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Combining ion pairing agents for enhanced analysis of oligonucleotide therapeutics by reversed phase-ion pairing ultra performance liquid chromatography (UPLC)

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ABSTRACT

The burgeoning field of oligonucleotide therapeutics is based upon synthetically derived biopolymers comprised of relatively simple RNA and DNA building blocks. Significant gains in knowledge around mechanisms of action (RNA interference, RNA splicing, etc.) and oligonucleotide design (ASO, siRNA, DsiRNA, miRNA, locked nucleic acid, etc.) have been the main drivers of recent investment for this field [1,2]. As therapeutics, there is currently great interest in oligonucleotides due to the reduced time required to achieve lead molecules and to their potential for treating previously untractable diseases. One of the more challenging areas for the field of oligonucleotide therapeutics is the development of high-quality analysis schemes for the determination of purity in drug substance and product. This, in part, is due to the fact that the synthesis of oligonucleotides results in a significant number of closely related impurities that are not easily removed during purification [1]. As a result, these macromolecules (4000–8000 MW on average, depending on chain length) and their soup of closely related impurities are typically not well resolved from one another via conventional chromatographic approaches. One of the more common chromatographic techniques used for oligonucleotide analysis is reversed phase-ion pairing liquid chromatography (RP-IP). Our research led us to the discovery that the use of multiple ion pairing agents combined in the mobile phase can improve the overall chromatographic resolution and peak shape of the oligonucleotide analytes over the use of a single ion pairing agent alone, resulting in enhanced purity analysis and the opportunity to identify related impurities with greater certainty. In addition, the use of combined ion pairing agents allowed for the development of a "universal" method which has provided superior chromatography for several different oligonucleotide compounds and their related impurities regardless of differences in nucleotide sequence. The RP-IP UPLC method conditions are ESI-MS compatible and have allowed for the mass identification of five positional isomeric impurities chromatographically resolved and present at less than 1% of the nominal parent peak area.

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1. Introduction

The burgeoning field of oligonucleotide therapeutics is based upon synthetically derived biopolymers comprised of relatively simple RNA and DNA building blocks. Significant gains in knowledge around mechanisms of action (RNA interference, RNA splicing, etc.) and oligonucleotide design (ASO, siRNA, DsiRNA, miRNA, locked nucleic acid, etc.) have been the main drivers of recent investment for this field [1,2]. As therapeutics, there is currently great interest in oligonucleotides due to the reduced time required to achieve lead molecules and to their potential for treating previously untractable diseases. One of the more challenging areas for the field of oligonucleotide therapeutics is the development of high-quality analysis schemes for the determination of purity in drug substance and product. This, in part, is due to the fact that the synthesis of oligonucleotides results in a significant number of closely related impurities that are not easily removed during purification [1]. As a result, these macromolecules (4000–8000 MW on average, depending on chain length) and their soup of closely related impurities are typically not well resolved from one another via conventional chromatographic approaches. One of the more common chromatographic techniques used for oligonucleotide analysis is reversed phase-ion pairing liquid chromatography. The use of ion-pairing (IP) agents in reversed phase chromatography was first introduced in 1976 and became widely used by the early 1980s [3]. An IP agent refers to a chemical modifier added to the mobile phase which forms an electrostatic attraction with the analyte at the given pH of the mobile phase. In addition to a charged

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functional group, IP agents typically have a hydrophobic component to their structure. The overall effect is an increased retention of the analyte via the combination of electrostatic attraction between the analyte and IP agent and hydrophobic attraction of the IP agent to the non-polar stationary phase.

Reversed phase ion pairing chromatography has become a standard separation platform for the analysis of oligonucleotides [4]. Because of the numerous negatively charged phosphate or phosphorothioate groups along the oligonucleotide backbone; they can be well retained by RP-IP chromatography. The IP agent is cationic and attracts the negatively charged phosphates. Triethylamine (TEA) is one of the most common IP agent used for the analysis of oligonucleotides [4–11]. The triethyl portion of the molecule provides the hydrophobic component for retention. As a result of this retention mechanism, oligonucleotides which differ in length by as few as one nucleotide, hence one phosphate group, can typically be resolved by RP-IP chromatography for oligonucleotides of 30mer or less [5]. The shorter the oligonucleotide, the greater the difference in retention one phosphate group plays, and hence the better the chromatographic resolution. The concentration of the ion pairing agent used in the mobile phase is critical to the amount of retention experienced by the oligonucleotide. The more ion pairing agent used the greater the retention. However, there is a negative side to the use of ion pairing agents and that is they can significantly suppress the electrospray ionization efficiency of oligonucleotides for mass spectrometric (MS) detection/analysis. The more ion pairing agent used the more ion suppression is experienced [7]. The coupling of mass spectrometry with liquid chromatography analysis of oligonucleotides is highly desirable from the standpoint of peak identification/characterization. As a result, optimization of the ion pairing agent concentration is commonly performed to enable mass spectrometric detection [6–8]. In addition to the use of TEA as an ion pairing agent, other ion pairing agents have been explored based of their differences in structure and overall hydrophobicity [6,8,9,12–15]. The use of ion pairing agents with greater hydrophobicity can enable a lower concentration to be used, improving MS ionization efficiency.

Our research led us to the discovery that the use of multiple ion pairing agents combined in the mobile phase can improve the overall chromatographic resolution and peak shape of the oligonucleotide analytes over the use of a single ion pairing agent alone, resulting in enhanced purity analysis and the opportunity to identify related impurities with greater certainty. In addition, the use of combined ion pairing agents allowed for the development of a “universal” method which has provided superior chromatography for several different oligonucleotide compounds and their related impurities regardless of differences in nucleotide sequence. The RP-IP UPLC method conditions are ESI-MS compatible and have allowed for the mass identification of five positional isomeric impurities chromatographically resolved and present at less than 1% of the nominal parent peak area.

2. Experimental

2.1. Instrumentation

All of the chromatography was performed on a Waters ACQUITY Binary UPLC system with Waters tunable UV (TUV) detector with an analytical flow cell. The specific UPLC conditions such as column type, mobile phase composition, gradient, flow rate, column temperature, and injection volume were varied throughout the stages of method development and are highlighted in Section 3. For all of the analyses reported, a wavelength of 260 nm was used for UV detection. For mass spectrometric detection and analysis, an Agilent ion trap 6300 SL MSD with a standard electrospray source was used. The ESI-MS conditions are listed in Table 1.

Table 1
Agilent ion trap MSD optimized ESI and MS settings for RP-IP-UPLC-MS analysis.

Parameter	Setting
ESI	Negative mode
LC flow rate post split	~35 μL/min
Capillary volt	4500
Nebulizer	20 psi
Dry gas	10 L/min
Dry temp	350 °C
Cap exit	−160 V
Skimmer	−15 V
Oct 1 DC	−2.25 V
Oct 2 DC	−1.35 V
ICC	Off

2.2. Materials and reagents

Five different custom short interfering RNA molecules (siRNA) were used during the development of this methodology and are referred to as siRNAs #1–5. The siRNAs are comprised of two 21mer complementary oligonucleotide strands (sense and antisense) that are hybridized via Watson–Crick base pairing to form an alpha helical duplex conformation with two nucleotide overhangs on the 3′ end of each strand. The specific nucleobase sequences of the siRNA discussed in this paper are not shared since they are associated with therapeutic targets of potential development interest to GlaxoSmithKline. All siRNA molecules used for UPLC RP-IP method development were chemically modified to enhance in vivo efficacy and stability. Fig. 1 shows these chemical modifications, as additions of fluorine or O-methyl to the 2′ position of the ribose (A) or as the use of inverted abasic end caps (B). The abasic endcaps (deoxyribose + phosphate) are only used at the 3′ and 5′ ends of the sense strand and the O-methylation modifications are only used on the antisense strand. It is important to note that none of the oligonucleotides used in this study had any phosphorothioate backbone modifications, a fairly common type of modification for oligonucleotide therapeutics that results in the formation of diastereomers during synthesis. All of the siRNA samples were prepared in 1× phosphate buffered saline (prepared from 10× PBS, Sigma Aldrich, part number P5493) for analysis.

The following ion pairing agents were use throughout the study; dibutylamine – Sigma Aldrich, part number 471232, 99.5+% purity, triethylamine – VWR, part number EM-TX-1202-5, HPLC grade, diethylamine – Sigma Aldrich, part number 471216, 99.5+% purity, propylamine – Sigma Aldrich, part number 240958, 99+% purity (first one used with baseline disturbance in Fig. 2), propylamine – Sigma Aldrich, part number 82100, purum 99+% purity (no baseline disturbance, Fig. 3 through the end of the article).

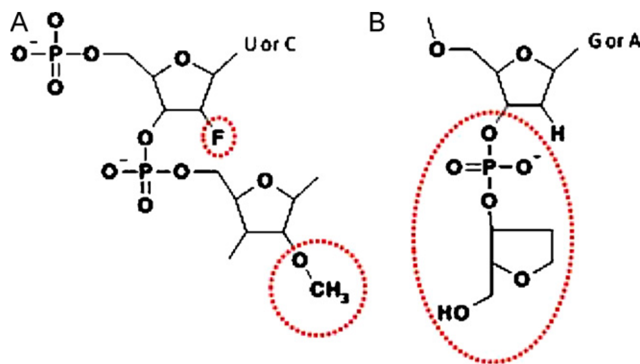

Fig. 1. Chemical modifications to siRNA backbone (A) 2′ ribose modification to F or O-methyl. For the five different siRNAs, the O-methyl modifications were only incorporated on the antisense strand. (B) shows the inverted abasic group endcaps which are located at both the 3′ and 5′ ends of the sense strand.

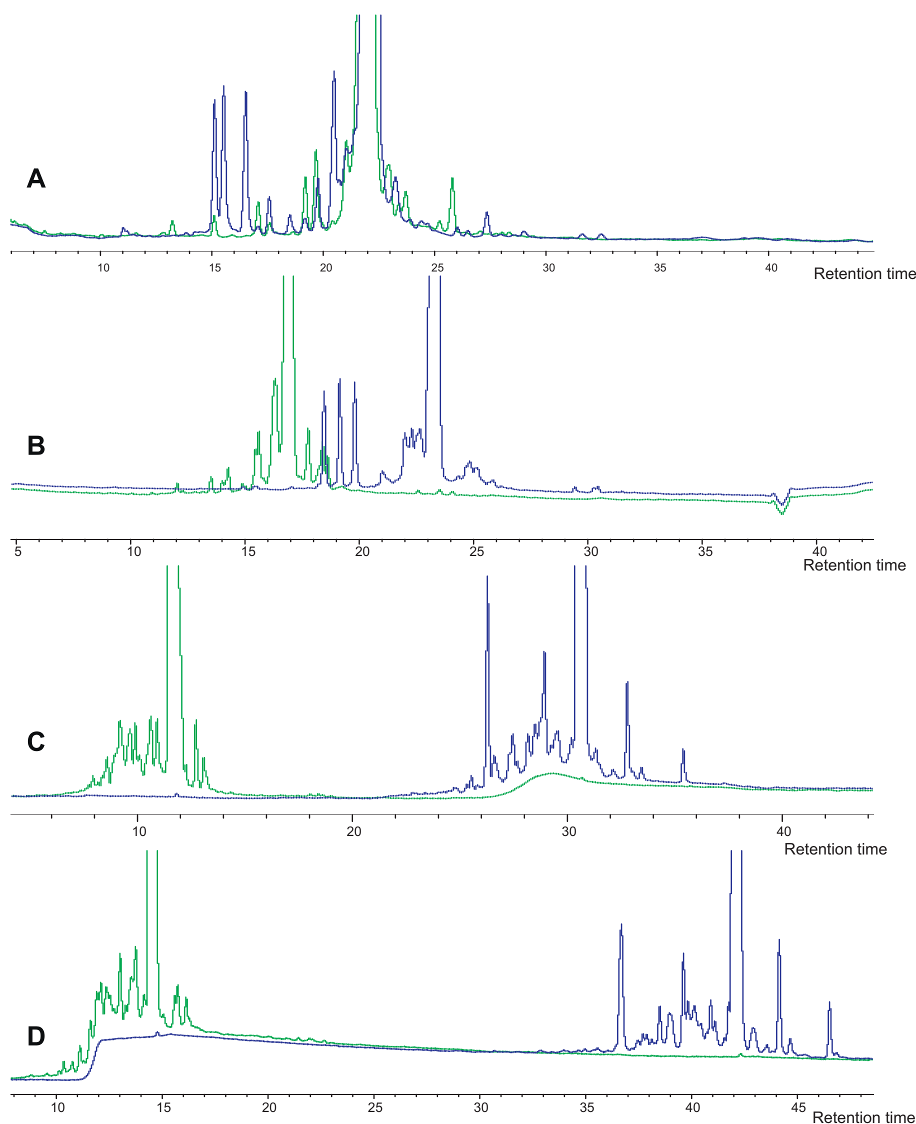

Fig. 2. Chromatography for sense (green) and antisense (blue) strand markers of siRNA #1 with methods containing either 20 mM DBA (A), TEA (B), DEA (C), and PA (D).

HPLC grade water and methanol from Sigma Aldrich were used for all of the mobile phase preparations. The pH of the mobile phase was adjusted with 0.1 N acetic acid and 0.1 N ammonium hydroxide as needed.

2.3. Procedures

Method procedures and conditions are described along with their corresponding chromatography throughout in Section 3.

3. Results and discussion

3.1. Evaluation of ion pairing agents

The following section details our research around evaluating various ion pairing agents as a single mobile phase additive. The oligonucleotides used for the development of these techniques were double stranded siRNA oligonucleotides, however the methodology can be exploited for other classes of oligonucleotides. Our goal was to develop a platform methodology which would denature the double stranded oligonucleotide on column, allowing for the separation and purity analysis of both the sense and antisense strands and their related impurities (variants) for several siRNA drug substances in development. Gilar et al. demonstrated that a reduction in column particle size results in improved mass transfer and peak capacity for synthetic oligonucleotides analyzed via RP-IP LC and UPLC [5,16]. As a result, a Waters ACQUITY UPLC system, along with 1.7 μm particle C18 columns were selected as the ideal platform to develop a method with the goal of maximizing resolution and peak capacity. When discussing synthetic oligonucleotide variants, such as full length product minus one or two nucleotides (i.e. $n-1$, $n-2$, etc.), it should be noted that these are commonly comprised of envelopes of numerous molecules. That is, $n-1$ variants can form for each of the nucleotides used in the sequence (i.e. $n-$G, $n-$C, $n-$A, $n-$U, as well as any chemically modified nucleotides) and at each of their various locations in the sequence. As described previously, oligonucleotide retention by RP-IP typically correlates with the number of phosphate groups, such that resolution of impurities within a variant envelope (i.e. all of the same length) will be limited. In that regard, having a separation where the retention mechanism is dominated by ion pairing is not ideal if the goal is to maximize resolution of all the impurities present. The research highlighted involves the screening of various ion pairing agents to achieve an optimal balance of ion pairing

based retention along with conventional reversed phase retention such that oligonucleotide resolution is based on length as well as hydrophobic differences in chemical sequence and modifications.

3.1.1. Dominant retention – ion pairing vs. reversed phase

Various IP agents at different concentrations were evaluated to identify denaturing RP-IP UPLC method parameters which maximized resolution of impurities and peak shape quality. With denaturing chromatography of double stranded oligonucleotides, the denatured state of the siRNA duplex is confirmed by the presence of both the individual sense and antisense peaks and lack of a duplex peak. The combination of increased column temperature and organic modifier in the mobile phase is used to dissociate or "melt" the duplex into individual strands and eliminate secondary structures amongst the single strands. The abasic endcap modifications on the sense strand result in two additional phosphate groups over the 20 found in the antisense strand. If no additional modifications are present within an RNA based siRNA then you would expect greater retention for the sense strand compared to the antisense strand when performing RP-IP chromatography, as demonstrated by Beverly et al. [17]. However, with a mobile phase containing 20 mM TEA, we find that the antisense strand elutes after the sense strand (see Fig. 2), indicating that the reversal of elution order is attributable to the 2′ O-methyl chemical modifications that are incorporated only on the antisense strand as detailed in Section 2.2. The O-methyl modifications increase the hydrophobic nature of the antisense strand and subsequently its affinity for the non-polar stationary phase.

Four different IP agents, with significant differences in hydrophobicity were evaluated. For each IP agent a concentration of 20 mM in water (adjusted to pH 8) was used for mobile phase A, and mobile phase B was neat methanol. Table 2 shows the method conditions used for the evaluation of the four IP agents. Due to the differences in retention exerted by each IP agent, the starting percent mobile phase B was adjusted such that the sense strand was not eluted in the void. Differences in the chromatographic retention caused by the various IP agents are shown in Fig. 2. Individual markers of the sense (green trace) and antisense (blue trace) strands of siRNA #1 were used and are shown at nominal concentrations of 0.25 mg/mL. Fig. 2(A)–(D) shows the IP agents in order of decreasing

Table 2
Method parameters used to screen the four different IP agents shown in Fig. 2.

Column	Waters ACQUITY UPLC OST C18, 2.1 × 100 mm, 1.7 μm
Flow rate	0.25 mL/min
Injection volume	5 μL
Column temperature	60 °C
Starting % mobile phase B	14–30% (depending on strength of IP agent)
Gradient slope	0.25% methanol/min

hydrophobic nature as follows: dibutylamine (DBA), TEA, diethylamine (DEA) and propylamine (PA). The correlation between the alkyl content of each IP agent and their corresponding influence on chromatographic retention can be seen. The use of DBA reduced the chromatographic capability to separate the sense and antisense strands based on differences in hydrophobicity between the two strands, resulting in co-elution of both strands and many of their related impurities. As the hydrophobic nature (strength) of the IP agent is reduced, an increase in the separation of the strands occurs due to exploitation of their differences in hydrophobicity. The individual use of both DEA and PA demonstrated the most favourable balance of IP and RP retention mechanisms, such that co-elution of the main peaks and their related impurities is minimized.

It is important to notice the baseline disturbances in the chromatograms of the DEA and PA containing mobile phases in Fig. 2(C) and (D), respectively. It was determined that an impurity contained within each IP agent was responsible for these disturbances and an alternative supplier of PA was found that resolved this baseline disturbance issue, as demonstrated in the remaining figures.

In addition to controlling the dominant mode of separation (ion pairing vs. reversed phase retention) via selection of the IP reagent's hydrophobicity, it can also be mediated by the concentration of the ion pairing reagent in the mobile phase. In comparison to the co-eluting peaks with 20 mM DBA, when the DBA concentration is reduced to 10 mM, resolution is improved whereby the antisense strand elutes after the sense strand, reflecting a decreased ion pairing retention mechanism (data not shown).

The propylamine (PA) IP agent at a concentration of 20 mM was identified to provide superior resolution and peak shape than the other IP agents. However, when this method was tested with four

Table 3
Chromatographic peak quality values for siRNA #1 and siRNA #2 with the 20 mM PA method.

siRNA	Sense peak			Antisense peak		
	Tailing factor[a]	Asymmetry ratio[b]	Half width (s)	Tailing factor	Asymmetry ratio	Half width (s)
siRNA #1	2.6	4.4	21	1.9	2.8	20
siRNA #2	2	3.3	16	3.1	5.4	23

[a] Tailing factor = $w0.05/2d$, where $w0.05$ is the width of the peak at one-twentieth of the peak height, and d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.
[b] Asymmetry ratio = (at 10% of the peak height) ratio of the distance between the peak apex and the back side of the chromatographic curve to the distance between the peak apex and the front side of the chromatographic curve. A value >1 is a tailing peak, while a value <1 is a fronting peak.

Table 4
Optimized parameters for the TEA/PA platform denaturing RP-IP UPLC method.

Column	Waters ACQUITY UPLC BEH Shield RP18, 2.1 × 150 mm, 1.7 μm
Flow rate	0.175 mL/min
Injection volume	5 μL
Column temperature	55 °C
Mobile phase A	10 mM TEA, 10 mM PA in 90/10 water/methanol (v/v), pH 7.1
Mobile phase B	10 mM TEA, 10 mM PA in 60/40 water/methanol (v/v), pH 7.1

	Time (min)	% A	% B	% Methanol
Gradient	0	90	10	13
	77	45	55	26.5
	77.5	90	10	13
	83.5	90	10	13

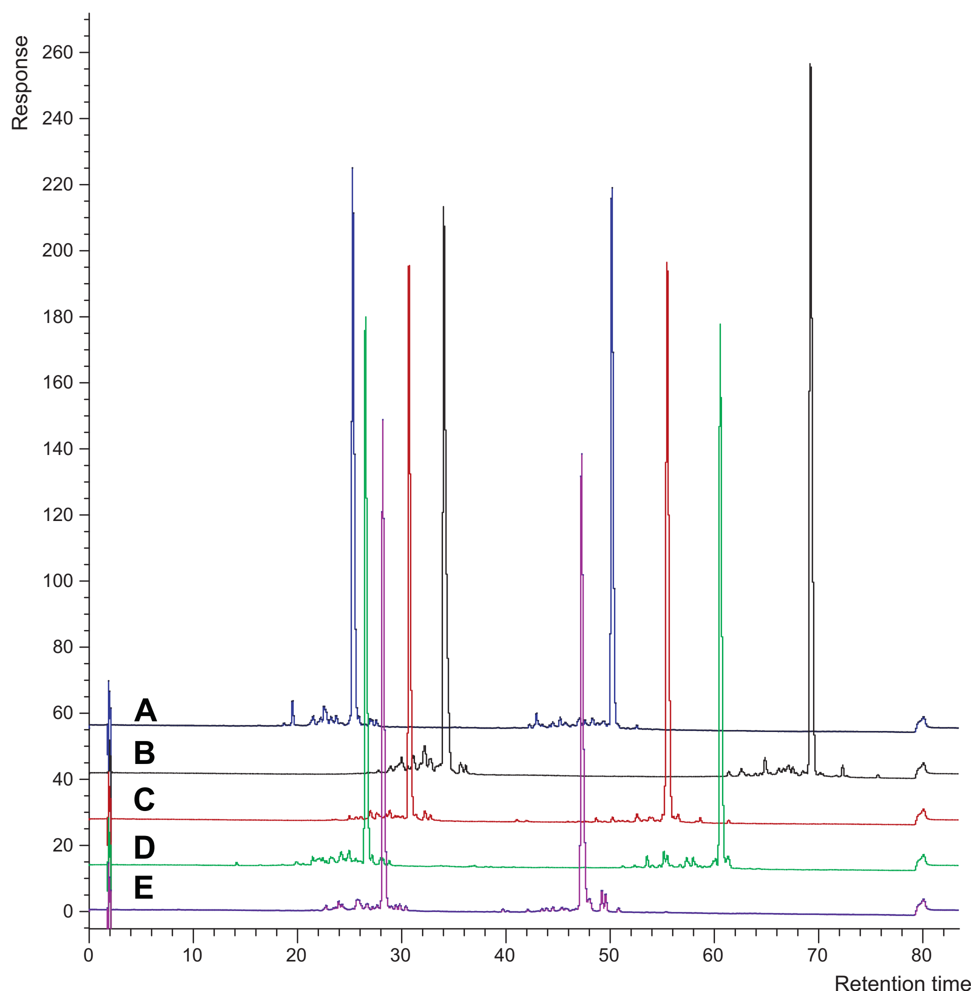

Fig. 3. Chromatography for five different siRNA compounds, siRNA #3(A, blue), siRNA #1 (B, black), siRNA #4 (C, brown), siRNA #5 (D, green) and siRNA #2 (E, purple trace) siRNA molecules with the optimized TEA/PA universal platform method detailed in Table 4.

additional siRNA compounds (#s 2–5) the chromatographic quality was not consistent (starting organic strength in MP adjusted to elute each siRNA, IP conc. and gradient slope unchanged). In particular, the antisense peak quality for siRNA #2 was very poor when considering factors such as tailing factor, asymmetry ratio and peak half width (s). These peak quality values for siRNA #1 and siRNA #2 as achieved with the 20 mM PA method are summarized in Table 3. As such, efforts to improve the chromatographic method continued and focused on the evaluation of mobile phases containing a mixture of two different IP agents.

3.2. Development of multiple ion pairing agent method

It was reasoned that combining two different IP agents may improve the chances of achieving acceptable chromatography for numerous siRNA molecules via one platform methodology. The hypothesis being that the presence of two different IP agents in the mobile phase would provide broader coverage for achieving the desired chromatography across oligonucleotides of varying sequence, length, and modifications. That is, some oligonucleotides may be more prone to having intra- or inter-molecular secondary structure interactions during the separation resulting in broader peak shapes. A more hydrophobic IP agent may increase the mass transfer rate of the oligo with the stationary/IP phase such that time for secondary structure formation is reduced and peak shape is improved. In addition, the use of a less hydrophobic IP agent where the steric shielding around the amine is minimized, such as propylamine, may enable stronger binding and slower on/off kinetics between the IP agent and the oligo, potentially providing more direct interaction between the oligo and the C18 column stationary phase, improving the ability to exploit chemical differences amongst closely related oligonucleotides. Although several different IP agent combinations were evaluated, the combination of triethylamine and propylamine demonstrated the most promise. As such, various concentrations of TEA and PA mixed were evaluated to achieve the best possible chromatographic peak shape and resolution for all five of the siRNA molecules. After evaluating several different concentrations, it was found that an equi-molar amount of TEA/PA at 10 mM each resulted in the best chromatography. The optimized parameters developed for the TEA/PA method are presented in Table 4. It is important to note that the 10 mM concentrations of TEA/PA were held constant across mobile phases A and B to improve overall chromatographic quality and reproducibility. An evaluation of MP pH ranging from 8.2 to 6.9 was carried out with the TEA/PA method. No change in selectivity of the method was observed across this pH range, however peak shape and robustness/reproducibility of the MP preparation was improved at a pH of 7.1 (data not shown).

Representative chromatography for the method outlined in Table 4 is presented in Fig. 3. The "universal" platform capabilities of the TEA/PA combination method are highlighted in Fig. 3, in which five different siRNA molecules are shown at nominal concentrations of 0.5 mg/mL. All five siRNAs used to develop this method are equivalent in oligonucleotide length as well as types of

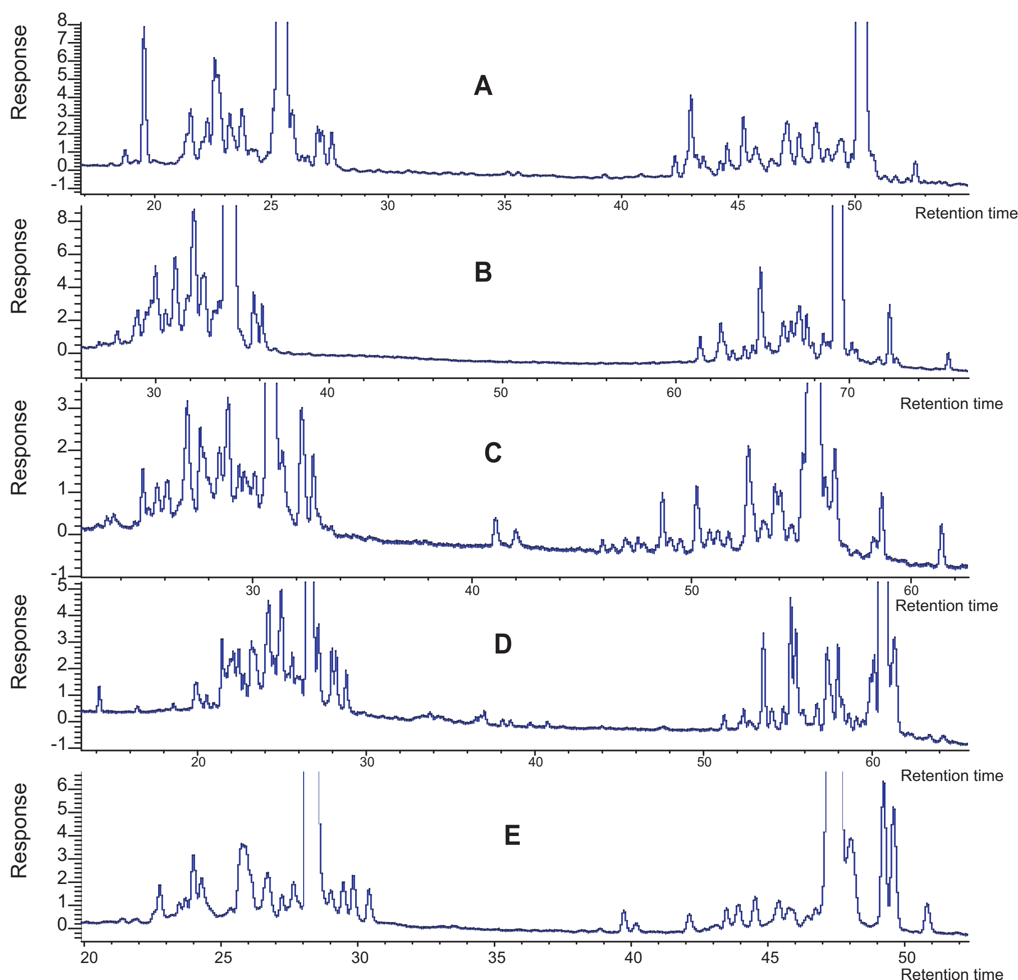

Fig. 4. Chromatographic impurity profile for the five different siRNA compounds, siRNA #3(A), siRNA #1 (B), siRNA #4 (C), siRNA #5 (D) and siRNA #2 (E) siRNA molecules with the optimized TEA/PA universal platform method detailed in Table 4.

chemical modifications. Fig. 4 shows a zoomed in view of the chromatographic impurity profile for all five siRNAs shown in Fig. 3. On average, the half width of a 0.25% (a/a) impurity peak is 11 s. Given that there is 77 min of separation gradient time, this platform method has a peak capacity of approximately 420.

The peak quality values for the five different siRNA molecules analyzed by the universal platform TEA/PA method are shown in Table 5. All of the peak quality values appear suitable for the intended use of the method. It is important to note that the quality values for the siRNA #2 antisense peak shown in Table 5 are significantly improved compared to those originally generated with the propylamine only method detailed in Table 3.

3.3. Interrogating method specificity

As discussed previously, the goal of the method development activities was to find a balance between ion pairing and reversed phase retention mechanisms such that oligonucleotides of the same base sequence but with small chemical differences could be separated to allow for greater control over purity analysis and impurity identification. The RP-IP UPLC method outlined in Table 4 was interfaced to an Agilent ion trap mass spectrometer (MS) to enable molecular weight determination of the sense strand related impurities for siRNA #3. While the focus of this article is on the chromatographic separation, the authors realize the importance of MS analysis for the characterization of oligonucleotide therapeutic impurities. Based on the extensive use of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in the literature as a mobile phase additive for RP-IP LC-MS analysis of oligonucleotides [5–9,13,15–17], its use was investigated both in the mobile phase and via post column addition. As a mobile phase additive it resulted in poor chromatographic peak shape and resolution compared to not using it. When added via post column addition a significant amount of HFIP adducts was observed in the MS spectra with no apparent increase in ionization efficiency for the oligonucleotide (data not shown). Several other organic solvents and bases were evaluated as modifiers via post column addition with the intent of improving the ESI efficiency; however none showed any significant benefit to warrant their use (data not shown). Nonetheless, the method outlined in Table 4 when interfaced to an Agilent ion trap system, with a post column split down to 35 μL/min, provided ample ESI efficiency and MS sensitivity for impurity characterization.

The RP-IP UPLC-MS analysis of the siRNA #3 sense strand enabled the mass identification of all the major impurities present, including a key impurity, the loss of HF from the 2′ ribose position of the fluorinated cytosine nucleotide [18–21]. Since the fluorinated cytosine nucleotide is used repeatedly throughout the sequence, the loss of HF can occur at any of the various positions resulting in the formation of several isomeric impurities. Chromatographic resolution of such single strand isomers with identical nucleobase sequence but differing only in the 2′ location of the de-fluoro modification is not expected to be achievable via previously reported RP-IP techniques for oligonucleotide analysis [4–17]. However; the increased selectivity offered by the combined TEA/PA method

Table 5
Chromatographic peak quality values for the siRNAs analyzed by the TEA/PA denaturing RP-IP platform method.

siRNA	Sense peak			Antisense peak		
	Tailing factor	Asymmetry ratio	Half width	Tailing factor	Asymmetry ratio	Half width
siRNA #1	2.4	3.7	16	1.4	1.8	17
siRNA #2	1.8	2.4	14	1.8	2.4	15
siRNA #3	1.8	2.5	14	1.5	1.9	14
siRNA #4	1.7	2.3	14	1.3	1.6	16
siRNA #5	1.8	2.4	13	1.5	2	15

enabled the separation of these five isomeric impurities. Fig. 5 shows the UV trace of the siRNA #3 sense strand and related impurities (A), followed by an overlay of four extracted ion chromatograms (EIC) corresponding to the −4H charge state of all the major impurities present in the UV trace; 1708.9 *m*/*z*, 1714.0 *m*/*z*, 1721.4 *m*/*z*, and 1669.0 *m*/*z* (B), as well as the representative MS spectrum observed across the five impurity peaks identified in the 1709 *m*/*z* EIC (C). The siRNA #3 sense strand has a MW of 6860, resulting in a −4H charge state ion at *m*/*z* 1714. As can be seen in the 1714 *m*/*z* EIC, two isomeric impurities (or of mass difference within the resolution of the MS detector) are resolved on either side of the sense strand peak. In addition, the 1721 *m*/*z* EIC trace shows the separation of two isomer impurities eluting after the sense strand with a MW of 6890. Of the most significance though is the 1709 *m*/*z* EIC, where five isomer peaks are resolved. Fig. 5(C) shows the −4H, −5H, and −6H charge state ions in the MS spectrum taken at peak #2 and observed across the other 4 isomer peaks. Deconvoluting the multiple charge states provides a MW of 6840 for this impurity, matching the loss of HF from the sense strand. The sense strand for siRNA #3 incorporates the 2′ fluorinated cytosine at five different positions in the sequence, corresponding perfectly to the five isomer impurity peaks chromatographically resolved and identified via MS. As can be seen in Fig. 5, MS sensitivity was sufficient to determine the MW of all the major impurities, where individual peak areas were within 0.3–0.9% (a/a) of the main peak via the UV trace.

Additional investigations into the selectivity offered via the combined ion pairing agent methodology were performed by having closely related impurity markers made and analyzed via this methodology. For the siRNA #3 compound, five different types of impurities were manufactured and are listed in Table 6. Minor modifications (not shown) were made to the gradient method as given in Table 4 to allow reduced analysis times for the siRNA #3 antisense strand and impurity markers. Fig. 6 shows the chromatographic separation of the siRNA #3 antisense strand (top trace) at a 0.3 mg/ml nominal concentration along with three individual impurity markers (at 0.0025 mg/ml each), an $n-1$ RNA guanine, $n+1$ RNA guanine, and replacing a single 2′ O-methyl for an OH group. All three impurities are well resolved from the main antisense peak, demonstrating that even a small change in hydrophobicity ($-CH_2$)

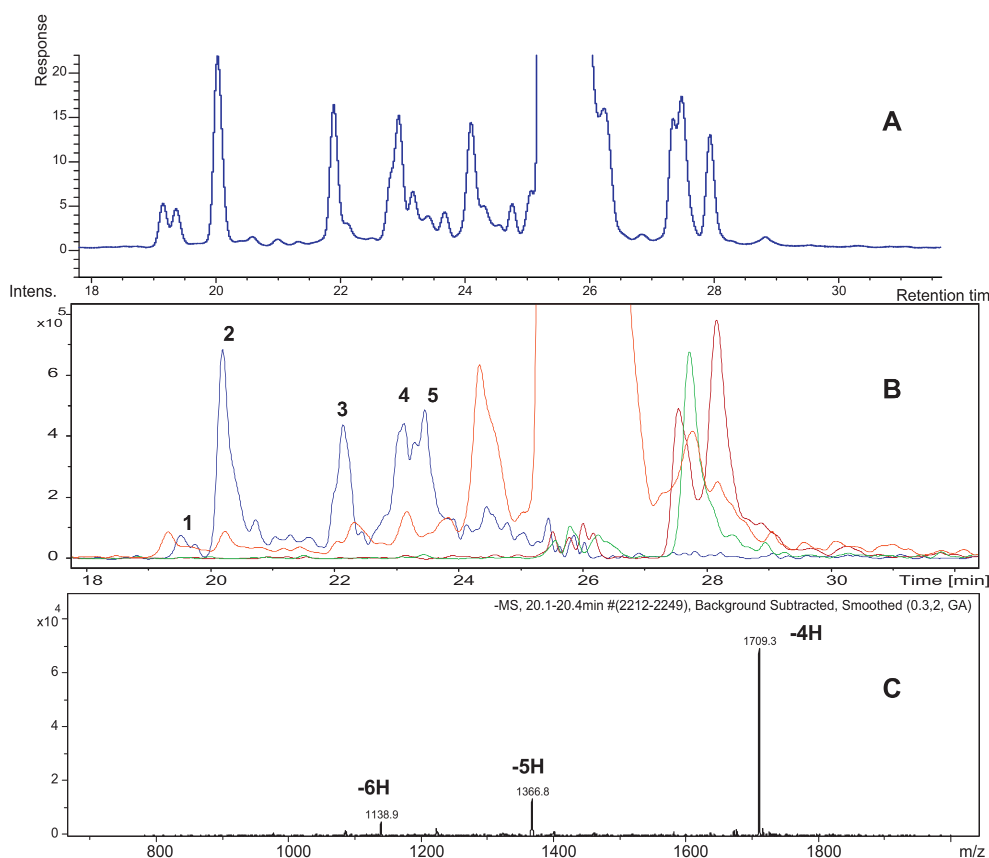

Fig. 5. TEA/PA combined RP-IP UPLC separation showing the UV trace of the siRNA #3 sense strand and related impurities (A), followed by an overlay of four extracted ion chromatograms (EIC) corresponding to the −4H charge state of all the major impurities present in the UV trace; 1708.9 *m*/*z* (blue), 1714.0 *m*/*z* (orange), 1721.4 *m*/*z* (dark red), and 1669.0 *m*/*z* (green) (B), as well as the representative MS spectrum observed across the five impurity peaks identified in the 1709 *m*/*z* EIC (C).

Table 6
Closely related impurity markers synthesized to evaluate the selectivity of the combined ion paring agent methodology.

Name of impurity marker	Mass difference from parent	Description
$n-1$	−345	Removal of an RNA Guanine nucleotide from the 5′ end of the antisense strand (most common location for $n-1$ impurity)
$n+1$	+345	Addition of an RNA Guanine nucleotide to the 5′ end of the antisense strand (most common $n+1$ impurity)
$O–CH_3$ to OH	−14	Changed a single Guanine nucleotide in the middle of the antisense strand sequence from a methoxy to a hydroxyl at the 2′ ribose position
3′–5′ swap sense strand	0	Swapping of a 2′ fluoro uracil and 2′ deoxy thymine at the 3′ and 5′ terminal positions of the sense strand
3′–5′ swap antisense strand	0	Swapping of a 2′ O-methyl uracil and RNA guanine at the 3′ and 5′ terminal positions of the antisense strand

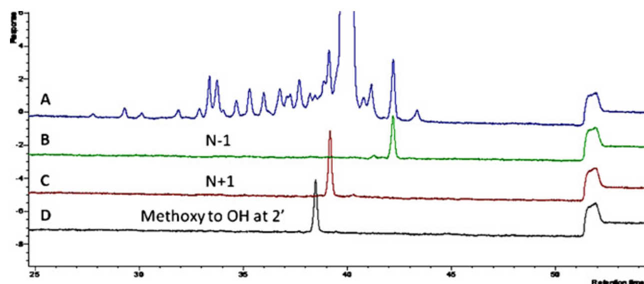

Fig. 6. Overlay of TEA/PA denaturing RP-IP UPLC separation of the siRNA #3 antisense strand impurity profile (A, blue) with the $n-1$ (B, green), $n+1$ (C, red), and 2′ methoxy to OH swap (D, black) impurity markers.

for a molecule with a molecular weight of greater than 6000 Da can be easily resolved with this methodology. The selectivity of the method is also highlighted by the fact that the $n-1$ marker elutes after the main peak and the $n+1$ elutes before. This is opposite to what is expected when the selectivity is dominated by ion pairing and the number of phosphate groups in the oligonucleotide. The elution order of these two markers with our method is due to the increased polarity of ribose compared to the other types of sugars on the nucleotides (2′ F, 2′ O-methyl) used heavily throughout the siRNA #3 antisense sequence. When an RNA nucleotide is added to the sequence it decreases its overall hydrophobicity resulting in earlier elution, and the opposite when an RNA nucleotide is removed. Fig. 7 demonstrates the TEA/PA RP-IP UPLC resolution obtained between the isomeric impurity markers described in Table 6 and their respective parent sense and antisense strands. As can be seen a significant degree of selectivity is achieved between the isomeric impurity markers, comprised of simply swapping the terminal 3′ and 5′ nucleotides, from their respective parent sense and antisense strands.

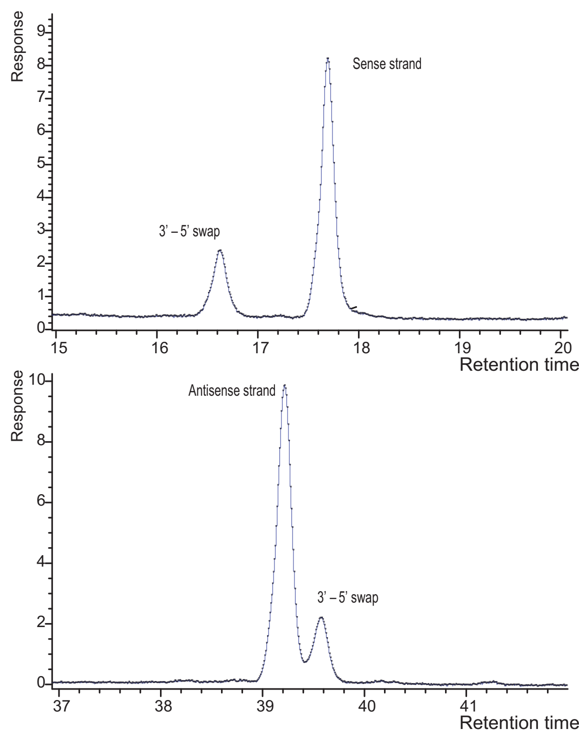

Fig. 7. TEA/PA denaturing RP-IP UPLC separation of isomer impurity markers (3′–5′ end nucleotide swap) spiked with excess amount of the parent sense or antisense strand.

While separation of isomers can be beneficial for control of impurities, it can be detrimental when the parent compound is comprised of several diastereomers, as is the case for phosphorothiolated oligonucleotides. We analyzed a 16mer oligonucleotide where 8 of the nucleotides were phosphorothiolated, resulting in the potential formation of 256 diastereomers during synthesis. The chromatography (not shown) for this oligonucleotide via our TEA/PA combined IP methodology, resulted in a span of over 15 min of unresolved peaks, assumed to be the various diastereomers. For phosphorothiolated oligonucleotides it is recommended to have a method with a very dominant IP retention mechanism to collapse the diastereomers into a single peak.

4. Conclusions

The development of oligonucleotide therapeutics is in its infancy with regards to establishing chemistry, manufacturing, and control (CMC) guidances from both industry and World Wide regulatory agencies. In particular, achieving suitable control over drug substance and/or drug product assay and purity can be analytically challenging. The intent of the research highlighted in this article was to develop a reversed phase-ion pairing methodology that pushed the limits of chromatographic specificity and peak quality for oligonucleotide therapeutics and their related impurities. The method development activities revealed the need for a balance between an ion pairing based retention mechanism vs. a purely reversed phase retention mechanism in order to increase selectivity based on small chemical differences amongst oligonucleotides. As a result, we found that the use of a second ion pairing agent combined in the mobile phase can improve the overall chromatographic resolution and peak shape of the oligonucleotide analytes over the use of a single ion pairing agent alone, resulting in enhanced purity analysis and impurity identification capabilities. However, the benefits in resolution achieved via this approach can be detrimental when analyzing phosphorothiolated oligonucleotides, as separation of the diastereomers results in poor chromatography for purity or assay determination.

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