Polar Modified Stationary Phases are Ideal for the Analysis of Nucleotides

Application Note

BioPharma

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Introduction

Nucleotides are the structural units of RNA, DNA, and several cofactors. In the cell, they play important roles in metabolism and signaling. They are very polar analytes that consist of a nitrogenous base, a sugar, and one or more phosphate groups. The base is a derivative of purine or pyrimidine, and the sugar is the pentose deoxyribose or ribose. Figure 1 shows the structural features of the most common nucleotides.

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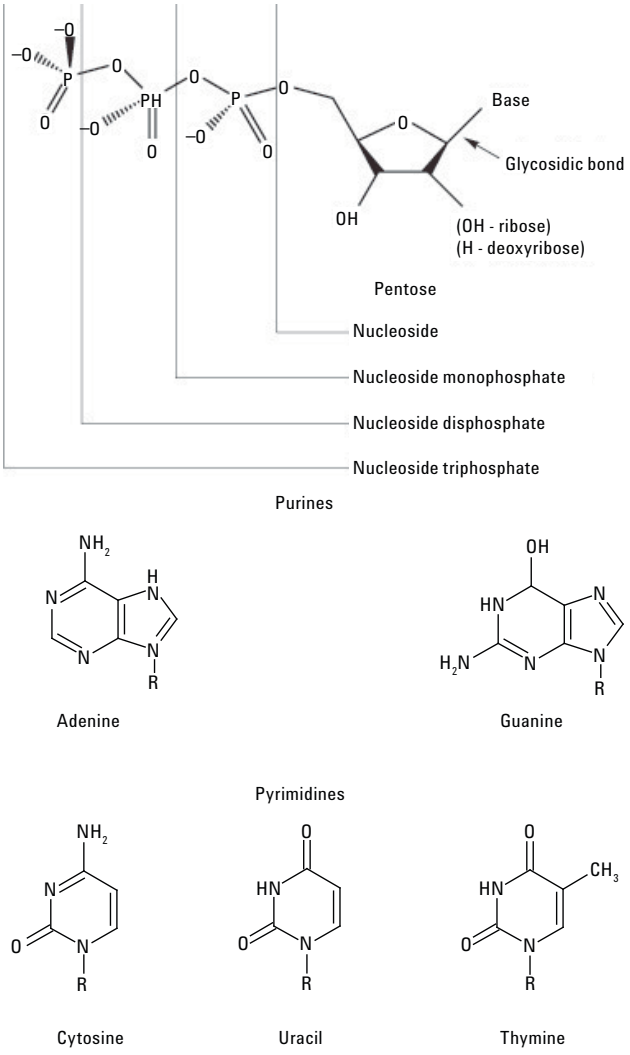

Figure 1. Structural elements of common nucleotides.

There are several options available for the analysis of hydrophilic compounds such as nucleotides. Due to the presence of phosphate groups in their structure, they can be separated by anion exchange chromatography, or more routinely, by reversed phase methods. In the latter, they can be retained on conventional alkyl bonded phases with the aid of ion pairing agents, or on polar modified and fluorinated phenyl mixed mode phases.

Results and Discussion

This application note discusses the choices available to chemists trying to select the best solution for this class of compounds. Table 1 lists some features of polar-modified chemistries for the analysis of nucleotides, such as Agilent Polaris C18-A and Agilent Pursuit PFP reversed phase, Pursuit XRs Ultra 2.8 C18 pure alkyl bonded, and Agilent PL-SAX polymeric anion exchange.

Table 1. Features of bonded phases for nucleotide analysis.

	Reversed-phase			
	Polar-modified		**Alkyl-bonded**	**Anion-exchange**
	Agilent Polaris C18-A	**Agilent Pursuit PFP**	**Agilent Pursuit XRs Ultra 2.8 C18**	**Agilent PL-SAX**
Strength	Improved water wettability, which supports use of high aqueous eluents Increased resistance to phase collapse Longer chain C18 ligand provides resolution of complex mixtures	Improved water wettability, which supports use of high aqueous eluents Increased resistance to phase collapse No ion pairing agent needed	Fast separations	Easy to retain Use 100% aqueous, nonorganic No use of ion-pairing agents Chemically stable from pH 1 to 14
Consideration	Ion pairing still required for separation of polar nucleotides	Ligand is less dense in PFP, and so resolution of complex mixtures not as great as C18	Will not retain polar nucleotides under high aqueous eluents without phase collapse Ion pairing agent required	High salt gradients affect instrument lifetime Salts may limit detection methods (for example, evaporative light scattering or mass spec cannot be used if nonvolatile salts present)

Reversed-phase

Polaris C18-A

Mixed mode polar-modified stationary phases such as 200Å silica based Polaris C18-A, in general, can help retain polar analytes under highly aqueous conditions. The polar functionality allows increased resistance to hydrophobic collapse under these conditions. Figure 2 shows the separation of 10 deoxyribonucleotides on this column. The gradient starts with a 95% aqueous eluent, indicating a high aqueous environment. This is compatible with the design of the chemistry, such that minimal reduction in retention times can be expected. Despite the presence of the polar group, ion pairing through the use of tetrabutyl ammonium hydroxide was used to create a more hydrophobic complex of each analyte, which would retain on the column.

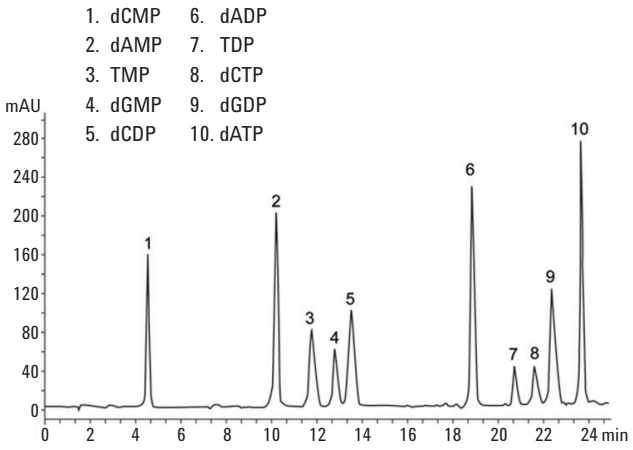

Figure 2. Deoxyribonucleotide separation on an Agilent Polaris C18-A.

Conditions

Column:	Agilent Polaris C18-A, 4.6 × 150 mm, 5 µm (p/n A2000150X046)
Eluent:	A: H_2O + 10 mM TBAH + 10 mM KH_2PO_4 TBAH
	B: CH_3OH +10 mM TBAH (tetra butyl ammonium hydroxide)
Flow rate:	1.5 mL/min
Gradient:	Time (min) % B 0 5 25 50 27 50 27.1 95 30.1 95 30.2 5 32.2 5
Temperature:	Ambient
Detector:	UV at 254 nm

Pursuit PFP

Pursuit PFP is another mixed mode 200Å reversed phase column suitable for polar compound retention under pure aqueous/high aqueous eluents, without being susceptible to phase collapse. By incorporating dipole-dipole, as well as *pi-pi* and hydrogen bonding mechanisms, this pentafluorophenyl phase delivers optimal separation of polar analytes. Figure 3 shows an example of seven nucleotides/nucleosides run on this column using 100% aqueous MS-compatible conditions.

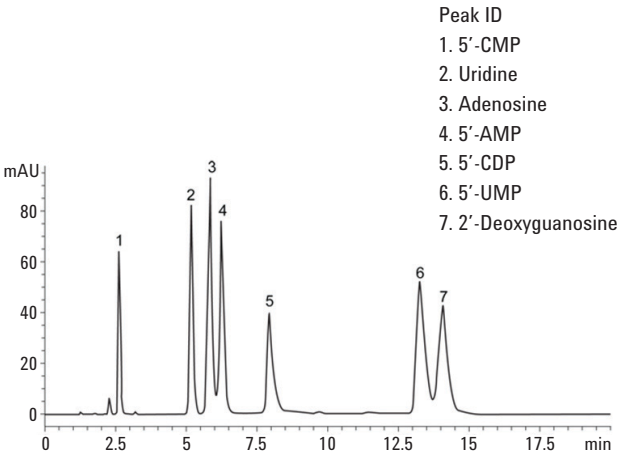

Figure 3. Nucleotides and nucleosides on an Agilent Pursuit PFP.

Conditions

Column:	Agilent Pursuit PFP, 4.6 x 150 mm, 5 µm (p/n A3050150X046)
Eluent:	H_2O + 0.1% HCOOH
Flow rate:	1.0 mL/min
Temperature:	Ambient
Detector:	UV at 254 nm

Since the PFP ligand involved in this bonded phase is less dense than the corresponding C18 phase (both 200Å products), resolution of complex mixtures is not as great as that seen on a Polaris C18-A.

Pursuit XRs Ultra 2.8 C18

Nucleotides can also be analyzed through conventional alkyl-bonded chemistries using ion pairing agents. Figure 4 illustrates a high speed separation of a mix of adenine nucleotides within 3 minutes on this Fast LC column. Table 2 provides details of MS transitions used for each analyte. These 2.8 µm columns are based on a combination of 100Å, high surface area silica and high ligand density, making them suitable for high speed, high resolution separations.

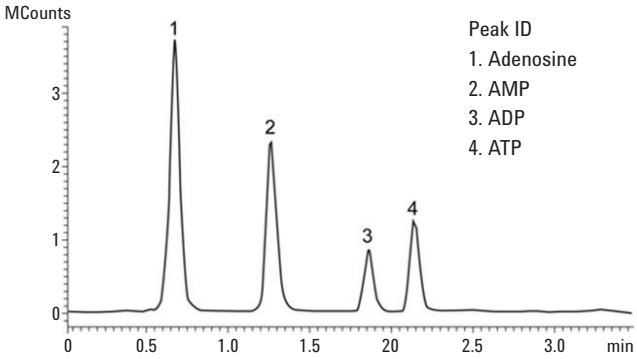

Figure 4. Fast LC/MS/MS separation of adenine nucleotides on an Agilent Pursuit XRs Ultra 2.8 C18.

Conditions

Column:	Agilent Pursuit XRs Ultra 2.8 C18, 2 × 50 mm, 2.8 µm (p/n 7501050X020)
Eluent:	A: H_2O + 0.1% formic acid + 20 mM TBAH, pH 4.3 B: CH_3OH + 0.1% formic acid + 20 mM TBAH
Flow rate:	0.5 mL/min
Gradient:	Time (min) % B 0 20 2 50 3 50 3.06 5 4 5
Temperature:	Ambient
Detector:	Triple quadrupole LC/MS
Source:	ESI
Ionization mode:	Negative

Table 2. MS/MS details of adenine nucleotides.

Compound	Parent ion	Daughter ion	Collision energy (V)	Dwell time (s)
Adenosine	266.0	133.0	18.0	0.1
AMP	346.0	133.0	34.5	0.2
ADP	426.0	133.0	18.5	0.2
	426.0	157.8	27.5	
ATP	506.0	157.8	27.0	0.2

Pure aqueous or highly aqueous eluents cannot be used on these columns without the possibility of phase collapse. Traditional C18 and C8 bonded phases are not very popular for the analysis of polar analytes.

Anion exchange

By using a hydrophilic, strong anion exchange resin based packing material like PL-SAX 1000Å 8 µm, a mix of 12 standard mono-, di- and triphosphate nucleotides can be resolved in under 20 minutes with a salt and pH gradient (Figure 5).

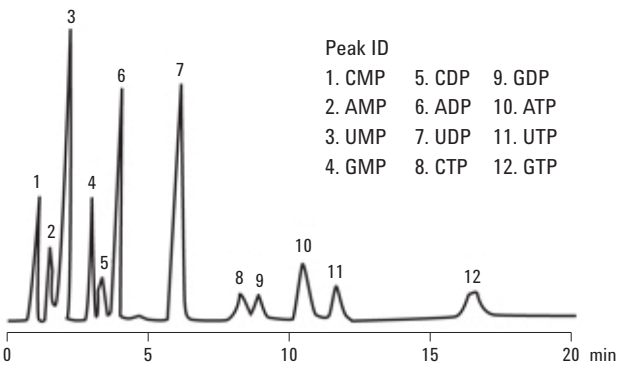

Figure 5. Nucleotide analysis on an Agilent PL-SAX.

Conditions

Column: Agilent PL-SAX 1000Å, 4.6 × 50 mm, 8 µm (p/n PL1551-1802)
Eluent: A: 0.02 M KH_2PO_4, pH 2.6
B: 0.5 M KH_2PO_4, pH 3.5
Flow rate: 1.0 mL/min
Gradient: Linear 0 to 100% B in 20 minutes
Detector: UV at 260 nm

Due to the high concentration of salts used in ion exchange methods, instrument lifetime can be affected as a result of salt precipitation. In addition, nonvolatile salts may not be compatible with certain detectors, such as evaporative light scattering or MS.

Conclusion

Polar analytes, such as nucleotides and nucleosides, can be analyzed in a number of ways, including ion exchange and reversed phase mechanisms. Polar modified bonded phases, such as Agilent Polaris C18-A, are ideal candidates for their analysis due to the improved water wettability of the column, allowing use of high aqueous eluents, increased resistance to phase collapse, and offering improved resolution of fairly complex mixtures.

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