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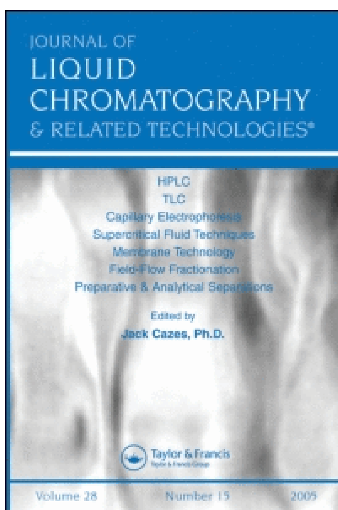

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Reverse Phase High Performance Liquid Chromotography of the 2'- and 3'-Nucleotide Monophosphates

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REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMOTOGRAPHY OF THE 2'- AND 3'-NUCLEOTIDE MONOPHOSPHATES

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SUMMARY

A radial compression reverse phase column in conjunction with a high performance liquid chromotographic system has been developed for the separation of nucleotides, with special emphasis on the separation of the 2'- and 3'-nucleotide monophosphates which are produced by the base hydrolysis of RNA. The complete separation of the eight 2'- and 3'-isomers (as well as cyclic AMP) can be accomplished in twenty-five minutes using radial compression chemical bonded alkyl (C-18) reverse phase column by eluting with a reverse ionic strength gradient. Previous separation of these 2'- and 3'- isomers required considerably longer time using the ion-exchange high performance liquid chromatography methods.

INTRODUCTION

The early separation of nucleotides was accomplished through the use of radiolabeling technique combined with paper chromatography or electrophoresis, [1,2] but because of the length of analysis time, several methods for the analytical high performance liquid chromatography (HPLC), separation of bases, [3,5] nucleosides, [4,5] and nucleotides [6,13] were developed. These methods were based on anion and cation exchange or liquid-exchange techniques using either isocratic or gradient elution. The separation of free bases, nucleotides, and nucleosides was necessary for the determination of nucleic acid composition of various RNA's, the elucidation of specific gene sequences, and the analysis of the synthesis of specific polynucleotides. Several separation techniques were developed to separate the free bases but because of the similarities in physical and chemical properties. Separation of the nucleotide monophosphates isomers (2'- and 3'-) obtained from the base hydrolysis of RNA was a difficult analytical problem.

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This paper describes using the rapid (25 minutes), quantitative separation, using reverse phase radial compression chemically bonded alkyl C-18 HPLC by a reverse ionic strength gradient of the 2'- and 3'- isomers of the nucleotide monophosphates which result from the base hydrolysis of RNA.

MATERIAL AND METHODS

Apparatus

The Waters Associate (Milford, MA. U.S.A) HPLC system was employed for all studies. The system consisted of two 6000A solvent delivering systems, U6K injector, 440 fixed wavelength (254 nm) detector, 450 variable wavelength UV-visible detector, 660 solvent programmer and 720 Data Module (for plotting and analysis of peak area and retention time). A radial-Pak A (reverse phase permanently bonded octadecylsilane column, 8 cm length, 10 μ particle size) was used as part of the Water's Radial Compression System. This separation system offers several benefits in comparison to the prepacked stainless steel columns including: (1) elimination of column voiding: (2) enhanced reproducibility and reliability, and (3) consistent high efficiences.

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Nucleotides and Chemicals

Potassium dihydrogen phosphate (Lot no. 715829) was purchased from Fisher Scientific (Fairlawn, N.J., U.S.A). The highly purified water was obtained by triple distillation in our laboratory. The 2'- and 3'- nucleotide monophosphates were obtained from Sigma (St. Louis, MO., U.S.A). Cytidine 2'-and 3'- monophosphoric acid (free acid-lot no. 39c-7670), adenosine 2'- and 3'-monophosphoric acid (free acid-lot no. 39c-7750), guanosine 2'- and 3'-monophosphoric acid (sodium salt-lot no. 99c-7540), uridine 5'- diphosphate (sodium salt; U4125), and uridine 5'- triphosphate (sodium salt, U6500) with solution of 5 nmole/10μl being used as standard solutions of each. The cyclic AMP was obtained from Boehringer Mannheim GmbH (W. Germany) - lot no. 118230. The buffers were filtered through 0.5 Millipore filters to remove solid particles and degassed in order to avoid bubble upon decompression after elution from the column.

RESULTS

The purpose of these studies was to investigate the separation of the 2'-

and 3'- nucleotide monophosphates which are obtained from the base hydrolysis of various RNA polymers. Initial studies with a 10 micron radial compressed C-18 reverse phase column using water as the elution buffer resulted in the elution pattern shown in Figure 1. The 2'-, 3'- CMP, 2'-, 3'- UMP, 2'- GMP, UTP, and UDP eluted in the void volume of the column with only cAMP and uridine being retained by the column.

After the elution profile obtained using water as the solvent demonstrated very little separation of the isomers, the ionic strength was increased using a potassium dihydrogen phosphate buffer. By increasing the initial ionic strength with 0.01 M KH_2PO_4 at pH = 4.4 using an isocratic elution procedure, the 2'-and 3'- nucleotide monophosphates were resolved better than with the water elution (Figure 2). The 2'-, 3'- GMP and 2'-, 3'- AMP isomers were separated,

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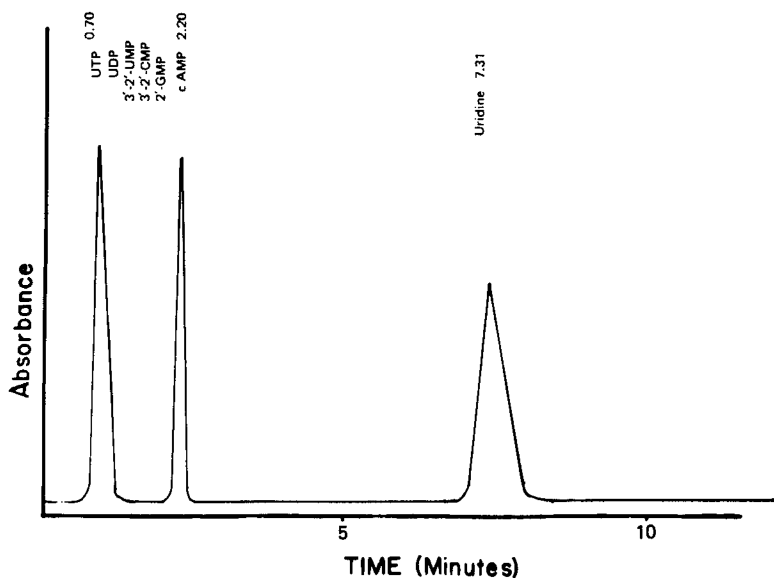

Figure 1: The HPLC separation of 2'- and 3'- nucleotide monophosphates using H_2O as the eluting solvent on a C-18 (10μ) reverse phase radial compression column. Solvent A = water. Isocratic elution with a run time of 20 minutes at a flow rate of 3.0 ml/min is used. The relative retention times are shown above each peak with the identity of each peak given below the retention time.

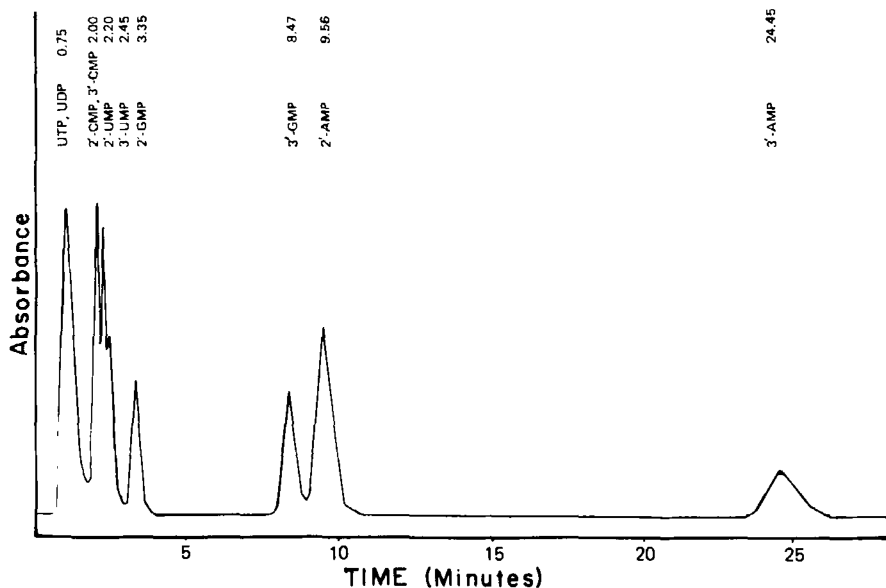

Figure 2: The HPLC separation of 2'- and 3'- nucleotide monophosphates using 0.01 M KH_2PO_4 pH = 4.4 as the eluting solvent in a C-18 (10μ) reverse phase radial compression column. Solvent A = 0.01M KH_2PO_4 pH = 4.4. The isocratic elution with Solvent A is run for 30 minutes at a flow rate of 3.0 ml/min. The relative retention times are shown above each peak with the identity of each peak given below the retention time.

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the CMP isomers co-eluting, and the 2'- and 3'- UMP separating only by 0.25 minutes. These results indicated that by increasing the ionic strength of the elution buffer that the 2'- and 3'- nucleotide monophosphate isomers were more separable, and if the ionic strength was increased even further, the resolution of the 2'- and 3'- isomers of UMP and CMP might be increased.

The ionic strength was therefore increased with 0.20M KH_2PO_4, pH = 4.4, this resulted in the 2'- and 3'- CMP and 2'- and 3'- UMP eluting as separable peaks but the 2'- and 3'- isomers of GMP and AMP did not elute in thirty minutes (data not shown). Since it was shown previously that the 2'- and 3'- isomers of

AMP was obtained from Boehringer Mannheim GmbH (W. Germany) - lot no. 118230. The buffers were filtered through 0.5 μ Millipore filters to remove solid particles and degassed in order to avoid bubble upon decompression after elution from the column.

RESULTS

The purpose of these studies was to investigate the separation of the 2'- and 3'- nucleotide monophosphates which are obtained from the base hydrolysis of various RNA polymers. Initial studies with a 10 micron radial compressed C-18 reverse phase column using water as the elution buffer resulted in the elution pattern shown in Figure 1. The 2'-, 3'- CMP, 2'-, 3'- UMP, 2'- GMP, UTP, and UDP eluted in the void volume of the column with only cAMP and uridine being retained by the column.

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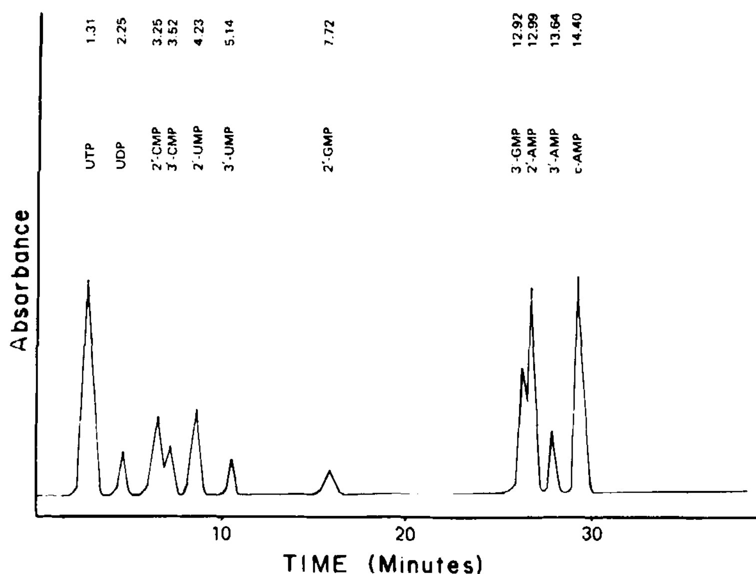

Figure 3: The HPLC separation of 2'- and 3'- nucleotide monophosphates using 0.20 M KH_2PO_4 pH = 4.4/H_2O as the eluting solvents on a C-18 (10μ) reverse phase radial compression column. Solvent A = 0.20 M KH_2PO_4 pH = 4.4. Solvent B = H_2O. A linear gradient starting at 100 % A and increasing to 100 % B over a 10 minute period at a flow rate of 3.0 ml/min is used. The relative retention times are shown above each peak with the identify of each peak given below the retention time.

Figure 3. The UTP, UDP, 2'-, 3'- CMP, UMP, GMP and AMP were separated using this system, with only the 3'- GMP and 2'- AMP found to elute in an almost identical position. The UTP eluted initially followed by the UDP. The 2'- CMP and 3'- CMP eluted separated by about 0.30 minutes, the 2'- UMP and 3'- UMP by 0.90 minutes, with the 2'- and 3'- GMP separated by five minutes with the 2'-and 3'- AMP peaks resolved by 0.50 minutes. In addition, a sample of cyclic AMP was found to elute after the two isomers of AMP at 14.40 minutes. This system seemed to separate well all of the 2'- and 3'- isomers except the 2'- AMP and the 3'- GMP, so two methods were employed in order to try to resolve these two

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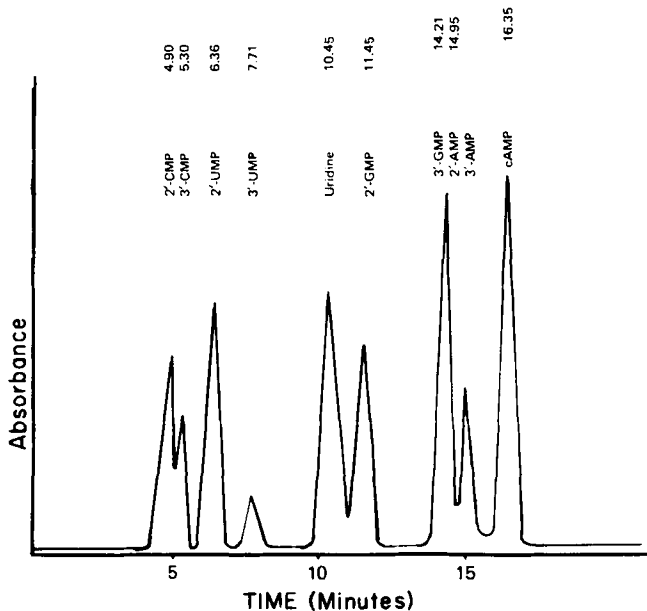

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Figure 4: The HPLC separation of 2'- and 3'- nucleotide monophosphates using 0.20 M KH_2PO_4 pH = 4.4/H_2O as the eluting solvents on a C-18 (10μ) reverse phase radial compression column. Solvent A = 0.20 M KH_2PO_4 pH = 4.4. Solvent B = H_2O. A linear gradient starting at 100 % A and increasing to 100 % B over a 10 minute period at a flow rate of 2.0 ml/min is used. The relative retention times are shown above each peak with the identifity of each peak given below the retention times.

peaks even further: (1) decreasing the overall flow rate or (2) increasing the ionic strength of the latter buffer (Buffer B) of the gradient. First, the flow rate was first decreased to 2.0 ml/minute and this resulted in better separation of 2'- and 3'- isomers of CMP and UMP but the 3'- GMP and 2'- AMP merged into one single peak (Figure 4) rather than becoming more resolved. From this data it was concluded that decreasing the flow rate was not effective in resolving the GMP and AMP, 2'- and 3'- isomers.

The second method of separating these isomers was then employed. The ionic strength of the second buffer (B) was increased to 0.002 M KH_2PO_4 pH = 4.4.

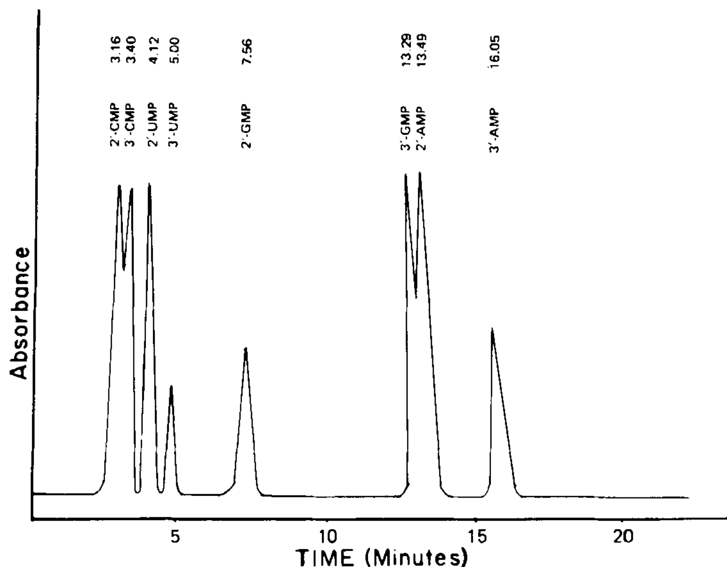

Figure 5: The HPLC separation of 2'- and 3'- nucleotide monophosphates using 0.20 M KH_2PO_4 pH = 4.4/0.002 M KH_2PO_4 pH = 4.4 as the eluting solvents on a C-18 (10μ) reverse phase radial compression column. Solvent A = 0.20 M KH_2PO_4 pH = 4.4. Solvent B = 0.002M KH_2PO_4 pH = 4.4. A linear gradient starting at 100 % A and increasing to 100 % B over a 10 minute period at a flow rate of 3.0 ml/min is used. The relative retention times are shown above each peak with the identity of each peak given below the retention time.

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The results of this separation are shown in Figure 5 with the 3'- GMP and 2'-AMP resolved by 0.25 minutes with the 2'-, 3'- isomers pair of GMP being separated by about seven minutes, and the AMP pair by 2.5 minutes. Therefore by increasing the ionic strength of the second buffer with 0.002M KH_2PO_4, the resolution of the 3'- GMP and 2'- AMP was 0.25 minutes compared 0.04 minutes when water was used.

These results indicated that the ionic strength could be increased further for better resolution of the 2'-, 3'- isomers of AMP and GMP but earlier studies (Figure 2) indicated that the 3'- AMP isomer was eluted in 30 minutes using 0.01 M KH_2PO_4 buffer so a concentration between 0.01 and 0.002 was tried. The

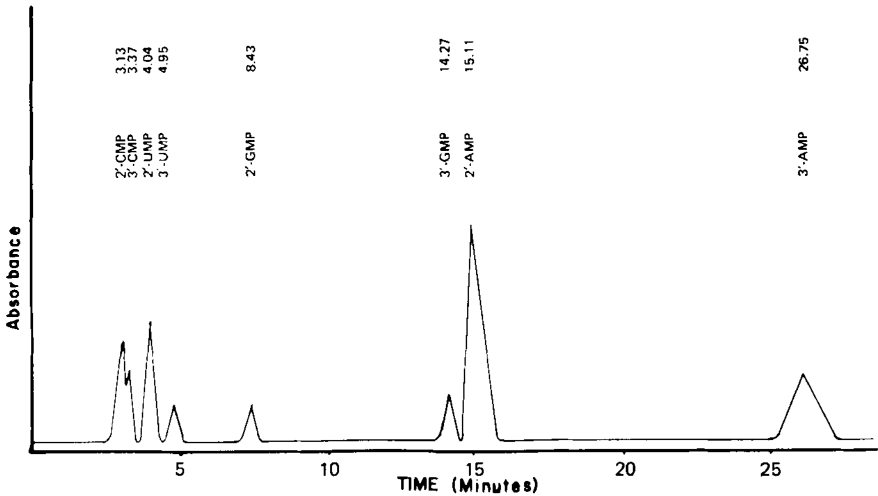

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Figure 6: The HPLC separation of 2'- and 3'- nucleotide monophosphate using 0.20 M KH_2PO_4 pH = 4.4/0.006 M KH_2PO_4 as the eluting solvent on a C-18 (10μ) reverse phase radial compression column. Solvent A = 0.20 M KH_2PO_4 pH = 4.4. Solvent B = 0.006 M KH $_2PO_4$ pH = 4.4. A linear gradient starting at 100 % A and increasing to 100 % B over a 10 minute period at a flow rate of 3.0 ml/min is used. The relative retention times are shown above each peak with the identity of each peak given below the retention times.

buffer concentration of 0.006M KH_2PO_4 was employed with the results shown in Figure 6. As can be seen the elution of the 2'-, 3'- isomers of CMP or UMP were not affected whereas the 2'-, 3'- AMP isomers were separated by 11.5 minutes and the 2'- AMP and 3'- GMP by 0.85 minutes. Therefore, the second buffer concentration of 0.006 M separated these isomers and thus producing an excellent HPLC system elution condition for the separation of the XTP, XDP group of nucleotide from the 2'-, 3'- isomers of CMP, UMP, GMP, AMP and cyclic-AMP.

This technique of the separation of the 2'- and 3'- isomers of nucleotide monophosphates by radial compression reverse phase C-18 has recently been used in this laboratory to study the composition of the cellular RNA obtained from rat Sertoli cells in culture. The RNA was acid extracted, base hydrolyzed and

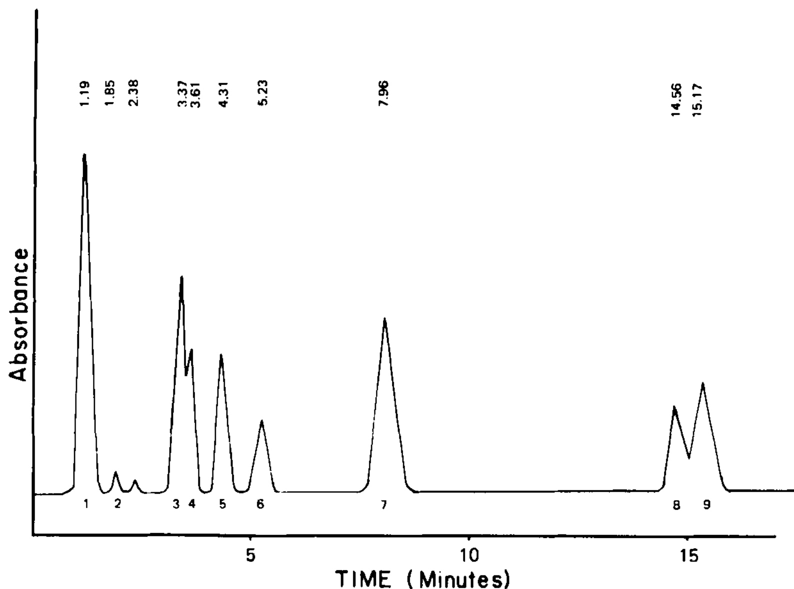

Figure 7: The HPLC separation of 2'- and 3'- nucleotide of RNA isolated from Sertoli cell cultures prepared from Long-Evans rats. The separation uses a 0.20 M KH_2PO_4 pH = 4.4/0.006 M KH_2PO_4 pH = 4.4 as the elution solvent using the same conditions described in Figure 6. The relative retention times are shown above each peak. The numbers below each peak represent the elution positions of various standard nucleotides: (1) XTP;(2) XDP; (3) 2'-CMP;(4) 3'-CMP; (5) 2'-UMP; (6) 3'-UMP; (7) 2'-GMP;(8) 3'-GMP;(9) 2'-AMP.

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the amount of each 2'-and 3'-XMP was determined using the 0.20 M KH_2PO_4 pH = 4.4 buffer system described previously. The relative amount of each isomer was quantitated by measuring the total absorbance area for each (manuscript in preparation) using the reverse phase column. A representative elution pattern using the reverse phase column is shown in Figure 7. Therefore, this method of 2'-and 3'-XMP separations after base hydrolysis of RNA, by reverse phase chromatography can be used directly to determining the nucleotide ratio in RNA samples. This separation using the reverse phase column (0.20 M KH_2PO_4 elution) compared to the ion exchange column is much faster and the special

purchase of an ion-exchange column is not required. The reverse phase column can be used for steroid, peptide, and XMP nucleotides without the purchase of a special column for any of these separations.

DISCUSSION

The initial separation and quantitation of nucleotide isomers was done using paper chromatography or electrophoresis techniques [1,2]. With the development of HPLC, a variety of ion-exchange columns and experimental conditions were used in the separation of nucleotide monophosphate isomers by HPLC. A partial resolution of AMP isomers was obtained using anion-exchange chromatography [14] in about 15 hours using a Dowex-1 (formate) column. The typical problem was the separation of the 2'- and 3'- isomers of each XMP from each other. Recently, Edelson, et. al. (1979) [16] reported the separation of the 2'- and 3'- isomers of the monophosphorylated nucleotide on an AX-10 column (a functional weak anion-exchange bond phase prepared by Lichrosorb Si-60 silica). This system allowed the resolution of some of the 2'-, 3'- monophosphorylated nucleotides in a single run of about 60 minutes using 0.01M KH_2PO_4 pH = 2.25. A similar system using Whatman Partisil 10 SAX column resolved the 2'- and 3'- isomers of the nucleotide monophosphates in 1.5-2 hours.

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In addition, some early studies showed that several nucleotides but not the 2'-, 3'-nucleotide monophosphates could be separated by using reverse-phase HPLC [17,18]. The present study demonstrates that 2'-and 3'- nucleotide monophosphates, which would result from the base hydrolysis of RNA, can be separated on a reverse phase column by adjusting the ionic strength of the buffer and using a reverse ionic strength gradient to elute the C-18 radial compression column.

These compounds are probably being separated on the basis of their differential partitioning between the hydrophobic packing and the hydrophilic solvents. Most compounds will have a particular affinity for both the nonpolar hydrocarbon phase of the column and for the mobile phase. Elution characteristics of this system can be changed by either changing the compounds affinity for the stationary phase (state of ionization of the compound) or by altering the affinity for the solvent (ionic strength or polarity changes in solvent). The

column affinity is fixed by the base moiety, with the mobile phase affinity increase with the addition of phosphate residues. Thus, the triphosphates residues are more soluble in the mobile phase and they elute faster. This is further illustrated when water is used as the eluting buffer, then almost all of the nucleotides elute with the solvent front and shown no separation but as the ionic strength is increased the isomers are retained longer. Therefore, the counter ion on the phosphate is very important in determining its solubility in the mobile phase but further investigation needs to be done.

Further investigations are now being conducted to determine if the XMP, XDP, and ATP and bases can be separated by changing the pH, changing the ionic strength, determining if the cation or anion is important in determining the separation of the various nucleotides or what other parameters determine the separation of these compounds on the reverse phase column. It must also be noted that a new 5 radial compression column is now available which should theoretically give better resolution in less time. This column is being tried in order to achieve better resolution in less time for separation of total base hydrolized RNA nucleotides from various cells of testicular origins stimulated by various gonadotropins and steroids.

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