Fast acquisition of DDA-MS/MS of oligonucleotides and the novel spectral merging algorithm for better assignment of sequence information

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Overview

- There are inherent challenges in developing oligonucleotide therapeutics given the large number of nucleic acid chemistries, delivery technologies and therapeutic modalities. Characterising synthetic oligonucleotide therapeutics requires complete workflows integrating LC-MS/MS analysis and data processing.
- In this work, we present a novel algorithm to help accelerate the characterization of oligonucleotide products and impurities by enhancing the processing of DDA-MS/MS high resolution mass spectra.
- The outcome is an enhanced sequence coverage and high data quality in oligonucleotide characterization.

1. Introduction

Oligonucleotides are nucleic acid polymers with the potential to change the treatment for a wide range of diseases, with several oligonucleotide drugs recently gaining approval by regulatory bodies. However, developing oligonucleotide therapeutics is not without significant challenges and a barrier for success is to find new ways to achieve an efficient delivery to specific target organs and tissues (other than the liver). A common strategy to increase the effectiveness of drug delivery is to include chemical modifications. Whilst there has been significant advances in solid phase oligomerization chemistry, characterizing synthetic oligonucleotides still presents obstacles in achieving complete sequence coverage for the full-length product (FLP) and impurities. To help accelerate data review and increase data quality a novel algorithm for DDA-MS/MS processing has been applied to analysis of Inotersen (antisense oligonucleotide therapeutic drug) and synthetic impurities.

2. Materials and Methods

2.1 LC-MS/MS method

The LC-MS/MS method was applied to the analysis of full-length product impurities in a sample of Inotersen [2'-O-(2-methoxyethyl) (2'-MOE) antisense oligonucleotide therapeutic]; C230 H318 N69 O121 P19 S19; monoisotopic mass 7179.06324].

LC Separation and Detection.

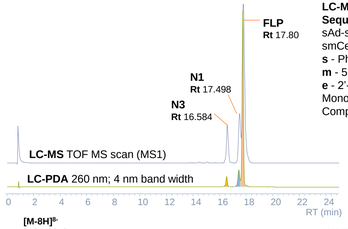
- **Inert LC system**. Shimadzu Nexera™ XS inert ultra-high performance liquid chromatograph (bioinert flow path).
- Shimadzu C18 column (Shim-pack Scepter Claris C18-120 2.1x100 mm; the column has a bioinert coating is applied to the column body and stainless-steel frit to reduce hydrophilic oligonucleotide adsorption).
- Binary gradient; water and water/methanol = 1:1, both containing 100 mM HFIP and 10 mM TEA; flow rate 0.3 mL/min.

- **Mass Spectrometry Detection**. Shimadzu QTOF LCMS™-9050 using external mass calibration. Data acquired in profile mode.
  - MS mass scan m/z 550-2500; 400 msecs; negative ion mode.
  - DDA-MS/MS mass scans m/z 100-2800; collision energy spread 10-75V.
  - DDA-MS/MS; 10 dependent MS/MS mass scans (no exclusion or inclusion list); precursor intensity threshold set to 1000; 100 msecs for each mass scan.

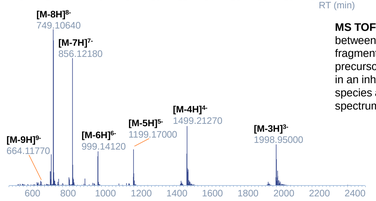
3. Results

3.1 Data processing

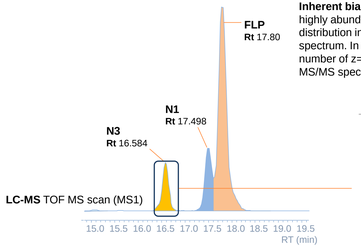
Conventional multiply charged data processing workflows. Processing MS TOF scan and precursor triggered DDA-MS/MS mass scan data.



LC-MS analysis of inotersen
Sequence 5'-Te-smCe-sTe-sTe-sGe-sGd-sTd-sTd-sAd-smCd-sAd-sTd-sGd-sAd-sAd-sAe-sTe-smCe-smCe-smCe-3'
s - Phosphorothioate backbone linker
m - 5-methyl
e - 2'-O-(2-MethoxyEthoxy)
Monoisotopic mass; 7178.05597
Components; FLP, N-1(C2:C20), N-3(T4:C20)=10:1:1



MS TOF scan for N-3 of inotersen; charge states between -3 and -9 are observed. MS/MS fragmentation is triggered for the most abundant precursor ions in the TOF MS mass scan resulting in an inherent bias toward more abundant ion species and a less comprehensive MS/MS spectrum.



Inherent bias in DDA-MS/MS for N-3 of inotersen; highly abundant charge states will bias the spectrum distribution in a weighted averaged DDA-MS/MS spectrum. In this example, the N-3 has a higher number of z=7,8 charges in the averaged DDA-MS/MS spectrum:

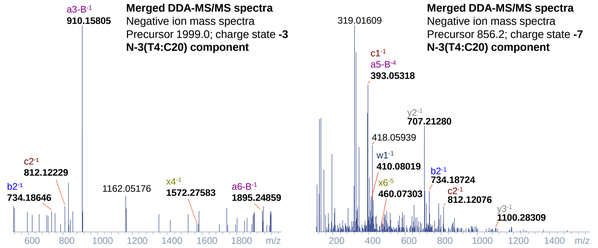
Charge state	#Fragment spectra
[M-8H]8-	25
[M-7H]7-	25
[M-6H]6-	12
[M-5H]5-	11
[M-4H]4-	14
[M-3H]3-	12

Figure 1. The separation of inotersen and two impurities N-1(C2:C20):N-3(T4:C20)=10:1:1 using the inert LC system with a PDA detector and high resolution QTOF mass spectrometer. N-3 of inotersen has a monoisotopic mass of 5996.86006 resulting in multiple charge states between z=3 to z=9 with different ion intensities for each charge state. As DDA-MS/MS precursor selection is stochastic in nature there is a bias towards the higher intensity charge state (z=3) and will lead to a loss of information particularly for low abundant charge states.

The merged spectrum algorithm has been developed to help reduce the impact of a weighted average spectrum in characterizing oligonucleotide products and impurities to enhance sequence coverage.

3.2 Merged spectrum processing

As an alternative to processing an averaged spectrum in determining sequence coverage, a novel algorithm has been developed for merging DDA-MS/MS spectrum data. The algorithm considers every fragment ion in every DDA-MS/MS mass scan for all charge states and registers the most intense value into a merged spectrum. This approach differs from an averaged DDA-MS/MS mass spectrum which is inherently biased to charge states with a higher signal intensity resulting in a weighted MS/MS spectra.



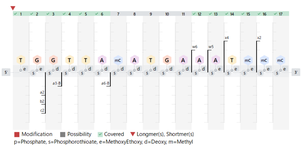
Merged DDA-MS/MS spectra
Negative ion mass spectra
Precursor 1999.0; charge state **-3**
N-3(T4:C20) component

Merged DDA-MS/MS spectra
Negative ion mass spectra
Precursor 856.2; charge state **-7**
N-3(T4:C20) component

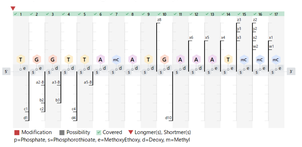
Figure 2. Merged DDA-MS/MS spectrum for N-3(T4:C20) [Rt 16.584 mins] for two separate charge states, z=3 and z=7 highlighting the marked difference in fragment ion distributions.

In the example shown above, the DDA-MS/MS spectra have been generated by applying a charge state filter in two separate data acquisitions specific to each charge state with a collision energy spread set to 30-50 V. The sequence coverage for two separate charge states are shown below.

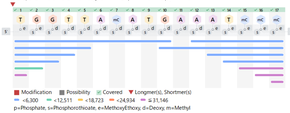
Branch mode display
Sequence coverage N-3(T4:C20); **z=3**



Branch mode display
Sequence coverage N-3(T4:C20); **z=7**



Fill mode display
Sequence coverage N-3(T4:C20); **z=3**



Fill mode display
Sequence coverage N-3(T4:C20); **z=7**

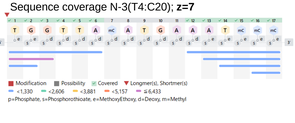
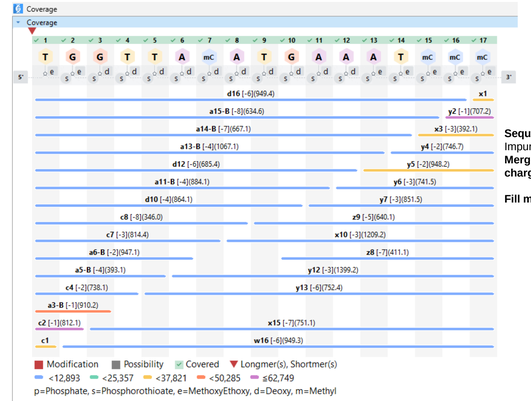


Figure 3. Sequence coverage for the impurity N-3(T4:C20) determined using merged DDA-MS/MS at two specific charge states; z=3 and z=7 showing incomplete coverage.

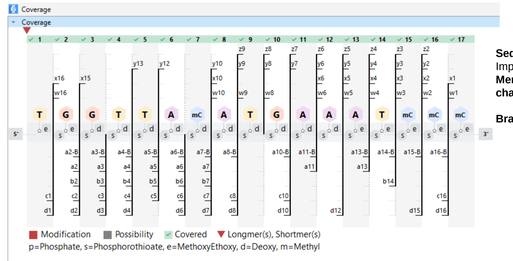
3.2 Increasing sequence coverage

The novel algorithm for merging spectra has been applied to the sequence coverage of the impurity N-3(T4:C20) resulting in a complete coverage.



Sequence coverage
Impurity N-3(T4:C20)
Merged spectra for all charge states.

Fill mode view



Sequence coverage
Impurity N-3(T4:C20)
Merged spectra for all charge states.

Branch mode view

Figure 4. Sequence coverage for the impurity N-3(T4:C20) determined for all charge states showing complete coverage.

4. Conclusions

- The merged spectrum algorithm for processing DDA-MS/MS spectrum data has resulted in higher sequence coverage.
- It is unbiased (it is not a weighted averaged MS/MS spectrum biased towards the most abundant charge states) and registers a single DDA-MS/MS spectrum for each detected component.