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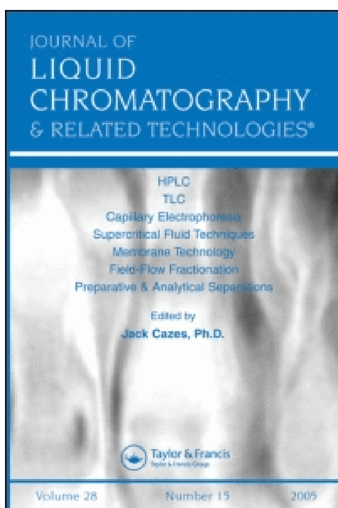

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DETERMINATION OF WATER SOLUBLE VITAMINS BY LIQUID CHROMATOGRAPHY WITH ORDINARY AND NARROW-BORE COLUMNS

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ABSTRACT

A reversed phase high performance liquid chromatographic method is described for the simultaneous determination of nicotinic acid, nicotinamide, pyridoxine, thiamine, folic acid and riboflavin. The vitamins were separated on a C_8 bonded phase column and eluted with a dihidrogen phosfate buffer, hexanesulphonate and methanol as the eluent. A rapid spectral detector Diode Array was used to optimise the detection of each vitamin. All vitamins were separated in less than 12 minutes. This method was applied to the determination all the vitamins in multivitamin pharmaceuticals. Recovery studies showed good results for all solutes (93.4%-106.5%) and some day coefficients of variation ranging from 0.91% to 4.95%.

Narrow bore columns were recommended because this alternative provided a good separation efficiency, plus greater economy and sensibility. Finally, the performance of an ordinary UV detector and that of a rapid spectral detector in this type of determination were critically compared.

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INTRODUCTION

The current increased interest in good eating habits has meant greater awareness of the vital role vitamins play in normal growth, development and health. Their measurement is of interest to those involved in biochemistry, pharmaceuticals and the food sciences. These considerations, together with regulations about food labelling, lead us to the necessity to have available rapid and reliable analytical methods for the simultaneous analysis of vitamins.

Vitamins are determined separately using widely different techniques-chemical, physicochemical, microbiological and biological (1-3). These methods are frequently time-consuming and are often limited by the number of interferent compounds found in the sample matrix. HPLC techniques could offer advantages of specificity and speed using the adequate equipment. In fact, the use of HPLC for water soluble vitamin analysis is not new; on the contrary, various liquid chromatography procedures have been described for their determination (4-6). Some workers have used amino-bonded phases (7,8) or a cation exchange column for the determination of nicotinic acid and its derivates (9) owing to the ionic nature of these water soluble vitamins. However, reversed phase high performance liquid chromatography methods are employed most generally, and its use for the analysis of formulations containing vitamins has gained wide acceptance from Quality Assurance laboratories of pharmaceutical industries (10-12).

The procedure proposed for the separation of the several water soluble vitamins in this paper makes use of hexanesulphonate as ion interaction reagent, since the high water solubility and structural dissimilarity (Figure 1) of these analytes makes it necessary to use the ion-pair technique (13-15) sometimes involving gradient elutions.

The objective of the present work was to develop a method for the simultaneous determination of B_1, B_2, B_6, nicotinic acid, nicotinamide and

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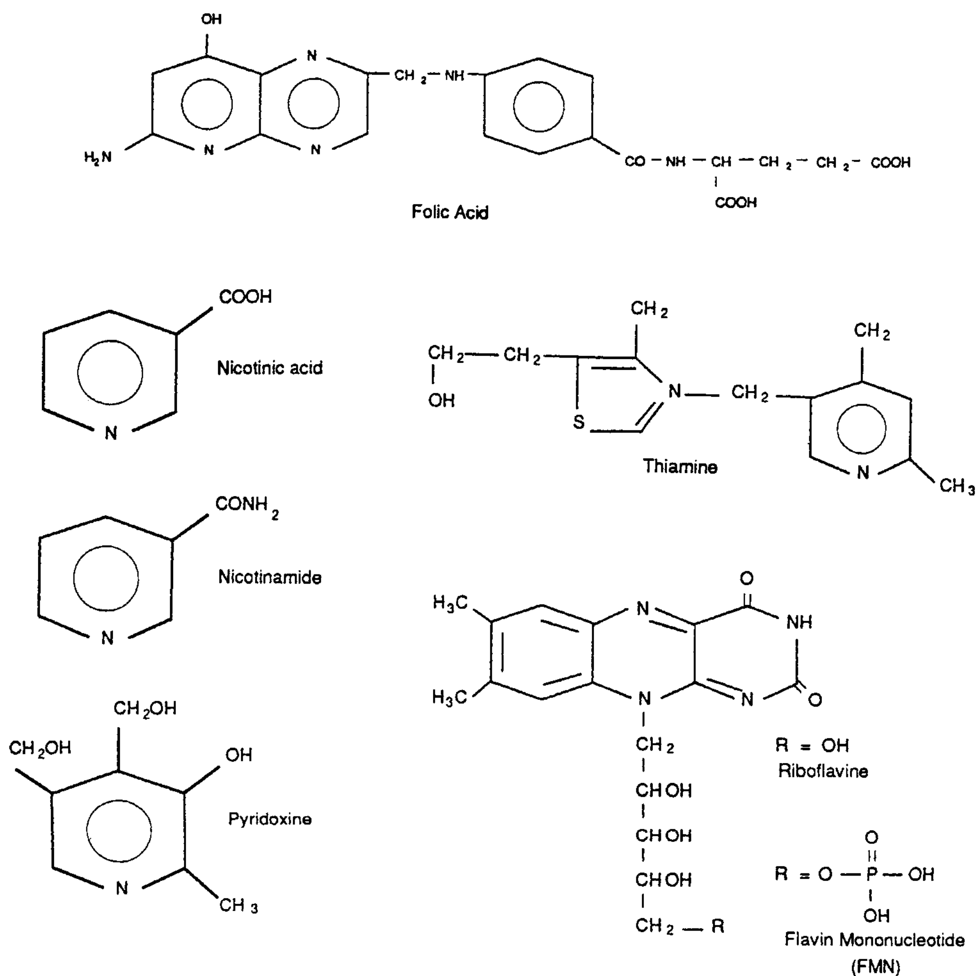

FIGURE 1. Chemical structures of the investigated water soluble vitamins.

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folic acid and was applied to the analyst of multivitamin pharmaceuticals. Alternatively these separations could been carried out over narrowbore columns with the aim of reducing the actual cost and increasing the sensibility.

EXPERIMENTAL

Apparatus and conditions

The HPLC system consisted of a Hewlett-Packard HP 1090 liquid chromatograph, a Rheodyne 7010 injection valve with a 5 µL loop, a Hewlett-Packard (HP) 79881A filter photometric detector, a HP 85B personal computer and a HP 3390A integrator. Column effluents were monitored at 254 nm for the nicotinic acid, nicotinamide, thiamine and riboflavin, and 280 nm for pyridoxine and folic acid.

Alternatively, a LKB (Bromma, Sweden) liquid chromatograph was used. The chromatograph was equipped with a model 2150 pump, a Rheodyne 7125 injection valve with a 20 µL loop, a LKB 2140 rapid spectral detector and IBM PS/2 data station.

The HPLC columns used were as follows: Lichrosorb RP-8 (250 x 4 mm i.d., 5 µm), Lichrospher 100 RP-18 (125 x 4 mm i.d., 5 µm) and Spherisorb ODS-2 (100 x 2.1 mm i.d., 3 µm).

For pH measurements, a PW 9422 Philips pHmeter equipped with a combined glass-Ag/AgCl electrode was employed.

Materials and reagents

All solvents used were HPLC grade and were employed as supplied by manufacturers. High purity water was obtained through a Millipore Milli-Q system.

Gradients were performed between two solvent mixtures. Solvent A was composed of 5 mM sodium hexanesulphonate (HSA) (Sigma, Saint

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Louis, MO) by adjusting the pH value of the solution to 2.8 through addition of 10^{-2} M potassium dihydrogen orthophosphate/phosphoric acid (Merck, Darmstadt, Germany). Solvent B was methanol (Romil Chemicals, Sps). Before being used both solvents were vacuum-filtered to 0.45 µm nylon filter and degassed with helium.

Analytical grade nicotinic acid, nicotinamide, pyridoxine, thiamine, riboflavin, flavin mononucleothide (FMN), folic acid and theobromine (IS) standards supplied by Sigma were used. Individual stock solutions of each vitamin were prepared every third day in water to provide a concentration of 1 mg mL^{-1}, except riboflavin (0.1 mg mL^{-1}) and folic acid (prepared in sodium bicarbonate 1 M). These solutions were degassed with helium and stored in dark glass flasks, in order to protect them from light, under -18°C refrigeration (folic acid solutions were stored at 3°C).

The working standard was prepared by adding aliquots of individual stock solutions and diluting with water. Then, an aliquot was taken and diluted with internal standard solution to give a concentration about 50 µg mL^{-1} of vitamins. The solution was filtered through a 0.45 µm membrane (Millex-HV_{13}, Millipore) before being injected into the system.

The samples of the liquid multivitamin products were injected immediately after adequate dilution and filtration (Millex, 0.45 µm) to remove impurities that might be present. The application of Sep Pak C_{18} clean-up cartridge in sample preparation for these formulations was not essential.

Complete triplicate analysis was performed on all samples to allow the calculation of average deviations as a measurement of chromatographic reproducibility.

RESULTS AND DISCUSSION

Standards of the vitamins were chromatographed separately in order to determine the retention time for each of them. The vitamins were then

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chromatographed as a mixture and chromatographic conditions (pH, ionic strength, hexanesulphonate concentration, percentage of the organic modifier, temperature and flow-rate) were altered to maximize peak resolution.

Using previous studies as a basic, preliminary separations were obtained on a C_{18} column, but, as consequence of ion-pair polarity the C_8 column offered best resolutions.

On the basis of resolution the following operating conditions for the HPLC system were chosen as the optimum:

Flow rate: 1 mL min^{-1}
pH=2.8
Ionic strength: 10^{-2}M KH_2PO_4
Hexanesulphonate concentration: 5 mM
λ detection: 272 nm

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The gradient run conditions were programmed as follows using methanol as phase B:

Time in minutes	0	4	5.5	12	17	22
%B	10	28.2	28.2	50	50	10

Figure 2 demonstrates the separation achieved from a standard vitamin mixture. As can be seen, the majority of vitamins present in the mixture have a baseline separation and elute as sharp peaks. Using the system described the retention times were found to be very consistent from one chromatogram to another. Table 1 gives the retention times of investigated vitamins and the coefficients of variation based on eleven sequential runs of standards.

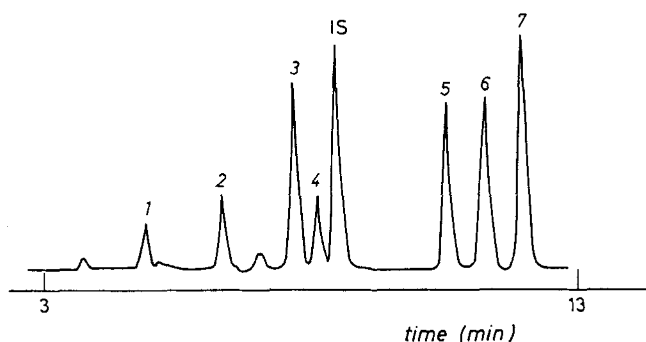

FIGURE 2. Chromatogram obtained from standard solutions of vitamins by using a Lichrosorb RP-8 (250x4 mm I.D., 5 μm) column. For gradient programme and conditions, see text. Peaks; 1.- Nicotinic acid; 2.- Nicotinamide; 3.- FMN; 4.- Pyridoxine; 5.- Thiamine; 6.- Folic acid; 7.- Riboflavin; I.S.- Teobromine.

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TABLE 1

Retention Times and its Repetibility in the investigated Vitamins.

Vitamin	Retention time (min)	CV %
Nicotinic acid	4.73	0.36
Nicotinamide	6.05	0.22
FMN	7.30	0.28
Pyridoxine	7.65	0.31
Thiamine	9.90	0.41
Folic acid	10.49	0.69
Riboflavin	11.12	0.97

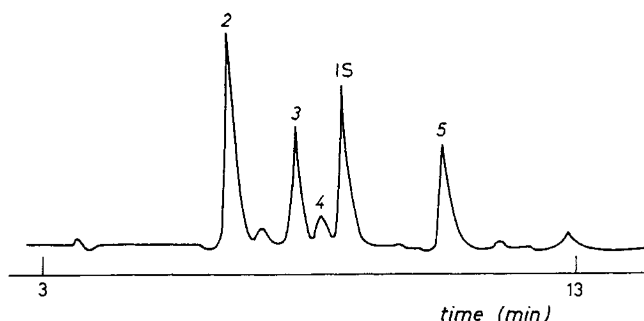

FIGURE 3. Typical chromatogram of water soluble vitamins in an oral liquid multivitamin product. Column and chromatographic conditions as in Figure 2. For peak identification see also Figure 2.

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Figure 3 illustrates a typical chromatogram of the water soluble vitamins in an oral liquid multivitamin product, obtained using the operating conditions specified. The sample peaks were identified by comparing either the relative retention times and the spectrum of each one with those of the standard reference vitamins. Four vitamins were present in the sample and they were quantitatively determined.

The quantification of vitamins was achieved by using the internal standard method. The calibration curve data were generated by repeated injections of a fixed volume, 20 µL, of standard solutions of vitamins covering a broad concentration range. The resulting peak areas data ($A_{vitamin}/A_{IS}$) were determined, plotted against concentration and stored in the data module. Injection volumes of 20 µL were employed to analise the multivitamin sample and the amount of vitamins was directly obtained from the data module. In order to determine the accuracy of the method, recovery studies were carried out. Known amounts of each vitamin were added to a variety of samples and the resulting spiked samples were subjected to the entire analytical sequence. All analytes were carried out in triplicate at four concentrations levels. The results are given in Table 2. The

TABLE 2

Recovery Studies of Water Soluble Vitamins added to Multivitamin Sample

Vitamin	Amount in sample µg/ml	Amount added µg/ml	Amount found µg/ml	%Recovery
Nicotinamide	455.2	50	511.4	101.2 ± 0.1
		100	555.6	100.3 ± 0.1
		150	619.5	104.3 ± 0.5
		200	661.8	100.8 ± 0.2
FMN	32.4	5	37.7	106.0 ± 1.5
		10	42.6	102.2 ± 0.3
		15	48.0	104.0 ± 2.1
		20	53.9	106.5 ± 1.7
Piridoxine	18.0	5	22.6	93.4 ± 2.7
		10	27.4	94.1 ± 2.3
		15	32.4	96.0 ± 3.5
		20	38.7	103.5 ± 2.9
Thiamin	61.5	10	71.9	104.1 ± 1.1
		20	82.4	104.8 ± 0.7
		30	92.6	100.3 ± 1.3
		40	100.8	99.5 ± 0.9

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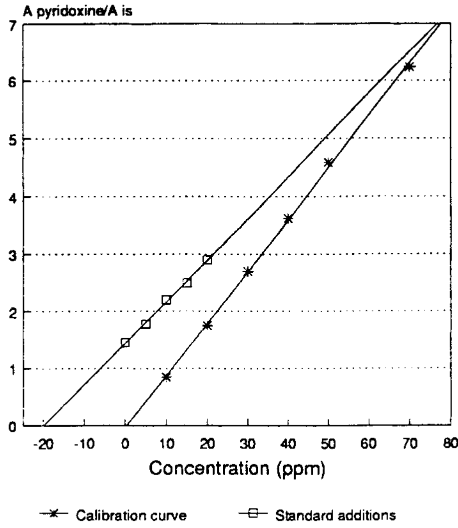
recovery data for the pyridoxine were obtained using 290 nm as wavelength of detection, because with the values corresponding to 272 nm (optimised for all vitamins) matrix effects were present, as can be seen in Figure 4 which presents the calibration graph with standards and the standard additions graph with the multivitamin sample. The average recoveries between 93.4 and 106.5 indicate that the method has an adequate degree of accuracy for the analysis of these substances. The results obtained were in accordance with the values specified by the manufacturer, as the maximun deviation was 10% (in the case of pyridoxine).

In order to increase the quality of the proposed method, with respect to sensibility and cost, microcolumns with 2.1 mm I.D. for the vitamin separation were used.



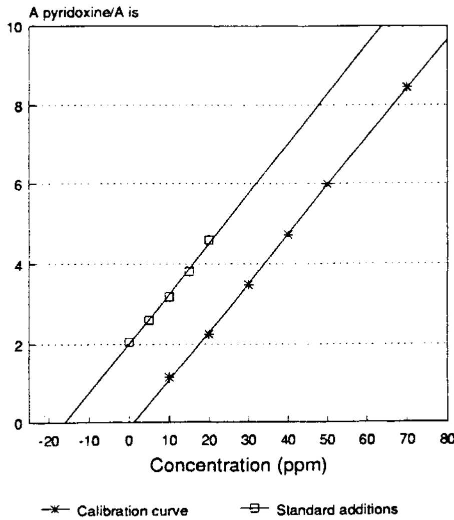


FIGURE 4.(*) Calibration graph with standards and (□) the standard additions graph with the multivitamin sample for the pyridoxine.

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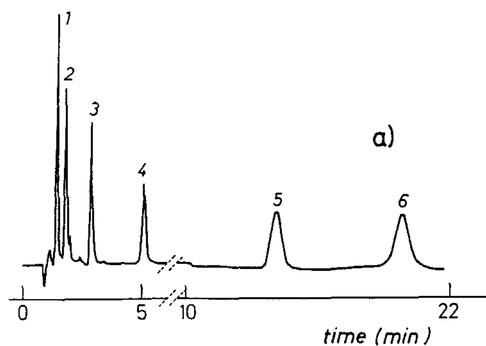

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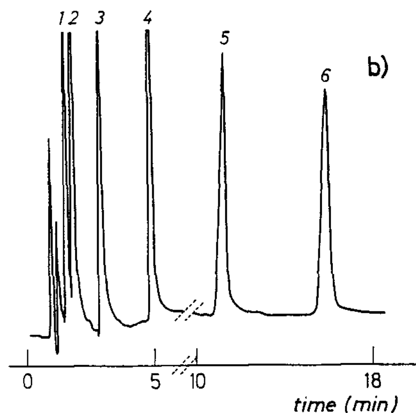

FIGURE 5. Chromatograms obtained from standard solutions of water soluble vitamins by using the following columns:
a) Lichrospher 100 RP-18 (125 x 4 mm i.d., 5 μm).
b) Spherisorb ODS-2 (100 x 2.1 mm i.d., 3 μm).
Mobile phase: 5 mM HSA, 20% Methanol, 0.1% Triethilamine and 0.01 M KH_2PO_4/H_3PO_4 pH=2.8.
Flow rate: a) 1 mL min^{-1}
b) Flow 0.2 and 0.3 mL min^{-1}
Peaks: 1.- Nicotinic acid; 2.- Nicotinamide; 3.-Pyridoxine; 4.- Thiamine; 5.- Folic acid; 6.- Riboflavin.

TABLE 3

Detection Limits (ng) of Water Soluble Vitamins determined by using Narrow-Bore and Normal-Bore Columns with Ordinary Photometric and Diode Array Detection.

Vitamin	Narrow-bore column	Normal-bore column	
	Photometric detection	Photometric detection	Diode array detection
Nicotinic Acid	0.125	0.38	16.3
Nicotinamide	0.185	0.56	21.0
Pyridoxine	0.260	0.62	22.7
Thiamine	0.430	1.26	26.9
Folic Acid	0.465	1.70	23.6
Riboflavin	0.465	1.80	32.5

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To do this, and because of our instrumental limitations, the separations were carried out in isocratic mode according to the following conditions:

Mobile Phase: 5 mM HSA, 0.01 M KH_2PO_4/H_3PO_4 pH=2.8, 20% Methanol, 0.1% triethilamine

Temperature: 25°C

Flow gradient:

t(min)	Flow (mL min^{-1})
0	0.2
5	0.2
5.5	0.3
18	0.3

λ detection: 254 and 280 nm

Figure 5 compares a chromatogram on the 125 x 4.6 mm I.D. column (A) and on the 100 x 2.1 mm I.D. column (B) and shows the benefits of the microcolumn packed with 3µm particles. Figure 5 also shows an increase in sensitivity for the microcolumn.

Table 3 compares the detection limits of nicotinic acid, nicotinamide, pyridoxine, thiamine, folic acid and riboflavin, obtained with these columns by using the ordinary filter detector and the diode array detector (DAD). As can be seen, the limits affored by narrow-bore column are lower than those by ordinary column. Analysts are increasingly using DAD in order to facilitate optimisation of separation and detection of analytes. However, the detection limits provided by the DAD used with the fotodiodes at room temperature, were between 14 and 43 times higher than those provided by an ordinary photometric detector.

Finally, the use of less solvent (flow = 0.25 mL min^{-1}) decreases the cost of disposal.

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CONCLUSIONS

This method provides a reliable means of analysing a number of water soluble vitamins simultaneously in multivitamin pharmaceuticals. Good results were obtained with baseline resolved peaks and chromatograms without interferences in less than 12 min.

Reversed-phase chromatography with narrow-bore columns packed with 3 µm particles makes an interesting alternative to the separation and determination of water soluble vitamins, through its higher sensibility and lower analysis time and cost.

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