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Critical Insight

Current State of Oligonucleotide Characterization Using Liquid Chromatography–Mass Spectrometry: Insight into Critical Issues

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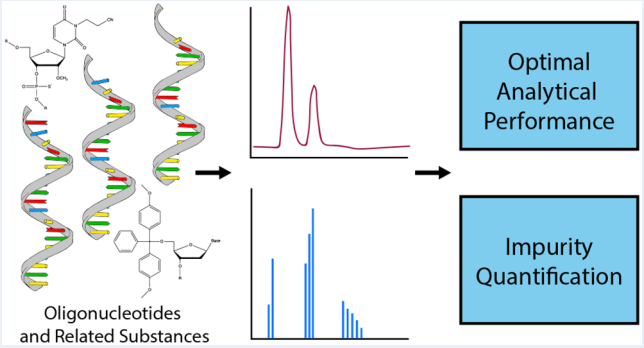
ABSTRACT: As interests increase in oligonucleotide therapeutics, there has been a greater need for analytical techniques to properly analyze and quantitate these biomolecules. This article looks into some of the existing chromatographic approaches for oligonucleotide analysis, including anion exchange, hydrophilic interaction liquid chromatography, and ion pair chromatography. Some of the key advantages and challenges of these chromatographic techniques are discussed. Colloid formation in mobile phases of alkylamines and fluorinated alcohols, a recently discovered analytical challenge, is discussed. Mass spectrometry is the method of choice to directly obtain structural information about oligonucleotide therapeutics. Mass spectrometry sensitivity challenges are reviewed, including comparison to other oligonucleotide techniques, salt adduction, and the multiple charge state envelope. Ionization of oligonucleotides through the charge residue model, ion evaporation model, and chain ejection model are analyzed. Therapeutic oligonucleotides have to undergo approval from major regulatory agencies, and the impurities and degradation products must be well-characterized to be approved. Current accepted thresholds for oligonucleotide impurities are reported. Aspects of the impurities and degradation products from these types of molecules are discussed as well as optimal analytical strategies to determine oligonucleotide related substances. Finally, ideas are proposed on how the field of oligonucleotide therapeutics may improve to aid in future analysis.



INTRODUCTION

Oligonucleotide (OGN) therapeutics are an exciting new class of treatments for human disease, with more than ten now approved and dozens more in clinical trials. They operate through a variety of molecular mechanisms designed to interfere with the processing of genetic information. Oligonucleotide therapeutics may be composed of either a single antisense strand or can be double stranded with both sense and antisense strands. The individual strands tend to be between 13 and 30 nucleotides, although there is no maximum size. To increase their resistance to catabolism and avoid *in vivo* cleavage at the phosphodiester linkage, improve distribution, and also to increase their potency, there are a wide variety of chemical modifications introduced into these structures. These modifications can occur on the base, sugar, or the phosphate backbone. To enhance the stability of oligonucleotides upon administration to the body, the phosphate groups in the backbone are often replaced by phosphorothioate (PS) groups or morpholino moieties.[1,2] Methylations of the bases (particularly cytosine) and modification of the 2′ hydroxyl position of the ribose can also increase nuclease resistance:[3−5]

- 2′-fluoro-nucleic acids
- 2′-*O*-Me-nucleic acids (OMe)
- 2′-*O*-(2-methoxy) ethyl (MOE)-nucleic acids
- locked nucleic acids at the 5′ and 3′ ends
- morpholino oligonucleotides where the ribose sugar moiety is replaced with morpholine rings and the anionic phosphodiester linkage is replaced with nonionic phosphorodiamidate groups

One of the more recent strategies has been the addition of carbohydrate targeting groups onto one of the ends of an oligonucleotide strand. All of these potential chemical alterations result in a significant degree of complexity in the final structures of these molecules. These molecules can also have higher-order structure. In the case of aptamers, this is part of their main mechanism of action. Due to these features,

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oligonucleotide therapeutics are highly specific and have significant potency toward their targets. The impact of secondary structures are generally minimized during analysis by using elevated chromatographic temperatures to prevent the formation of double strands, loops or hairpins.

Oligonucleotides are commonly analyzed through hybridization techniques such as ELISA (enzyme-linked immunosorbent assay and PCR (polymerase chain reaction).[6] These techniques have greater sensitivity over mass spectrometry (MS), but they lack in specificity. Mass spectrometry is required to analyze impurities, degradants, and other biological/chemical modifications that are invisible to hybridization techniques. Mass spectrometry techniques often utilize liquid chromatography (LC) on the front end of the analysis to separate the unique molecules from one another. Historically, the mobile phases for LC alkylamine and acetate counterion resulted in unsatisfactory sensitivity. Recently, advances in the mobile phase to include alkylamines and fluorinated alcohol counterions have resulted in significant improvements in sensitivity. However, these new mobile phases suffer some mechanistic drawbacks. This critical insight aims to review and propose alternative retention mechanisms involved in LC-MS analysis of oligonucleotides, to evaluate current challenges involved in their MS analysis, and to report optimal approaches to determine oligonucleotide impurities. The current status of accepted thresholds for oligonucleotide impurities are discussed.

■ ION-PAIR CHROMATOGRAPHY OF OLIGONUCLEOTIDES

Ion-pair reversed phase chromatography (IP-RP) has become the LC method of choice for OGNs. Mobile phases used for chromatographic separation of oligonucleotides initially consisted of combinations of alkyl amines with corresponding carboxylic acid counterions.[7] These organic carboxylic acids were superseded by hexafluoroisopropanol (HFIP), which had ideal physiochemical properties for use in LC-MS assays. It is quite volatile and due to its pK_a is ionized at the high pH values typically used, therefore serving well as a counterion. Following this change, there has been significant research done with respect to the choice of the alkylamine. The various alkylamines confer differing amounts of hydrophobicity to the oligonucleotides. Therefore, the extent of chromatographic retention can be modulated by varying the alkylamine used. To this day, the combination of alkyl amines and fluoroalcohols remain the most widely used mobile phase composition. While there are many choices of ion pairs, it has been proposed that the best choice is highly dependent on the composition of the oligonucleotides being studied. The best choice of fluoroalcohol appears to be dependent on the choice of alkylamine.[8]

One of the unique challenges of using LC-MS to analyze oligonucleotides is the stability of the mobile phases. However, this is only hinted at in most studies which usually note the need to remake mobile phases, often daily. This is somewhat unexpected because this has not previously been noted when using alkylamines for ion pair chromatography. In fact, the tributylamine mobile phase, lacking HFIP, used for oligonucleotide impurity analysis by Roussis et al. at Ionis appears to be quite stable.[9] It appears that the combination of the alkylamine and fluoroalcohol creates an outstanding yet transient environment for conducting high quality LC-MS.

When alkylamines are used in combination with fluorinated alcohols, the general principle of separation has been that the alkylamine acts as an ion pairing agent by both shielding the negatively charged phosphate backbone, making the analyte more hydrophobic, and interacting with the stationary phase to introduce an additional ionic retention mechanism. The fluoroalcohol has also been noted to improve the distribution constant of the oligonucleotides between the mobile phase and stationary phase.[10] Recent studies have revealed that this mobile phase is far more complex than has been previously understood. Studies by Li et al. have shown that at relatively low concentrations of the alkylamine (below 10 mM), there is a linear increase in retention with increasing alkylamine concentration.[2] However, between 20 and 100 mM, there is a massive decrease in analyte retention. The change in retention was also found to correlate with the solution pH. The higher the pH, the lower the retention. Solution pH adjustments using formic acid were found to restore analyte retention. While this is useful for demonstrating the impact of pH on retention, formic acid, along with many other common organic acids, cause massive ion suppression in LC-MS of oligonucleotides. Therefore, there appears to be a need to find ways to adjust mobile phase pH while maintaining high electrospray desorption efficiency. The current solution to this issue has been to use a relatively low concentration of alkylamine (15−25 mM) with 100 mM of the fluoroalcohol. Fluoroalcohols such as HFIP and HFMIP have a pK_a around 8, which means they behave as weak acids and have a positive impact on the overall pH of the mobile phase.

■ PROPOSED ION-PAIR CHROMATOGRAPHIC MICELLE MECHANISM

Further analysis of the alkylamine/fluoroalcohol mobile phase system was conducted using transmission electron microscopy, which observed that there are micelles or colloids formed as the ion pairing reagent's concentration and the solution temperatures are raised.[3] The positive charge on these alkylamine-containing colloids appear to decrease the interaction between the oligonucleotides and the stationary phase. It does appear that the fluoroalcohol plays a role in colloid formation, but the mechanism of this interaction is not well-understood. However, the aging of the mobile phases appears to be well-connected to the time dependent formation of these colloids, and therefore, methods to eliminate this issue would be of significant value.

There are many common practices that are frequently used in the making of mobile phases. A few of the important ones include preparing them fresh, checking the pH value, and using clean containers and lab equipment. Apart from these, it has been seen that lower pH values have less micelle formation, making the mobile phase more stable and prevent an unwanted loss of retention.[2]

Considerable care is required in choosing the types of solvents and additives to be used in mobile phases; this diminishes the chances of ion suppression of the additives/solvents that have a relatively high proton affinity. Studies at Ionis have suggested that removing the fluoroalcohol from the mobile phase has eliminated some of these issues.[9] This suggests that segregation of fluoroalcohols from alkylamines may in fact prove beneficial because the highest sensitivities are obtained when using optimized alkylamines and fluoroalcohols. The role of the organic solvent has also not been fully explored with respect to mobile phase optimization. To date, most studies have used methanol because the fluoroalcohols have the greatest solubility in this solvent. Earlier work by

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Chen et al. showed that using ethanol provided twice the LC-MS response of methanol, and work by Moderna has used acetonitrile.[11,12] It is unclear how using these solvents might impact the formation of micelles or colloids, and perhaps this is part of the reason that these solvents have shown promise in limited use.[3]

ALTERNATIVE CHROMATOGRAPHIC APPROACHES

Alternative chromatographic methods such as hydrophilic interaction liquid chromatography (HILIC) and anion exchange have been used in the analysis of oligonucleotides. HILIC methods provide different selectivity and in some applications have shown similar performance to IP-RP methods.[13] HILIC methods try to address one of the major limitations of IP-RP methods, which is eliminating the need for the alkylamines, which create significant carry-over ion suppression if there is a need to run in positive ion mode. However, to date, the HILIC conditions to surpass the performance of IP-RP methods have yet to be found. Even under the current state of knowledge, HILIC methods should still be pursued because their selectivity may be better suited for a particular application. Overall, HILIC methods for oligonucleotide analysis remain new with a myriad of unexplored possibilities that could make HILIC an appealing alternative to IP-RP methods.

Anion exchange methods have shown some of the most efficient separations of oligonucleotides. Results showing baseline separation of four 21 mer oligonucleotides defy the idea that anion ion exchange works purely as a function of the number of charges and bases contained within an oligonucleotide.[14] While this method provides great separation, the mobile phase is incompatible with mass spectrometry due to the abundant use of nonvolatile salts. There have been studies showing the implementation of two-dimensional analysis where the use of a trap column allows for the mobile phase to be exchanged, therefore enabling mass spectrometry implementation.[15,16] Overall, there appear to be yet unexplored secondary interactions with polymeric ion exchange phases that may be capable of being further exploited for even greater separation power.[3,17]

IP METHOD OPTIMIZATION: IMPACT ON MASS SPECTROMETRY PERFORMANCE

Optimization of ion pair chromatography has a direct impact on mass spectrometry performance. Historically, reversed-phase ion pair utilizing a base (alkylamine) and acidic counterion are used for oligonucleotide analysis. The incompatibility of the acetate counterion with MS was the driving factor in the switch to fluoroalcohol-based mobile phase combinations. Acetate mobile phases provide better chromatographic resolution when compared to HFIP, but the decreased MS signal is a major drawback. Systematic optimization of fluoroalcohol and alkylamine combination have been shown to increase sensitivity and even reduce the amount of cationic adduction.[8,11,18−21] Matching the hydrophobicity of the alkylamine and the oligonucleotide being analyzed increases the MS sensitivity significantly.[8,20] However, there are many other sensitivity challenges.

MASS SPECTROMETRY SENSITIVITY CHALLENGES

Sensitivity is a challenge in utilizing mass spectrometry for oligonucleotide analysis. The best methods can accurately quantitate down to high picogram/low nanogram/mL levels when compared to other hybridization techniques (ELISA, PCR, fluorescence), which can do high femtogram/low picogram/mL levels. The advantage of mass spectrometry in comparison to these hybridization techniques is the specificity of the measurement.[22] Hybridization techniques typically require amplification or an indirect measurement, unlike mass spectrometry, which measures the oligonucleotide directly. The advantage of this is that amplification or indirect measurements can be skewed by measuring full length oligonucleotides as well as impurities and metabolites. For example, PCR and ELISA often utilize hybridization of a complementary strand, but if there is a missing base on the strand that does not inhibit binding, then the oligonucleotide minus base is included in the measurement for the full-length oligonucleotide. In mass spectrometry, this would not be the case because the mass of the oligonucleotide minus the base will be less than the full-length oligonucleotide, and it is easier to differentiate what is being measured.

Electrospray ionization is used for oligonucleotides because of the ability to "softly" ionize biomolecules. However, electrospray creates a charge-state envelope that distributes total ion signal of the full-length oligonucleotide over at least 10+ charges, which is again subdivided into isotopic distributions. Moreover, polyanions like oligonucleotides show a tendency to exchange the H^+ of the phosphate or phosphorothioate groups of the oligonucleotide backbone for Na^+ and K^+, which adds additional challenge for sensitive ESI-MS analysis of oligonucleotides.[20,21,23] Having multiple exchangeable H^+ in the OGN backbone leads to $[M-mH+m\text{alkali}-nH]^{n-}$ exchange ions in negative-ion mass spectra. As a result of the H+−alkali+−exchange, the multiply charged oligonucleotide ions will be further dispersed among multiple cation-containing species with an m/z $22/n$ difference for sodium exchange and an m/z $38/n$ difference for potassium exchange (with n being the charge states of the ion). Taking the multiple charge, the isotopic distribution and the alkali exchange together means that the MS signal is distributed over at least 50+ channels.

MASS SPECTROMETRY IONIZATION CONSIDERATIONS

Ionization in electrospray for oligonucleotides has had a lack of clarity in regard to the mechanisms involved.[24,25] It is generally accepted that the charge residue model (CRM), which states that there is one analyte per droplet until the droplet is so small that the biomolecule is ionized, is the mechanism of ionization.[26,27] The competing theory is the ion evaporation model (IEM) that states before a droplet gets too small for one analyte per droplet, the analyte is desorbed.[28,29] The chain ejection model (CEM) states that for an unfolded analyte, one "tail" of the analyte can be charged and ejected from the droplet. The analyte is gradually ejected and through H^+ migration charged to be in equilibrium with the droplet while simultaneously leaving the droplet.[30] The CEM may explain why unfolded oligonucleotides are highly charged upon ionization versus folded oligonucleotides. Oligonucleotides contain hydrophilic phosphate groups that tend to associate with water in the interior of the droplet, which is why the

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leading theory is the CRM; however, when you add an ion pair, you create an environment where the hydrophilic portion of the ion pair interacts with the phosphate, and the hydrophobic end of the ion pair tends to move toward the droplets surface.[11,20,21] It is therefore plausible that if enough of the ion pair is at the edge of the droplet, then the oligonucleotides could be close to the edge of a droplet and be desorbed via the IEM or through the CEM. Therefore, it is highly probable that desorption of oligonucleotides employs a combination of ionization mechanisms. Improved understanding of ways to advantageously exploit these mechanisms is a significant challenge which must be addressed to continue to improve ionization efficiency and therefore method sensitivity. One possibility to increase sensitivity is increasing the amount of organic present, like with HILIC chromatography or organic vapor assisted electrospray.[31] The organic is known to play an important role in the desorption of oligonucleotides by decreasing droplet surface tension via changing the organic solvent or increasing the concentration of organic present.[11]

MS IMPURITY/RELATED SUBSTANCES CHALLENGES

Mass spectrometry is the method of choice to directly obtain structural information from therapeutic oligonucleotides and their impurities. It is also indispensable for determining impurity levels. As several modified full-length impurities, the $n-1$ deletion and $n+1$ addition sequences (see also below at section synthesis impurities) oftentimes cannot be baseline separated chromatographically, it is a challenge to quantitate these impurities at 0.2% (Table 1).

Table 1. Reporting, Identification, and Qualification Threshold for Oligonucleotide Impurities According to ICH Guidelines, EU Guidelines, and Leading Oligonucleotide Researchers

	Reporting threshold	Identification threshold	Qualification threshold
ICH Q3A and Q3B	0.05%	0.10%	0.15% or 1 mg
Impurities in peptides European Pharmacopeia (Ph. Eur) 2034	0.1%	0.5%	1.0%
Oligonucleotide community Capaldi[32]	0.2%	1.0%	1.5%

To distinguish these impurities with molecular masses close to its parent compound, high resolution mass spectrometry is essential. A classic example of this is the deamination impurity (see also the Synthesis Impurities section) that converts the amine ($-NH_2$) group from either a cytosine base or a 5-methyl cytosine to a carbonyl, thereby removing the double bond on the nitrogen by adding a hydrogen, which converts the base to uracil or thymine, respectively. This deamination is only a 1 Da difference in mass.

OPTIMAL ANALYTICAL STRATEGY TO DETERMINE OLIGONUCLEOTIDE RELATED SUBSTANCES

Presently, the combination of IP-HPLC-UV-MS is required to obtain a full overview of all related substances of phosphorothioated oligonucleotides, as illustrated in Figure 1. LC-UV (large blue outlined peak) is sufficient to determine deletion sequence impurities up to $n-2$, addition sequence impurities from $n+2$, and their related emerging impurities. LC-MS (colorful peaks inside the large blue UV peak) is required to determine the coeluting impurities of the parent compound (n), i.e. mono-PO, n, $n-1$, $n+1$, n + protection groups.

REPORTING THRESHOLDS FOR RELATED SUBSTANCES OF THERAPEUTIC OLIGONUCLEOTIDES

Currently there is no generally accepted reporting threshold for oligonucleotide therapeutics. Capaldi et al. cautiously used a reporting threshold for related substances of therapeutic oligonucleotides based on small molecule and peptide drugs.[32] As listed in Table 1, for most small-molecule drugs, the reporting threshold is set to 0.05% (ICH Q3A and Q3B) and for impurities in peptides set at 0.1% (Pharmacopeia). For setting the reporting threshold, it must also be taken into account that most oligonucleotide impurities comprise a mixture of several components, so the reporting threshold applies not to a single impurity, but rather to a group of several related components. Therefore, when the decision is made to disregard an impurity, the individual components are present at only a fraction of the reporting threshold.[32] Moreover, it is important to realize that therapeutic oligonucleotides have a relatively high molecular weight and thus, in comparison to small molecules, much less molecules per weight unit are present, as illustrated in Table 2. Based on the facts that related compounds are mixtures of relatively high molecular weight, a reporting threshold of 0.2% was considered to be attainable in the majority of cases.[32]

SYNTHESIS IMPURITIES

Related substances of therapeutic oligonucleotides originate from the synthetic route and its chemical transformations, starting materials, and its impurities and degradation routes.[32]

To start with the first item, related substances of phosphorothioate oligonucleotides arising from synthesis and manufacturing can be generally subdivided in three classes: (i) shorter chain length (deletion sequences), (ii) sequences of longer chain length (addition sequences), and (iii) modified full length impurities. An overview of the general synthesis impurities are provided in Table 3.

DELETION SEQUENCES

The most common impurities found in oligonucleotides include the $n-1$ deletion sequences ($n-x$).[33−35] Deletion sequences, also called shortmers, typically arise from incomplete base coupling at the extending 5′-end followed by a failed capping reaction. Any residual 5′-hydroxyl containing material is capped as an isobutyryl ester prior to the detritylation step and thus cannot participate in further chain elongation: $n-x$ species are formed as a consequence. Incomplete capping can also lead to carryover of a deletion sequence that will be further chain extended and thus contributes to the so-called $n-1$ impurity, which is a mixture of small amounts of materials with different single internal deletions plus material arising from incomplete coupling in the final solid-phase synthesis cycle. Although thorough process development work leads to a robust scalable manufacturing process, nonetheless, reactions do not all reach 100%, and impurities generated by this nonperfect process accumulate throughout the synthesis. Fortunately, the use of anion-

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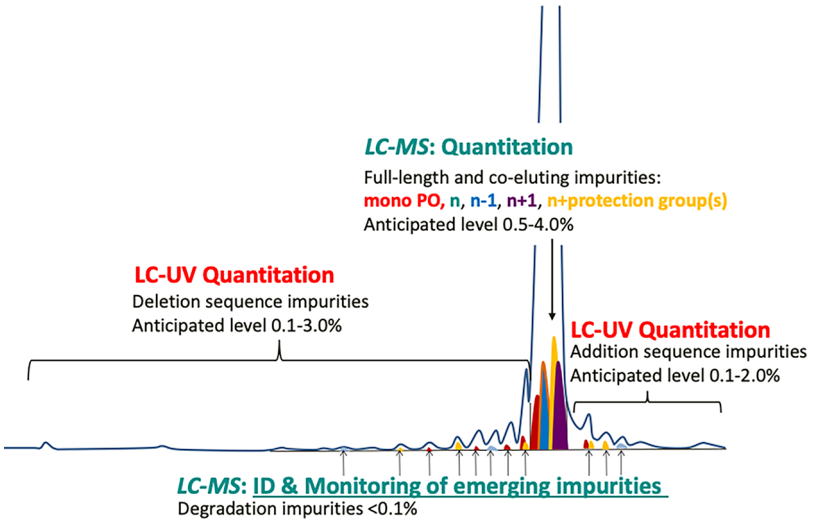

Figure 1. Analytical strategy to comprehensively determine related substances of therapeutic oligonucleotides using LC-UV-MS.

Table 2. Concentration Determination of ICH Q3A Thresholds for Oligonucleotide Impurities from the Given ICH 0.15% for Small Molecule vs Two Different Length Oligonucleotides

Impurity qualification threshold (Q3A)				
type of molecule	MW (Da)	%	mg	μM
small molecule	700	0.15	1.0 mg	1.43
oligonucleotide 18-mer	7000	0.15	1.0 mg	0.14
oligonucleotide 30-mer	11 000	0.15	1.0 mg	0.09

Table 3. General Scheme for Oligonucleotide Synthesis with Possible Side Product Impurities and Their Type of Related Substance

Step in synthesis	Side product	Type of related substance
deprotection DMT	$n - x$ ($n - 1$, $n - 2$, etc.)	deletion sequences
	depurination	full length
coupling	$n + x$ ($n + 1$, $n + 2$, etc.)	addition sequences
	impurity in amidite	+PS
capping	$n - x$	deletion sequences
sulfurization	PO	full length
	DMT phosphonate	longmers ($n + x$)
	$n - x$	deletion sequences
final cleavage and deprotection	protecting group remaining (CNEt)	addition sequences
	incomplete cleavage linker (Unylinker)	+PS

exchange purification enables removal of most of the shorter oligonucleotide impurities, whereas removal of $n - 2$ and $n - 1$ impurities is more challenging, particularly for longer therapeutic oligonucleotides having a full phosphorothioate backbone. LC-MS analysis of process development batches as well as the nonclinical and clinical batches of DS and stability samples show, in many cases, measurable levels of $n - x$ impurities down to $n - 20$.

MODIFIED FULL LENGTH IMPURITIES

Capaldi et al.[32] listed several impurities from modification at a single internucleotide linkage, i.e. phosphate diester impurity in phosphorothioate diester oligonucleotides,[36] trichloroacetaldehyde modified oligonucleotides,[37] C-phosphonate oligonucleotides,[38] ethylene phosphodiester impurities,[39] and phosphorodithioate diester impurities in phosphorothioate diester oligonucleotides.[40]

Modified full length impurities have the correct length but contains one or more modified nucleobases or phosphorothioate linkages.

Among others, several impurities with modified nucleobases were also listed by Capaldi:[32]

- Oligonucleotides containing a urea- or aminofuran residue in place of a pyrimidine nucleotide.[41] Oligonucleotides containing a spiroiminodihydantoin[42] (Hill KW, 2007, DIA Conference, Washington, DC) or isobutyryldiaminopurine in place of guanine,[43,44] 5-amino-4-pyrimidinylimidazole in place of adenine,[43,45] and oxopropylimidazopyrimidinone in place of cytosine.[39]
- Addition of acrylonitrile on the N^3 of thymine[46] or uridine (N+CNEt, characterized by a +53 Da peak in mass spectra) during deprotection, see Figure 2a above for uridine.
- Incomplete deprotection of the N^2-isobutyryl groups on the guanine residues (characterized by a + 70 Da peak in mass spectra).
- N+isobutyryl and N+benzoyl denote incompletely deprotected species that contain a residual N^2-isobutyrylguanosine and a residual N^6-benzoyladenosine, respectively. N^2-isobutyrylguanosine can be subsequently converted into an acetyl-modified diaminopurine moiety or an N^2-acetyl-2,6-diaminopurine impurity during the capping reaction.[37,44]
- The presence of trace amounts of trichloroacetaldehyde (chloral) in dichloroacetic acid used in the detritylation steps in the solid-phase synthesis can lead to a full-length oligonucleotide impurity that is a family of species each with a single different modified internucleotidic linkage and observed as a +148 Da peak in the MS. The modified linkage is illustrated in Figure 2b.
- Among the other classes of impurities, several different types can occur at the base residues, such as deamination or depurination of adenine, cytosine, or guanine residues, which can be induced by thermal, basic, and

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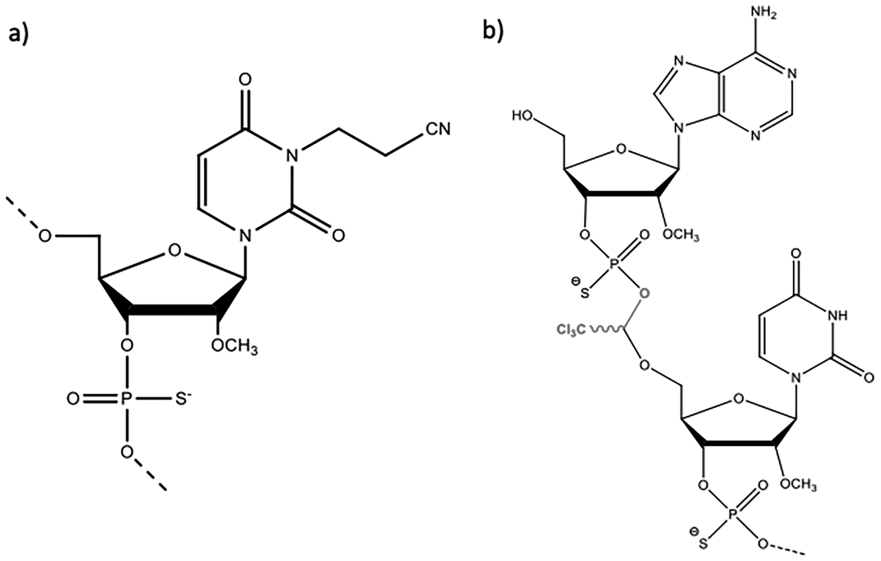

Figure 2. (a) Structure of an internal N^3-(2-cyanoethyl)-2′-*O*-methyluridine residue. (b) Structure of a modified internucleotide linkage caused by the presence of chloral in dichloroacetic acid and illustrated between a 2′-*O*-methyladenosine and a 2′-*O*-methyluridine.

acidic conditions;[9,32] and (iii) oxidation, primarily occurring at guanine residues.[32,42]

- A special category of modified full-length impurity is represented by a variant that arises by incomplete sulfurization and/or possible side reactions related to the reagent used,[47] partial oxidation during the solid-phase synthesis, or by partial desulfurization during the deprotection step.[36] This leads to a mono-phosphodiester variant (mono-PO) of phosphorothioate oligonucleotides, characterized by $n - 16$ Da peak in the MS and comprises a mixture of n (n = number of nucleotide residues in oligo) individual species. This variant can be (partially) resolved from the full phosphorothioate species by analytical anion-exchange HPLC (IEX-HPLC). On IPRP-HPLC, this species conveniently coelutes with the nonmodified species and is classified as part of the active API. Phosphorus NMR spectroscopy gives an estimate of the level of the mono-PO variant.

■ ADDITION SEQUENCES

Addition sequences ($n + x$) or longmers are produced during the coupling step, whereby the incoming phosphoramidite species is added twice during a single coupling step. Addition sequences are mostly restricted to $n + 1$ and a small amount of $n + 2$.[48] Because these impurities elute just after the full-length product, they are, depending on their length, less or more difficult to remove by anion-exchange chromatography.

■ DEGRADATION IMPURITIES

As mentioned above, chemically modified oligonucleotides are usually stable at standard *in vitro* and *in vivo* conditions.[3] The most observed degradation impurities during shelf life studies are mono-PO oxidation products of n and deletion and addition sequences that can already present after solid-phase synthesis.[36] This was confirmed by stress testing with hydrogen peroxide, where oxidation of the phosphorothioate linkage PO analogues was determined to be the main degradation pathway. At higher concentrations of hydrogen peroxide, deletion sequences, depurination products, terminal phosphorothioates, ribose, ribophosphorothioates, and phosphoribophosphorothioates were also observed.[3,49]

Several forced degradation studies have been performed to determine the stability of oligonucleotides at different pH conditions.[3,49−51] For four phosphorothioate oligonucleotides (1 deoxyribose, and 3 with MOE 2′ ribose chemistry and methyl cytosine modifications), it was determined that at acidic pH values, significant degradation was observed. For pH 1.5 within 1 h, all full-length products were completely degraded, whereas for pH 3.0, degradation took more than 48 h.[3] For pH 5.0, 7.0, 9.0, and 11.0, the full-length products were degraded by less than 20% within 48 h for all four oligonucleotides.[3] Acid treatment of DNA oligonucleotides resulted in loss of adenine and guanine followed by the addition of water.[49] In contrast, acid hydrolysis of oligonucleotides containing a mixture of deoxy- and 2′-MOE or 2′-OH and 2′-modified nucleotides (2′-OMe, 2′- F) at pH 2−3 at room temperature for several hours did not result in the cleavage of the internucleotide linkages.[50,51] This was confirmed by Pourshahian et al., who demonstrated that 2′-OH, 2′-MOE, 2′- OMe, and 2′-F modified residues made oligonucleotides resistant to acid treatment.[49]

El Zahar et al. observed that the sequence of oligonucleotides may have an impact on the rate of the degradation: both a higher pyrimidine content and the presence of sequences of AT increased the rate of degradation.[3] Finally, Shishkina and Johnson reported that oligonucleotides are susceptible to degradation during evaporation to dryness.[52]

■ CONCLUSIONS

To properly analyze therapeutic oligonucleotides, a combination of the techniques and strategies proposed here must be used. Adequate chromatographic resolution and MS sensitivity must be achieved to quantitate the impurities mentioned above. The constant trade-off between chromatographic resolution and mass spectrometry sensitivity remains a big challenge in LC-MS analysis of oligonucleotides. Ion-pair chromatography has been the most popular technique, but alternative approaches such as anion exchange and HILIC

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methods have presented unique results for specific applications.

Here, we describe several of the issues with mass spectrometry related to oligonucleotides. There is a dire need for increased sensitivity and resolution for routine analysis. Perhaps different mobile phase compositions can accomplish this without a need for significant instrumental improvements. Mobile phases can influence the charge state distribution, salt adduction, and the major ionization pathway.

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Notes

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REFERENCES

(1) Tamm, I.; Dorken, B.; Hartmann, G. Antisense therapy in oncology: new hope for an old idea? *Lancet* **2001**, *358*, 489−497.

(2) Li, N.; El Zahar, N. M.; Saad, J. G.; van der Hage, E. R. E.; Bartlett, M. G. Alkylamine ion pairing reagents and the chromatographic separation of oligonucleotides. *Journal of chromatography. A* **2018**, *1580*, 110−119.

(3) El Zahar, N. M.; Magdy, N.; El-Kosasy, A. M.; Bartlett, M. G. Chromatographic approaches for the characterization and quality control of therapeutic oligonucleotide impurities. *Biomed. Chromatogr.* **2018**, *32*, e4088.

(4) Mansoor, M.; Melendez, A. J. Advances in Antisense Oligonucleotide Development for Target Identification, Validation, and as Novel Therapeutics. *Gene Regul. Syst. Biol.* **2008**, *2*, 275−295.

(5) Wilson, C.; Keefe, A. D. Building oligonucleotide therapeutics using non-natural chemistries. *Curr. Opin. Chem. Biol.* **2006**, *10*, 607−614.

(6) Wang, L.; Meng, M.; Reuschel, S. Regulated bioanalysis of oligonucleotide therapeutics and biomarkers: qPCR versus chromatographic assays. *Bioanalysis* **2013**, *5*, 2747−2751.

(7) Fritz, H. J.; Belagaje, R.; Brown, E. L.; Fritz, R. H.; Jones, R. A.; Lees, R. G.; Khorana, H. G. Studies on polynucleotides. 146. High-pressure liquid chromatography in polynucleotide synthesis. *Biochemistry* **1978**, *17*, 1257−1267.

(8) Basiri, B.; van Hattum, H.; van Dongen, W. D.; Murph, M. M.; Bartlett, M. G. The Role of Fluorinated Alcohols as Mobile Phase Modifiers for LC-MS Analysis of Oligonucleotides. *J. Am. Soc. Mass Spectrom.* **2017**, *28*, 190−199.

(9) Roussis, S. G.; Pearce, M.; Rentel, C. Small alkyl amines as ion pair reagents for the separation of positional isomers of impurities in phosphate diester oligonucleotides. *Journal of chromatography. A* **2019**, *1594*, 105−111.

(10) Apffel, A.; Chakel, J. A.; Fischer, S.; Lichtenwalter, K.; Hancock, W. S. Analysis of Oligonucleotides by HPLC-Electrospray Ionization Mass Spectrometry. *Anal. Chem.* **1997**, *69*, 1320−1325.

(11) Chen, B.; Mason, S.; Bartlett, M. The Effect of Organic Modifiers on Electrospray Ionization Charge-State Distribution and Desorption Efficiency for Oligonucleotides. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 257−264.

(12) Jiang, T.; Yu, N.; Kim, J.; Murgo, J.-R.; Kissai, M.; Ravichandran, K.; Miracco, E. J.; Presnyak, V.; Hua, S. Oligonucleotide Sequence Mapping of Large Therapeutic mRNAs via Parallel Ribonuclease Digestions and LC-MS/MS. *Anal. Chem.* **2019**, *91*, 8500−8506.

(13) Lobue, P. A.; Jora, M.; Addepalli, B.; Limbach, P. A. Oligonucleotide analysis by hydrophilic interaction liquid chromatography-mass spectrometry in the absence of ion pair reagents. *Journal of chromatography. A* **2019**, *1595*, 39−48.

(14) McGinnis, A. C.; Cummings, B. S.; Bartlett, M. G. Ion exchange liquid chromatography method for the direct determination of small ribonucleic acids. *Anal. Chim. Acta* **2013**, *799*, 57−67.

(15) Husser, C.; Brink, A.; Zell, M.; Müller, M. B.; Koller, E.; Schadt, S. Identification of GalNAc-Conjugated Antisense Oligonucleotide Metabolites Using an Untargeted and Generic Approach Based on High Resolution Mass Spectrometry. *Anal. Chem.* **2017**, *89*, 6821−6826.

(16) Ahmad, I. A. H.; Blasko, A.; Clarke, A.; Fakih, S. Two-Dimensional Liquid Chromatography (2D-LC) in Pharmaceutical Analysis: Applications Beyond Increasing Peak Capacity. *Chromatographia* **2018**, *81*, 401−418.

(17) Biba, M.; Jiang, E.; Mao, B.; Zewge, D.; Foley, J. P.; Welch, C. J. Factors influencing the separation of oligonucleotides using reversed-phase/ion-exchange mixed-mode high performance liquid chromatography columns. *Journal of Chromatography A* **2013**, *1304*, 69−77.

(18) Chen, B.; Bartlett, M. G. Evaluation of mobile phase composition for enhancing sensitivity of targeted quantification of oligonucleotides using ultra-high performance liquid chromatography and mass spectrometry: Application to phosphorothioate deoxyribonucleic acid. *Journal of Chromatography A* **2013**, *1288*, 73−81.

(19) McGinnis, A. C.; Grubb, E. C.; Bartlett, M. G. Systematic optimization of ion pairing agents and hexafluoroisopropanol for enhanced electrospray ionization mass spectrometry of oligonucleotides. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 2655−2664.

(20) Basiri, B.; Murph, M. M.; Bartlett, M. G. Assessing the Interplay between the Physicochemical Parameters of Ion-Pairing Reagents and the Analyte Sequence on the Electrospray Desorption Process for Oligonucleotides. *J. Am. Soc. Mass Spectrom.* **2017**, *28*, 1647−1656.

(21) Sutton, J. M.; Bartlett, M. G. Modeling Cationic Adduction of Oligonucleotides Using Electrospray Desorption Ionization. *Rapid Commun. Mass Spectrom.* **2020**, *34*, No. e8696.

(22) Wang, L.; Meng, M.; Reuschel, S. Regulated bioanalysis of oligonucleotide therapeutics and biomarkers: qPCR versus chromatographic assays. *Bioanalysis* **2013**, *5*, 2747.

(23) van Dongen, W. D.; Niessen, W. M. A. Bioanalytical LC-MS of therapeutic oligonucleotides. *Bioanalysis* **2011**, *3*, 541−564.

(24) Nguyen, S.; Fenn, J. B. Gas-phase ions of solute species from charged droplets of solutions. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 1111.

(25) Kebarle, P.; Peschke, M. On the mechanisms by which the charged droplets produced by electrospray lead to gas phase ions. *Anal. Chim. Acta* **2000**, *406*, 11−35.

(26) Hogan, C. J.; Carroll, J. A.; Rohrs, H. W.; Biswas, P.; Gross, M. L. Combined Charged Residue-Field Emission Model of Macromolecular Electrospray Ionization. *Anal. Chem.* **2009**, *81*, 369−377.

(27) Fernandez de la Mora, J. Electrospray ionization of large multiply charged species proceeds via Dole's charged residue mechanism. *Anal. Chim. Acta* **2000**, *406*, 93−104.

(28) Fenn, J. B. Ion formation from charged droplets: Roles of geometry, energy, and time. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 524−535.
(29) Iribarne, J. V.; Thomson, B. A. On the evaporation of small ions from charged droplets. *J. Chem. Phys.* **1976**, *64*, 2287−2294.
(30) Metwally, H.; Duez, Q.; Konermann, L. Chain Ejection Model for Electrospray Ionization of Unfolded Proteins: Evidence from Atomistic Simulations and Ion Mobility Spectrometry. *Anal. Chem.* **2018**, *90*, 10069−10077.
(31) Weng, G.; Liu, Z.; Chen, J.; Wang, F.; Pan, Y.; Zhang, Y. Enhancing the Mass Spectrometry Sensitivity for Oligonucleotide Detection by Organic Vapor Assisted Electrospray. *Anal. Chem.* **2017**, *89*, 10256−10263.
(32) Capaldi, D.; Teasdale, A.; Henry, S.; Akhtar, N.; den Besten, C.; Gao-Sheridan, S.; Kretschmer, M.; Sharpe, N.; Andrews, B.; Burm, B.; Foy, J. Impurities in Oligonucleotide Drug Substances and Drug Products. *Nucleic Acid Ther.* **2017**, *27*, 309−322.
(33) Chen, D.; Yan, Z.; Cole, D. L.; Srivatsa, G. S. Analysis of internal (n-1)mer deletion sequences in synthetic oligodeoxyribonucleotides by hybridization to an immobilized probe array. *Nucleic Acids Res.* **1999**, *27*, 389−395.
(34) Fearon, K. L.; Stults, J. T.; Bergot, B. J.; Christensen, L. M.; Raible, A. M. Investigation of the 'n-1' impurity in phosphorothioate oligodeoxynucleotides synthesized by the solid-phase beta-cyanoethyl phosphoramidite method using stepwise sulfurization. *Nucleic Acids Res.* **1995**, *23*, 2754−2761.
(35) Temsamani, J.; Kubert, M.; Agrawal, S. Sequence identity of the n-1 product of a synthetic oligonucleotide. *Nucleic Acids Res.* **1995**, *23*, 1841−1844.
(36) Bergot, B. J.; Egan, W. Separation of synthetic phosphorothioate oligodeoxynucleotides from their oxygenated (phosphodiester) defect species by strong-anion-exchange high-performance liquid chromatography. *Journal of Chromatography A* **1992**, *599*, 35−42.
(37) Gaus, H.; Olsen, P.; Sooy, K. V.; Rentel, C.; Turney, B.; Walker, K. L.; McArdle, J. V.; Capaldi, D. C. Trichloroacetaldehyde modified oligonucleotides. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4118−4124.
(38) Capaldi, D. C.; Gaus, H. J.; Carty, R. L.; Moore, M. N.; Turney, B. J.; Decottignies, S. D.; McArdle, J. V.; Scozzari, A. N.; Ravikumar, V. T.; Krotz, A. H. Formation of 4,4′-dimethoxytrityl-C-phosphonate oligonucleotides. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4683−4690.
(39) Rentel, C.; Wang, X.; Batt, M.; Kurata, C.; Oliver, J.; Gaus, H.; Krotz, A. H.; McArdle, J. V.; Capaldi, D. C. Formation of Modified Cytosine Residues in the Presence of Depurinated DNA. *J. Org. Chem.* **2005**, *70*, 7841−7845.
(40) Capaldi, D. C. Detecting and controlling oligonucleotide impurities. In *Podium Presentation at 14th Annual EuroTides Conference*, Berlin, Germany, November 17−19, 2014.
(41) Hill, K. W. Manufacturing oligonucleotide API's: identification of process-related impurities. In *Podium Presentation at DIA/FDA 1st Oligonucleotide-Based Therapeutics Conference*, Bethesda, MD, April 19−20, 2007.
(42) Fleming, A. M.; Muller, J. G.; Dlouhy, A. C.; Burrows, C. J. Structural context effects in the oxidation of 8-oxo-7,8-dihydro-2′-deoxyguanosine to hydantoin products: electrostatics, base stacking, and base pairing. *J. Am. Chem. Soc.* **2012**, *134*, 15091−15102.
(43) Rodriguez, A. A.; Cedillo, I.; McPherson, A. K. Conversion of adenine to 5-amino-4-pyrimidinylimidazole caused by acetyl capping during solid phase oligonucleotide synthesis. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 3468−3471.
(44) Rodriguez, A. A.; Cedillo, I.; Mowery, B. P.; Gaus, H. J.; Krishnamoorthy, S. S.; McPherson, A. K. Formation of the N(2)-acetyl-2,6-diaminopurine oligonucleotide impurity caused by acetyl capping. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3243−3246.
(45) Lorenz, S.; Przybytek, J.; Snobel, K. Synthesis of oligonucleotides or phosphorothioate oligonucleotides with a capping reagent of N-methylimidazole free of 1,3,5-trimethylhexahydro-1,3,5-triazine. Patent WO WO2009052034, 2009.
(46) Capaldi, D. C.; Gaus, H.; Krotz, A. H.; Arnold, J.; Carty, R. L.; Moore, M. N.; Scozzari, A. N.; Lowery, K.; Cole, D. L.; Ravikumar, V. T. Synthesis of High-Quality Antisense Drugs. Addition of Acrylonitrile to Phosphorothioate Oligonucleotides: Adduct Characterization and Avoidance. *Org. Process Res. Dev.* **2003**, *7*, 832−838.
(47) Xu, Q.; Musier-Forsyth, K.; Hammer, R. P.; Barany, G. Use of 1,2,4-dithiazolidine-3,5-dione (DtsNH) and 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) for synthesis of phosphorothioate-containing oligodeoxyribonucleotides. *Nucleic Acids Res.* **1996**, *24*, 1602−1607.
(48) Krotz, A. H.; Klopchin, P. G.; Walker, K. L.; Srivatsa, G. S.; Cole, D. L.; Ravikumar, V. T. On the formation of longmers in phosphorothioate oligodeoxyribonucleotide synthesis. *Tetrahedron Lett.* **1997**, *38*, 3875−3878.
(49) Pourshahian, S. Therapeutic oligonucleotides, impurities, degradants, and their characterization by mass spectrometry. *Mass Spectrom. Rev.* **2019** DOI: 10.1002/mas.21615.
(50) Calvitt, C. J.; Levin, D. S.; Shepperd, B. T.; Gruenloh, C. J. Chemistry at the 2′ position of constituent nucleotides controls degradation pathways of highly modified oligonucleotide molecules. *Oligonucleotides* **2010**, *20*, 239−251.
(51) Capaldi, D. C. *Stress testing of oligonucleotides*; Baertschi, S. W., Alsante, K. M., Reed, R.A., Eds.; CRC Press: Boca Raton, FL, 2011; pp 391−425.
(52) Shishkina, I. G.; Johnson, F. A New Method for the Postsynthetic Generation of Abasic Sites in Oligomeric DNA. *Chem. Res. Toxicol.* **2000**, *13*, 907−912.