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Application Note

Quantification of Biopharmaceuticals in Plasma Using a Multiple Charge Stage Calibration Line

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This is an Application Brief and does not contain a detailed Experimental section.

Abstract

The accurate quantification of biologics-based therapeutics such as proteins, oligonucleotides, and peptides in biofluids is critical to support candidate selection, preclinical and human volunteer studies. When analyzed by electrospray mass spectrometry (LC-MS/MS), these biotherapeutic molecules form ions with multiple charge states, distributing the analyte signal across multiple MRM transitions which can impact both assay sensitivity and dynamic range. The ability to sum the signal from multiple MRM transitions to form one consolidated calibration line has the potential to improve overall data quality and bioanalytical assay sensitivity. The "SUM MRM" function in waters_connect™ MSQuan Software provides a simple and easy approach to combining several MRM channels. Allowing rapid methods evaluated, optimized and data processed of data in one easy step.

Benefits

- Simple creation of combined charge state calibration line for rapid data processing.
- Address the challenge of charge state distribution variation as concentration of biopharmaceutical changes.
- Increase assay sensitivity by combining LC-MS signal from several charge state precursor → product ion pair combinations

Introduction

Reversed-phase liquid chromatography coupled with Electrospray Ionization (ESI) Mass Spectrometry (RPLC-MS/MS) operated in multiple reaction monitoring (MRM) mode is the primary technique for the qualification of pharmaceuticals in biofluids (***e.g.,*** plasma, serum, urine, CSF etc).[1] When analyzed by ESI LC-MS protein and peptide-based pharmaceuticals such as GLP-1 receptor agonists (Ras), monoclonal antibodies (mAb), and oligonucleotide can form several multiply charged precursor ions, ***e.g.,*** $M\text{-}3H^{3-}$, $M\text{-}4H^{4-}$ etc.[2,3] This results in the analyte signal being distributed across multiple precursor ion → product ion pairs, which can impact the overall assay sensitivity, dynamic range and accuracy.[2,3] This is further complicated by the fact that the charge state distribution is dynamic and can change across the concentration range of the bioanalytical assay.[4]

To address this challenge, bioanalysts often combine the signal from several charge states to create a single, unified calibration line. This has the advantage of addressing the charge state variation, improving accuracy and potentially increasing sensitivity. This application note demonstrates how the waters_connect Quantitation Software can combine the data from multiple charge state calibration lines to create a single calibration line and how this capability has been applied for the analysis of the antisense oligonucleotide (ASO) nusinersen in dog plasma.

Experimental

Nusinersen, an ASO used for the treatment of spinal muscular atrophy, ($C_{234}H_{340}N_{61}O_{12}8P_{17}S_{17}$ average MW 7127.21 $g{\cdot}mol^{-1}$), was isolated from rat plasma using solid phase extraction (SPE) and analyzed via ion-pair reversed-phase chromatography with detection by -ve ESI tandem quadrupole Mass Spectrometry in MRM mode.

The methodology for this analysis was previously reported by Twohig *et-al.*[5] Briefly, a calibration curve for nusinersen was prepared in control rat plasma (Wistar Hannover) over the range 0.1–1000 ng/mL with quality control (QCs) concentrations between 0.375–750 ng/mL. A 100 µL aliquot of the plasma samples were denatured, reduced and digested prior to isolation via SPE using an OligoWorks™ SPE Microplate (2 mg).[5] The resulting sample aliquots were diluted 1:1 with distilled water to give a final volume of 100 µL. A 20 µL aliquot of the diluted extracts were loaded onto an ACQUITY™ Premier Oligonucleotide Chromatography Column, 2.1 x 50 mm C_{18} 1.7 µm, (p/n: 186009484 <https://www.waters.com/nextgen/global/shop/columns/186009484-acquity-premier-oligonucleotide-c18-column-130a-17--m-21-x-50-mm.html>) maintained at 60 ˚C and eluted with a 5–45 % aqueous buffer (A) – organic solvent (B) gradient over 3.25 minutes at 600 µL/min, where mobile phase A = 1% HFIP and 0.1% DIPEA in H_2O and mobile phase B = 0.75% HFIP, 0.0375% DIPEA in 65:35 ACN:H_2O. The column effluent was monitored by -ve ESI LC-MS/MS, the MRM transitions employed for the quantification of nusinersen are listed in Table 1. The capillary voltage was 2.5 kV, desolvation temperature = 600 °C, source temperature = 150 °C, desolvation gas flow = 1000 (L/hr) and cone gas flow = 150 L/hr.

Charge State	Precurs *m/z*	Product *m/z*	Cone (V)	Collision (eV)
Nusinersen-[M-8H]$^{-8}$	889.95	393.10	45	30
Nusinersen-[M-7H]$^{-7}$	1017.2	393.20	50	40
Nusinersen-[M-9H]$^{-9}$	791.00	393.10	40	30

Table 1. Multiple reaction monitoring precursor → production ion pair conditions employed for the analysis of nursinersen in extracted rat plasma.

The resulting LC/MS data was collected using waters_connect MSQuan Software version 2.7.0 and analyzed using waters_connect MSQuan 2.4.0 Software.

Results and Discussion

The target charge state *m/z* for optimization of nursinersen was determined from the UNIFI™ integrated Mass Calculator and verified on the MS tune page in the system console. The MRM transitions were then evaluated

across multiple precursor charge states and product ions combinations to select the transitions with most intense signal. The precursor → product ion pair combinations and associated cone–collision energy settings were further evaluated and optimized by LC-MS injection.[5] The full optimization procedure and resulting optimized conditions is reported in Table 1. Following LC-MS optimization, the signal from nursinersen was monitored for three different charge states M-7H^{-7}, M-8H^{-8}, M-9H^{-9} using tandem quadrupole MS operated in -ve ESI MRM mode (Table 1).

Nursenersen eluted from the chromatography column with a retention time of t_R=1.9 minutes (Figure 1). The resulting LC-MS/MS data from the various MRM transitions was used to create calibration lines for each charge states using waters_connect MSQuan Software. The data for each individual charge state showed a linear response, using 1/x weighting, over the calibration range 0.1–1000 ng/mL, with regression coefficients of R^2 = 0.9977, 0.9992 and 0.9988 for the charge stages M-7H^{-7}, M-8H^{-8}, M-9H^{-9} respectively (Figure 2).

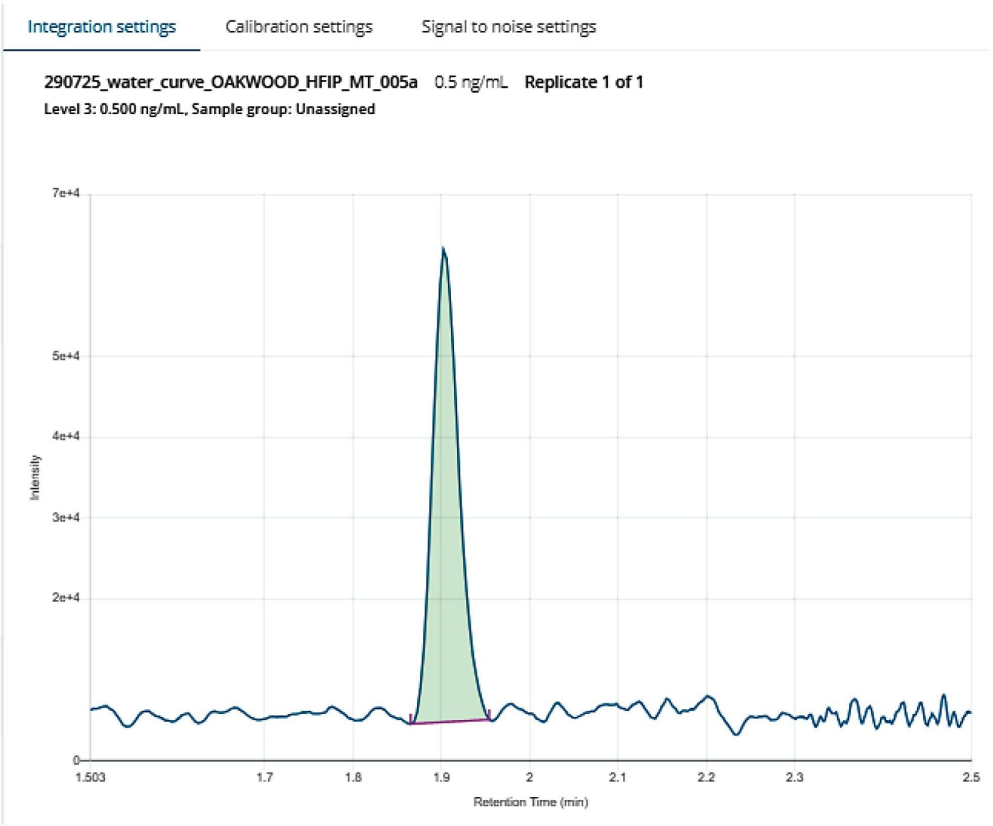

Figure 1. Extracted ion chromatogram for nursenersen using -ve ESI MS/MS operated in MRM mode for the precursor product ion pair 889.95 → 393.10.

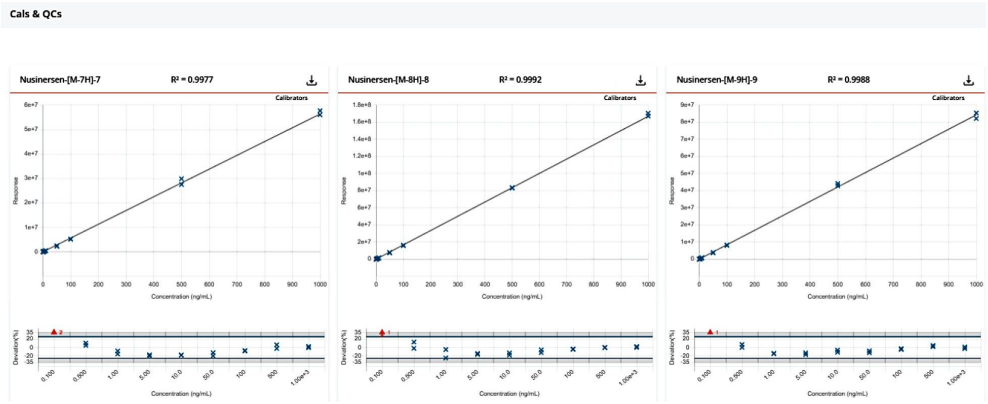

Figure 2. Calibration lines for nusinersen plasma extracts for the charge states M-7H-7, M-8H-8, M-9H-9.

As previously discussed, electrospray MS analysis of biotherapeutics such as proteins, peptides and oligonucleotides form ions with multiple charge states (***e.g.,*** $M\text{-}7H^{-7}$, $M\text{-}8H^{-8}$, $M\text{-}9H^{-9}$), distributing the MS signal between several precursor ions and reducing the overall signal response. This issue is further confounded by the fact that the charge state distribution of these biomolecules can vary with analyte concentration, solvent composition, or pH etc. To address this issue, bioanalysts have combined the signals obtained from multiple precursor ions different charge MRM transitions to generate a more robust calibration with a wider dynamic range.[4]

Analyte quantification is performed in waters-connect via the MSQuan Application. The use of MSQuan for the quantification of pharmaceuticals in plasma and serum has been previously described,[5,6] and combining MRM data from multiple charge state precursor ions is supported within the MSQuan Application. A combined MRM channel can be quickly created in MSQuan Application by adding a new analyte channel to the MS processing method (Figure 3). The "Data Type" used for quantification in MSQuan are MRM, PDA or Sum MRMs, the designation of Sum MRMs indicates that this data channel will use the signal from multiple MRM transitions to quantify the samples. The MS processing method is then edited to select the MRM channels to be summed dialogue box (Figure 3).

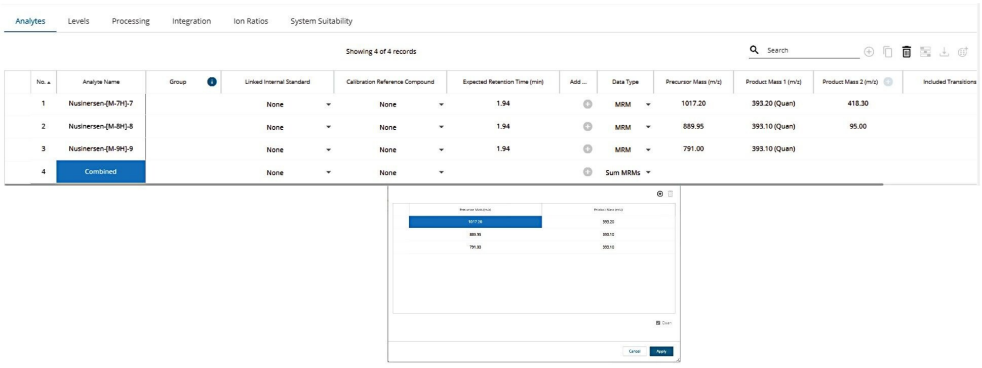

Figure 3. Construction of combined MRM function in waters_connect MSQuan Software.

The MSQuan Software will then create a new data channel using the combined data from all of the selected MRMs. The number of MRMs employed for the summed MRMs channel is only limited to the MRM channels acquired. The signal from all of the selected MRM channels is then merged into one data set and the resulting data processed in the same manner as would be employed for a single MRM channel (Figure 4). The calibration weighting and internal standard are also selected in the same manner as that employed for an individual MRM channel. If the MRM signal from several multiple charge states have been collected, they can be evaluated in various different groups to determine the most robust and appropriate combination for the analysis.

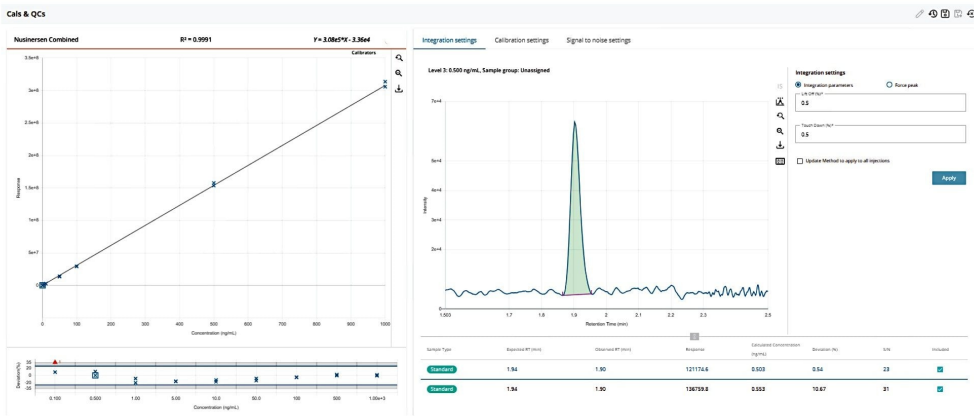

Figure 4. Calibration line and 0.5 ng/mL extracted plasma standard for nusinersin using combined MRM channels, for the charge states M-7H^{-7}, M-8H^{-8}, M-9H^{-9}.

Conclusion

The accurate quantification of biopharmaceuticals such as proteins, oligonucleotides, antibody drug conjugates, and peptides is confounded by the fact that these molecules form multiple charge states when analyzed using electrospray mass spectrometry thus, distributing the analyte signal across multiple MRM transitions. The charge state distribution can vary accordingly with mobile phase composition and analyte concentration. The ability to sum the signal from multiple MRM transitions to form one consolidated calibration line can improve overall data quality, address the issue of charge state variation and improve bioanalytical assay sensitivity. The “SUM MRMs” function in waters_connect MSQuan Software provides a simple and easy approach to combining the data from several MRM channels allowing the data from multiple channels to be combined and evaluated in one easy step enabling rapid processing method evaluation and optimization.

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