Application of the Fused-Core® Penta-HILIC Column for High Performance Separations of Nucleobases, Nucleosides and Nucleotides

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Summary

During the past few years superficially porous silica particles have emerged as preferred materials for high efficiency and high speed separations in HPLC. Superficially porous (also known as core-shell or Fused-Core®) silica particles can be obtained with a variety of bonded phases, pore sizes, particle sizes, and shell thicknesses. Columns packed with 2.7 µm diameter, 90Å pore size Fused-Core® particles have been shown to exhibit surprising efficiency, particularly with small molecule separations. Novel hydroxylated bonded-phases are observed to be highly hydrophilic, exhibiting typical HILIC retention properties. A selected material, commercialized as Halo Penta-HILIC, contains five hydroxyl groups on the bonded ligand and shows high efficiency and improved ionic interactions with ionizable compounds, including bases, acids, and zwitterions. We demonstrate applications of this highly polar bonded phase in HILIC mode of operation for efficient separations of nucleobases and nucleosides, using mobile phases that are compatible with mass spectroscopic (MS) detection. High efficiency separations of mono-, di- and tri-phosphate nucleotides are also observed, although to date the best results have been obtained with phosphate buffers, which have limited MS compatibility. We continue to move towards the goal of high speed separations of complete mixtures of the nucleobases and nucleotides, with improved MS-capable mobile phase conditions.

Objectives

Evaluate HILIC as a method for the separation of nucleobases, nucleosides, nucleotides, and their derivatives to establish:

- Ability to resolve structurally similar compounds
- Measures of column efficiency and peak shape (Tailing Factor) and peak widths.
- Compatibility of the separation conditions with LC-MS operation.

Approach

The Fused-Core® Penta-HILIC bonded phase column packing material was applied to this task with the expectations that:

- HILIC may possess high selectivity for resolving structurally similar polar compounds
- Fast separations could be accomplished for complex samples.
- A neutral bonded-phase HILIC column material may permit the use of low ionic strength and volatile mobile phase modifiers that are preferred for effective ESI-MS detection.
- Isocratic separations with varying acetonitrile concentration (AcN) and buffer systems were initially employed to provide retention and peak shape data for DryLab (Molar Institute, Berlin, DEU) modeling and optimization of gradient elution conditions.

Materials and Methods

The LC experiments were run on a Shimadzu Nexera LC equipped with a SPD-M30A diode array detector. LC-MS detection was scouted using the LCMS2020 single quadrupole MS, with Electrospray Ionization (ESI). Unless otherwise stated the column used was a 4.6x100mm, 2.7µm Penta-HILIC prepared in-house (Advanced Materials Technology). Standard nucleobases, nucleosides and nucleotides were obtained from Sigma and prepared in either 0.1% formic acid, or in 50% AcN/water mixtures. Mobile phases are expressed as concentration of buffer in final solution, with pH in the aqueous component.

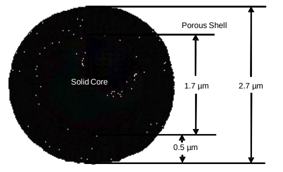
Fused-Core Particles

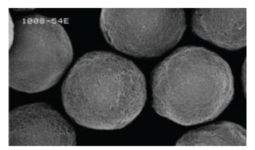
Particle Characteristics

- **Silica: High purity, Type B**
- **Pore Size: 90 Å, 160 Å, 400 Å**
- **Particle Size Distribution: 5% RSD**
- **pH range: 2–9**
- **Efficiency: > 230,000 plates/m**

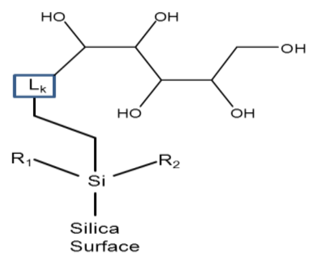
Features and Benefits

- **Ultrafast separations save time and improve productivity**
- **UHPLC performance without the need for UHPLC equipment**
- **Low pressures enable the coupling of columns for high efficiency/high resolution**





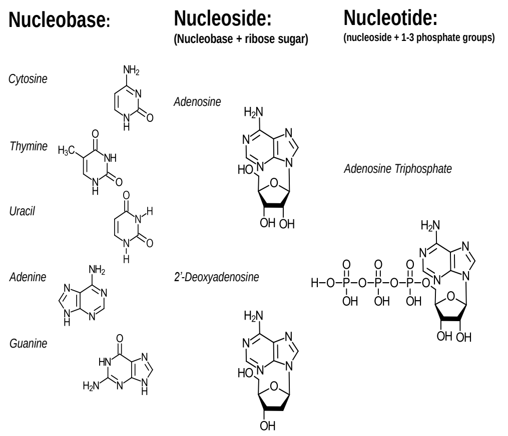
Bonded Phase Ligand



Halo Penta-HILIC

Polar ligand with a proprietary linker and silane side chains, 90 A pore size, 2.7 µm particles

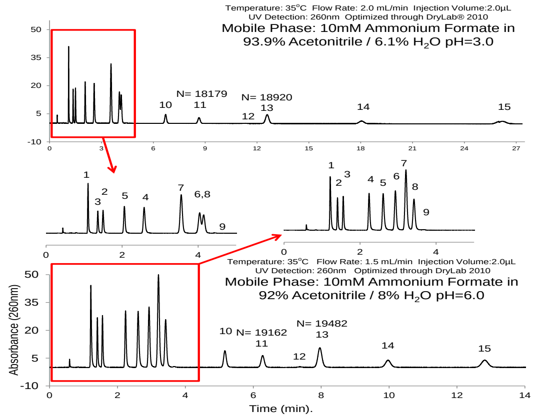
Nucleotide Components



Nucleobase and Nucleoside Separations

Initial scouting using ammonium acetate, formate and hydroxide at pH 3, 6, and 9 identified the most promising conditions as ammonium formate at pH 6. Using DryLab for acetonitrile optimization resulted in conditions that separated all 15 compounds (minimum resolution of 2.0 for adenine [5] and uridine [6]). Isocratic elution followed by gradient of water (strong solvent) permits separation of early eluting compounds with a separation time under 9 minutes. Equilibration at elevated flow permits reproducible run-to-run analyses of less than 20 minutes. The method was evaluated using LC/MS with a 2.1 mm ID column. The ability to observe positive and negative molecular ions suggests a manageable path to development of a multistage MS assay for purines, pyrimidines and nucleosides.

High Resolution Isocratic Separation of Complex Mixture with Ammonium Formate

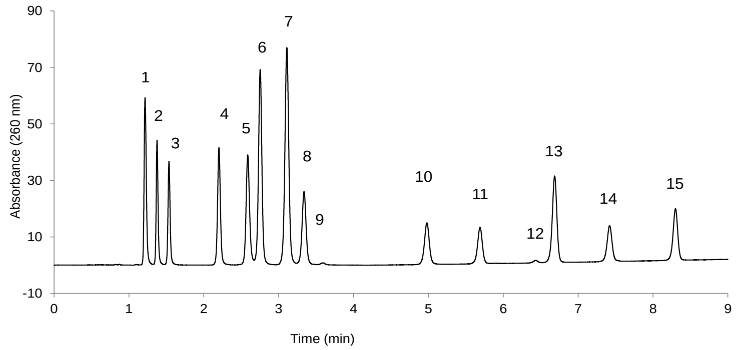


Temperature: 35°C Flow Rate: 2.0 mL/min Injection Volume:2.0µL
UV Detection: 260nm Optimized through DryLab® 2010
Mobile Phase: 10mM Ammonium Formate in 93.9% Acetonitrile / 6.1% H_2O pH=3.0
N= 18179
N= 18920
Temperature: 35°C Flow Rate: 1.5 mL/min Injection Volume:2.0µL
UV Detection: 260nm Optimized through DryLab 2010
Mobile Phase: 10mM Ammonium Formate in 92% Acetonitrile / 8% H_2O pH=6.0
N= 19162
N= 19482
Absorbance (260nm)
Time (min).

	Compounds
1	Thymine
2	Uracil
3	Thymidine
4	2'-Deoxyadenosine
5	Adenine
6	Uridine
7	Adenosine
8	Hypoxanthine
9	Xanthine
10	Cytosine
11	2'-Deoxycytidine
12	Guanine
13	2'-Deoxyguanosine
14	Cytidine
15	Guanosine

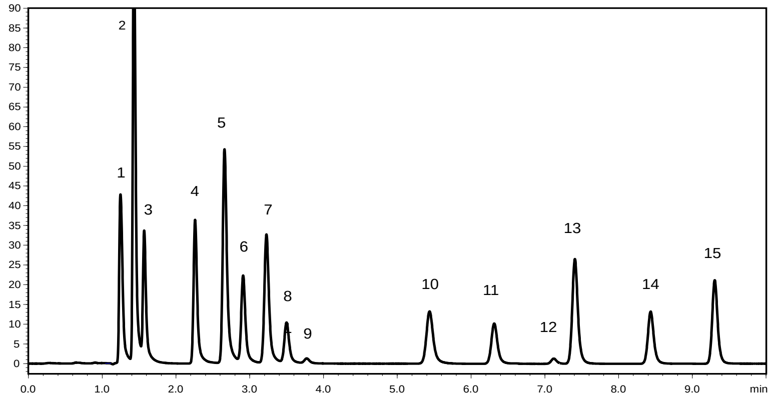
LC-MS Compatible Gradient with Ammonium Formate pH=6

Temperature: 35°C Flow Rate: 1.5mL/min Injection Volume:3.0µL UV Detection:260nm
Mobile Phase: Pump A= 10mM Ammonium Formate in 85% Acetonitrile / 15% H_2O pH=6,
Pump B= 10mM Ammonium Formate in 93% Acetonitrile / 7% H_2O pH=6,
Gradient Conditions: Isocratic 6% A from 0-3 minutes, 6-100% A from 3-9 minutes



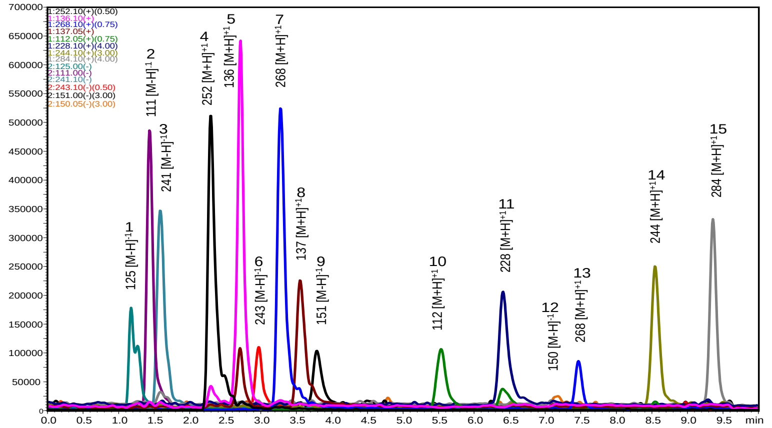
HILIC Separation with Coupled UV and MS Detection of Nucleosides and Nucleobases

Shimadzu Nexera LC: 2.1 mm ID x 100 mm Halo Penta HILIC column; 35°C ; Abs. 260 nm
Gradient: 6% B for 3.5 min, 65-100% B 3.5 to 9.5 min., hold 100% B for 0.5 min;
A – 10mM Ammonium Formate in 93% AcN/7% H_2O, pH 6.0; B – A at 85% AcN
Flow 0.31 mL/min 0-10 min, 1.0 mL/min (6%B) 10-16 min, return to initial
Sample: 2 uL of 15 purine and pyrimidine bases and –sides (as above)



HILIC Separation with LC-ESI/MS Detection of Nucleoside and Nucleobase Molecular Ions

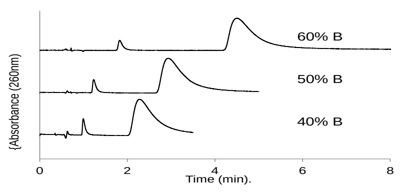
Shimadzu LCMS-2020 Single Quadrupole: ESI (+) 4.5 kV, (-) 3.5 kV; SIM 1 amu; 0.25 sec each SIM Event (2 points/sec sample rate); 1.2 LPM N_2 neb; 12.5 LPM N_2 dry gas; Cap DL 300°C; Heat Block, 400°C



Nucleotide Separations

Nucleotides Have Poor Peak Shape Using Standard Conditions

Temperature: 35°C Flow Rate: 1.5 mL/min Injection Volume: 10.0µL UV Detection: 260nm
Mobile Phase: Pump A= 100mM Ammonium Formate pH=6.0
Pump B= Acetonitrile



% B	Tailing Factor 10%	Width Half-Height (min)
60	3.062	0.576
50	2.782	0.523
40	2.417	0.480

Nucleotides are subject to increased secondary interactions due to the high polarity and multiple negative charges associated with each phosphate group. Lowering the acetonitrile concentration (< 75%) helped with retention, but new mobile phase additives are required to obtain satisfactory peak shape.
Literature suggested using:

a) Pre-wash with Ethylenediaminetetraacetic acid (EDTA)[2]
b) Ammonium Bicarbonate[1]
c) Ammonium Phosphate[3]

1. Asakawa, Y.; Tokida, N.; Ozawa, C.; Ishiba, M.; Tagaya, O.; Asakawa, N. Suppression effects of carbonate on the interaction between stainless steel and phosphate groups of phosphate compounds in high-performance liquid chromatography and electrospray ionization mass spectrometry. *Journal of Chromatography a* **2008**, *1198*, 80-86.
2. Liu, S.; Zhang, C.; Campbell, J.; Zhang, H.; Yeung, K.; Han, V.; Lajoie, G. Formation of phosphopeptide-metal ion complexes in liquid chromatography/electrospray mass spectrometry and their influence on phosphopeptide detection. *Rapid Communications in Mass Spectrometry* **2005**, *19*, 2747-2756.
3. St Claire, R. Positive ion electrospray ionization tandem mass spectrometry coupled to ion-pairing high-performance liquid chromatography with a phosphate buffer for the quantitative analysis of intracellular nucleotides. *Rapid Communications in Mass Spectrometry* **2000**, *14*, 1625-1634.

EDTA Shows Minimal Improvement of Nucleotide Peak Shape

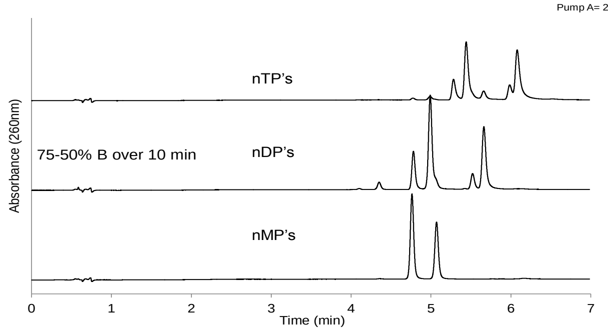
Temperature: 35°C Flow Rate: 1.5mL/min Injection Volume:10.0µL UV Detection: 260nm
Mobile Phase: Isocratic 50%B / 50%A, Pump A= specified buffer in table at pH=6.0, Pump B= Acetonitrile
Solute: Guanosine Monophosphate (GMP), Data Averaged from Duplicate Runs
EDTA Conditions: 2 hours of flow through the column of 10mM EDTA in specified pump A mobile phase at 50%B / 50%A

Pre-EDTA			
Buffer	Ret. Time (min)	Width Half-Height (min)	Tailing Factor 10%
100mM Ammonium Bicarbonate	1.68	0.150	2.95
25mM Ammonium Phosphate	1.08	0.039	1.87
10mM EDTA in Buffer			
Buffer	Ret. Time (min)	Width Half-Height (min)	Tailing Factor 10%
100mM Ammonium Bicarbonate	1.38	0.060	2.23
25mM Ammonium Phosphate	1.02	0.027	1.34
Post-EDTA			
Buffer	Ret. Time (min)	Width Half-Height (min)	Tailing Factor 10%
100mM Ammonium Bicarbonate	1.64	0.120	2.49
25mM Ammonium Phosphate	1.05	0.034	1.52

The addition of EDTA and post EDTA show an improvement in peak shape for the bicarbonate mobile phase, but a modest improvement for the phosphate mobile phase. EDTA improvements are not additive to the use of phosphate buffer.

Ammonium Bicarbonate Improves Peak Shape but with Inadequate Selectivity

Temperature: 35°C Flow Rate: 1.5 mL/min
Injection Volume: 3.0µL
UV Detection: 260nm Mobile Phase:
Pump A= 200mM Ammonium Bicarbonate pH=8.0
Pump B= Acetonitrile



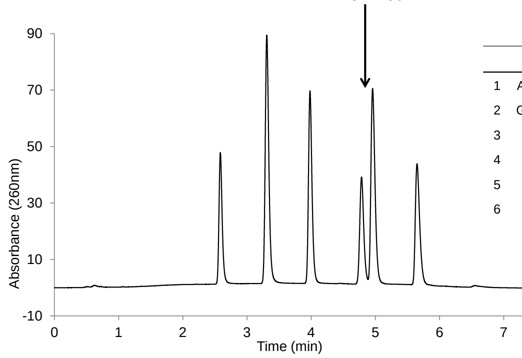
- Resolution of IMP and UDP= 0.26
- Resolution of GMP and ADP= 0.96.
- Resolution of CTP and GTP= 0.92

Elution Order- **nTP's:** Uridine Triphosphate, Adenosine Triphosphate, Cytidine Triphosphate, Guanosine Triphosphate;
nDP's: Uridine Diphosphate, Adenosine Diphosphate, Cytidine Diphosphate, Guanosine Diphosphate;
nMP's: Inosine Monophosphate, Guanosine Monophosphate

Ammonium bicarbonate shows a positive improvement in the peak shape of the nucleotides. Unfortunately ammonium bicarbonate has limited stability and solubility in the acetonitrile/water. This mobile phase does not adequately resolve all nucleotides.

Ammonium Phosphate Gradient of Adenosine and Guanosine Nucleotides

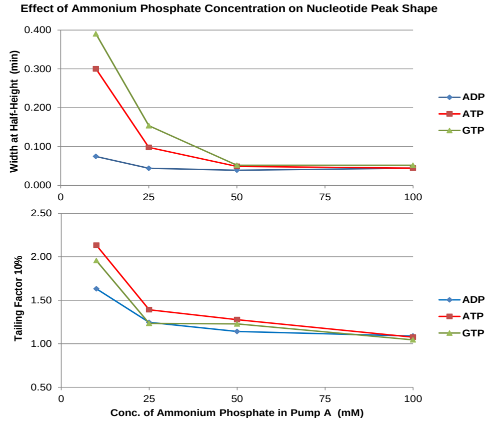
Column: 2.1x100mm, 2.7µm Penta-HILIC
Temperature: 35°C Flow Rate: 1.5mL/min Injection Volume: 1.0µL UV Detection:260nm
Mobile Phase: Pump A= 12.5mM Ammonium Phosphate in 50% Acetonitrile / 50% H_2O pH=6,
Pump B= 6.25mM Ammonium Phosphate in 75% Acetonitrile / 25% H_2O pH=6,
Gradient Conditions: 90-40% B over 8 min



	Elution Order	Tailing Factor 10%
1	Adenosine Monophosphate	1.265
2	Guanosine Monophosphate	1.225
3	Adenosine Diphosphate	1.244
4	Guanosine Diphosphate	1.198
5	Adenosine Triphosphate	1.294
6	Guanosine Triphosphate	1.397

Retention of nDP's and nTP's overlap. Above is an example for limiting resolution for GDP and ATP (Rs=1.5). Overall the ammonium phosphate buffer shows excellent peak shape and allows resolution between the purine mono-,di-, and trinucleotides.

Effect of Ammonium Phosphate Concentration on Peak Shape



Temperature: 35°C, Flow Rate: 1.5mL/min, Injection Volume: 2.0µL, UV Detection: 260nm
Mobile Phase: Isocratic elution of 60% Acetonitrile / 40% x mM Ammonium Phosphate pH=6

Under isocratic conditions, ammonium phosphate is effective in improving the peak shape of nucleotides over a range of concentrations.

Due to solubility issues of the 50 and 100mM buffers, the lowest effective concentration is preferred, 25mM.

Conclusions

Mobile Phase Considerations:

- Ammonium formate has proven useful as a mobile phase modifier for the separation of nucleobases, nucleosides and derivatives, using increasing water as the strong solvent.
- Use of ammonium formate with online MS detection shows promise, with clear detection of molecular ions for all compounds studied.
- Ammonium phosphate has produced the best separation results for the phosphorylated nucleotides, but has limitations in use for MS detection.

Stationary Phase Considerations :

- The Penta-HILIC bonded phase is shown to efficiently resolve a mixture of 15 compounds, with good selectivity and high efficiency (>180,000 plates/meter).

Ongoing efforts are directed to uncovering mobile phase modifiers that can bridge the useful HILIC nucleobase and nucleoside separations to the nucleotide separations, while maintaining a LC-MS friendly eluent.

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