Table 7: Best Practices for Analyzing Oligonucleotides Using MS

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Scope:

The use of LC-MS, particularly ion-pair (IP) LC-MS, has become more widespread in recent years in the analysis of therapeutic oligonucleotides. However, many challenges remain, including; hurdles in the implementation of MS in a QC environment, inadequate MS resolution to resolve structurally closely related impurities and insufficient MS sensitivity to profile the entire complement of impurities. Thus, establishing best practices for MS analysis of oligonucleotides and identifying the optimal protocols for data interpretation and reporting is a key element of quality control of oligo-based therapeutics. The focus of this roundtable will be on the use of MS to analyze synthetic oligonucleotides and enhanced capability to identify complex impurities resulting from oligo synthesis. This roundtable aims to discuss the practical application of MS in QC and manufacturing environments and to examine the technical aspects desired for obtaining, interpreting, and reporting product-related impurity data.

Questions for Discussion:

1. What are the challenges associated with implementing mass spectrometry in a QC environment? What release tests can MS be used for?
2. What are the challenges associated with implementing mass spectrometry in a manufacturing environment?
3. Is implementation of high-resolution accurate mass (HRAM) mass spectrometry beneficial? Technical and regulatory hurdles?
4. What are the best practices for analyzing product-related oligo impurities (structurally closely related components) using MS? At which steps are you assessing impurity identification and/or impurity profiling or quantification? How to address the abundance difference between API and impurities? (e.g., fractionation?) Is it necessary to differentiate composite impurities (e.g., positional isomers)?
5. How do you handle impurity datasets? What software do you use to process impurity data? How do you report impurity data?
6. What are the best practices for analyzing hybrid nucleic acid molecules (small-molecule-conjugated oligos and peptide nucleic acid constructs) using MS?

Discussion Notes:

1. What are the challenges associated with implementing mass spectrometry in a QC environment for assessing therapeutic oligonucleotides?
    - How to validate a QC MS based assay?
    - System suitability?
    - What release tests can MS be used for?

    Many impurities can be tested for using LC-UV as this is easier to implement, more robust, less expensive. Some were concerned with minor impurities under UV peak. MS ion source-related fragmentation creates a possibility of false positive detection of impurities, which is avoided with UV-only detection.

2. What are the challenges associated with implementing mass spectrometry in a manufacturing environment?
    - In process monitoring and process improvement?
    - In in-line testing?
    - What product quality attributes (PQA) or critical quality attributes (CQA) can MS monitor?

    Some impurities can be related to synthesis processes or storage.

    Quality concerns and batch-to-batch comparisons are currently driven more by toxicology and bioactivity assays and less by analytical measurements.

3. Is implementation of high-resolution accurate mass (HRAM) mass spectrometry beneficial?
    - Advantages?
    - Disadvantages?
    - Technical and regulatory hurdles?

    Reasons for avoiding high resolution MS are high cost associated with high-end instrumentation and lack of obvious demonstrated need. Patient safety issues have been infrequent for oligonucleotide drugs, which may only be related to the low number of oligo-based drugs being tested in clinical settings.

    LC-MS of oligos hasn't progressed much in recent years. Most RP and anion exchange columns currently on the market have been designed and optimized for proteins.

    MALDI is a low-resolution technique for QC of small oligos which offers the benefit of being very fast. MALDI negative mode is best for MS1 screening. Positive mode combined with in-source decay is best for oligo sequencing.

    For very large oligos, including mRNA-related molecules, there are additional challenges. Low resolution MS such as MALDI may not provide enough detail or any useable data. High resolution MS instruments such Thermo Orbitrap UHMR are very expensive. In addition to high mass range requirements, desalting or metal adducts presents a massive challenge to analysis of very large oligos.

4. What are the best practices for analyzing product-related oligo impurities (structurally closely related components) using MS?
    - At which steps are you assessing impurity identification and/or impurity profiling or quantification? (e.g., final product, in process samples)
    - How to address the abundance difference between API and impurities? (e.g., fractionation?)
    - How to address coeluting impurities?
    - Is it necessary to differentiate composite impurities (e.g., positional isomers)?

    Current software solutions need more flexibility to adapt to industry needs. Current and future software should support custom modifications of sugars on bases, API modifications, and synthesis-related impurities (e.g., N – 1).

    Anion exchange and reversed phase with ion pairing are two popular techniques for impurity analysis of dsDNA, ssDNA, and ssRNA oligos up to 25 bp in length. Double stranded (duplex)

oligos can be denatured to allow analysis of separated strands. Heat and pH can be used to create denaturing conditions for LC separation. These harsh(er) LC conditions can be combined with high(er) ion source energy conditions to further separate duplexed strands which are coeluting.

Sodium adducts can be very tricky. Care should be taken to avoid additional sodium, potassium, or other common metals in sample preparation as these adducts are highly visible in mass spectra and not easily removed with any desalting techniques.

5. How do you handle impurity datasets?
    - What software do you use to process impurity data? (e.g., impurity identification (molecular mass only or including sequencing?), product-specific impurity database?)
    - How do you report impurity data? (e.g., individual impurity abundances, grouping by formation mechanism, grouping by retention time range)

An analytical pipeline can be quite robustly designed by setting a UV peak threshold for impurities, which will only trigger follow-up by LC-MS analysis when an unknown peak (e.g., > 0.5% relative abundance level) is detected in significant quantities.

MS/MS sequence can be confirmed using vendor-supported GMP-compliant solutions such as Waters Synapt with UNIFI software or Thermo Q Exactive + Novatia's Promass software. Water's UNIFI software allows integrated workflow for acquisition through data analysis. Similarly, Thermo's Chromeleon software is a newer GMP-compliant software which allows deconvolution, acquisition, and analysis.

There is currently a lack of vendor-supported options for MS/MS analysis of short < 25 bp synthetic oligo sequences. Mongo Oligo software has existed for many years and can aid in manual analysis of oligo sequence MS/MS. Protein Metrics and Thermo (BioPharma Finder software) may have these features soon.

6. What are the best practices for analyzing hybrid nucleic acid molecules (small-molecule-conjugated oligos and peptide nucleic acid (PNA) constructs) using MS?

MS/MS fragmentation nomenclature is a difficult aspect for vendors in creating a comprehensive software platform for oligo analysis. Pharmaceutical research has created a rapidly evolving chemical space, resulting in countless possibilities for custom chemical modifications of classical nucleotide bases as well creation of PNAs and new oligo-related moieties.

Custom software is likely needed as a solution for customized/specialized oligo chemistries, and would likely require some degree of customization of MS/MS fragmentation nomenclature, such as Agilent" oligo analysis software.