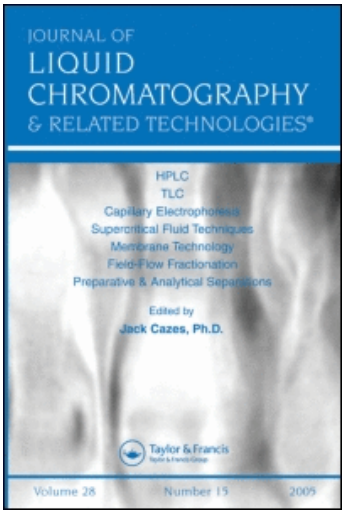

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Separation of Biological Pyridines by High Pressure Liquid Chromatography

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SEPARATION OF BIOLOGICAL PYRIDINES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation of the six pyridine compounds which comprise the pyridine nucleotide cycle, nicotinamide adenine dinucleotide phosphate and para-aminobenzoic acid, a compound biologically related to these pyridines, can be achieved rapidly utilizing high pressure liquid chromatography. Optimum separation is accomplished using ion-ion pairing in reverse phase chromatography with a C_{18} stationary phase and an aqueous mobile phase of 5 mM pentanesulfonic acid and 25 mM KH_2PO_4. The effect of temperature on the separation is minimal. As little as 10 ng of these compounds is detected via absorption of ultraviolet light at a wavelength of 254 nm.

INTRODUCTION

The pyridine nucleotide cycle has been found in some form, in every living organism in which it has been searched for (1). This cycle allows the conservation and recycling of the pyridine ring in cellular metabolism. The cycle itself is central to all of cellular metabolism since the intermediates of the cycle are involved in over 300 oxidation-reduction reactions (2), repair of UV light-induced damage to DNA (3), synthesis of vitamin B_{12} (4) and control of cellular differentiation via the synthesis of poly adenosine diphosphate ribose (5). Many physiological studies require the quantitation of one or more components of this cycle. In order to study the cycle

quantitatively, a rapid procedure for the isolation of the various components of the cycle is necessary. Earlier work on the separation on these intermediates relied on the use of multiple paper chromatographic separations. Thus, Withold (6) had to run multiple solvents to separate the various intermediates. Averett and Tritz (7) published a two-dimensional paper chromatographic method which uniquely separated the various pyridines but the process required a total of 24 h for complete development. In each of these separation procedures quantitation was difficult owing to the necessity of eluting the compound of interest from the paper prior to quantitation.

This report describes a high pressure liquid chromatography procedure which separates the six components of the pyridine nucleotide cycle and allows their quantitation, all in a matter of minutes. In addition, the system separates out p-aminobenzoic acid, a biologically related and sometimes contaminating substance and nicotinamide adenine dinucleotide phosphate, a metabolic product of one of the components of the pyridine nucleotide cycle.

EXPERIMENTAL

Instrumentation

A Waters Associates (Milford, MA) ALC/GPC Model 244 high pressure liquid chromatograph with a M6000A solvent delivery system, U6K injector and Model 440 dual wavelength UV absorbance detector was used in the separation system. The columns evaluated were commercial stainless steel columns pre-packed with C_{18}-μ-Bondapak (30 cm x 4.6 mm, 10 μ) for reverse phase chromatography or with ZIPAX SCX (30 cm x 4.6 mm pellicular) for ion exchange chromatography (both purchased

from Alltech Associates, Arlington Heights, IL) or with spherosorb (25 cm x 4.0 mm, 5 μ) for ion exchange chromatography utilizing functional nitrile groups (Chromanetics Corp., Baltimore, MD).

Retention times and concentrations of eluants were electronically calculated using a CSI Supergrator-3A (Columbia Scientific Industries, Austin, TX). The program used to determine these values is shown in Figure 1. The signal from the UV detector was fed through the integrator to a Fisher Recordall dual pen recorder (Fisher Scientific Co., Pittsburgh, PA).

PROGRAM

RUN PARAMETERS

initial peak width	0.1
minimum peak size	0.
sensitivity	3.

TIMED EVENTS

type	time	value
1	15.00	–

CALCULATION PARAMETERS

type calculation	3.
type results	2.
system factor	1.
sample amount	2.
minimum report	0.

IDENTITY PARAMETERS

Calibration Table Number	1.
Time Reference peaks	
minimum value	1.
percent window	1.
minimum window	0.01
Unknown factor	1.
Calibration Table Percent Update	
normal run	0.
calibration run	50.

Figure 1. Program used to determine retention times and concentration of eluants.

Chemicals and Reagents

The mobile phase for reverse phase chromatography consisted of reagent grade potassium dihydrogen phosphate, and distilled water to which was added either 1-pentanesulfonic acid sodium salt or 1-heptanesulfonic acid sodium salt (Eastman Organic Chemicals, Rochester, NY) to a concentration of 5 mM. The pH of these solutions was adjusted with phosphoric acid to either 2.5 or 3.5. The mobile phase used in ion exchange chromatography with both ion exchange columns consisted of a 2.5 mM phosphate buffer containing the proper ratio of KH_2PO_4 and K_2HPO_4 to give a pH of 2.5, 3.5 or 7.5. At the lower pH ranges it was necessary to adjust the pH with phosphoric acid. All solvents were filtered through a 0.45 µm pore size nitrocellulose filter (Millipore Corp., Bedford, MA).

Stock solutions of the pyridine nucleotide cycle intermediates, obtained from Sigma Chemical Co., St. Louis, MO,were made up in distilled water at a concentration of 1 µg/µl and were stored at -20° C until used. At the time of use, each solution was diluted 1:50 with distilled water and 0.2 µg samples (10 µl) were chromatographed to obtain retention times. Finally, a mixture of the compounds was chromatographed and the retention times compared.

Preparation of C_{18} Column

Prior to use, the reverse phase column was prepared by washing with 50 ml of 0.1 M oxalate followed by 150 ml distilled water and then 50 ml of 0.1% triethanolamine. Finally, the column was equilibrated with the solvent system to be used in the separation for 30 minutes. Equilibration was established by obtaining similar results

in duplicate runs at a 15 minute interval.

RESULTS AND DISCUSSION

The pyridine nucleotide cycle consists of the intermediates nicotinic acid, nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide, nicotinamide adenine dinucleotide, nicotinamide mononucleotide, and nicotinamide. Nicotinamide adenine dinucleotide phosphate, although not in the pyridine nucleotide cycle, is a metabolic product of nicotinamide adenine dinucleotide; p-aminobenzoic acid, although not a pyridine, has biologic effects which influence the concentration of the pyridine nucleotide cycle components.

Initial attempts to separate the above mentioned eight compounds were carried out using ion exchange high pressure liquid chromatography. Neither cation exchange chromatography using a nitrile column and mobile phases of phosphate buffer at pH values of 2.5, 3.5 or 7.5 nor cation exchange chromatography with a ZIPAX SCX column and mobile phases of phosphate buffer at pH values of 2.5, 3.5 or 7.5 successfully separated the compounds of interest. It became evident that ion exchange chromatography would result in a separation of these compounds only with gradient elution techniques or with elution by multiple solvent systems. In order to attempt to develop a more simple system, the separation of these compounds via ion-ion pairing in reverse phase high pressure liquid chromatography with isocratic elution was investigated.

Preliminary attempts at reverse phase chromatography of these compounds met with difficulty due to the inability to separate nicotinic acid adenine dinucleotide, nicotinamide adenine dinucleotide

and nicotinamide; nicotinamide adenine dinucleotide phosphate, nicotinamide mononucleotide and nicotinic acid mononucleotide were also not resolved. This initial attempt at separation utilized pentanesulfonic acid at a pH of 3.5. Addition of 5 mM KH_2PO_4 to the pentanesulfonic acid or use of heptanesulfonic acid, pH 3.5, with 5 mM KH_2PO_4 did not enhance the separation (Figure 2, panels (a) and (b) respectively). These data were interpreted to mean that the pH of 3.5 was not low enough to suppress a majority of the ionization of these compounds and, therefore, we were not dealing with a complete ion pair system. This problem was overcome by lowering the pH to 2.5. Figure 3, panels (a) and (b), illustrates the result of lowering the pH of the mobile phase.

Evaluation of these results led to the postulation that the optimum separation would be achieved with a mobile phase of pentanesulfonic acid at a pH of 2.5 with added KH_2PO_4. The function of the phosphate ions was to increase the polarity of the mobile phase with the concomitant increase in retention time of the pyridine compounds. In order to determine the most efficient concentration of phosphate, the separation of nicotinamide mononucleotide from nicotinamide adenine dinucleotide phosphate was investigated; these two compounds had been the most difficult to separate. Table I tabulates the results of the effect of phosphate ions on this separation. As is evident, a solvent system of 5 mM pentanesulfonic acid, pH 2.5, containing 25 mM KH_2PO_4, separated nicotinamide mononucleotide from nico-

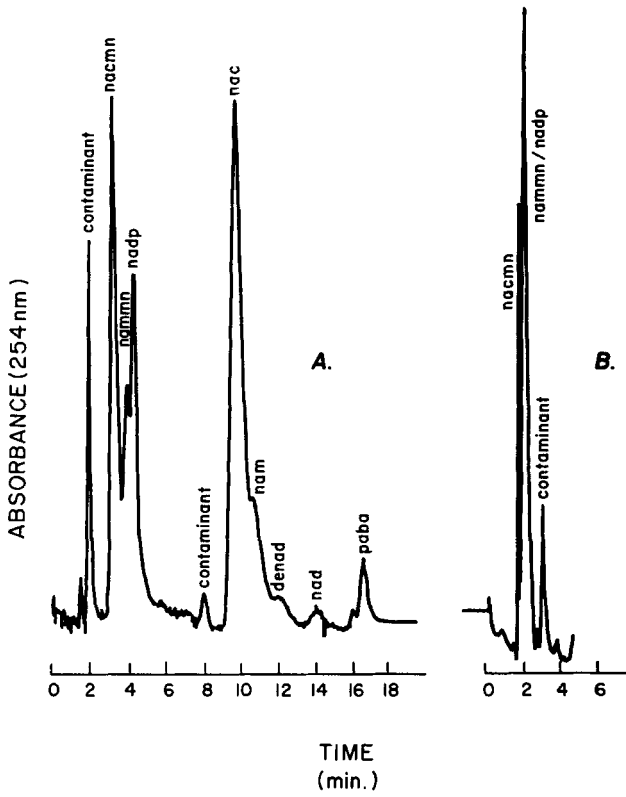

Figure 2. Separation of biological pyridines in reverse phase high pressure liquid chromatography. Conditions: column, C_{18}-μ-Bondapak (30 cm x 4.6 mm, 10 μ); temperature, ambient; mobile phase, 5 mM pentane sulfonic acid containing 5 mM KH_2PO_4, pH 3.5 (panel A) or 5 mM heptane sulfonic acid containing 5 mM KH_2PO_4, pH 2.5 (panel B).

Abbreviations: nacmn, nicotinic acid mononucleotide; nammn, nicotinamide mononucleotide; nadp, nicotinamide adenine dinucleotide phosphate; nac, nicotinic acid; nam, nicotinamide; denad, nicotinic acid adenine dinucleotide; nad, nicotinamide adenine dinucleotide; paba, para-aminobenzoic acid.

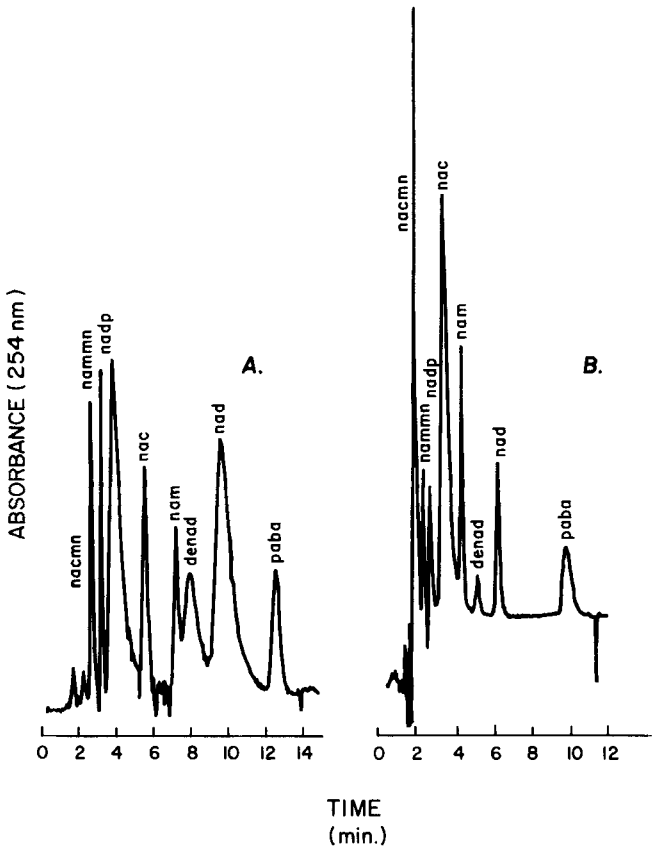

Figure 3. Effect of pH 2.5 on the separation of biological pyridines in reverse phase high pressure liquid chromatography. Conditions: same as described in Fig. 2 except that the pH was lowered to 2.5; abbreviations, same as listed in Fig. 2.

TABLE I

Variation in Minutes of Retention Time of NAMMN[1] and NADP[2] with Changing Phosphate Concentrations.

Intermediate	Phosphate Concentration 0 mM	5 mM	10 mM	25 mM	50 mM
NAMMN	2.93	2.79	2.75	2.73	2.73
NADP	3.23	3.07	3.16	3.52	3.51

Conditions were identical to those listed in Figure 1 except only the aqueus mobile phase of 0.005 M pentanesulfonic acid at pH 2.5 was used.

[1]NAMMN - Nicotinamide mononucleotide
[2]NADP - Nicotinamide adenine dinucleotide phosphate

tinamide adenine dinucleotide. Using this mobile phase, all of the biological pyridines of interest plus p-aminobenzoic acid were chromatographed. The resulting separation is shown in Figure 4.

The effects of temperature upon the separation shown in Figure 4 was investigated. The original separation was run at ambient temperature. At no temperature tested (0°, 10°, 15°, 20° C) was there sufficient enhancement of the separation to warrant the use of closely controlled temperatures.

The final parameter investigated was the limits of detectability via absorption of UV light. By calibrating the integrator with known concentrations of the various compounds and obtaining peak areas, it was possible to estimate the ultimate limits of detection. For all of the compounds except nicotinic acid, the level of detection was approximately 10 ng. For nicotinic acid this value was 1 ng.

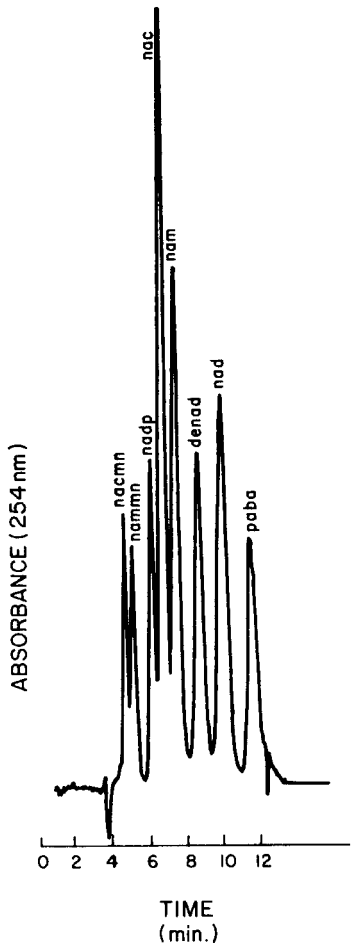

Figure 4. Effect of 25 mM KH_2PO_4 on the separation of biological pyridines in reverse phase high pressure liquid chromatography. Conditions: same as described in Fig. 2 except that the solvent system contained 5 mM pentane sulfonic acid and 25 mM KH_2PO_4, pH 2.5; abbreviations, same as listed in Fig. 2.

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