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Separation of Impurities from a Fully Phosphothioated and 2'-O-methylated RNA Using Ion-Pair Reversed-Phase Chromatography

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ABSTRACT

Purpose: Demonstrate separation of impurities from a fully phosphothioated and 2'-O-methylated RNA using ion-pair reversed-phase chromatography.

Methods: A 40mer fully phosphorothioated and 2'-O-methylated RNA molecule (nucleic acid polymer) was separated from impurities formed by oxidation of one or more phosphorothioate (PS) linkage to the phosphodiester (PO) and truncation impurities (n-1, n-3, n-5) using ion-pair reversed-phase (IPRP) HPLC. Key chromatographic parameters including temperature, pH, ion-pair reagents, organic modifier, and addition of EDTA were investigated on a polymer based reversed-phase column.

Results: n-1 and 1 PO impurities were baseline separated from the full length product using TEA as ion-pair reagent at 100 ºC and pH 7.0, acetonitrile as organic modifier, and in the presence of 5 mM EDTA in mobile phase. n-1 and 1 PO peaks did not separate at this condition.

INTRODUCTION

Oligonucleotides or longer nucleic acid polymers (NAPs) have emerged as promising drug candidates for various diseases such as cancer, viral infections, Alzheimer's disease and cardiovascular disorders. Antisense oligonucleotides, aptamers, and small-interfering RNAs (siRNAs) are currently under evaluation in clinical trials. To ensure efficacy and eliminate possible side effects, regulatory agencies require thorough and effective characterization and quality control of synthetic oligonucleotides and nucleic acid polymers. Often these molecules are chemically modified to improve hydrolytic stability, reduce enzymatic degradation or alter hydrophobicity property for a specific therapeutic activity. The most common modifications to RNA molecules are replacement of PO with PS linkages and addition of 2'-O-methyl to ribose. The PS linkage introduces a chiral center at phosphorus. In combination with the chiral centers in D-ribose this produces diastereoisomer pairs at each PS linkage making characterization of these molecules more challenging. In addition, PS linkages may undergo oxidation to form PO groups, which could affect the stability of the therapeutic agent. Therefore it is important to develop effective separation methods to detect and quantify PO impurities. Several studies have been reported to separate the diastereomers of PS oligonucleotides using IPRP chromatography either linked to MS or UV and anion exchange chromatography (AEX).[1,2] However there are limited number of reports separating PO linkage impurities from fully phosphorothioated product.

Here, we report the investigation of ion-pair reversed phase chromatography methods for the separation of an antiviral NAP targeting Hepatitis B (REP 2139) from its PO and truncation impurities formed during synthesis or storage. The antiviral NAP is a fully phosphothioated and 2'-O-methylated 40mer RNA comprised of alternating adenosine and 5-methylcytidine nucelotides.[3] A polymer based reversed-phase column which can withstand high temperature and high pH was used to separate the impurities. Several ion-pair reagents, different temperature and pH values, acetonitrile and methanol as organic modifier, and addition of EDTA to mobile phases were investigated to collapse the diastereoisomers as one peak and increase the separation between the full length product and the 1 PO and n-1 impurities.

MATERIALS AND METHODS

Sample: Nucleic acid polymer (NAP), REP 2139 and impurity samples were donated by Replicor.

- REP 2139, full length product (FLP): 5'-[A*MeC*]$_{20}$-3'
- REP 2139, n-1: 5'-MeC*-[A*MeC*]$_{19}$-3'
- REP 2139, n-3: 5'-MeC*-[A*MeC*]$_{18}$-3'
- REP 2139, n-5: 5'-MeC*-[A*MeC*]$_{17}$-3'
- REP 2139, 1 PO: 5'-[A*MeC*]$_{19}$-A*-MeC-3'
- REP 2139, 2 PO: 5'-[A*MeC*]$_{19}$-A-MeC-3'
- REP 2139, 3 PO: 5'-[A*MeC*]$_{18}$-A*-MeC-A-MeC-3'

A = Adenine
MeC = 5-methyl-Cytosine
** = phosphothioate linkage*
All ribose are 2'-O-methylated.

Column: DNAPac RP, 4 µm, 3.0 × 100 mm (P/N 088919)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ Vanquish™ F system equipped with:

- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A) equipped with an active pre-column heater (P/N 6732.0110) and a post-column cooler (P/N 6732.0510).
- Diode Array Detector HL (P/N VH-D10-A)
- Chromatography was controlled by Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System.

RESULT

For convenient method development, a quaternary pump was used equipped with following solutions-Line A: water, line B: organic modifier, line C: ion-pair reagent, line D: additive. The REP 2139 sample was found to contain some impurities-mostly 1PO impurity and small amount of n-1 impurity-based on the comparison of chromatograms of REP 2139 (FLP), 1 PO and n-1. This allowed us to compare some conditions with just the REP 2139 (FLP) sample.

Effect of Temperature

Higher temperature of the column has been shown to improve resolution of single-stranded DNA molecules.[4] Therefore temperatures from 80 ºC to 110 ºC were investigated for the separation of n-1 and 1PO impurities from the FLP (Figure 1). As the temperature increased from 80 ºC to 100 ºC, the peaks became sharper which resulted in higher resolution of the 1 PO impurity from the FLP and the n-1 from the FLP. However at 110 ºC, the peak width of FLP increased therefore resulted in lower resolution between the FLP peak and the impurities. The elution order was n-1, 1PO, then FLP up to 100 ºC while at 110 ºC, the elution order of 1PO and n-1 peak reversed. The resolution between the 1PO and the n-1 peak decreased as the temperature increased. The most important aspect of this study was to increase the separation between the impurities and the FLP, thus the following experiments to evaluate other parameters were performed at 100 ºC.

Figure 1. Effect of temperature on the separation of REP 2139 and impurities. FLP, 1 PO and n-1 chromatograms at different temperatures. were overlaid.

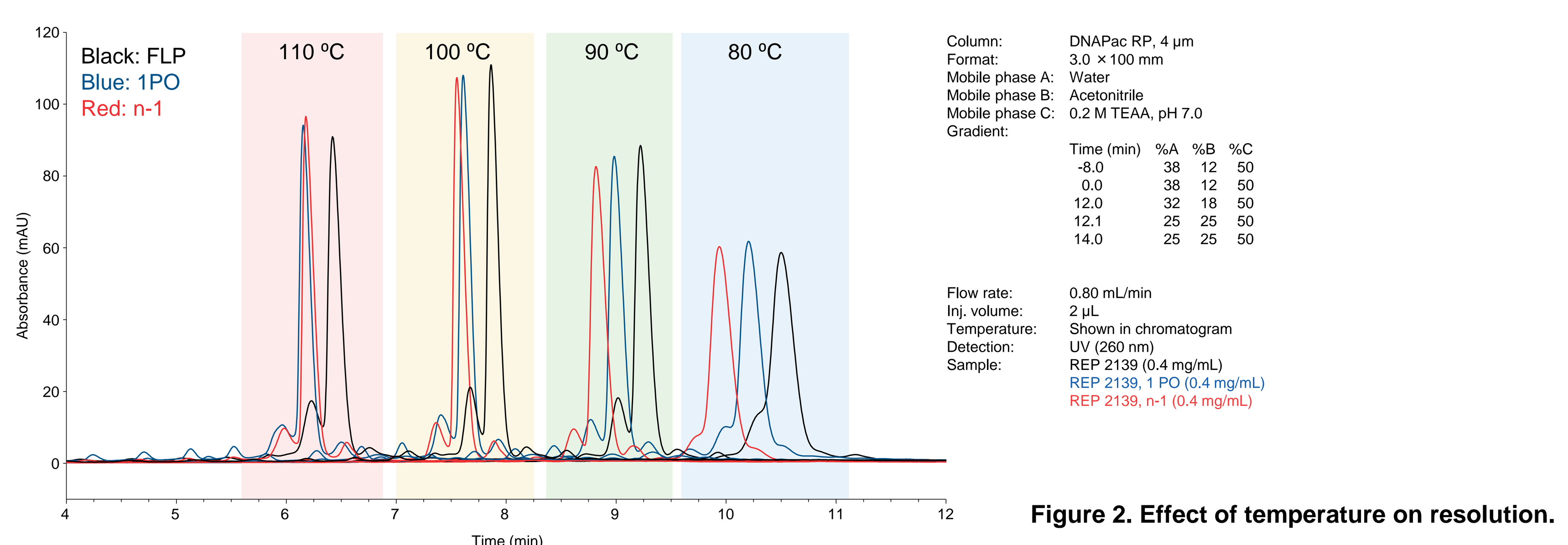
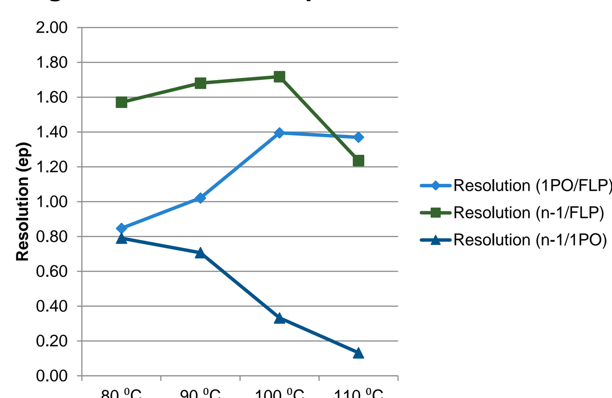


Table 1. Retention time, peak width at half height and resolution of REP 2139 FLP, 1PO and n-1 at different temperatures.

	Retention Time (min)			Peak width (50%)			Resolution (ep)		
	FLP	1 PO	n-1	FLP	1 PO	n-1	1 PO/FLP	n-1/FLP	n-1/1 PO
80 ºC	10.50	10.20	9.94	0.22	0.19	0.20	0.85	1.57	0.79
90 ºC	9.22	8.98	8.82	0.14	0.13	0.14	1.02	1.68	0.71
100 ºC	7.86	7.61	7.55	0.11	0.11	0.11	1.40	1.72	0.33
110 ºC	6.42	6.15	6.18	0.12	0.11	0.11	1.37	1.24	0.13

Figure 2. Effect of temperature on resolution.



Effect of pH

Higher and lower pHs were explored for the separation of impurities from the FLP. At higher pH, the diastereomers of the phosphothioate groups start to separate making the peak split into two or three peaks (Figure 3c). This partial resolution of diastereomers hinder the separation of impurities since, the impurities also contain diastereomers (Figure 4). Higher pH condition is thought to alter the charge density of oligonucleotides due to presence of G or T bases and therefore could alter the structure of the molecules resulting in different selectivity. However the NAP molecule does not contain G or T bases in its sequence. Therefore the difference in separation is not due to difference in charge state of bases but rather may be due to alteration in intra- or inter-molecular hydrogen bonding pattern. Lower pH condition at pH 5.9 was also investigated. The lower pH condition resulted in broader peaks, therefore the resolution between the FLP and the impurities were reduced (Figure 3a).

Figure 3. Effect of pH on the chromatogram of REP 2139.

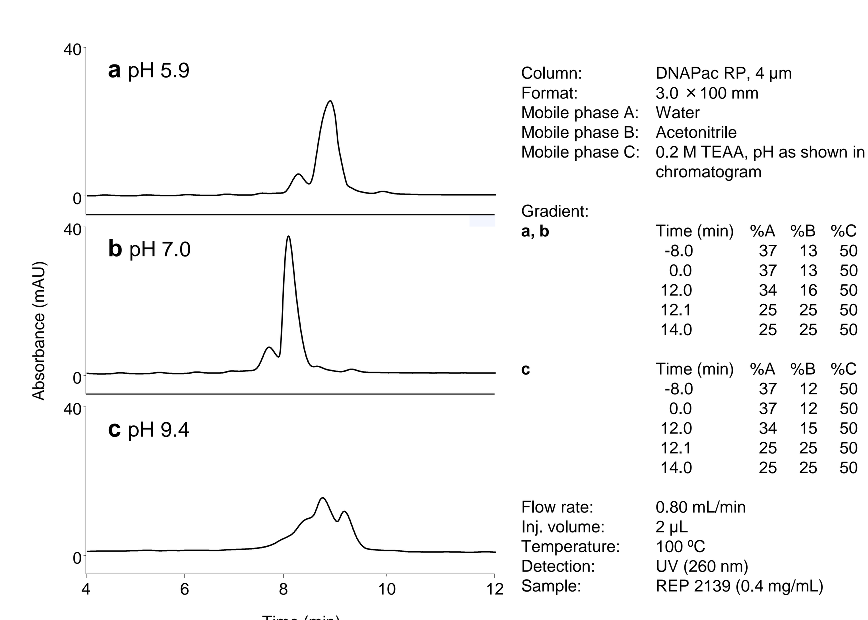
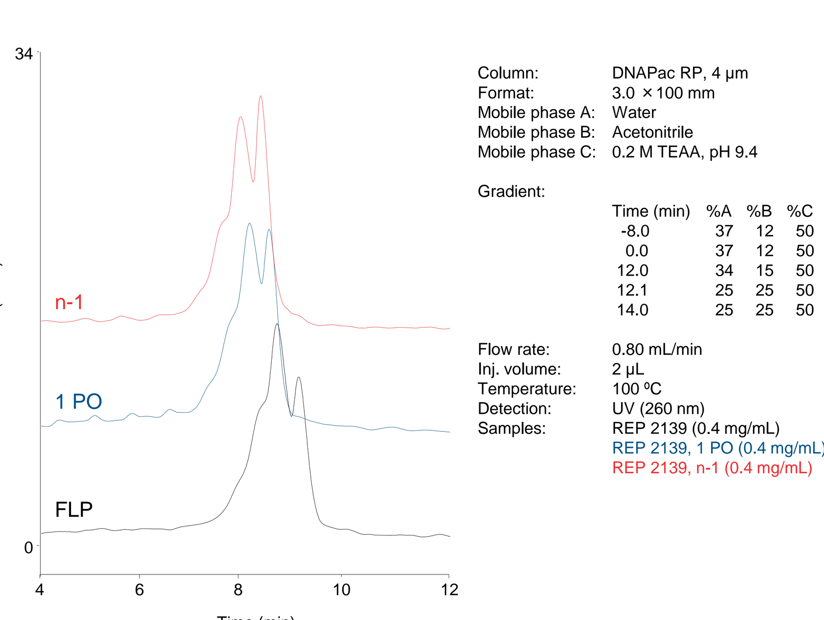


Figure 4. Separation of REP 2139 at higher pH.



Effect of Ion-Pair Reagents

The structure and the hydrophobicity of the ion-pair reagent has been shown to affect the retention and/or the selectivity of the oligonucleotides.[5] Here we have explored several different ion-pair reagents including triethylamine, hexylamine, dibutylamine, and diethylmethylamine at 100 ºC. For the separation of REP 2139 from its impurities, triethylamine with acetate as it's counter ion provided the best separation (Figure 5). Stronger (more hydrophobic) ion-pair reagents provided poor resolution of FLP and impurity peaks while weaker (less hydrophobic) ion-pair reagent, dimethylethylamine resulted in partial diastereomer separation which hinders accurate quantitation of impurities (Figure 6, 7, and 8).

Figure 5. Separation of REP 2139 and impurities using triethylamine as ion-pair reagent.

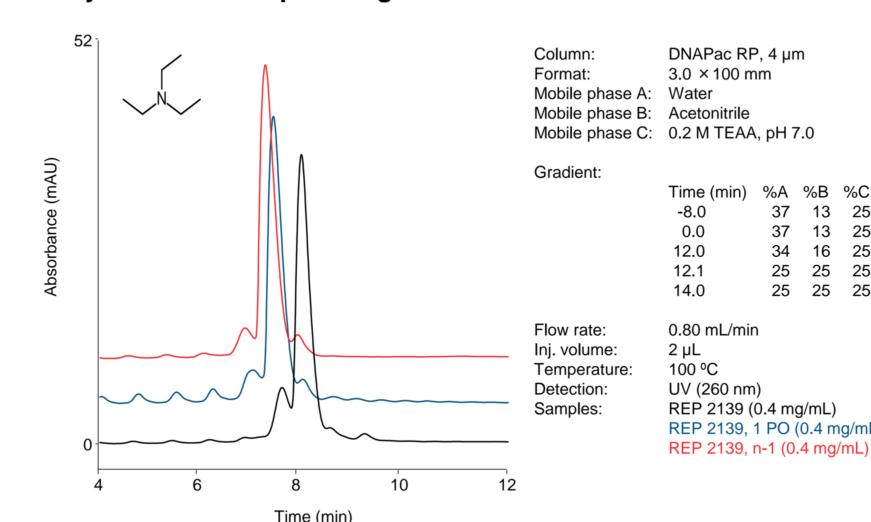


Figure 6. Separation of REP 2139 and impurities using hexylamine as ion-pair reagent.

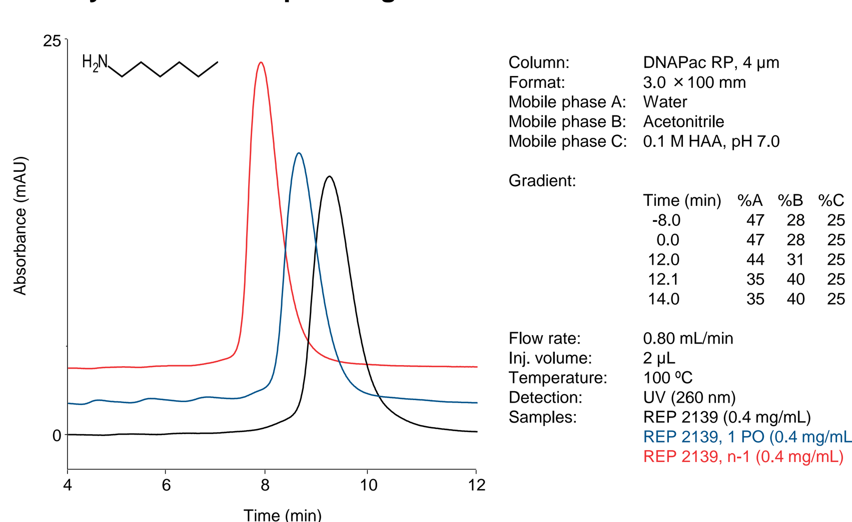


Figure 7. Separation of REP 2139 and impurities using diethylmethylamine as ion-pair reagent.

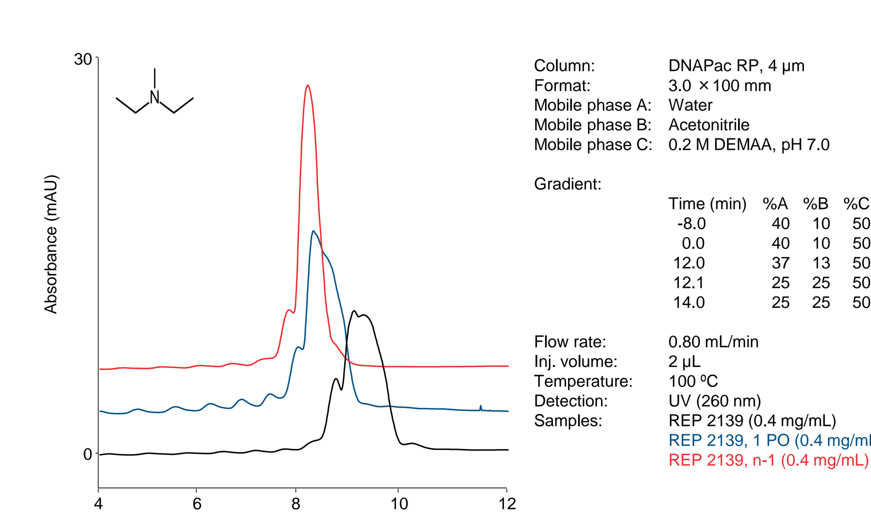
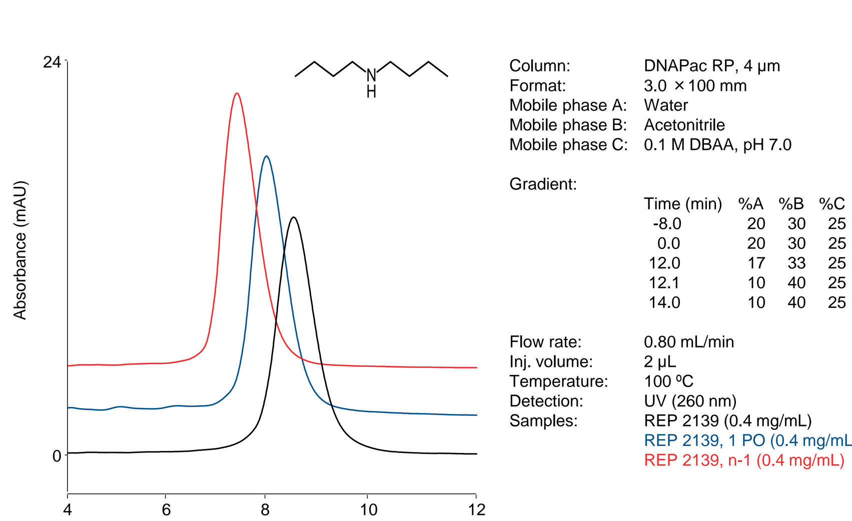


Figure 8. Separation of REP 2139 and impurities using dibutylamine as ion-pair reagent.



Effect of Adding EDTA to the Mobile Phase

Additives that could affect the structure of the nucleic acid polymer were considered. EDTA removes metal ions from oligonucleotides or nucleic acid polymers which in turn could alter the conformation. Addition of 5 mM EDTA to mobile phase resulted in improved resolution which enables one to more accurately quantify impurities based on peak area (Figure 9). Figure 10 shows the separation of 1, 2 and 3 PO impurities and truncation impurities (n-1, n-3, and n-5) from the FLP.

Figure 9. Separation of REP 2139 with or without EDTA in mobile phase.

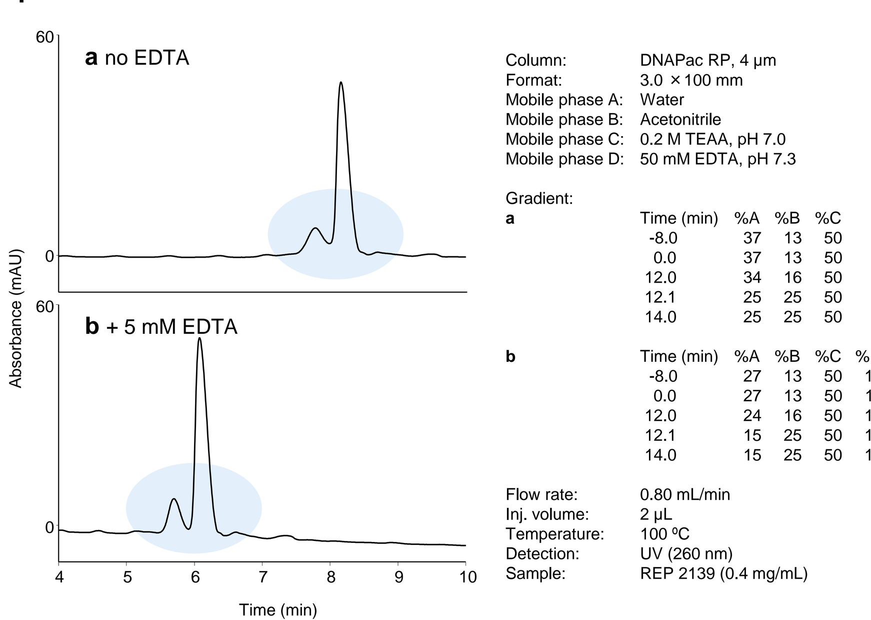
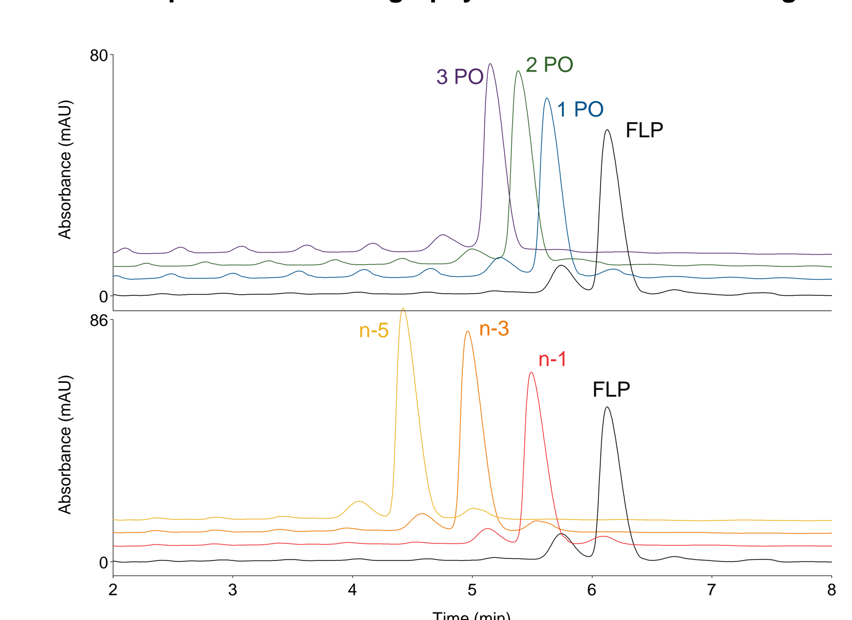


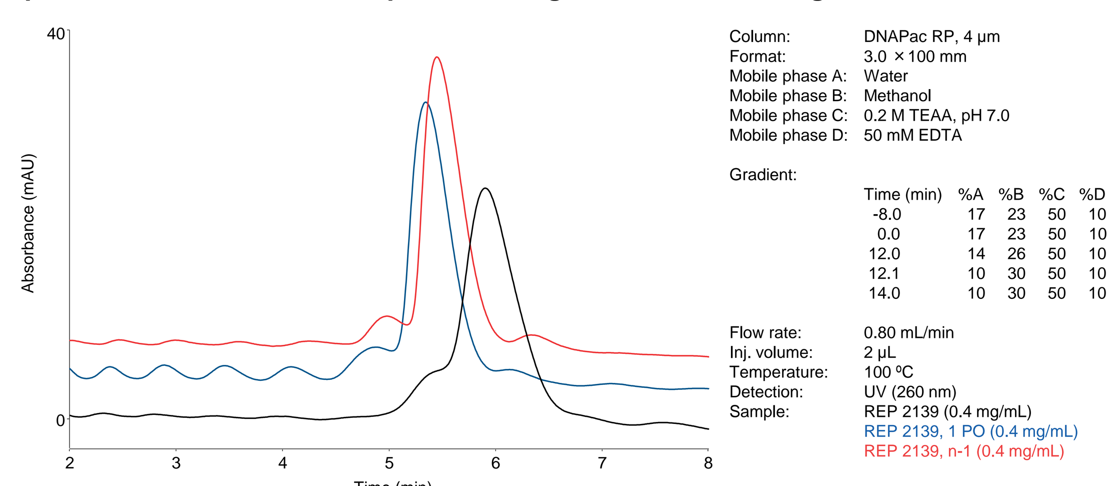
Figure 10. Separation of REP 2139 and impurities with 5 mM EDTA in mobile phase. Chromatography condition is same as Figure 9b.



Effect of Organic Modifier

Acetonitrile and methanol was evaluated as organic modifiers. Acetonitrile is aprotic and suppresses π-π interaction while methanol is protic and does not suppress π-π interaction. Therefore these two organic modifiers could provide different selectivity. When methanol was used instead of acetonitrile, the elution order of 1 PO and n-1 impurities reversed and the overall resolution between the FLP and impurities reduced (Figure 11, compared with Figure 10).

Figure 11. Separation of REP 2139 and impurities using methanol as the organic modifier.



CONCLUSIONS

The best separation of n-1 and 1 PO impurities from the FLP was achieved using TEA as ion-pair reagent at 100 ºC and pH 7.0, acetonitrile as organic modifier, and in the presence of 5 mM EDTA in mobile phase. This condition allows quantification of impurities (n-1 and 1 PO) in the sample but does not provide information on the quantity of each impurity due to co-elution of n-1 and 1 PO peaks.

- Higher temperature resulted in sharper peaks and higher resolution between the FLP and the impurities while lower temperature provided better resolution between n-1 and 1 PO.
- Higher pH condition partially separated the diastereomers which resulted in lower resolution between the FLP and the n-1 and 1 PO impurities.
- Stronger ion-pair reagents such as hexylamine and dibutylamine provided poor resolution of FLP and impurities while weaker ion-pair reagent diethylmethylamine partially separated the diastereomers.

REFERENCES

1. J.R. Thayer, Y. Wu, E. Hansen, M.D. Angelino, S. Rao, *J. Chromatography A,* 1218, 802-808 (2011).
2. L. Li, T. Leoneb, J.P. Foleyc, C.J. Welch, *J. Chromatography A,* 1500, 84-88 (2017).
3. A. Vaillant, *Antiviral Research,* 133, 32-40 (2016).
4. Thermo Fisher Scientific Application Note AN21476 (2016).
5. L. Gong and J.S.O. McCullagh, *Rapid Commun. Mass Spectrom.* 28, 339-350 (2014).

ACKNOWLEDGEMENTS

We acknowledge Dr. Andrew Vaillant at Replicor for providing samples and discussions about the sample.

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PO21721-EN 0517S

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