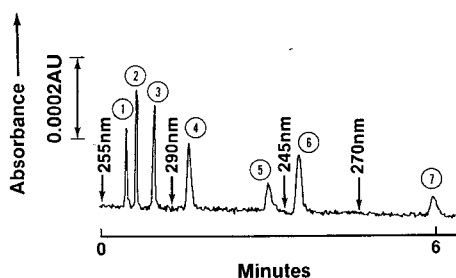


Fig. 9. High-sensitivity determination of trace level of WSVs using UV absorbance detection at optimized wavelengths. Peak identification: 1 = ascorbic acid (2.3 ng); 2 = niacin (2.5 ng); 3 = niacinamide (2.5 ng); 4 = pyridoxine (2.6 ng); 5 = folic acid (1.5 ng); 6 = thiamine (2.7 ng); 7 = riboflavin (0.7 ng).

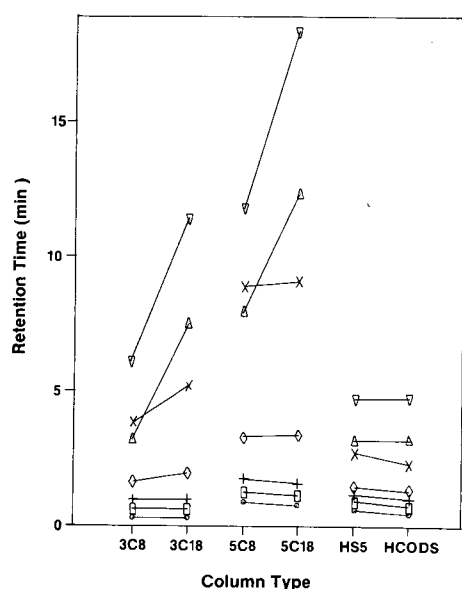


Fig. 8. Retention data of WSV on different C_8 and C_{18} columns. The mobile phase conditions are listed in Table III except the column used is varied. Symbols as in Fig. 2.

As shown in Fig. 8, the HS-5 (Pecosil-5 C_8 , 125 mm \times 4.6 mm I.D., packed with 5- μ m irregular C_8 particles) and the 5- μ m HCODS (Pecosphere HS-5 HCODS, 125 mm \times 4.6 mm I.D., packed with wide-pore (300 Å), spherical, polymerized C_{18} particles) have relatively low k' values for the WSVs. Both columns yield symmetrical peaks for thiamine without TEA in the mobile phase.

Effect of particle size and column length. Comparison of chromatograms from columns packed with similar material of different particle sizes (e.g., 3 C_8 and 5 C_8),

TABLE III

REPEATABILITY OF RETENTION TIME AND PEAK AREA

Column: Pecosphere-3CR C_8 at 2.0 ml/min, thermostatted at 35°C; mobile phase: methanol-water (15:85) containing 5 mM of alkylsulfonate, 0.13% TEA, and 1% acetic acid; number of injections in each experiment: 8; concentration of WSV standard ranged 2–5 μ g/ml; injection volume was 10 μ l.

Vitamin	Retention time (min \pm range)	C.V. of peak area	
		1 h (%C.V.)	8 h (%C.V.)
Ascorbic acid	0.54 \pm 0.00	3.6	9.8
Niacin	0.66 \pm 0.00	0.29	0.51
Niacinamide	0.92 \pm 0.00	0.33	0.52
Pyridoxine	1.32 \pm 0.00	0.44	0.55
Folic Acid	2.21 \pm 0.01	0.36	0.47
Thiamine	2.64 \pm 0.01	0.39	0.81
Riboflavin	4.65 \pm 0.01	0.56	0.77

analysis speed, in order of importance. While the data are derived from the specific column studied, the trends and mobile phase ranges might be extended to other similar reversed-phase columns.

The choice of hexanesulfonate at about 5 mM level as the ion-pairing reagent is rather straightforward and is based primarily on resolution considerations. The pH range of 2.8–3.2 is obtained by adding 1% acetic acid and 0.10–0.13% TEA to a 5 mM sodium 1-hexanesulfonate solution. Methanol is clearly the best organic modifier based on the better resolution of ascorbic acid and niacin. Lowering the percentage of methanol in the mobile phase increases resolution of the early eluting WSVs at the expense of analysis time. TEA controls primarily the retention and peak shape of thiamine. Since most coelution or peak crossing situations involve thiamine (Figs. 2–4 and 6), TEA concentration can be adjusted to avoid coelution problems. Elevated column temperature decreases the retention and resolution of all WSVs to various degrees. While thermostating the column increases precision, ambient column temperature operation is preferable for convenience and instrumental simplicity. Judging from Fig. 7, the separation appears to be rugged enough to withstand normal room temperature fluctuations between 22–28°C without major coelution problems.

For a particular column selected, the percentages of methanol and TEA are the two mobile phase factors that can be varied readily to control the separation. The percentage of methanol adjusts the overall solvent strength and retention, while the TEA controls the retention of thiamine and fine-tunes its resolution from other WSVs. In practice, thiamine can be kept midway between folic acid and riboflavin for optimum analysis. Based on these criteria, the optimum mobile phase for room temperature operation was found to be methanol–water (15:85) containing 5 mM sodium 1-hexanesulfonate, 1% acetic acid, and 0.10–0.13% TEA.

While the selection of the above mobile phase conditions involved a somewhat subjective decision, such compromises are necessary for complex separations that are influenced by many independent factors. Alternately, similar results could be derived through a systematic sequential solvent optimization search¹⁵. Further experiments were conducted to evaluate the suitability of the mobile phase conditions for routine analysis of WSVs. The evaluations included column selectivity effects and lifetime, precision studies, sensitivity determination, and sample analysis.

Column effects

Column selectivity effects were evaluated by collecting retention data on six different C₈ and C₁₈ columns under identical mobile phase conditions. Data are summarized in Fig. 8. These columns differ in dimension, physical characteristics of the base silica and bonding chemistries. While analysis times range from 4 to 18 min, baseline resolution of the seven WSVs is obtained on all the columns, demonstrating the ruggedness of the mobile phase conditions⁹.

Effect of alkyl chain length and selectivity. The effect of bonded-phase alkyl chain length (i.e., C₈ or C₁₈) can be delineated by data from two column sets (3C₈ and 3C₁₈, 5C₈ and 5C₁₈). Each set of columns is packed with identical 3- μ m or 5- μ m base silica but are bonded with either C₈- or C₁₈-monochlorosilane¹⁶. Data show substantially higher *k'* values of the hydrophobic WSVs (folic acid and riboflavin) on C₁₈ bonded phase. C₈ columns appear to be preferable since they yield comparable resolution in shorter assay times.

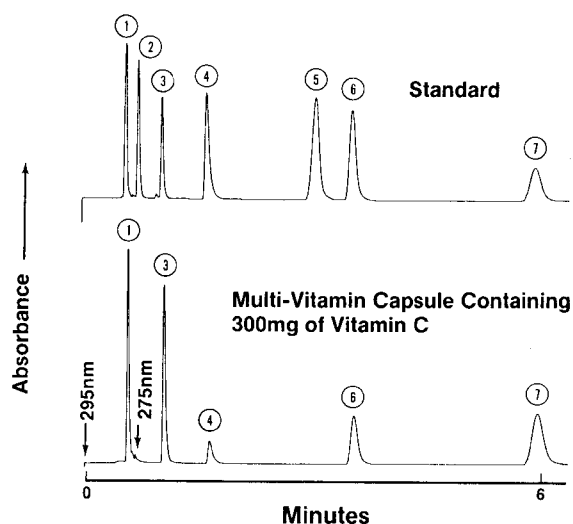


Fig. 10. The analysis of a multivitamin capsule containing 300 mg of vitamin C using the developed LC separation. Peak identification is the same as in Fig. 5.

set at a compromised wavelength in the range of 265–280 nm. Since wavelengths of maximum absorbance vary for each WSV, enhancements in detection limits up to a factor of 4 can be realized through wavelength programming. Fig. 9 illustrates a high-sensitivity analysis of WSVs under optimized absorbance wavelength conditions. The detection limits were estimated to be 0.5 ng for folic acid and thiamine, and 0.2 ng for the other WSVs.

Sample analysis

A multi-vitamin capsule was extracted according to a published method⁵ and analyzed by the developed LC method. Chromatograms are shown in Fig. 10. Because of the very high ascorbic acid level in the sample (300 mg), which caused detector saturation and low assay results (115 mg), the monitoring wavelength for ascorbic acid was changed to 295 nm. Table IV summarizes the data obtained which showed reasonably good agreement with the label claim. Further work is planned to

TABLE IV
COMPARISON OF ASSAYED VALUES WITH LABEL CLAIM

Vitamin	Assayed value (mg)	Label claim (mg)
Ascorbic acid	280	300
Niacin	0	0
Niacinamide	51.2	50
Pyridoxine	6.1	5
Folic acid	0	0
Thiamine	15.2	15
Riboflavin	10.8	10

uncover potential practical difficulties in applying this LC separation to more complex multi-mineral multi-vitamin tablets.

Addressing problem areas in WSV determination

The data generated in this study can be used to help resolve the many problems commonly encountered in LC determination of WSVs. While the absolute data pertain only to a specific column, the data trends found are significant to most C₈ and C₁₈ columns. Poor peak shapes of thiamine, pyridoxine, and folic acid are controlled by TEA and acetic acid in the mobile phase. Coelution problems and sensitivity of elution pattern to column-to-column variations are primarily caused by peak crossing of thiamine, which can be controlled by adjusting TEA concentration. Resolution of early eluting WSVs is improved by lowering the methanol concentration or using a longer column. Ascorbic acid has a very low k' value under the current operating conditions. Its k' value is unaffected by most mobile phase and column factors. While its separation might be satisfactory for simple mixtures, its resolution from interfering excipients in complex dosage forms can be a difficult challenge and might require different approaches, such as gradient elution⁵, anion pairing⁵, or spectroscopic deconvolution techniques¹⁹.

CONCLUSIONS

The systematic evaluation in this study confirmed the use of 5 mM hexanesulfonate and 1% acetic acid used in the mobile phase prescribed in previous reports. Methanol, ranging from 12.5–20% in the mobile phase, gives adequate resolution of seven WSVs in a reasonable analysis time. TEA controls the resolution and peak shape of thiamine and reduces the sensitivity to column-to-column variability. An optimum mobile phase (15% methanol–water containing 5 mM hexanesulfonate, 1% acetic acid, and 0.13% TEA) was found to yield complete resolution of 7 WSVs on many C₈ and C₁₈ columns with excellent precision, column lifetime, and sensitivity. C₈ columns were preferable since analysis times were substantially lower than comparable C₁₈ columns. Repeatability of retention time and peak area was found to be excellent for all WSVs except ascorbic acid. The LC separation was stable during the 4-month study period. Detector wavelength programming enhances analytical sensitivity and avoids detector saturation caused by high ascorbic acid levels in some formulations.

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