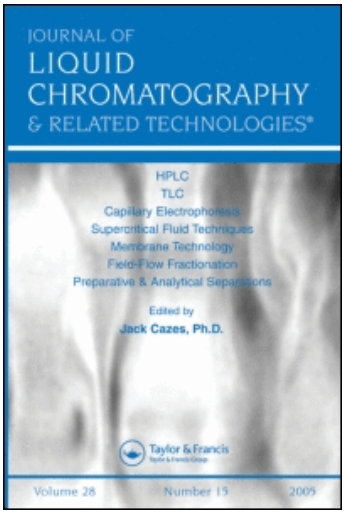

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ANALYSIS OF RIBONUCLEOTIDES BY REVERSE-PHASE HPLC USING ION PAIRING ON RADIALLY COMPRESSED OR STAINLESS STEEL COLUMNS

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ABSTRACT

A method is described for the analysis of 16 major nucleotides including NAD, IDP, GDP, cAMP, cGMP and succinyl AMP by reverse phase HPLC. The use of 65mM KH_2PO_4 at pH 3.2, low concentrations of the ion pairing reagent tetrabutylammonium phosphate and acetonitrile allowed the simultaneous separation of these nucleotides by isocratic or gradient elution in 18 and 28 minutes respectively. Stainless steel and radially compressed columns were compared and a similar separation profile was obtained. The latter columns increased retention and improved the efficiency of separation.

INTRODUCTION

Although several methods have been developed for the separation of nucleotides by HPLC, most have relied on the use of anion exchange chromatography (1-6). The introduction of reverse

phase columns has improved column stability and extended the technique to new applications. However, the nucleotides IDP, GDP, ADP, NAD and succinyl AMP were of special interest in our studies of helminth parasite metabolism and had not been adequately resolved or separated by either ion exchange or reverse phase chromatography. With the earlier technique IDP had not been fully resolved from ADP (1) and with the latter, Anderson and Murphy (7) could not demonstrate complete resolution of inosine, guanine and adenine nucleotides. Furthermore, IMP, GMP and ATP coeluted, as did, GTP and ITP, and GDP and IDP. Apart from these studies very little has been reported on the separation of IDP and succinyl AMP.

Hoffman and Liao (8) employed ion pair reverse phase chromatography, a relatively high concentration of tetrabutylammonium hydrogen sulphate (25 mM), various high ionic strength combinations of KH_2PO_4, NH_4Cl, CH_3COONH_4, CH_3COOH in the presence of 15 to 25% methanol. Moreover numerous pH conditions were required and the use of such mixtures is undesirable as precipitation is possible. More recently, Shaw et al (9) suggested the use of a combination of anion exchange and reverse phase columns for the the separation of major nucleotides. This system is more complex, time consuming and is unlikely to be versatile.

Using anion exchange HPLC, Riss et al (5) separated most major nucleotides, but NAD coeluted with AMP, and IDP and ITP were ignored. Furthermore, this analysis required approximately 60 minutes, an interrupted flow rate and discontinuous buffer gradient. On the other hand, Schwenn and Jender (10) used tetrabutylammonium hydroxide on a reverse phase C_{18} column to separate adenine nucleotides but required an extremely high pH (9.4 to 10). Unfortunately, high pH is known to dissolve the salica matrix of C_{18} columns and could mask the contribution of substituted groups on the purine or pyrimidine ring.

In the present study we investigated the ion pairing reagent tetrabutylammonium phosphate on reverse-phase stainless steel columns and the recently developed radially compressed cartridges (Waters Associates). The aim was to develop a simple, fast and practical method for the separation of adenine, guanine and inosine ribonucleotides under isocratic or gradient elution conditions.

MATERIALS AND METHODS

HPLC instrumentation

A Waters high pressure liquid chromatography system was used (Waters Associates, Milford, Mass., U.S.A.), which consisted of an M600A and

M45 solvent delivery system, model 440 absorbance detector, U6K Universal Liquid Chromatograph injector, model 660 solvent programmer and RCM-100 Radial Compression separation system. Absorbance was measured at 254nm and peak area and retention time were calculated using a Spectraphysics minigrator (Santa Clara, Cal., U.S.A.).

Columns

Two types of Waters reverse-phase columns, containing 10μ octadecysilane permanently bonded to unmodified silica were used, μ Bondapak C_{18} stainless steel columns (30 cm x 3.9 mm I.D.) and Radialpak A cartridges (10 cm x 8 mm I.D.).

Chromatographic conditions

The mobile phase was prepared by making a 65mM KH_2PO_4, 0.9 mM TBAP solution in glass distilled water with the pH adjusted to 3.2 All analyses were carried out at ambient temperature after filtration of solutions through a Millipore HAWP 0.45 μ filter (Bedford, Mass., U.S.A.). Isocratic or gradient elution modes were selected as required. Acetonitrile or methanol was used at 6% or less and all columns were equilibrated for at least 15 minutes. The radial compression module was used at 1600 to 2500 p.s.i. as recommended by the manufacturer. Column regeneration was achieved by washing with methanol, ethanol and then hexane.

Peak identification

Peaks were identified by observing retention time, addition of internal standards, absorbance ratios and with some nucleotides, by the enzymic shift method (2) or chemical hydrolysis of the pyrophosphate bond. Only those nucleotides of biochemical interest to us were subjected to enzymic identification.

Nucleotides and reagents

NAD and the mono-, di-, and tri-phosphate nucleosides of adenosine, cytidine, inosine and guanosine were purchased from Boehringer Mannheim, (Melbourne, Australia), succinyl AMP (adenyl succinic acid) from Calbiochem, (Sydney, Australia), and 3':5' cyclic AMP and GMP from Sigma Chemicals Co., (St. Louis, Mo., U.S.A.).

HPLC grade acetonitrile, hexane, methanol and TBAP as PIC Reagent A were obtained from Waters Assoc., Freon from DuPont Aust., (Sydney, Australia); tri-n-octylamine from ICN Pharm., N.Y., U.S.A. All other chemicals used were of analytical grade.

Preparations of standard solutions and tissue extract

The 0.1mM standard solutions were prepared by dissolving nucleotides in glass distilled water or the mobile phase buffer. The tissue used was adult Fasciola hepatica, which after collecting

from sheep liver was washed with saline solution at 37°C, homogenized in 10% TCA at 0°C and centrifuged at 3000 g in a refrigerated centrifuge. It was then filtered through a Millipore 0.45 μ filter. Neutralization and extraction of nucleotides was carried out according the Khym procedure (6) as optimized by Van Haverbeke and Brown (11). Some precipitation of the extract was observed on standing in ice or storage in liquid nitrogen, hence all solutions of standards and extracts were filtered again prior to HPLC analysis. The recovery of nucleotides by this procedure was periodically determined.

RESULTS AND DISCUSSION

Ion pair chromatography functions largely by exploiting the ionization characteristics of the compounds under investigation. Nucleotides are ionic species above pH3 and this property has allowed their separation initially by anion exchange and later by ion pair HPLC. Although ion pair partitioning is complex, some similarity can be assumed to exist between the phase equilibria of these two techniques (9, 12, 13). This similarity may be responsible for the apparent resemblence of some of the mobile phase buffers used in both cases.

Because aqueous buffers had been used with some success in anion exchange chromatography of

nucleotides (1-6) various solutions of phosphate, formate and acetate were investigated in the presence and absence of TBAP. Optimal conditions were greatly influenced by each of the mobile phase parameters. Phosphate in the presence of TBAP was found to be the most satisfactory buffer. Phosphate concentrations between 50 and 120 mM gave the best resolution, whilst the addition of TBAP at concentrations between 0.5 and 2.0 mM significantly increased resolution and retention time. However, concentrations higher than 2 mM increased retention without any apparent improved resolution.

Varying the pH considerably affected the resolution of closely related nucleotides. The resolution of IDP and GDP, for example, was lost above pH 3.9 and ADP and GDP coeluted at pH 2.7. For the nucleotide groups NAD, AMP, GMP and CTP, or IDP, GDP and ADP, optimal pH conditions were 3.1 to 3.4 at 60 to 65 mM KH_2PO_4 containing 0.8 to 1.5 mM TBAP. The increase in retention time, resulting from the addition of the ion pairing reagent, was overcome without loss of resolution by the addition of small proportions of methanol or acetonitrile. The optimal concentration of these organic solvents was dependent upon the nucleotides to be separated and the elution time required. Acetonitrile was found to maintain resolution better than methanol.

Suitable separations of nucleotides could be achieved with both, stainless steel and radially compressed columns. However, the latter columns required higher flow rates to achieve similar elution time but shorter time to equilibrate. The relative behaviour of the two types of columns was similar to the findings of Assenza and Brown (14). Under isocratic conditions elution time on the radially compressed columns could be more than twice that on stainless steel columns (compare Figures 1A and 1B)but the magnitude of this increase in retention was not constant. In the absence of acetonitrile mono- and diphosphate nucleosides and NAD could be readily detected in standard solutions or tissue extracts. However, under these conditions triphosphates were difficult to detect and quantitate because of long retention times (Figure 1A). On the other hand the presence of a low concentration of acetonitrile markedly reduced retention without significant loss of resolution (Figure 1C).

Simultaneous separation of nucleotides by gradient elution was also possible for both standard solutions and tissue extracts (Figure 2). However, any variations in the gradient conditions had noticeable effects on reproducibility and the elution profile of some nucleotides. For example, NAD was fully resolved in Figure 2A but coeluted with GMP in Figures 2B

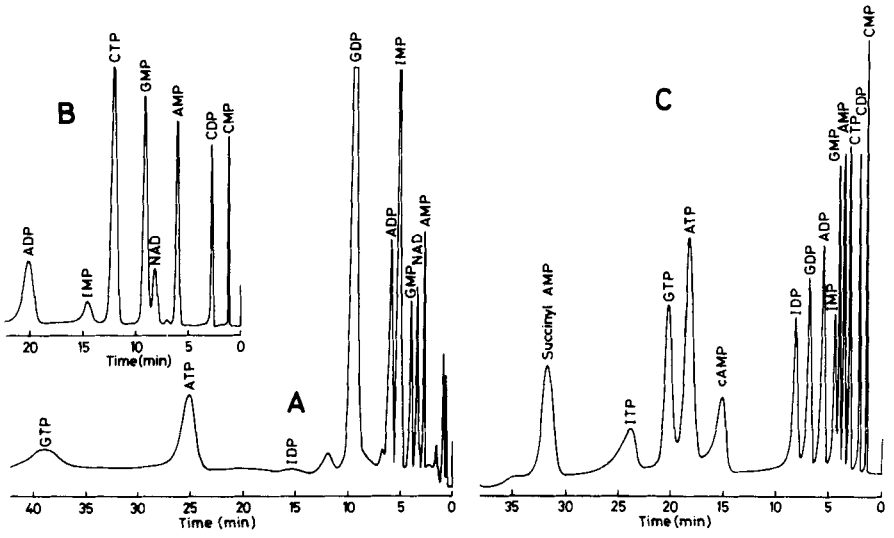

Figure 1: Isocratic elution in the absence (A + B) and presence (C) of acetonitrile using both columns and showing the difference in retention. Initial mobile phase, 65mM KH_2PO_4, 0.9 mM TBAP, pH 3.2.
A. Tissue extract with added GMP, IMP and GDP on μ Bondapak C_{18} column; flow rate 2.8 ml/min.
B. Separation of standard nucleotides on Radialpak A column; flow rate 3.5 ml/min.
C. Separation of nucleotide standards on Radialpak A column in the presence of 3.3% acetonitrile, flow rate 2.0 ml/min.

and 3B. The elution behaviour of CTP varied between isocratic and gradient conditions (compare Figures 1C and 3A with 2A and 3B).

The selection of an isocratic or gradient elution mode of separation was largely dependent on the type of nucleotides wanted, the time required to determine a suitable gradient or equilibrate the column, and the period for total elution. The contrast between these two modes is made in

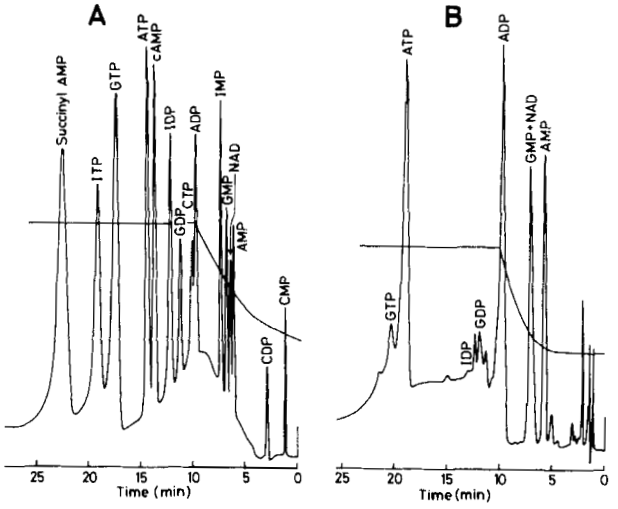

Figure 2: Gradient elution on Radialpak A column showing the difference in resolution of GMP and NAD caused by variations in acetonitrile conditions and gradient profile. Initial mobile phase as for Figure 1. A. Separation of 15 nucleotide standards with 0 to 5% acetonitrile gradient; flow rate 3.5 ml/min. B. Tissue extract with 1 to 6% acetonitrile gradient; flow rate 2.0 ml/min.

Figure 3 where isocratic elution of CTP, ATP, GTP, succinyl AMP, cAMP and cGMP was achieved in 18 minutes while gradient elution required 28 minutes. However, the latter gave better resolution of the mono- and diphosphate nucleosides, and the absence of significant base line fluctuation allowed the selection of high sensitivity attenuation.

The consistent increase in retention of nucleotides on the radially compressed column enhanced the resolution of CTP, AMP, NAD and GMP, or cAMP, cGMP, ATP and GTP. Varying the amount of acetonitrile and the flow rate allowed the

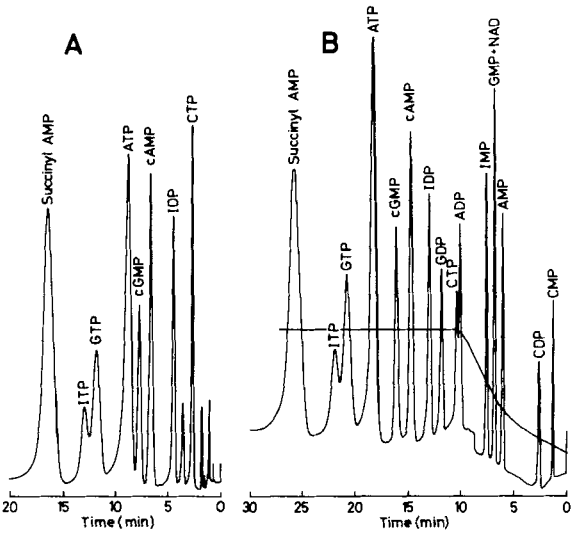

Figure 3: Isocratic versus gradient elution of standards including cAMP, cGMP and succinyl AMP in the presence of triphosphate nucleosides on Radialpak A column, initial mobile phase as for Figure 1. A. Isocratic elution with 5% acetonitrile; flow rate 2.5 ml/min. B. Gradient elution with 0 to 5% acetonitrile; flow rate 3.0 ml/min.

selective analysis of these related groups of nucleotides with very short elution time. This in turn eliminated peak broadening and made the detection of picamole levels possible.

From these observations it can be concluded that the use of relatively low ionic strength buffer, low concentrations of TBAP and acetonitrile and a pH of 3.1-3.4 gave good reproducibility, even with gradient elution, and permitted the analysis of very closely related nucleotides. Under these conditions low levels

of nucleotides in tissues can be determined isocratically or with gradient elution on stainless steel or radially compressed columns.

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APPENDIX

List of Abbreviations

ADP	Adenosine 5' -diphosphate
AMP	Adenosine 5'-monophosphate

ATP	Adenosine 5'-triphosphate
CDP	Cytidine 5'-diphosphate
CMP	Cytidine 5'-monophosphate
CTP	Cytidine 5'-triphosphate
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
IDP	Inosine 5'-diphosphate
IMP	Inosine 5'-monophosphate
ITP	Inosine 5'-triphosphate
cAMP	Adenosine 3':5'-cyclic monophosphate
cGMP	Guanosine 3':5'-cyclic monophosphate
TBAP	Tetrabutylammonium phosphate
TCA	Trichloroacetic acid
NAD	Nicotinamide-adenine dinucleotide