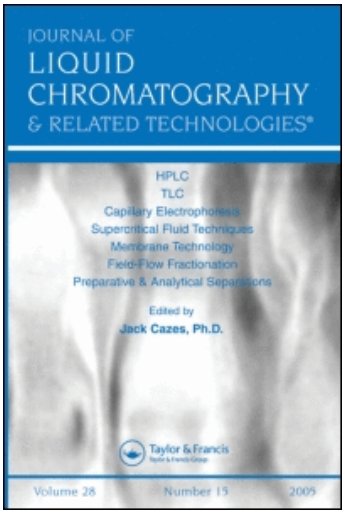

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F. L. Vandemark[a]; G. J. Schmidt[a]
[a] The Perkin-Elmer Corp., Main Avenue, Norwalk, CT

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Determination of Water Soluble Vitamins in Pharmaceutical Preparations Using Liquid Chromatography

F.L. Vandemark and G.J. Schmidt
The Perkin-Elmer Corp.
Main Avenue, Norwalk, CT 06856

ABSTRACT

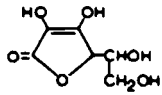
A liquid chromatography procedure is described for the simultaneous separation of seven water soluble vitamins in pharmaceutical preparations. The vitamins are separated on a C_8 bonded-phase column and eluted with a gradient solvent program. A microprocessor controlled variable wavelength ultraviolet detector is used to automatically change detection wavelength to optimize detection of each vitamin. Pharmaceutical preparations are briefly sonicated in a dimethyl sulfoxide-water mixture, centrifuged, and an aliquot injected directly onto the column.

INTRODUCTION

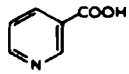
Vitamins are an important class of compounds and are essential for normal growth and development. Their measurement is of interest to those involved in biochemistry, food and the pharmaceutical sciences.

The water soluble vitamins differ greatly in their chemical structure and properties as shown in Fig. 1. The structures vary from the simple pyridine ring structure of niacin, to polyhydroxyl substitution in ascorbic acid, to the long chain dicarboxylic acids such as folic and pantothenic acid. Vitamin B-12 has a very complex structure with a molecular weight of 1355.

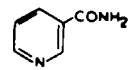
This diversity of chemical structure has in large part necessitated a wide variety of analytical techniques for their determination. Traditional methods have most often been based upon their physical or chemical properties. Many of these procedures



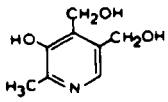
VITAMIN C



NIACIN



NIACINAMIDE



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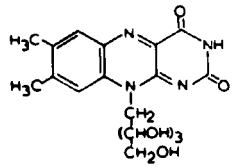

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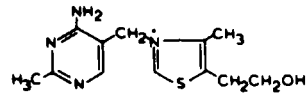

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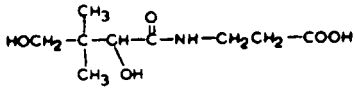
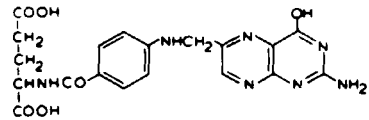
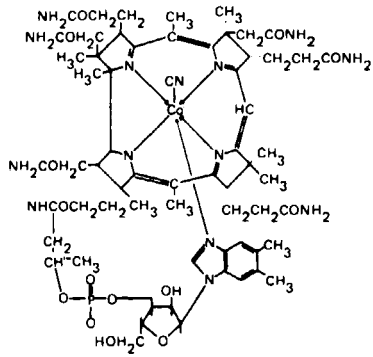


PANTOTHENIC ACID



FOLIC ACID



CYANOCOBALAMIN B-12

Fig. 1 Chemical structures of the major water soluble vitamins.

are not satisfactory due to limitations in sample throughput or inadequate specificity.

Liquid chromatography (LC) offers a useful alternative to the classical chemical manipulation commonly used for determining the water soluble vitamins. LC provides the capability for the simultaneous measurement of a number of the water soluble vitamins and is well-suited for the separation of hydrophilic compounds.

Various liquid chromatography procedures have been described for determining the water soluble vitamins. Methods developed for analyzing pharmaceutical preparations have typically made use of C_{18} bonded-phase columns with appropriate ion-pair reagents added to the mobile phase (1-3). Owing to the ionic nature of most water-soluble vitamins, other workers have chosen to use ion-exchange systems, either cation exchange resins (4,5) or amino bonded-phases (1,6). These systems do not typically provide the resolving power as compared to those chromatography systems using C_{18} columns.

Other workers have applied similar chromatography systems for determining water soluble vitamins in foodstuffs (7,8). In addition, Rouseff (9) used an anion exchange column for determining vitamins in selected citrus juices and Richardson et al. (10) suggested the use of silica columns for determining riboflavin in foods.

We have developed a liquid chromatography procedure using a C_8 bonded-phase column for separating simultaneously the major water soluble vitamins. These included ascorbic acid, niacin, niacinamide, pyridoxine, folic acid, riboflavin and thiamine. Provision was provided for incorporation of an internal standard and chromatography was complete in approximately 12 minutes.

MATERIALS AND METHODS

Apparatus

We used a Perkin-Elmer Series 3B liquid chromatograph equipped with a Model LC-100 column oven, a Model LC-75 variable wavelength ultraviolet detector and a Model LC-75 Autocontrol. Injections were made with a Rheodyne 7125 injection valve or a Model LC-

420 autosampler. A Model Sigma 10 chromatography data station was used in some instances for data reduction.

The chromatography column used was a Perkin-Elmer Analytical C_8 column, 0.46 x 25 cm, 10-µm C_8 packing, part no. 0258-1684. Other columns evaluated included a 0.46 x 25 cm column packed with 10-µm C_2 packing (Perkin-Elmer RP-2, part no. 0258-0658) and a 0.46 x 25 cm column packed with 10-µm amino phase packing (Perkin-Elmer -NH_2, part no. 0258-1489).

Other equipment included 16 x 100 mm tubes with PTFE-lined caps and a mortar and pestle. A bench-top centrifuge, a Millipore sample filtration and solvent filtration apparatus, a pH meter, a test tube rotator rack and a sonifier (Model W-185, Branson Scientific, Danbury, CT) were also used.

Reagents and Standards

Acetonitrile, methanol and dimethylsulfoxide, distilled in glass, UV grade, were used as obtained from Burdick and Jackson Labs (Muskegon, MI). Phosphoric acid, disodium hydrogen phosphate and ammonium carbonate were reagent grade. Hexadecyltrimethylammonium bromide was obtained from Eastman Kodak (Rochester, NY). Hexanesulfonic acid was obtained from Regis Chemical Co. (Morton Grove, Illinois). Water was purified, filtered and deionized by a carbon bed system from Continental Water Conditioning Corp (El Paso, TX).

Ascorbic acid, folic acid and pyridoxine were obtained from Pfaltz and Bauer (Stamford, CT). Riboflavin, thiamine, niacin, and niacinamide were obtained from Sigma Chemical Co. (St. Louis, MO). Standard solutions of each vitamin, except folic acid and riboflavin, were prepared daily in water to provide a concentration of 1 g/L. A 1 g/L standard solution of folic acid was prepared daily in 0.128 mol/L ammonium carbonate. Riboflavin was prepared daily in water to provide a concentration of 0.1 g/L.

The 0.128 mol/L ammonium carbonate solution was prepared by dissolving 10 g of reagent grade ammonium carbonate in 1 liter of deionized water and filtering through a 0.45-µm filter (Millipore Corp., Bedford, MA) prior to use.

The water used for sample pretreatment was deionized and contained 250 mg/L theobromine. The amount of theobromine added should be adjusted to give a peak height similar to those of the vitamin concentrations being determined.

Hexanesulfonic acid, 0.005 mol/L, was prepared by dissolving 0.95 g in 900 mL of deionized water and adjusting the pH to 2.8 with 20% phosphoric acid. The solution was diluted to 1 L with water and filtered through a 0.45 µm filter.

Sample Preparation

Accurately weigh and record the weight of the vitamin tablet. Place the tablet in a mortor and crush to a fine powder. Transfer the entire crushed tablet to a 25 mL beaker. Wash the mortar with two successive 5-mL volumes of DMSO, containing 10 mL of ammonium hydroxide per liter, and transfer to the beaker. Sonicate at 90 W (scale reading of 50) for 1 minute. Pour the contents of the 25-mL beaker into a clean 150-mL beaker. Wash the 25-mL beaker twice with 10 mL of water containing theobromine and transfer to the 150-mL beaker. Add an additional 60 mL of water containing theobromine to the 150-mL beaker and sonicate at 90W for 1 min. Filter a 5-mL aliquot through a Millipore sample filter apparatus. Inject 20 µL into the chromatograph.

CHROMATOGRAPHY

Figure 2 illustrates the mobile phase gradient used for the separation of the water soluble vitamins. The sample was

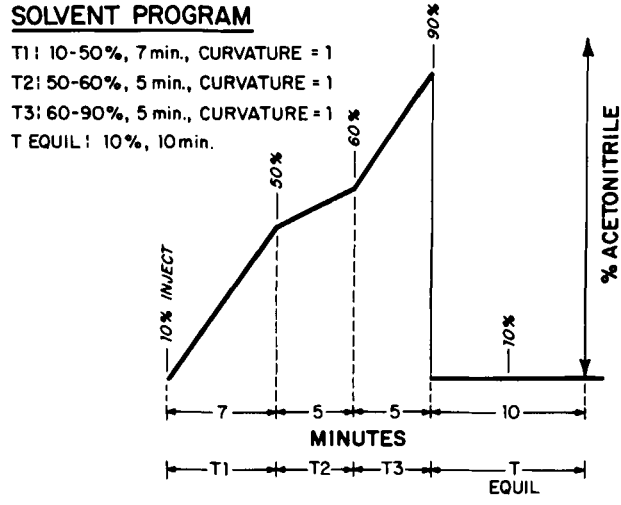


Fig. 2 Schematic showing the gradient profile used.

injected onto the column after the system was equilibrated with the initial mobile phase composition of 10% methanol. Upon injection, the mobile phase gradient was started. The gradient consisted of a 3-segment profile as shown. Upon completion of the last segment of the elution program, the column was re-equilibrated at 10% methanol for 10 min before injection of the next sample. A mobile phase flow rate of 2 mL/min was used at a separation temperature of 55 °C.

Figure 3 illustrates the separation of the major water soluble vitamin standards. Approximately 1.5 µg of each compound except thiamine was injected in the 20-µL injection aliquot. Thiamine was injected at a concentration of 0.75 µg in the 20-µL injection aliquot. Theobromine was used as the internal standard since it has similar water solubility as compared to the

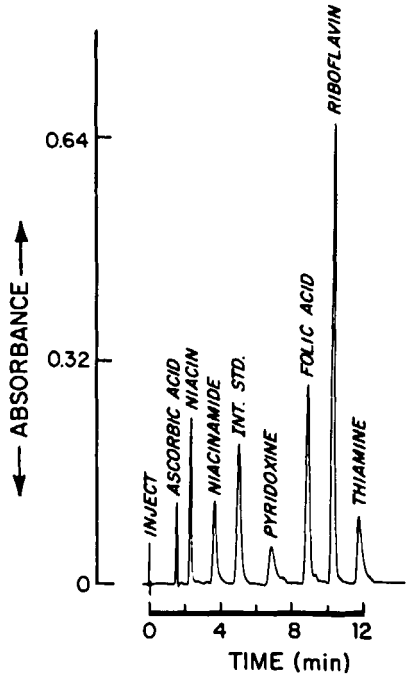


Fig. 3 Chromatogram illustrating the separation of seven water soluble vitamins and the internal standard. The UV detector was set to 272 nm.

compounds of interest, is chromatographically resolved from the other compounds and has a typical xanthine absorption spectra with a maximum at 272 nm, the detection wavelength set when the wavelength program was not used. Retention time, and relative retention (r_{is}) data for each vitamin is given in Table I.

The variable wavelength ultraviolet detector was operated at a single wavelength or with wavelength programming to permit optimum detection of each vitamin. For those experiments where a single detection wavelength was used, the detector was set to 272 nm. This wavelength was chosen because it offered a compromise detection wavelength where all the test compounds exhibited some absorption. For those experiments where several wavelengths were used, the wavelength change program illustrated in Figure 4 was used. This was achieved using the LC-75 Autocontrol, entering by keyboard the appropriate time and wavelength change set points.

Figure 5 shows the separation of the vitamins using the detector set to the wavelength change program. The detector sensitivity was the same as that used in Figure 3.

CALIBRATION AND QUANTITATION

Quantitation of the water soluble vitamins was done by comparison of the peak area of vitamin unknowns in the pharmaceuti-

TABLE I

Retention Time and Relative Retention Data

Vitamin	Retention Time (min)	Relative Retention*
Ascorbic acid	1.65	0.161
Niacin	2.41	0.349
Niacinamide	3.72	0.673
Theobromine	5.04	1.000
Pyridoxine	6.74	1.421
Folic Acid	8.87	1.948
Riboflavin	10.28	2.297
Thiamine	11.80	2.673

*Relative to theobromine

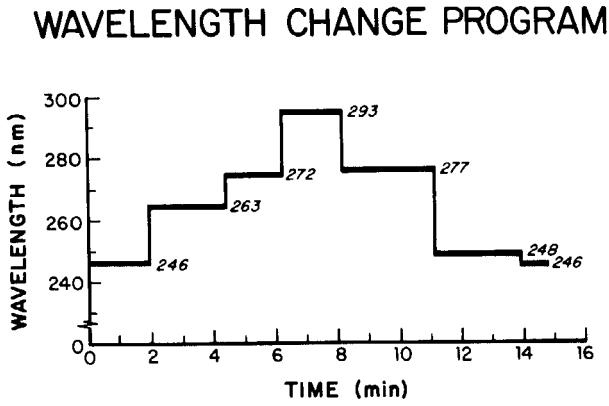


Fig. 4 Schematic showing the detector wavelength program used.

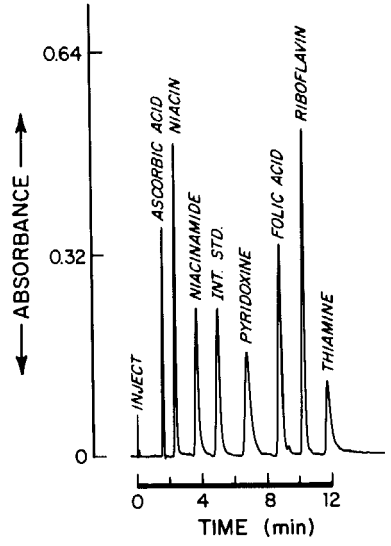


Fig. 5 Chromatogram illustrating the separation of seven water soluble vitamins and the internal standard. The detector wavelength was programmed as shown in Figure 4.

cal preparations with the peak areas of known amounts of injected pure standards. To compensate for injection variability, the peak area ratio using the internal standard and each vitamin, either unknowns or standards, may be used.

Since the amount of each vitamin added to the tablet is presumably known, a standard should be prepared at approximately the same concentration. This standard should then be treated similarly to the unknown, as noted in the section on sample pretreatment, i.e. the final volumes of both standard and unknown solutions should be equal to 100 mL. By preparing a standard of similar concentration to that expected in the unknown, the detection wavelength can be chosen to avoid detector saturation.

RESULTS AND DISCUSSION

A number of different chromatography systems were evaluated for separating the water soluble vitamins. Our use of C_{18} bonded-phase columns did not provide sufficient resolution to achieve separation of all the vitamins in a single run. In an effort to improve the number of vitamins which could be separated simultaneously, a number of other separation systems were evaluated.

Figure 6 illustrates the separation obtained on different bonded-phase columns. The use of an amino bonded-phase column as previously suggested (1,6) did not provide adequate resolution, as shown in Figure 3A. Using an isocratic mobile phase of 80% acetonitrile in water buffered to pH 3, partial separation of six vitamins was obtained in approximately 8 minutes. The amino bonded-phase column exhibits poor retention characteristics and adequate separations were difficult to obtain in our hands. Due to the high water solubility of the vitamins, increasing the percentage of acetonitrile in the mobile phase increased the retention. This improved the separation of the early eluting vitamins, however folic acid and thiamine had exceedingly long retention times. A reverse gradient, increasing the percentage of the more polar solvent, gave better separations, however, the separations were obtained by solubility differences of the vitamins in the mobile phase and not by active partitioning with the column packing. This was indicated by large variations in retention time reproducibility.

The use of a C_2 bonded-phase column permitted the separation of six vitamins in a reasonable length of time, as shown in Figure

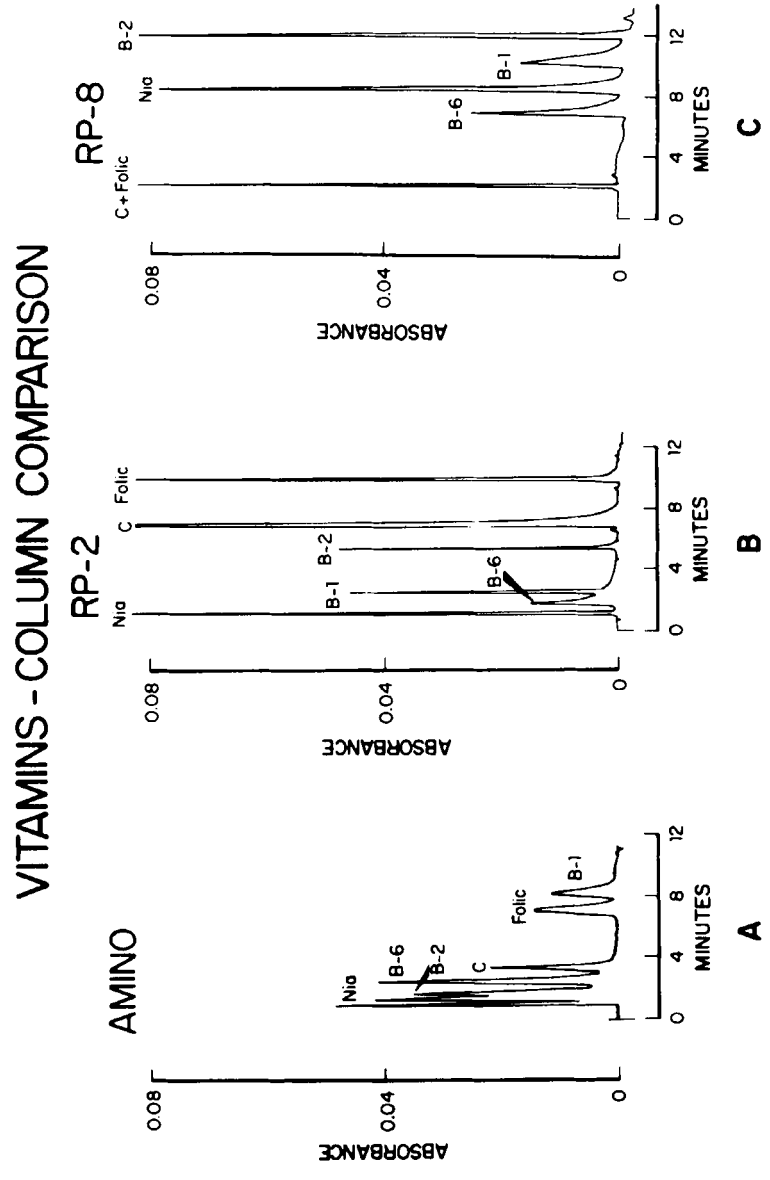


Fig. 6 Separation of water soluble vitamins by various chromatography systems.

6B. A gradient was used from 10% to 60% methanol in water containing 0.5% hexadecyltrimethylammonium bromide at pH 5.5. Pyridoxine exhibited poor peak quality, preventing low concentrations from being acceptably quantitated. In addition, niacinamide had a very fast retention and co-eluted with niacin.

Figure 6C illustrates our preliminary separations as obtained on a C_8 column using gradient elution to 70% methanol in water. Reproducible separations of five vitamins were obtained in twelve minutes however, ascorbic acid and folic acid co-eluted and pyridoxine continued to exhibit poor peak quality.

It was observed that pyridoxine gives poor peak shape when chromatographed using neutral or alkaline mobile phase solutions. Peak shape is improved considerably when acidic mobile phases are used, however, the retention time of the vitamins are quite low. The addition of hexanesulfonic acid to the mobile phase and control of the pH at 2.8 as previously noted results in increased retention due to ion-pair interaction and excellent peak shape of all the vitamins as shown in Figure 5.

To determine the optimum detection wavelength stop-flow spectra were obtained for each vitamin. Each vitamin was injected into the chromatograph and the mobile phase flow was stopped at the appropriate time to trap the eluting vitamin in the detector flow cell. The wavelength was scanned from 200 to 350 nm to provide the spectra for each vitamin. Each spectrum was automatically corrected for mobile phase absorption. Table II lists the absorbance maximum for each compound.

TABLE II

Ultraviolet Wavelength Maxima of Water Soluble Vitamins

Vitamin	Wavelength (nm)
Ascorbic Acid	246
Niacin	260
Niacinamide	263
Theobromine	272
Pyridoxine	293
Folic Acid	286
Riboflavin	269
Thiamine	248

Due to the relatively high concentrations of most vitamins in a pharmaceutical matrix, a single detection wavelength may often be used successfully. In those instances where lower concentrations are required, especially for preparations containing low concentrations of pyridoxine, the use of the optimum wavelength may be advantageous.

In addition to the use of the optimum detection wavelength for detecting each individual component of the mixture, there is another important advantage to the wavelength selection when pharmaceutical preparations are analyzed. Very often, large amounts of ascorbic acid are present in tablet preparations and detection at the optimum wavelength will cause detector saturation. This problem can be avoided by using a detection wavelength removed from the wavelength where the absorbance maxima occurs. By using fully variable wavelength selection, a detection wavelength can be chosen to select the sensitivity to be appropriate for each compound in the pharmaceutical preparation.

Figure 7 illustrates chromatograms from the analysis of a pharmaceutical tablet. Chromatogram A is a tablet processed exactly according to the procedures outlined using the automatic wavelength change program. The ascorbic acid peak was off-scale due to the large amount present in the tablet causing detector saturation. In chromatogram B, the wavelength program was the same except that the ascorbic acid peak was monitored at 285 nm. By monitoring at a wavelength coinciding with an absorption shoulder, off the absorption maximum of 246 nm, the problem of detector saturation was not observed.

The vitamins in this tablet were quantitated using peak area ratios as previously described. The values determined as compared to those listed are shown in Table III.

Six samples of a vitamin preparation were analyzed according to the complete procedure to determine within-run precision. The coefficients of variation for each of the vitamins present in the preparation are listed in Table IV. These values represent precision of both the sample pretreatment and chromatography.

CONCLUSION

Liquid chromatography has proven to be extremely useful for separating vitamin compounds of interest from a wide variety of analytical matrices. Our work thus far has indicated that

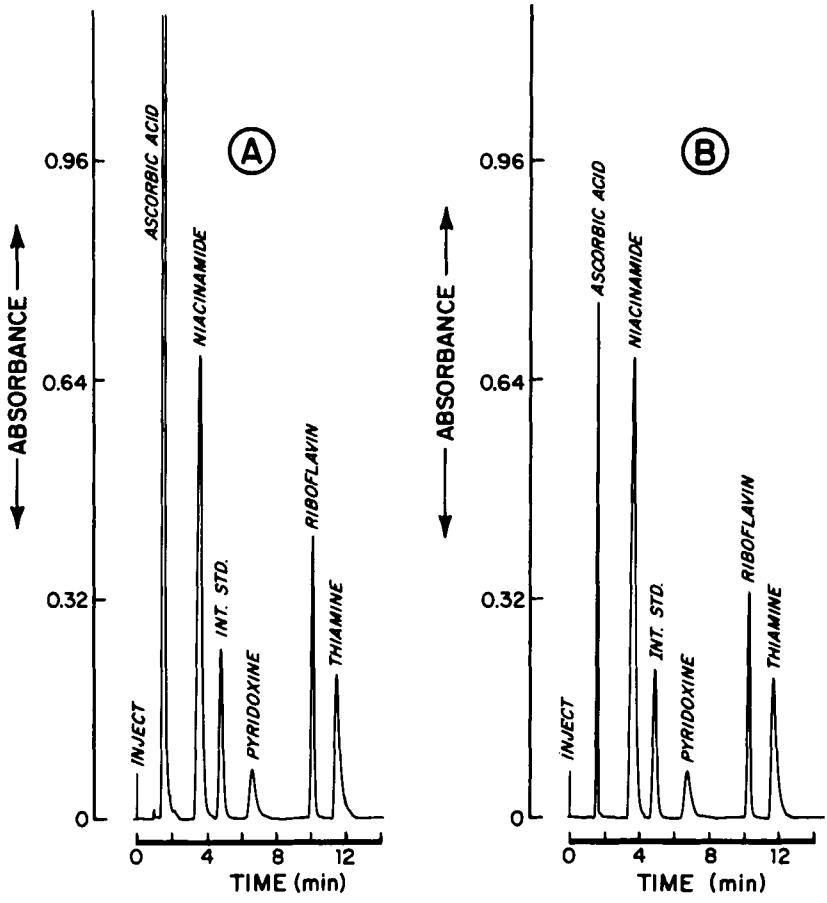


Fig. 7 Chromatograms of the same vitamin tablet analyzed according to the procedures. Chromatogram A was detected using the suggested wavelength program. Chromatogram B uses the same detector program, however ascorbic acid is detected at 285 nm.

this technique is well-suited and conveniently applied to the separation of a large number of the major water soluble vitamins in tablet preparations.

Preparations in the form of split capsules were most convenient to work in that they simply required opening and subse-

TABLE III

Quantitated Values of Water Soluble Vitamins

Vitamin	Listed Value (mg)	Calculated Value (mg)
Ascorbic Acid	300	314.1
Niacinamide	50	47.2
Pyridoxine	5	5.1
Riboflavin	10	10.1
Thiamine	15	14.6

TABLE IV

Within-run Precision Data for Vitamins (n = 6)

Vitamin	$\bar{x}$	SD	CV(%)
Ascorbic Acid	405.7	9.668	2.38
Niacinamide	376.0	9.633	2.56
Pyridoxine	26.0	0.633	2.43
Riboflavin	108.8	6.463	5.94
Thiamine	69.5	2.588	3.72

quent pretreatment. Encapsulated tablets required manual crushing to a fine powder before pretreatment. We have not observed any interferences with this analysis by the encapsulating material or filler materials within the capsules themselves. The extent of these interferences will be determined however, upon the particular materials used in the tablet manufacture.

The determination of two other water soluble vitamins, pantothenic acid and vitamin B-12, have not been included in the present study. Preliminary work indicates that the system described here will not provide adequate sensitivity for the very low concentrations required for these determinations.

Due to the high water solubility and structural dissimilarity of these vitamins, we found it necessary to use ion-pair chromatography in conjunction with gradient elution. By use of accurate gradient formation and a constant equilibration time between runs, we observed reliable retention time reproducibility.

Several different C_8 columns of the same type have been evaluated and found to be suitable for the analysis. Therefore,

small variations in column to column performance should not have a serious effect on the routine application of the procedure.

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