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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 m*M*), methanol (15%), acetic acid (1%), and triethylamine (0.10–0.13%) yielded excellent separations with several C_8 and C_{18} 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 B_1, B_2, B_6, and B_{12}, 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 Formulatory* (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 phenyl[4], C_2 (ref. 5), C_8 (ref. 5), and C_{18} columns[6–8] were reported. The best separations were obtained with 1-hexanesulfonate as the ion-pairing reagent and

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either a C_8 or C_{18} column[5–8]. The LC analyses are fast (<40 min), amenable to several WSVs simultaneously, and yield accurate quantitative results[6–8]. However, the use of LC for routine assay remains difficult because of the following problem areas: (i) peak coelution; (ii) poor peak shapes of thiamine, pyridoxine, and folic acid; (iii) sensitivity of elution pattern to column-to-column variations, temperature, and column age; (iv) low retention excipient materials that interfere with the determination of ascorbic acid; (v) poor column lifetimes.

In this paper, we attempt to address these problem areas through a systematic study of factors influencing the ion-pair chromatography of WSVs. The factors evaluated were ion-pair reagent, pH, organic modifier, mobile phase additives, temperature, alkyl chain length and particle size. The objective was to develop a robust isocratic LC separation that is immune to minor compositional changes in the mobile phase, temperature fluctuations, and column-to-column variability[9]. Seven WSVs were separated including ascorbic acid, niacin–niacinamide, pyridoxine, folic acid, thiamine, and riboflavin. Other WSVs were not included in this study because their low dose levels (cyanocobalamin) or their low UV absorptivity (calcium pantothenate and biotin) made a simultaneous LC assay with other WSVs impractical. Further experiments were performed to delineate the column compatibility, precision, column lifetime and analytical sensitivity of the developed separation.

EXPERIMENTAL

Apparatus

The LC system used consisted of a Series 410 quaternary pump, an LC-235 photodiode array detector, a Model 7125 manual injector or an ISS-100 automatic sampler, and an LCI-100 integrator. Spectral data from the photodiode array detector aided in solute identification and eliminated the need to inject individual standards. All LC equipment was from Perkin-Elmer (Norwalk, CT, U.S.A.). Other equipment included a Neslab refrigerated circulating water bath for column temperature control, a pH meter (Orion Research, Cambridge, MA, U.S.A.), and an ultrasonic bath (Model W-185, Branson Scientific, Danbury, CT, U.S.A.) for sample extractions.

Columns

A C_8 column, Pecosphere-3CR C_8, 83 mm × 4.6 mm I.D., was the primary column used. This column is packed with 3-μm, end-capped spherical C_8 bonded-phase material with a carbon loading of 10.7%, surface area of 170 m^2/g, and 80 Å pore diameter[10]. This column was selected because of its high efficiency (150000 plates/m) and because its packing typifies the reversed-phase materials used in many other columns. Five other C_8 and C_{18} columns were evaluated for column selectivity effects. A detailed description of these columns has been published elsewhere[10].

A low-capacity guard column (30 mm × 2.1 mm I.D., dry-packed with 40-μm C_{18} pellicular material) and a scavenger column (33 mm × 4.6 mm I.D., packed with 10-μm C_{18} particles and connected between the pump and the injector) were used to protect the analytical column from mobile phase and sample contaminants. These precolumns are effective devices to maintain column performance during the experiment and to prolong column lifetime[11]. The retention characteristics of solutes

were not affected by the guard column because of its small volume (< 0.1 ml) and the very low surface area of its packing.

Chemicals and reagents

Vitamins, chemicals, and reagents were of the highest purity grade available and were obtained from various suppliers including: Aldrich (Milwaukee, WI, U.S.A.), Sigma (St. Louis, MO, U.S.A.), Mallinckrodt (St. Louis, MO, U.S.A.), and Fisher Scientific (Fair Lawn, NJ, U.S.A.). 1-Hexanesulfonic acid, sodium salt (HPLC grade), was from Eastman-Kodak (Rochester, NY, U.S.A.). All organic solvents were HPLC grade obtained from EM Science (Cherry Hill, NJ, U.S.A.). Water was filtered and purified by passage through mixed-bed ion-exchange and activated charcoal cartridges. Vitamin capsules and tablets were purchased from the open market in Connecticut.

Procedure for preparing the mobile phase

The optimum mobile phase was found to be methanol–water (15:85) containing 5 m*M* sodium hexanesulfonate, 1% acetic acid, and 0.10–0.13% triethylamine (TEA) at a pH of 3.2. This mobile phase was prepared by dissolving 941 mg of sodium 1-hexanesulfonate, and adding 10 ml of glacial acetic acid and 1.0–1.3 ml of TEA in one liter of water. The pH was found to be 3.2. An 850-ml fraction of this aqueous solution was mixed with 150 ml methanol followed by further degassing via helium sparging. Other mobile phases used during method development were prepared similarly using the appropriate reagents and were mixed with organic solvents either manually or by the LC pump. Prior to sample injection, the column was washed with at least 20 times its column volume of the mobile phase.

Procedure for preparing standards and samples

Folic acid solution (1 g/l) was prepared in 0.1 *M* sodium carbonate solution (adjusted to pH 7)[5]. Riboflavin (0.1 g/l) and all other WSVs (1 g/l) were prepared in water. Fresh standard solutions were prepared weekly in water and were kept refrigerated in amber bottles until used. Mixed WSV standard solutions in capped vials remained stable at room temperatures for at least 1 day, with the exception of ascorbic acid which degrades rapidly in aqueous solution. For the analysis of a pharmaceutical product, the extraction procedure published by Vandemark and Schmidt[5] was followed.

RESULTS AND DISCUSSION

Mobile phase factors

Fig. 1 shows the structures of the seven WSVs separated in this study. They differ greatly in their chemical structures and properties. The structures vary from a simple pyridine ring in niacin to the multiring structure found in folic acid. Water solubilities differ from the very water-soluble ascorbic acid to the hydrophobic, sparingly water-soluble riboflavin. Thiamine and pyridoxine are cations which ion-pair with the alkylsulfonates under acidic pH values.

The effect of various factors on the retention and resolution of WSVs under reversed-phase ion-pair chromatography conditions was evaluated by performing a series of experiments. Results are described below.

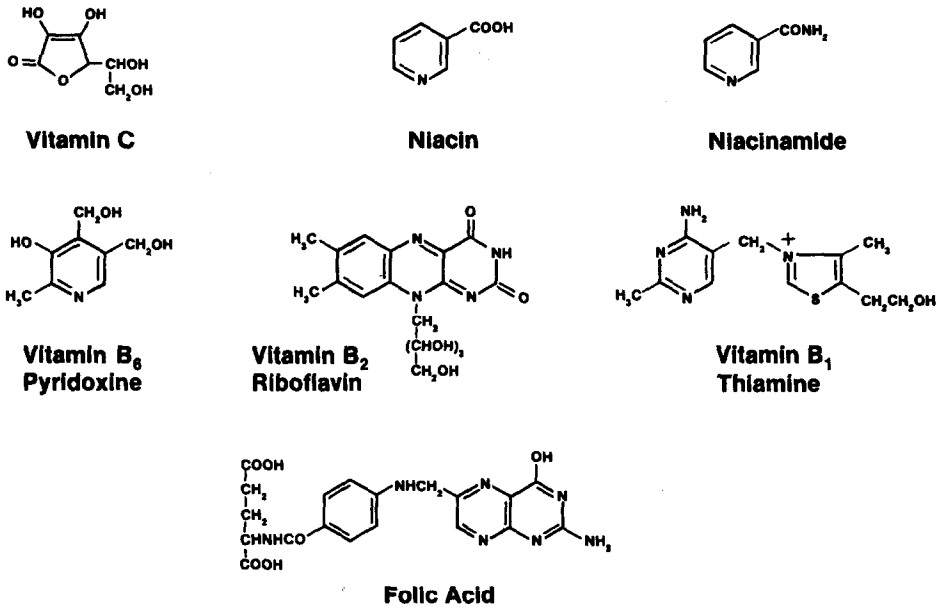

Fig. 1. Chemical structures of the seven water-soluble vitamins separated in this study.

Ion-pairing reagent

Although sodium 1-hexanesulfonate was most commonly used in the literature, the effect of C_5–C_{10} alkylsulfonates was evaluated in this study to search for the optimum reagent. Retention data for 7 WSVs *vs.* alkylsulfonate concentration levels of 0–20 m*M* in the mobile phase are listed in Table I. Data for C_5, C_6, and C_7 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 m*M*), confirming several reports using the same reagent at a 5 m*M* 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 m*M* 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 m*M* level is problematic due to the very sharp increase of thiamine retention. Higher alkylsulfonates (C_8 and C_{10}) 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 understood[12]. Fig. 3 shows the effect of mobile phase pH on WSV retention. The pH of the mobile phase containing 1% acetic acid and 5 m*M* sodium hexanesulfonate was adjusted from pH 2.4

TABLE I

RETENTION TIME OF WSVs *vs.* ALKYLSULFONATE

Column: Pecosphere-3CR C_8 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*	*Niacin-amide*	*Pyri-doxine*	*Folic acid*	*Thiamine*	*Ribo-flavin*
	0	0.52	0.61	0.86	0.67	3.24	0.52	5.95
C_5	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	4.98
	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	4.59
	2	0.50	0.65	1.03	2.63	2.36	>30	4.11
C_{10}	1	0.51	0.65	0.94	1.96	2.32	>40	4.26

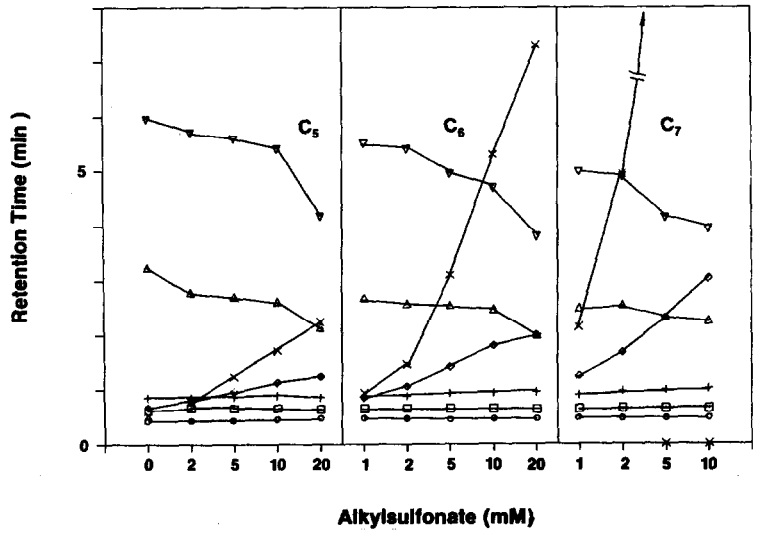

Fig. 2. The effect of alkylsulfonate on WSV retention. Column: Pecosphere-3CR C_8; mobile phase: methanol–water (15:85) containing various concentrations of alkylsulfonate, 1% acetic acid, and 0.13% TEA; flow-rate 2 ml/min. (○) Ascorbic acid; (□) niacin; (+) niacinamide; (◇) pyridoxine (Vit. B_6); (△) folic acid; (×) thiamine (Vit. B_1); (▽) riboflavin (Vit. B_2).

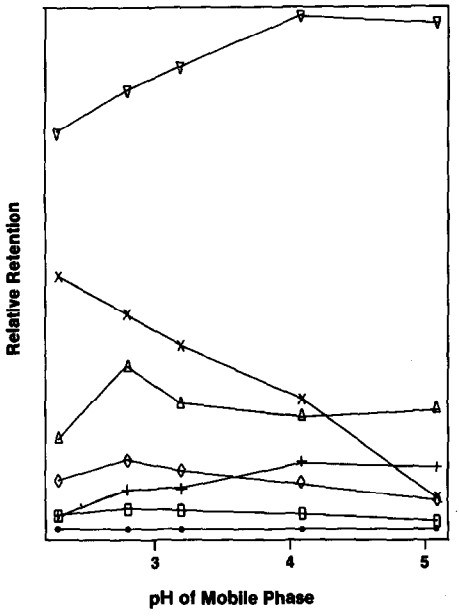

Fig. 3. The effect of pH on WSV retention. The aqueous portion of the mobile phase contains 5 m*M* hexanesulfonate and is adjusted to various pHs. Other mobile phase parameters and symbols are the same as in Fig. 2.

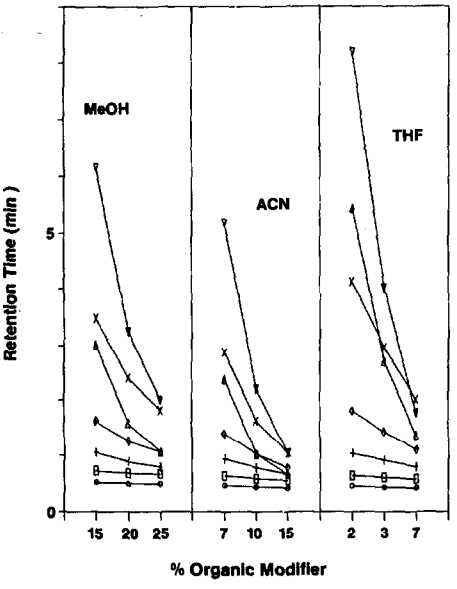

Fig. 4. The effect of organic modifier on WSV retention. Mobile phase contains various levels of organic modifier in 5 m*M* hexanesulfonate, 1% acetic acid, and 0.13% TEA. Symbols as in Fig. 2. MeOH = Methanol, ACN = acetonitrile, THF = tetrahydrofuran.

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 pH[12]. 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 eluotropic strength of each solvent. As expected, the eluotropic 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

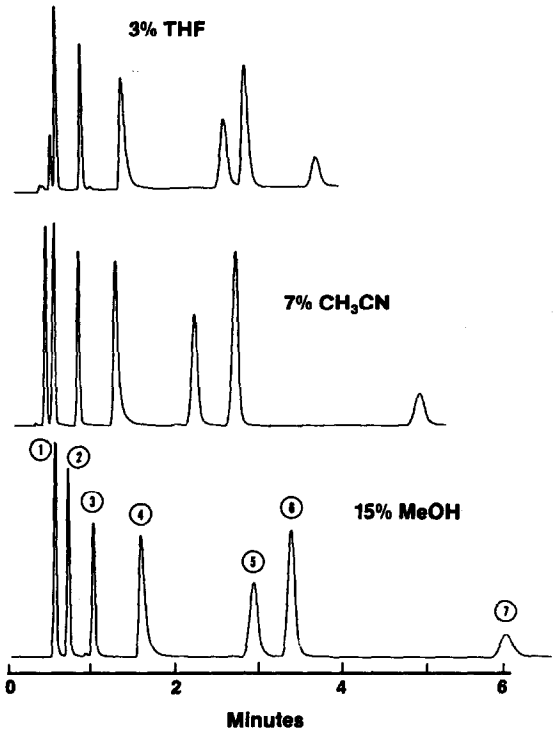

Fig. 5. Chromatograms showing the separation of WSVs using different organic modifiers. See the experimental section for column and mobile phase conditions. Peak identification: 1 = ascorbic acid; 2 = niacin; 3 = niacinamide; 4 = pyridoxine; 5 = folic acid; 6 = thiamine; 7 = riboflavin.

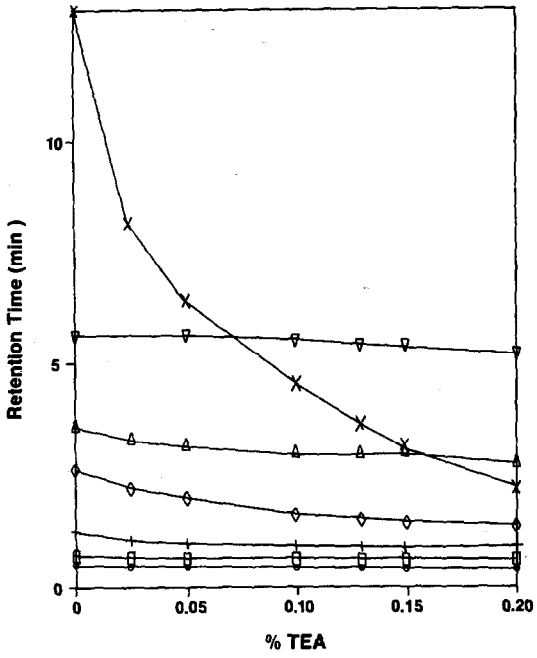

Fig. 6. The effect of TEA on WSV retention. The mobile phase conditions are listed in Table III except TEA% is varied. Symbols as in Fig. 2.

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 documented[12]. 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.

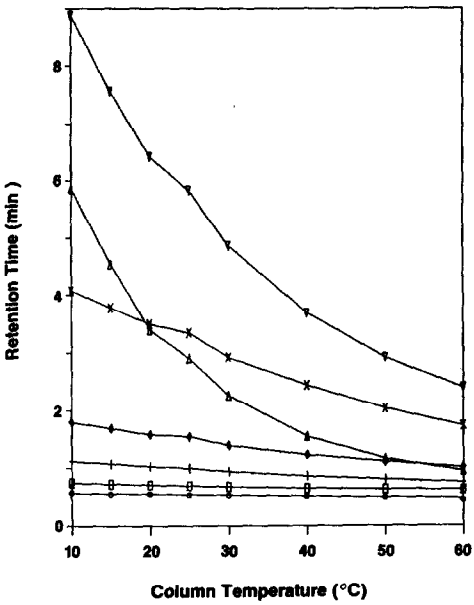

Fig. 7. The effect of column temperature on WSV retention. The mobile phase conditions are listed in Table III except column temperature is varied. Symbols as in Fig. 2.

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

Factors	*Optimum range*
Ion-pairing reagent	4–7 m*M* 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

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 m*M* 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 m*M* 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 thermostatting 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 m*M* 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 search[15]. 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 C_8 and C_{18} 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 conditions[9].

Effect of alkyl chain length and selectivity. The effect of bonded-phase alkyl chain length (*i.e.*, C_8 or C_{18}) can be delineated by data from two column sets ($3C_8$ and $3C_{18}$, $5C_8$ and $5C_{18}$. Each set of columns is packed with identical 3-μm or 5-μm base silica but are bonded with either C_8- or C_{18}-monochlorosilane[16]. Data show substantially higher k' values of the hydrophobic WSVs (folic acid and riboflavin) on C_{18} bonded phase. C_8 columns appear to be preferable since they yield comparable resolution in shorter assay times.

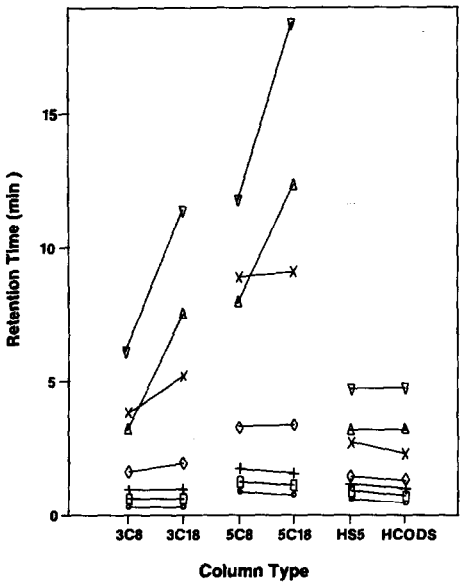

Fig. 8. Retention data of WSV on different C_8 and C_{18} columns. The mobile phase conditions are listed in Table III except the column used is varied. Symbols as in Fig. 2.

As shown in Fig. 8, the HS-5 (Pecosil-5 C_8, 125 mm × 4.6 mm I.D., packed with 5-μm irregular C_8 particles) and the 5-μm HCODS (Pecosphere HS-5 HCODS, 125 mm × 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.*, $3C_8$ and $5C_8$),

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 m*M* of alkylsulfonate, 0.13% TEA, and 1% acetic acid; number of injections in each experiment: 8; concentration of WSV standard ranged 2–5 μg/ml; injection volume was 10 μl.

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

shows that smaller particles (*i.e.*, 3-μm) generate higher performance by delivering higher efficiency per unit time[17]. Pecosphere-3CR C_8 (83 mm long, 3-μm particles) yields similar resolution of the seven WSVs in 6 min similar to what Pecosphere-5CR C_8 (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 C_8 column[15,17]. However, considering both resolution and performance, the best choice appears to be a longer 3-μm C_8 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 solution[18].

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 10200–10400 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

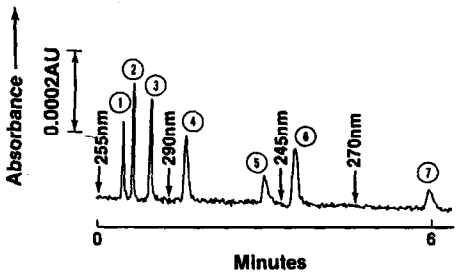

Fig. 9. High-sensitivity determination of trace level of WSVs using UV absorbance detection at optimized wavelengths. Peak identification: 1 = ascorbic acid (2.3 ng); 2 = niacin (2.5 ng); 3 = niacinamide (2.5 ng); 4 = pyridoxine (2.6 ng); 5 = folic acid (1.5 ng); 6 = thiamine (2.7 ng); 7 = riboflavin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

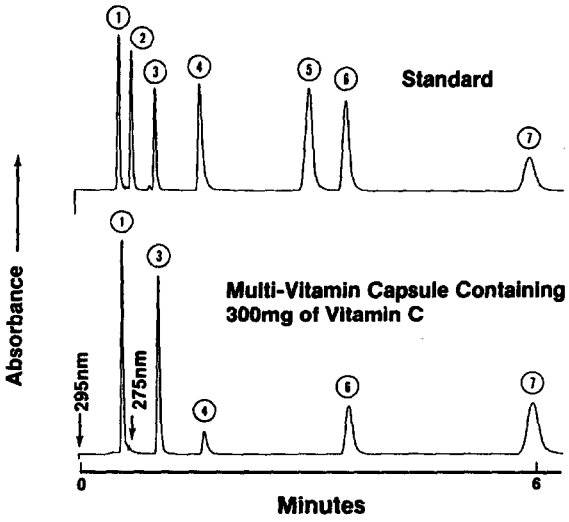

Fig. 10. The analysis of a multivitamin capsule containing 300 mg of vitamin C using the developed LC separation. Peak identification is the same as in Fig. 5.

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 method[5] 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 C_8 and C_{18} 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 elution[5], anion pairing[5], or spectroscopic deconvolution techniques[19].

CONCLUSIONS

The systematic evaluation in this study confirmed the use of 5 m*M* 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 m*M* hexanesulfonate, 1% acetic acid, and 0.13% TEA) was found to yield complete resolution of 7 WSVs on many C_8 and C_{18} columns with excellent precision, column lifetime, and sensitivity. C_8 columns were preferable since analysis times were substantially lower than comparable C_{18} 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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