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Note

Simultaneous separation of water-soluble vitamins and coenzymes by reversed-phase high-performance liquid chromatography

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In the course of analyzing purine and pyrimidine nucleotide pools from several complex biological systems on a reversed-phase C_{18} column, we noted that certain vitamins and cofactors eluted along with some nucleotides and nucleosides. This prompted us to examine whether reversed-phase column liquid chromatography could be used in the analysis of vitamins and cofactors. In this communication, we report the successful separation of water-soluble vitamins and cofactors using isocratic elution conditions. The identification and quantitation of vitamins in a commercially available multivitamin preparation and cofactor in a biological system is illustrated.

MATERIALS AND METHODS

Apparatus

For high-performance liquid chromatography (HPLC), a sample injector Model U6K, solvent delivery system M45, a fixed-wavelength detector Model 440 (Waters Assoc., Milford, MA, U.S.A.) and an automatic recorder OmniScribe (Houston Instruments) or Hewlett-Packard Model 3390A integrator were used. A prepacked reversed-phase column (30 × 0.4 cm I.D.) containing an C_{18} chemically bonded stationary phase (Waters Assoc.) was utilized. A precolumn (5 × 0.4 cm I.D.) also packed with C_{18} reversed-phase material was used to protect the main column.

Chemicals

Most of the vitamins and cofactors were obtained from Sigma (St. Louis, MO, U.S.A.) and a few from Nutritional Biochemical Corp. (Cleveland, OH, U.S.A.). A MEM vitamin concentrate preparation was obtained from Gibco (Grand Island, NY, U.S.A.). A 10 m*M* aqueous stock solution of each vitamin or cofactor was prepared using dilute hydrochloric acid or sodium bicarbonate as necessary, stored at −70°C, and suitably diluted before use. Ammonium hydrogen phosphate used in the buffer preparation was analytical-reagent grade (Fisher Scientific, Pittsburgh, PA, U.S.A.), and methanol of HPLC grade was from Waters Assoc. Ammonium phosphate buffer (0.2 *M*, pH 5.1) was freshly prepared each time in double-distilled water, prefiltered through a 0.2-μm Millipore filter, and degassed extensively before use.

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Chromatographic conditions

A μBondapak C_{18} (10 μm) column was used in these studies. The column was washed daily with double-distilled water, followed by methanol–water (30:70), and preserved in the latter in between use. Before use it was equilibrated with water and ammonium phosphate buffer (0.2 *M*, pH 5.1). The samples were chromatographed at room temperature at a flow-rate of 2 ml/min, 1000 p.s.i., chart speed of 1 cm/min and at 1.0 a.u.f.s. The resolution was monitored at 254 nm.

Silica cartridge chromatography

In order to avoid confusion among vitamins and their phosphorylated derivatives, an artificially prepared mixture of nicotinamide and nicotinamide–adenine dinucleotide phosphate (NADP) was fractionated according to Lothrop and Uziel's procedure[1] through a silica cartridge (Waters Assoc.). The resultant fractions were then independently chromatographed on a μBondapak C_{18} column. The water elutable fraction from the silica cartridge coeluted from the C_{18} column with NADP indicating that it was indeed NADP. To confirm this, NADP (5 μl, 10 m*M*) and the corresponding fraction were treated with 0.34 units of snake venom phosphodiesterase (Sigma) at 37°C, pH 8.8 for 4 h and the resultant reaction mixture chromatographed on a C_{18} column. The quantitative disappearance of NADP and appearance of two peaks, subsequently confirmed as nicotinamide mononucleotide (NMN) and adenosine 2′,5′-diphosphate, unequivocally confirmed NADP peak in standard and artificially prepared mixture.

RESULTS

Separation of vitamins

It is clear from Fig. 1 that folic acid, pyridoxine hydrochloride (vitamin B_6), nicotinamide, thiamine hydrochloride (vitamin B_1), cyanocobalamin (vitamin B_{12}), and riboflavin (vitamin B_2) are separated from each other in that order. Since vitamins B_{12} and B_2 are retained on the column quite firmly, they elute only with the use of 30% aqueous methanol, while the others elute with ammonium phosphate buffer. Immediately upon the elution of vitamin B_1, the eluent was changed from ammonium phosphate buffer to 30% aqueous methanol. Thus their differential retention permits distinct separation. Although day-to-day minor variations appeared in retention times, which were subject to temperature fluctuations, the sequence of elution remained unaltered.

Quantitation of vitamins

In order to test the applicability of this procedure to a commercial vitamin preparation, Gibco MEM vitamin concentrate was chromatographed as above. Integration of the separated peak areas was made with a Hewlett-Packard integrator and quantitation of each vitamin was achieved using the peak area–microgram relationship obtained with the standard vitamins. The results are summarized in Table I. It is obvious that the protocol affords reproducible quantitation of different vitamins with about 3–6% error. Furthermore, addition of a known amount of cyanocobalamin in the vitamin concentrate could be recovered quantitatively upon chromatography, indicating that the procedure is indeed reliable.

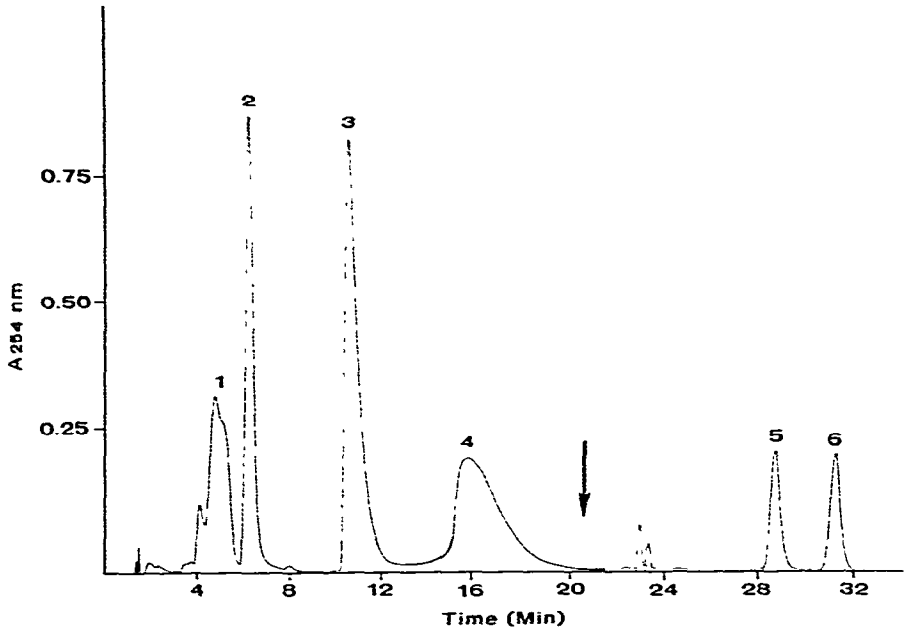

Fig. 1. Chromatogram of vitamin standards on a μBondapak C_{18} column. Peaks: 1 = folic acid; 2 = pyridoxine hydrochloride; 3 = nicotinamide; 4 = thiamine hydrochloride; 5 = vitamin B_{12}; 6 = vitamin B_2. The arrow indicates a change in the eluting agent from ammonium phosphate buffer to 30% aqueous methanol.

TABLE I

ANALYSIS OF GIBCO MEM VITAMIN CONCENTRATE

Besides the vitamins listed in the table, MEM vitamin concentrate also contained choline chloride (100 mg/l) and inositol (200 mg/l) which could not be analyzed by our method.

Vitamin	*Given amount (mg/l)*	*Experimentally derived amount (mg/l)*		
		Trial 1	*Trial 2*	*Trial 3*
Riboflavin	10.0	9.6	9.8	9.7
Folic acid	100.0	106.2	104.2	105.6
Pyridoxine hydrochloride	100.0	102.5	104.5	97.5
Nicotinamide	100.0	104.8	102.1	98.1
Thiamine hydrochloride	100.0	103.1	105.7	101.7

Separation of cofactors

NMN, its reduced entity ($NMNH^+$), thiamine pyrophosphate (TPP), pyridoxal phosphate, flavin adenine dinucleotide (FAD), NADP, its reduced entity ($NADPH^+$), oxidized nicotinamide–adenine dinucleotide (NAD) and its reduced entity ($NADH^+$) (see Fig. 2) and riboflavin monophosphate (FMN) elute in the given order, the last being eluted with 30% aqueous methanol (Fig. 3).

Uridine diphosphate galactose (UDP-Gal), UDP-glucose (UDPG) and adenosine diphosphate glucose (ADPG), eluted in the given order (Fig. 3). S-Adenosyl

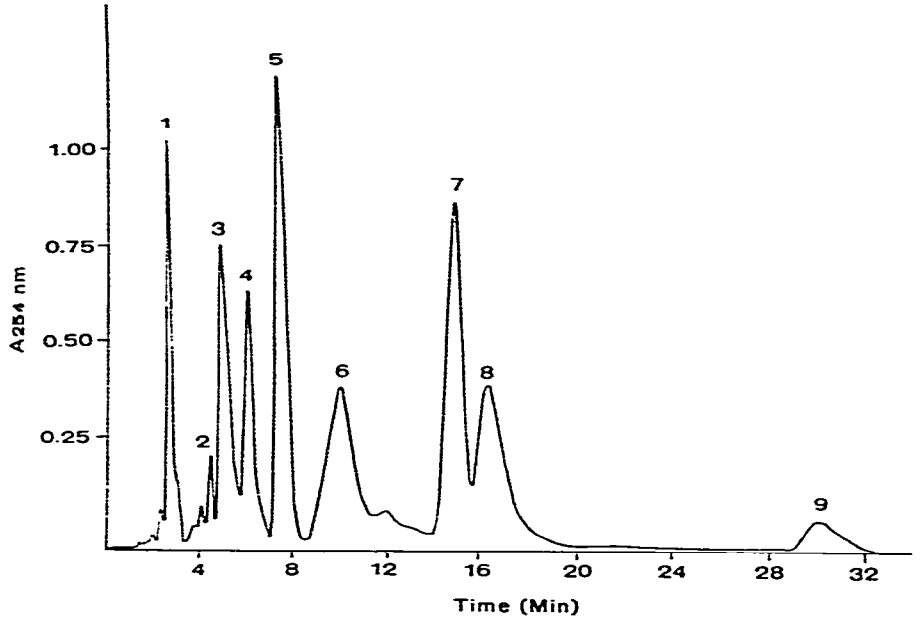

Fig. 2. Chromatogram of coenzyme standards on a μBondapak C_{18} column. Peaks: 1 = NMN; 2 = $NMNH^-$; 3 = TPP; 4 = pyridoxine phosphate; 5 = FAD; 6 = NADP; 7 = NAD; 8 = $NADPH^-$; 9 = $NADH^-$. Throughout, ammonium phosphate buffer was the eluent.

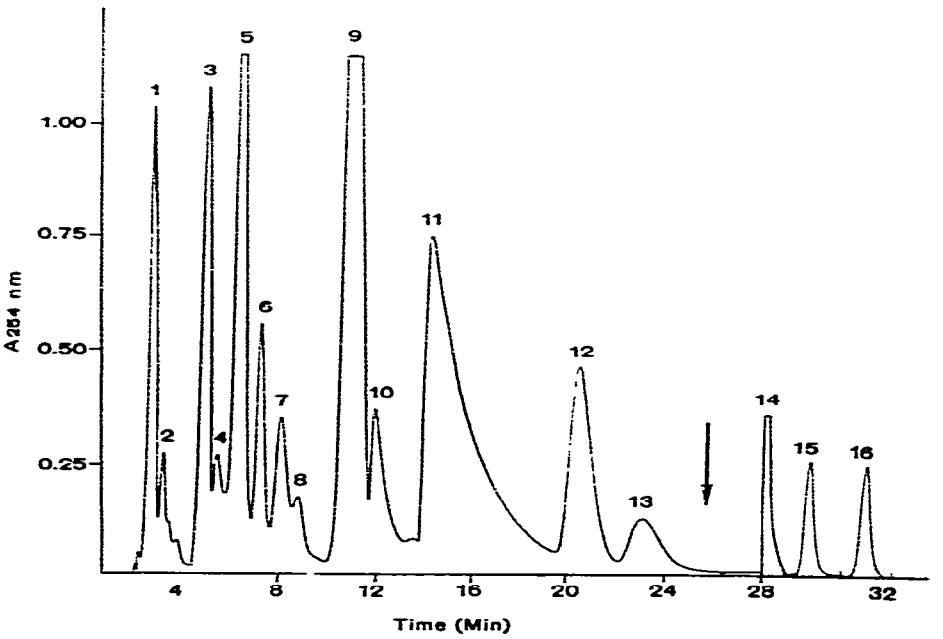

Fig. 3. Composite chromatogram of vitamin and coenzyme standards on a μBondapak C_{18} column. Peaks: 1 = UDP-glucose + UDP-galactose; 2 = NMN; 3 = ATP; 4 = folic acid; 5 = pyridoxine hydrochloride + TPP; 6 = adenosine diphosphoglucose; 7 = pyridoxine phosphate; 8 = FAD; 9 = NADP; 10 = Nicotinamide; 11 = thiamine hydrochloride + NAD; 12 = $NADPH^+$; 13 = $NADH^+$; 14 = FMN; 15 = vitamin B_{12}; 16 = vitamin B_2. The arrow indicates a change in the eluting agent from ammonium phosphate buffer to 30% aqueous methanol.

methionine eluted quite late (about 78 min retention time) and is therefore not shown in the figure. Although these entities are not phosphorylated derivatives of vitamins, traditionally they have been studied as cofactors due to their vital role in varied metabolic processes. During these studies, elution of ATP served as a marker point for comparison of elution patterns from batch to batch or day to day. Fig. 3 provides a composite elution pattern showing that the simultaneous separation of vitamins and coenzymes is feasible. Immediately upon the elution of $NADH^+$, the eluent was changed from ammonium phosphate buffer to 30% aqueous methanol. It may be noted from Figs. 2 and 3 that from day to day, the retention times of some components, notably that of pyridoxine phosphate, $NADPH^+$, and $NADH^+$, varied considerably, possibly as a function of temperature fluctuations. However, their sequence of elution remained unaltered as observed in the case of vitamins.

Cofactor detection in the tissue extract

Using the above procedure, it was possible to demonstrate the presence of NMN during a particular developmental stage in *Xenopus laevis*. Finite retention time, coelution, and characteristic $A_{280}/A_{254\,nm}$ ratio confirmed such a finding. Details on these development-related changes will be published elsewhere[2]. Under isocratic elution conditions used here, most of the common bases, nucleosides, and nucleotides retained on C_{18} column for 10–12 min (ref. 3), while many vitamins and cofactors eluted from the column between 12–50 min; therefore, their identification becomes relatively easy. Furthermore, silica cartridge chromatography and enzymatic tests, as discussed under Materials and methods, aid in their confirmation. A typical case to confirm the identification of NADP is discussed.

DISCUSSION

To our knowledge, this is the first comprehensive study on the resolution of both water-soluble UV-absorbing vitamins as well as cofactors. Some of the studies reported earlier are restricted to the separation and estimation of two or more derivatives of only one vitamin. For example, nicotinic acid and nicotinamide in multivitamin preparations[4] and human plasma and urine[5] are estimated upon separation using μBondapak C_{18} column and ion-pairing mode of elution. Similarly, separation of pyridoxine, pyridoxic acid, pyridoxal 5′-phosphate, and pyridoxine 5′-phosphate from human urine samples is reported using reversed-phase ion-pair HPLC[6]. Earlier, Wittmer and Haney[7] developed the procedure employing LiChrosorb SI 60 (E. Merck) column at 42°C and chloroform–methanol–0.4 *M* acetate buffer, pH 4.0 (60:25:4.5) as an eluting agent for the estimation of riboflavin in the presence of its degradation products in several multivitamin preparations. Recently, Gregory[8] has reported modification of his earlier procedures for the estimation of only vitamin B_6 in fortified breakfast cereals using C_{18} column and potassium phosphate buffer (0.033 *M*, pH 2.2).

In the light of these studies, procedures were developed for the simultaneous determination of thiamine, riboflavin, nicotinic acid, and/or nicotinamide and pyridoxine, in the commercial multivitamin preparations, by reversed-phase ion-pair HPLC[9,10]. Very recently, an analysis of these vitamins has been reported[11] from a protein supplement by reversed-phase gradient elution using coupled HPLC and 1-

heptanesulfonic acid as an ion-pairing eluting agent (in place of 1-hexanesulfonic acid used by previous workers[9,10]. Relatively, our procedure is (a) comprehensive, being useful in the estimation of folic acid as well as cyanocobalamin besides the above vitamins; (b) applicable in the analysis of cofactors in tissue fluids; (c) simple since it uses isocratic mode of elution and commonly available salt as an eluant; and (d) reproducible since it can quantitate different ingredients with reasonable accuracy. In these aspects, our findings on vitamin analysis are in conformity with those of Wills *et al.*[12] who have studied retention times of water-soluble vitamins as a function of different modifiers in aqueous methanol as an eluting agent.

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REFERENCES

1 C. D. Lothrop, Jr. and M. Uziel, *Anal. Biochem.*, 109 (1980) 160–166.
2 R. M. Kothari, M. W. Taylor and H. Martinez-Valdez, in preparation.
3 H. Martinez-Valdez, R. M. Kothari, H. V. Hershey and M. W. Taylor, *J. Chromatogr.*, 247 (1982) 307.
4 S. P. Sood, D. P. Wittmer, S. A. Ismaiel and W. G. Haney, *J. Pharm. Sci.*, 66 (1977) 40–42.
5 J. X. De Vries, W. Günthert and R. Ding, *J. Chromatogr.*, 221 (1980) 161–165.
6 E. Morita and N. Mizuno, *J. Chromatogr.*, 202 (1980) 134–138.
7 D. P. Wittmer and W. G. Haney, Jr., *J. Pharm. Sci.*, 63 (1974) 588–590.
8 J. F. Gregory, III, *J. Agr. Food Chem.*, 28 (1980) 486–489.
9 R. L. Kirchmeier and R. P. Upton, *J. Pharm. Sci.*, 67 (1978) 1444–1446.
10 R. P. Kwok, W. P. Rose, R. Tabor and T. S. Pattison, *J. Pharm. Sci.*, 70 (1981) 1014–1017.
11 J. A. Apffel, T. V. Alfredson and R. E. Majors, *J. Chromatogr.*, 206 (1981) 43–57.
12 R. B. H. Wills, C. G. Shaw and W. R. Day, *J. Chromatogr. Sci.*, 15 (1977) 262–266.