The Separation of oligonucleotides on reversed phase ion-pairing chromatography for highly polar compounds

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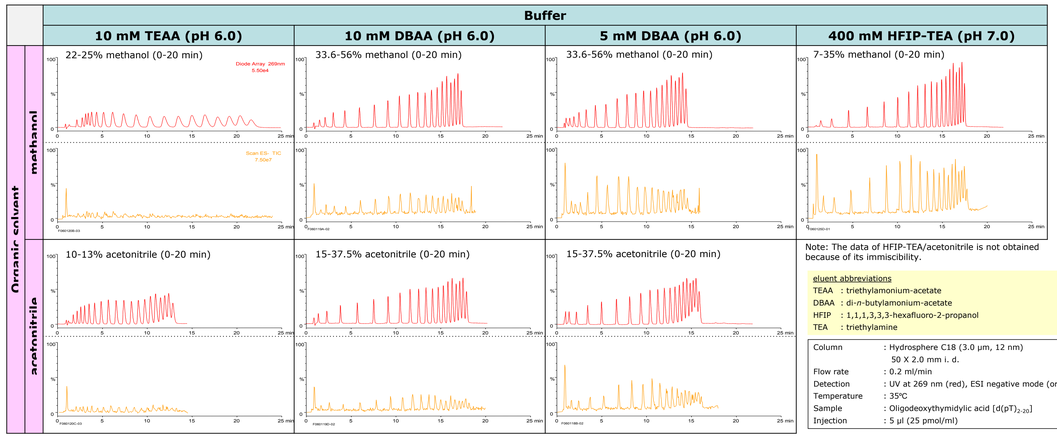
Introduction

Oligonucleotide therapeutics such as antisense, siRNA, aptamer, etc. are expected as next-generation pharmaceuticals products following antibody drugs. Reversed-phase ion-pairing chromatography is widely used for the analysis of oligonucleotide therapeutics and their metabolites. It is difficult to retain and separate highly polar compounds (such as short oligonucleotides) on ordinary reversed-phase columns. YMC offers a silica-based C18 column (Hydrosphere C18), which provides strong retention and excellent peak shape for these types of polar analytes.

"Denaturing HPLC" is a technique that utilizes high temperature to generate single-stranded RNA and is widely used in the field of gene mutation analysis. YMC-Triart C18 using inorganic/organic hybrid silica offers excellent durability at elevated temperature. Both Hydrosphere C18 and YMC-Triart C18 can be used with 100% aqueous mobile phases and are ideal for oligonucleotide analysis. In this poster, we introduce an efficient analytical method for short oligonucleotides using Hydrosphere C18 with an ion-pairing buffer, and high temperature analysis of oligonucleotides using YMC-Triart C18.

Effect of composition and salt concentration of ion-paring mobile phase on separation and intensity of oligonucleotides

Comparison of separation and ESI-MS signal intensity under different ion-pairing buffers and organic solvents



Note: The data of HFIP-TEA/acetonitrile is not obtained because of its immiscibility.

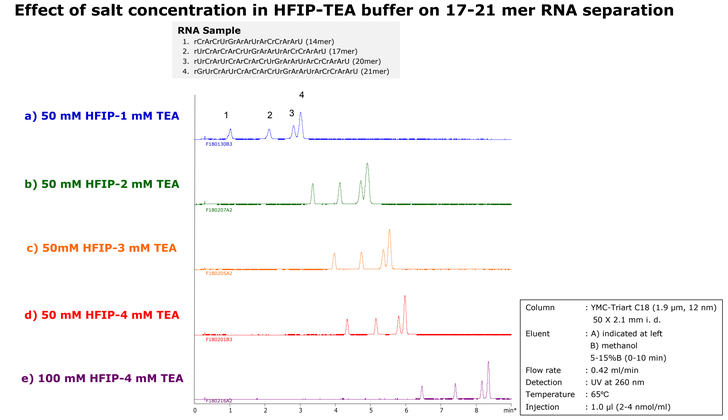
eluent abbreviations
TEAA : triethylamonium-acetate
DBAA : di-*n*-butylamonium-acetate
HFIP : 1,1,1,3,3,3-hexafluoro-2-propanol
TEA : triethylamine

Column	: Hydrosphere C18 (3.0 µm, 12 nm) 50 X 2.0 mm i. d.
Flow rate	: 0.2 ml/min
Detection	: UV at 269 nm (red), ESI negative mode (orange)
Temperature	: 35ºC
Sample	: Oligodeoxythymidylic acid [$d(pT)_{2\text{-}20}$]
Injection	: 5 µl (25 pmol/ml)

- The effect of mobile phase composition on the separation and signal intensity in electrospray ionization mass spectrometry (ESI-MS) are compared under seven different gradient conditions in $d(pT)_{2\text{-}20}$ analysis using a Hydrosphere C18 column. As shown in the upper UV chromatograms (red trace) of each condition, acceptable retention and resolution are achieved by optimizing the gradient slope of organic solvent regardless of the type of mobile phase.
- The ESI-MS intensity is significantly influenced by the type and concentration of ion-pairing buffer as shown in the lower MS chromatograms (orange trace),with the HFIP-TEA buffer/methanol providing the maximum MS intensity. Enhanced retention and MS intensity are obtained with 10 mM DBAA buffer versus 10 mM TEAA buffer, and a 5 mM decrease of DBAA concentration results in approximately 1.5-3 times the increase of intensity without a change in the concentration of organic solvent.

Effect of salt concentration in HFIP-TEA buffer on 17-21 mer RNA separation

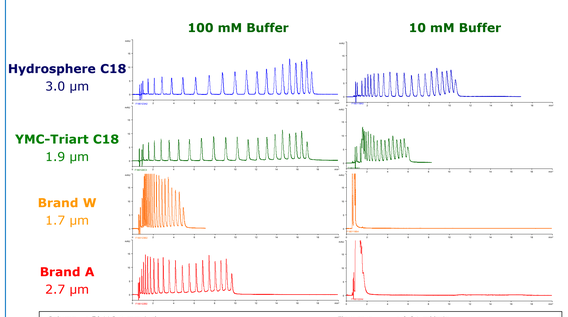
RNA Sample
1. rCrArCrUrGrArArUrArCrCrArArU (14mer)
2. rUrCrArCrArCrCrUrGrArArUrArCrCrArArU (17mer)
3. rUrCrArUrCrArCrCrUrGrArArUrArCrCrArArU (20mer)
4. rGrUrCrArUrCrArCrCrUrGrArArUrArCrCrArArU (21mer)



Column	: YMC-Triart C18 (1.9 µm, 12 nm) 50 X 2.1 mm i. d.
Eluent	: A) indicated at left B) methanol 5-15%B (0-10 min)
Flow rate	: 0.42 ml/min
Detection	: UV at 260 nm
Temperature	: 65ºC
Injection	: 1.0 µl (2-4 nmol/ml)

- The separation of 17-21mer RNA is compared using mobile phases that consist of different ratios of HFIP and TEA. Retention is increased approximately 2X when changing the TEA concentration from 1 to 4 mM at a constant 50 mM HFIP concentration (chromatograms a to d), and increased approximately 1.5 times when changing the HFIP concentration from 50 mM to 100 mM at a constant 4 mM TEA concentration (chromatograms d and e). These results indicate that slight changes in concentration of basic ion-pairing reagents such as TEA and DBA provide a great impact on oligonucleotide separations.

Comparison of d(pT)2-20 separation among commercially available C18 phases

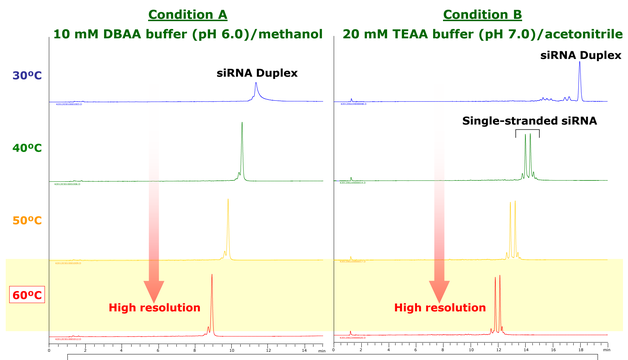


Column	: 50 X 2.1 mm i. d.	Flow rate	: 0.21 ml/min
Eluent	: A) 100 mM or 10 mM triethylamine-acetic acid (pH 6.0) B) Eluent A/acetonitrile (80/20) 50-65%B (0-20 min)	Detection	: UV at 260 nm
		Temperature	: 35ºC
		Sample	: Oligodeoxythymidylic acid [$d(pT)_{2\text{-}20}$]
		Injection	: 2 µl (5 nmol/ml)

- Separation of oligodeoxythymidylic acids: $d(pT)_{2\text{-}20}$ is compared with 100 mM and 10 mM triethylammonium-acetate (TEAA) buffer, under the same gradient condition. Hydrosphere C18 and YMC-Triart C18 show enhanced retention and resolution versus other commercially available C18 phases designed for oligonucleotide analysis, even at a low concentration such as 10 mM.
- Ion-pairing reagents containing both a positively charged group and a hydrophobic functional group, such as triethylamine (TEA) and dibutylamine (DBA) can interact with negatively charged phosphodiester groups of oligonucleotides *and* the hydrophobic stationary phase. The higher concentration provides stronger retention and superior resolution of oligonucleotides, while on the other hand the lower concentration has the advantage of increasing signal intensity and reducing system contamination in HPLC-MS analysis.

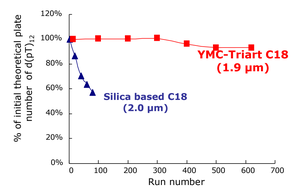
Effect of mobile phase and column temperature on separation of siRNA duplex

Crude synthetic siRNA duplex (19 bp) : 5'-CGU ACG CGG AAU ACU UCG AdTdT-3'
3'-dTdTGCA UGC GCC UUA UGA AGC U-5'



Column	: YMC-Triart C18 (1.9 µm, 12 nm) 100 X 2.0 mm i. d.	Detection	: UV at 269 nm
		Injection	: 1 µL (5 nmol/ml)
Flow rate	: 0.2 ml/min	System	: Agilent 1290
Condition A		**Condition B**	
Eluent	: A) 10 mM di-*n*-butylamine-acetic acid (pH 6.0) B) methanol 35-60%B (0-15 min)	Eluent	: A) 20 mM triethylamine-acetic acid (pH 7.0) B) acetonitrile 5-12%B (0-20 min)

Durability at pH 6.0 (DBAA buffer) and 65ºC

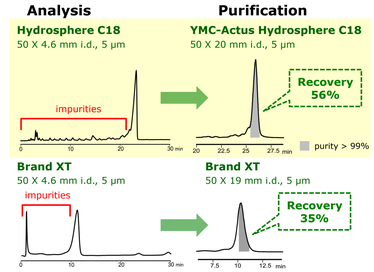


Test condition

Column	: 1.9 µm or 2.0 µm, 12 nm 50 X 2.0 mm i. d.
Eluent	: A) 10 mM di-*n*-butylamine-acetic acid (pH 6.0) B) methanol 30-50%B (0-20 min)
Flow rate	: 0.4 ml/min
Detection	: UV at 269 nm
Temperature	: 65ºC
Sample	: Oligodeoxythymidylic acid, [$d(T)_{2\text{-}20}$]
Injection	: 1 µl (5 nmol/ml)
System	: Agilent 1290

- The separation of siRNA duplex is compared under different mobile phase conditions at various temperatures on YMC-Triart C18. Under both condition A and condition B, peak shape and resolution between peaks is improved by increasing the column temperature.
- Due to the improvement of dispersion and distribution velocity when increasing column temperature, bio-macro molecules such as RNA and DNA generally exhibit sharper peak shape and improved resolution.
- Under condition B at 40°C or higher temperature, two peaks of single-stranded RNA generated by denaturation of siRNA duplex are observed. This HPLC technique utilizing high temperature to generate single-stranded RNA is called "Denaturing HPLC", and widely used in the field of gene mutation analysis.
- Denaturation of duplex DNA or RNA is also influenced by ionic strength (type and concentration), pH and polarity, as well as temperature. Those analysis conditions (temperature and mobile phase) are recommended to be optimized depending on the characteristics of target analytes and purpose of analysis.
- The combination of a neutral buffer containing amino ion-paring reagent and high temperature is useful for high-throughput analysis of oligonucleotides or denaturing HPLC. However, conventional silica-based reverse-phase columns are not recommended for use under such conditions due to poor durability.
- YMC-Triart C18 is based on inorganic/organic hybrid silica with thorough surface modification, offering excellent durability at elevated temperature and pH. This makes YMC-Triart C18 an ideal stationary phase for oligonucleotide analysis.

Purification of crude synthetic oligonucleotide with DBAA buffer



Synthetic oligonucleotide (30 mer)
5'-CCG CTC GAG CTA AAA AAA GCC TGT GTT ACC-3'

Buffer	: 10 mM di-*n*-butylamine-acetic acid (pH 6.0)
Eluent	: A) Buffer/methanol (60/40) B) Buffer/methanol (20/80) 15-35%B (0-30 min)
Flow rate	: 1.0 ml/min for analysis 19 ml/min for purification
Detection	: UV at 269 nm
Temperature	: ambient
Injection	: 5 µl for analysis 100 µl for purification

- Hydrosphere C18 was compared with competitor Brand XT for the separation of a synthetic oligonucleotide and related impurities. At the analytical scale, many impurities could be separated from the target product by one-nucleotide difference on Hydrosphere C18 (as shown in figures above, on left). At purification scale, YMC-Actus Hydrosphere C18 also exhibited superior separation and recovery (as shown in figures above, on right).
- TEAA and DBAA buffers can reduce mobile phase costs by eliminating HFIP use, and is suitable for cost effective purification of oligonucleotides.

Conclusions

- Hydrosphere C18 and YMC-Triart C18 phases, which are designed to have moderate hydrogen bonding capacity, provide adequate retention and separation of oligonucleotides even at low concentrations of ion-pairing reagent.
- YMC-Triart C18 based on inorganic/organic hybrid silica particles is applicable to denaturing HPLC analyzed at high temperature due to its outstanding thermal stability.
- It is possible to select a more appropriate condition for purpose in either case of purification and analysis with these C18 phases.