



Reversed-Phase Nucleotide Separations

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Abstract

Nucleotides are ubiquitous in cells, with major functions including the control of cellular energetics, cell signaling cascades, biosynthesis of nucleic acids, and involvement in countless enzymatic reactions. It is therefore of widespread interest to resolve and identify components of various such mixtures.

Traditionally, reversed-phase (RP) separations of nucleotides have relied on ion-pair reagents, typically salts of tetrabutylammonium. We sought to determine whether viable alternatives of RP nucleotide separations existed which would not be dependent on quaternary ammonium ion-pair (IP) reagents. However, this proved unsatisfactory; herein we demonstrate optimal RP nucleotide separations by use of alternative ion-pairing reagents on a silica-based alkyl phase.



Introduction

- **RP nucleotide separations typically are performed on alkyl phases and rely on quaternary amine IP reagents to provide adequate retention.**
- **Quaternary amine IP reagents present problems for MS detection.**
- **IP reagents can present problems of reproducibility, particularly where gradient elution is performed**
- **We sought to explore alternate stationary and mobile phases that might circumvent the need for quaternary amine IP reagents, or preferably any IP reagent.**
- **Regarding alternatives, zirconia-based stationary phases were of particular interest given the fact that phosphates behave as strong Lewis bases for coordination with the Zr surface, thus providing a means for the nucleotide analytes to be retained in the absence of any mobile phase modifier (IP reagent).**



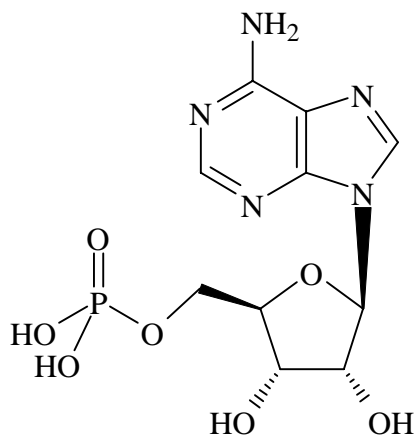
Initial Strategies

In our effort to find alternate RP separations of nucleotides which did not rely on traditional use of quaternary amine ion-pair reagents the following courses were initially pursued:

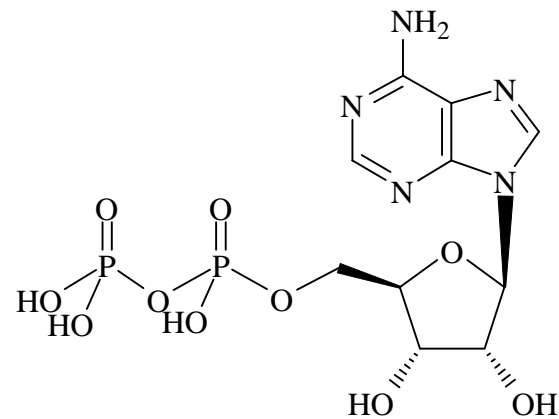
- 1. Perform the separation at pH 2 in which the negative charge on the phosphate moieties would be reduced to $\leq \sim 0.5$. TFA was included to ion-pair with any protonated amines of the purine/pyrimidine bases. Samples to be run on various phases (alkyl, polar-embedded, and fluorinated).**
- 2. Perform the separation at pH 1 on a zirconia column, at which phosphate moieties would have a net negative charge of only ~ 0.1 .**
- 3. Perform the separation at neutral pH (to enhance ion-pairing with phosphate moieties) on the various phases (alkyl, polar-embedded, and fluorinated) with triethylamine, a weak basic IP reagent. This might still permit MS detection in a negative-ion mode.**

Initial evaluations were with a sample mixture of AMP, ADP, and ATP.

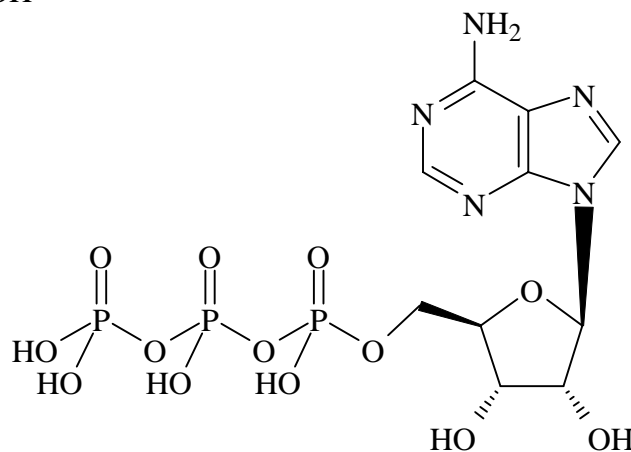
Structures of Initial Analytes



AMP



ADP



ATP



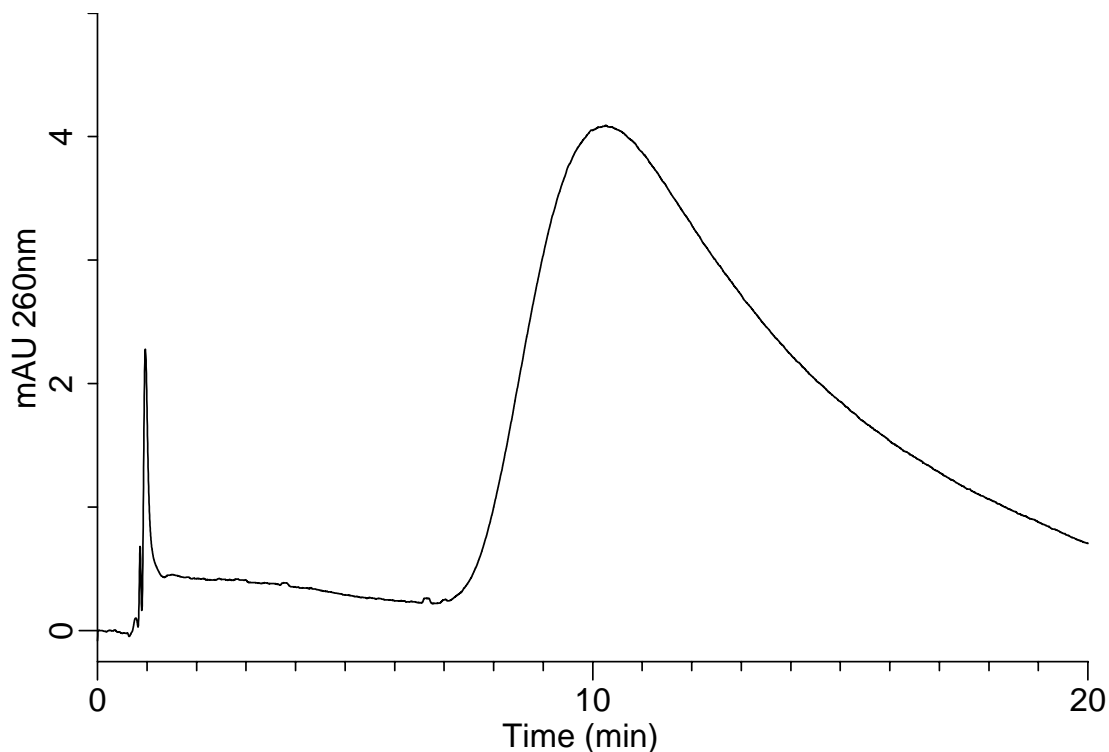
Results of Initial Strategies

Summaries from the initial strategies:

1. The TFA pH 2 mobile phase system provided inadequate retention and/or resolution on alkyl, polar-embedded, or fluorinated phases (data not shown).
 - a. Other perfluoro organic acids might still provide adequate retention while permitting mass-spectral detection.
 - b. Inclusion of phases with polar functionalities was to observe if polar interactions with analytes might contribute to unusual selectivity. The fluorinated phase was not expected to perform favorably considering its electronegative surface and the negative charge on the phosphate moieties.
2. Sample components appeared well retained on the zirconia support, but eluted in an unsatisfactory broad peak (see Figure A).
3. Triethylamine provided inadequate retention and/or resolution of the nucleotide mixtures (including the guanidyl-, cytosyl- and uridyl- sample mixtures of mono-, di- and triphosphates) on alkyl, polar-embedded, or fluorinated phases; data not shown.

Figure A. Chromatogram of Nucleotide Mixture on Zirconia-PDB Column

Column: Discovery Zr-PBD, 7.5cm x 2.1mm ID, 3 μ m
Mobile Phase: 80:20, (25mM H₃PO₄/HCl, pH 1.0) : MeOH
Flow: 0.21mL/min
Temp.: 50°C
Det.: 260nm
Inj.: 2 μ L, 0.2 μ g each analyte
Sample: AMP, ADP, & ATP



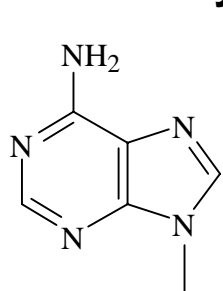


Optimization With Ion-Pair Reagents

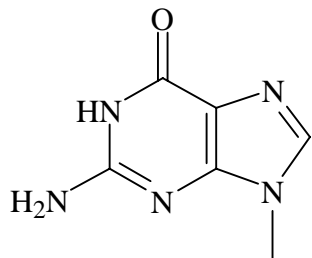
- None of the previous separation systems (no IP utilized) yielded satisfactory results.
- Tetraethylammonium (TEA) was initially studied as the IP reagent. With sample mixtures of adenylyl-, guanidyl-, cytosidyl- and uracyl- nucleotide mono-, di- and triphosphates, even at concentrations of ~15mM TEA, the volume fraction of methanol had to be as low as 1- 2% for good retention and resolution. This is not desirable for standard C18 phases as it could lead to problems with reproducibility due to inadequate “wetting” of the stationary phase.
- Tetrapropylammonium (TPA) was therefore studied as the IP reagent to see if it would provide adequate retention for the nucleotide analytes.

Structures of Additional Analytes

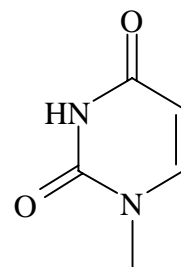
Purine / Pyrimidine Bases



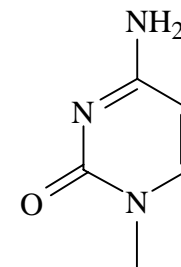
adenosine (A)



guanosine (G)

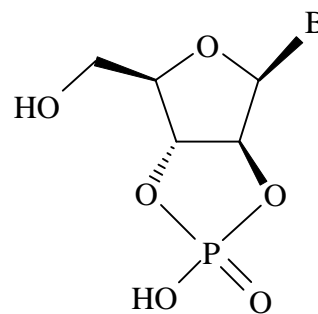
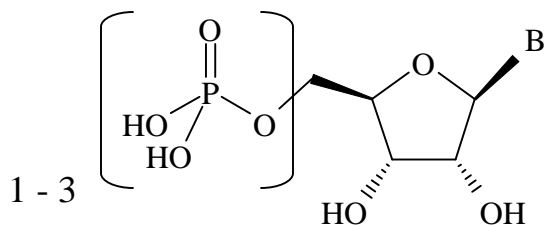


uridine (U)

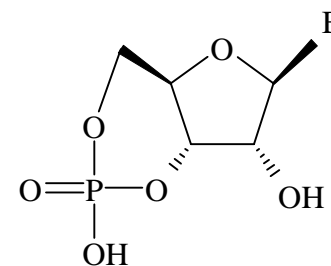


cytidine (C)

Nucleotides



2'-3'-cNMP



3'-5'-cNMP

Tetrapropylammonium (TPA) Mobile Phase System Methods

Two mobile phase buffers (pH 7.0) were explored:

1. phosphate because it is so commonly used, and
2. Bis-Tris, because phosphate at neutral pH is aggressive toward silica, and may present column lifetime issues.

Phosphate Mobile Phase System

Mobile Phase A: 10mM H_3PO_4 , 5mM TPAOH / NH_4OH , pH 7.0

Mobile Phase B: 75:25, (13.3mM H_3PO_4 , 6.7mM TPAOH / NH_4OH , pH 7.0) : MeOH

Bis-Tris Mobile Phase System

Mobile Phase A: 10mM Bis-Tris, 5mM TPAOH / HCO_2H , pH 7.0

Mobile Phase B: 75:25, (13.3mM Bis-Tris, 6.7mM TPAOH / HCO_2H , pH 7.0) : MeOH

For both mobile phase systems:

Various proportions of mobile phases A & B (isocratic)

Column: Discovery HS C18, 15cm x 2.1mm ID, 5 μm

Flow: 0.21mL/min

10 Temperature: 35°C

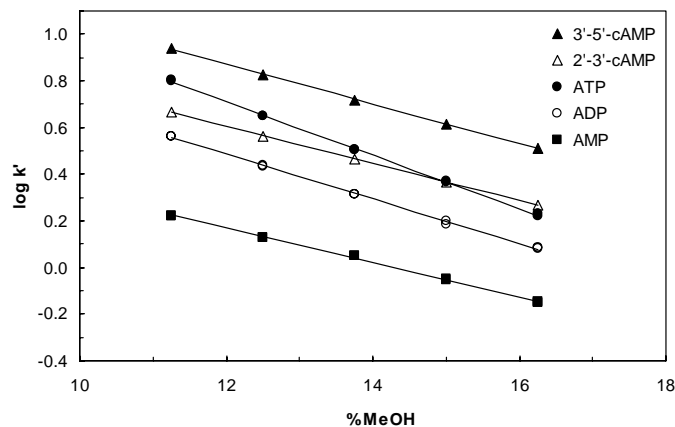
Injection: 2 μL

Detection: 260nm

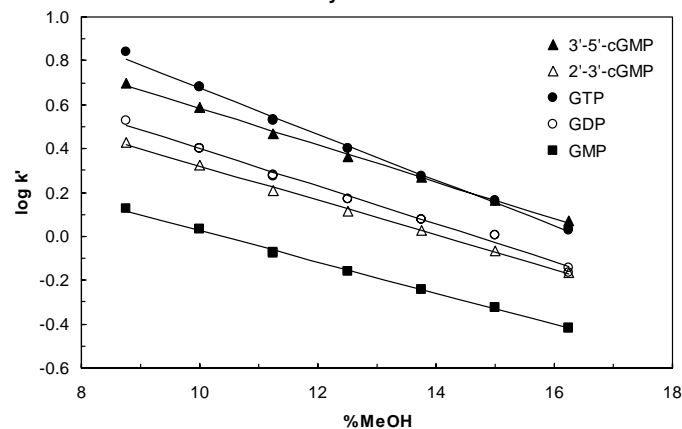
Sample: 0.1g/L each analyte

Phosphate Mobile Phase System: Results Summary

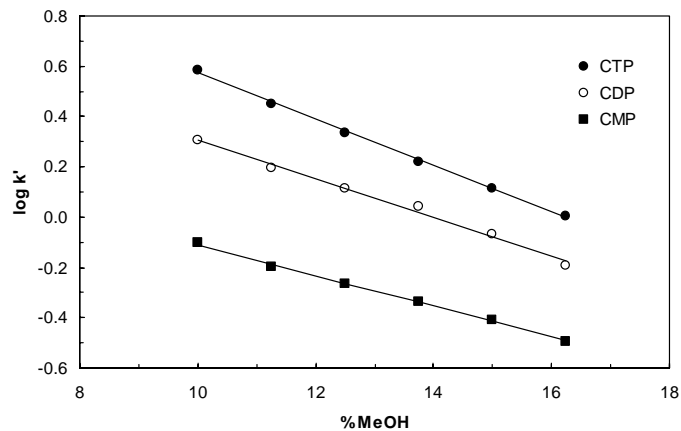
Adenosyl Nucleotides



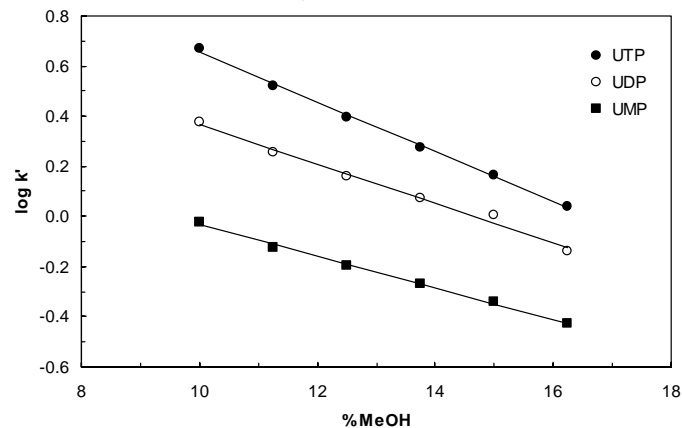
Guanidyl Nucleotides



Cytidyl Nucleotides

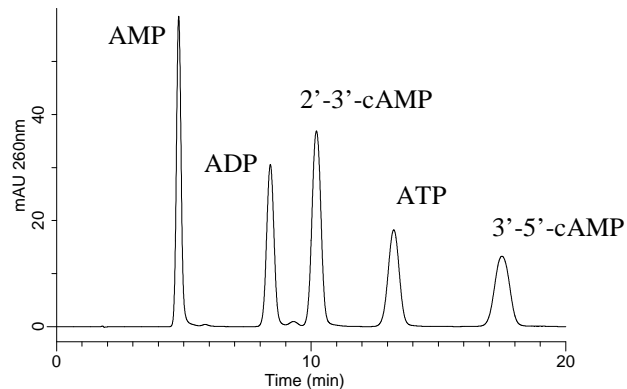


Uridyl Nucleotides

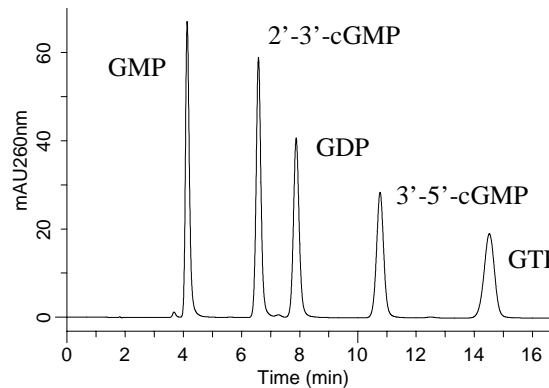


Phosphate Mobile Phase System: Sample Chromatograms

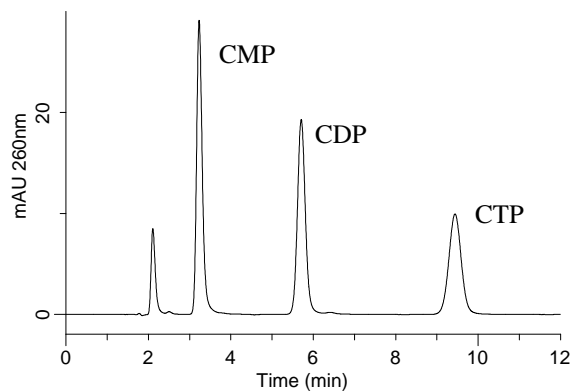
50%B (12.5% MeOH)



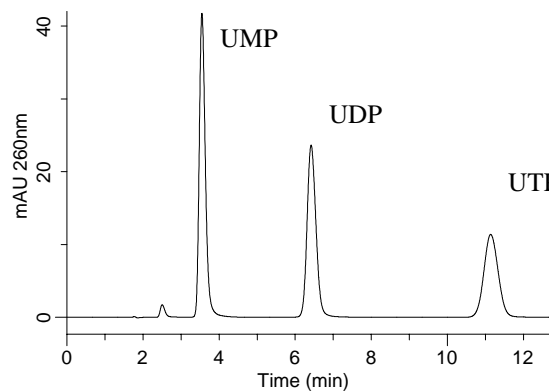
35%B (8.75% MeOH)



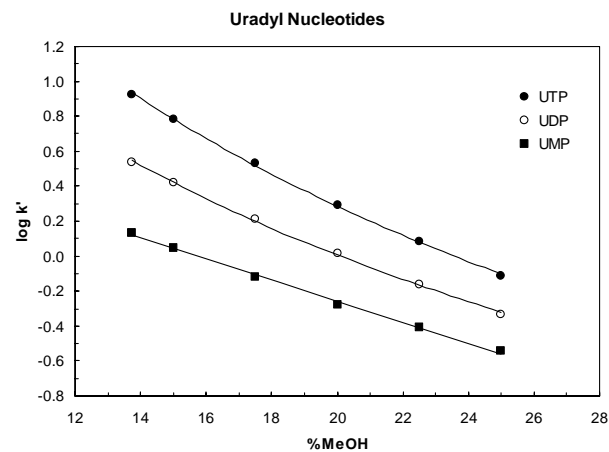
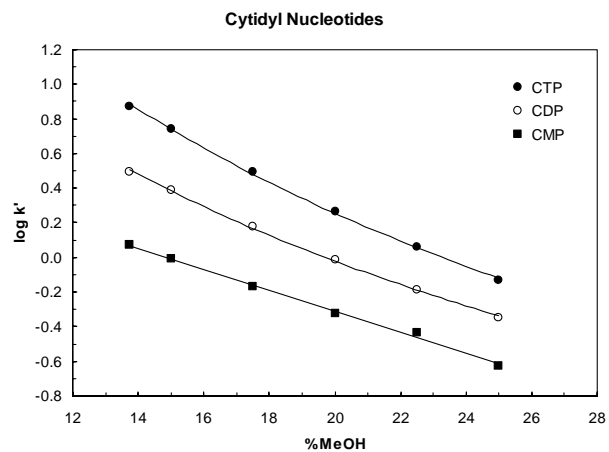
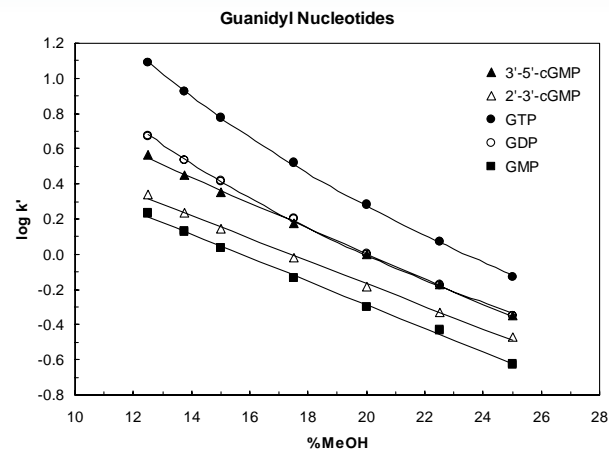
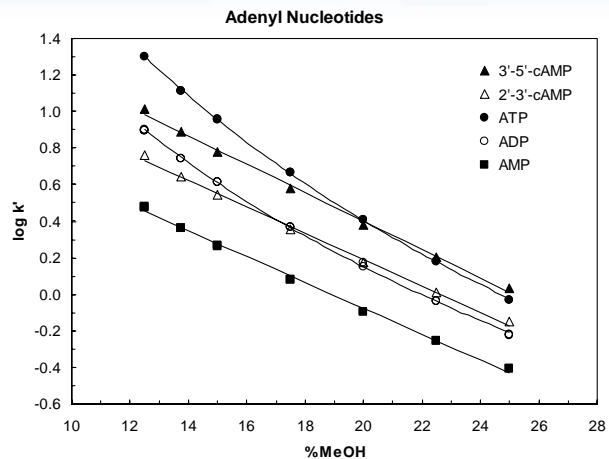
40%B (10% MeOH)



40%B (10% MeOH)

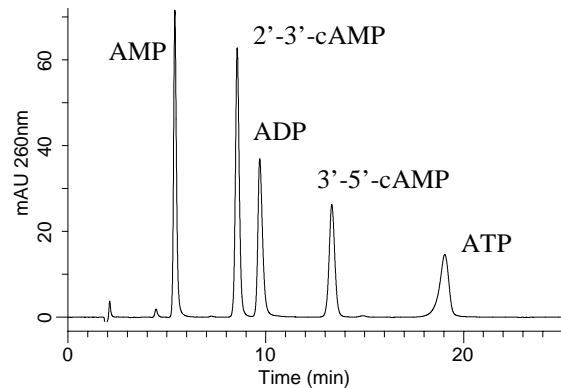


Bis-Tris Mobile Phase System: Results Summary

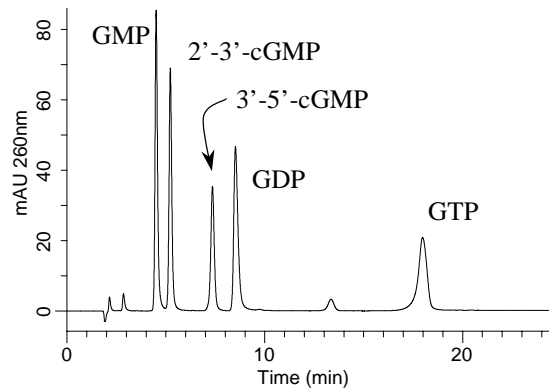


Bis-Tris Mobile Phase System: Sample Chromatograms

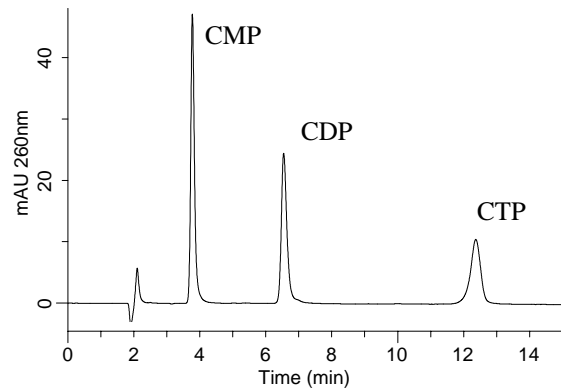
60% B (15% MeOH)



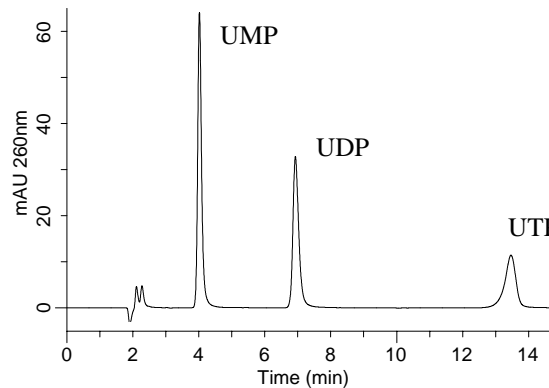
55% B (13.75% MeOH)



60% B (15% MeOH)



60% B (15% MeOH)





Observations

- Retention patterns of the NMP, NDP, and NTP series with IP reagents, do correlate with the number of phosphate moieties, which is consistent with an operational IP mechanism.
- Selectivities of the adenyl and guanidyl nucleotide sample sets vary not just between themselves but also between buffer systems.
- The Bis-Tris buffer system permits greater retention of the nucleotides.
- Of the various silica-bases phases investigated (alkyl, polar-embedded, fluorinated) with any of the IP reagents, the alkyl phase provided better retention and resolution (data not shown).



Conclusions

- **Quaternary amine-based IP reagents, as components of mobile phases, provide the necessary retention and selectivity for nucleotide separations.**
- **Reduced retention of the nucleotide analytes in the phosphate mobile phase system could be due to the scavaging of the IP reagent by the buffer, with a lower level of IP available to ion-pair with the analytes.**



Conclusions (cont'd.)

- **Conceivably, Bis-Tris, a tertiary amine, could also ion-pair with the analytes. However, its selection was based on its pK_a (6.5) and the observation that triethylamine provided inadequate retention. While triethylamine would be expected to carry a positive charge of 1 at pH 7 (even in the presence of 10-20% methanol, given its pK_a of 10.8), that of Bis-Tris would be ≤ 0.25 . Thus, as an IP reagent, it would be expected to exhibit a very minor role, if any.**
- **Silica-based alkyl phases appear to provide the optimum support for RP nucleotide separations under the conditions examined.**



Conclusions (cont'd.)

- While zirconia-based phases may display good retention for nucleotides without ion-pairing reagents, further investigations have yet to be conducted to determine conditions that could provide good chromatography.
- Other perfluoro organic acids will be investigated as IP reagents that might still permit MS detection.



Conclusions (cont'd.)

- **Methods have been demonstrated, that while necessitating the use of IP reagents, they are isocratic and thus not plagued by reproducibility issues that can occur with gradient methods (especially when the strong solvent does not contain any IP reagent as is common practice).**
- **An ideal chromatographic stationary phase for nucleotide separations would be hydrophobic, inert (display no other secondary interactions), and stable to pH 1 or 12. Thus chromatography at extremes of pH could neutralize either the phosphates or amines, with absence of secondary interactions permitting good chromatographic behavior.**