Assessing key attributes of adeno-associated viral proteins using UHPLC-FLD-intact accurate mass analysis

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ABSTRACT

Purpose: Rapid monitoring of key attributes of adeno-associated viral proteins for AAV product quality control using a HPLC-FLD-HRAM MS method.

Methods: A Thermo Scientific™ Orbitrap Exploris™ MX mass detector coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system and a Thermo Scientific™ Vanquish™ Fluorescence Detector F was used for the method development. The viral proteins from multiple AAV serotype samples were separated and detected using the developed HPLC-FLD-intact mass analysis method. The respective ratio of the AAV viral proteins (VP1:VP2:VP3) per AAV sample was assessed using the integrated peak area of FLD detection data. The viral protein identity and their proteoforms of AAV serotype samples were confirmed by the high resolution accurate intact mass analysis.

Results: The developed method allowed rapid assessment of the ratio of the AAV viral proteins VP1:VP2:VP3 using fluorescence detection. The excellent sensitivity and mass accuracy offered by the Orbitrap Exploris MX mass detector enabled confident viral proteins and their proteoforms confirmation.

INTRODUCTION

Recombinant Adeno-associated viral (rAAV) vectors have emerged as the leading gene delivery vehicles for gene therapy due to their high-efficiency transduction and safety. AAV viral proteins (VPs) are critical for viral infectivity and vector potency. The key attributes of AAV VPs, such as identity and relative ratio of VPs and their PTMs need to be fully characterized and monitored during the viral vector development and manufacturing to ensure the safety, quality, and efficacy of AAV products.

To address the analytical needs for supporting large scale AAV product manufacturing, we developed a UHPLC-FLD-HRAM MS method for simultaneous relative expression ratio assessment of VP1, VP2 and VP3 using FLD and direct accurate intact mass measurement of the VPs, their associated proteoforms and truncated protein forms using an HRAM Orbitrap mass spectrometer. The market-leading resolution, mass accuracy and sensitivity offered by the Orbitrap technology enabled excellent spectral quality for accurate AAV viral protein intact mass analysis. The HPLC-FLD-HRAM MS method was applied to analyze multiple AAV serotype samples. The analytical results are reported here.

MATERIALS AND METHODS

Sample Preparation

Two internal AAV samples (AAV6, AAV9) expressed via transient transfection in HEK293 cells using the Gibco™ AAV-MAX Helper-Free AAV Production and four commercially available AAV serotype samples (AAV1, AAV6, AAV8 and AAV9) were used for the HPLC-FLD-HRAM MS method development and evaluation. The AAV samples were buffer exchanged and concentrated into 80% H_2O/20% acetonitrile containing 5 mM TCEP and 0.1% formic acid using 30K centrifugal filter (Amicon® Ultra, 0.5 mL). The collected sample was incubated at room temperature for 2 hours. The estimated protein concentration per AAV sample was 0.2 µg/µL - 0.3 µg/µL based on the AAV titer information provided by the manufactures.

HPLC conditions

For all experiments, chromatographic separations were carried out using a C4 stationary phase column (1.0 x 100 mm, 300 Å, 1.7 µm) on the Vanquish Horizon UHPLC system coupled with the Vanquish Fluorescence Detector F. The solvent A was water with 0.1% difluoroacetic acid and the solvent B was acetonitrile with 0.1% difluoroacetic acid. The column temperature was set to 80 ºC. The flow rate was 80 µL/min. The gradient condition used was listed in Table 1. The fluorescence detector (FLD) settings are shown in Table 2.

Mass spectrometry

The Orbitrap Exploris MX mass spectrometer equipped with the BioPharma Option was used for MS data collection. The MS settings are shown in table 3.

Data Analysis

Thermo Scientific™ BioPharma Finder™ 5.0 software was used for the intact protein spectra deconvolution.

RESULTS

The denatured samples were analyzed using the HPLC-FLD- intact MS method. The VP1. VP2 and VP3 of the multiple AAV serotype samples were efficiently separated with the above chromatography conditions. Additional viral protein proteoforms and truncated protein forms were also detected. The average mass of each viral protein and associated proteoforms and truncated proteins were determined through deconvolution of the intact mass raw data. The high resolution, high sensitivity, and high desolvation efficiency offered by the Orbitrap MS allowed great spectral quality and, hence, precise measurement of average mass of each viral protein and associated PTMs.

Table 1. HPLC gradient condition

Time	Flow (ml/min)	%B
0	0.08	20
1	0.08	32
16	0.08	36
20	0.08	80
21.5	0.08	80
22	0.08	20
30	0.08	20

Table 2. FLD settings

Excitation wavelength	280 nm
Emission wavelength	350 nm
Lamp mode	High Power
Sensitivity	1
Data collection rate	5 Hz

Table 3. ESI and MS settings

ESI Source Settings	
Sheath gas (a.u.)	25
Aux gas (a.u.)	8
Sweep gas (a.u.)	0
Spray voltage (+V)	3400
Capillary temp. (°C)	320
Vaporizer temp. (°C)	100
MS conditions	**Intact**
Method type	Full MS
Scan range (m/z)	700-2800
Application mode	Intact
Pressure mode	Low
Resolution	30000 at m/z 200
RF lens (%)	150
AGC target value	75
Max inject time (MS)	100
Microscans	10
Source fragmentation (V)	25

Figure 1 shows the deconvoluted spectra of AAV viral proteins. Excellent mass accuracy was observed with three viral proteins and the VP3 proteoform with deamidation. Exceptional spectral quality was observed not only for VP3 but also for the less abundant VP1, VP2 and VP3 with deamidation, all measured with mass accuracy <10 ppm. The average masses of detected additional truncated VP proteins were shown in Figure 2.

Figure 1. The Full MS raw spectra and their deconvoluted results for VP1, VP2, VP3 and deamidated VP3 proteoform from the internal manufactured AAV9 sample. The mass accuracy was less than 5 ppm per VP protein.

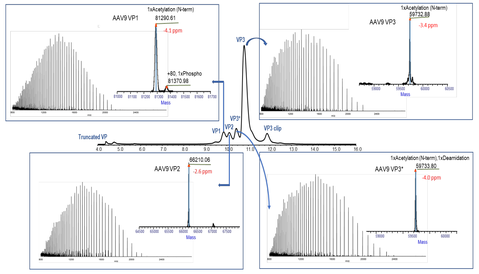
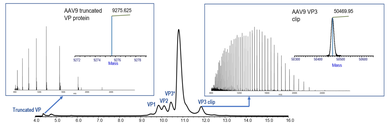
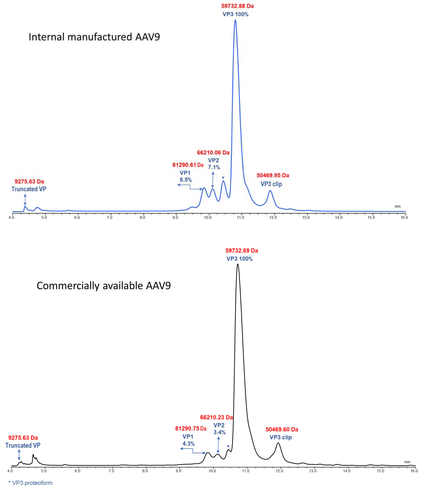


Figure 2. The Full MS raw spectra and their deconvoluted results for truncated VP proteins from the internal AAV9 sample.



The combined FLD optical data and the accurate intact mass measurement data can be used for quick monitoring of the critical quality attributes of viral proteins including relative ratio of viral proteins and identify conformation of viral proteins, providing a powerful tool for monitoring the AAV product quality attributes across different batches, manufacturing processes and over different AAV serotypes. As an example, a quick comparison of the quality attributes for the two AAV9 samples is shown in the Figure 3. The integrated FLD peak areas were used for relative quantitation of VPs and the intact protein raw spectra were used for the average mass measurement of VPs and associated proteoforms. Although both AAV9 samples showed comparable separation profiles, the observed relative ratios of VP1:VP3 and VP:VP3 were much lower for the commercially available AAV9 sample compared to the internal manufactured AAV9 sample. The observed average masses from the two truncated VP proteoform peaks are identical in both AAV9 samples, suggesting that they have the same identity.

Figure 3. FLD elution profile with observed average mass results from the two AAV9 denatured capsid protein mixtures. The integrated FLD peak areas were used for relative quantitation of viral proteins. The relative abundance of the detected components are calculated against the integrated peak area of VP3, which is assigned as 100%. The average mass per detected VP and associated VP proteoform peak was determined through the deconvolution of the raw full MS spectra.



The FLD elution profile and the accurate intact mass measurement data for other AAV serotype samples (AAV8, AAV6 and AAV1) are shown in the Figure 4, Figure 5 and Figure 6, respectively. The accurate intact mass data (< 10 ppm) allowed rapid conformation of AAV serotypes,rapid identification of PTMs on the viral proteins and a quick estimation about the PTM abundance compared to the unmodified viral proteins. For our AAV samples, the AAV8 had most abundant VP1 and VP2 with phosphorylation. AAV8 was also the only one that identified VP3 without and with acetylation. For the AAV6 and AAV1 samples. only VP3 with acetylation was identified.

Figure 4. The FLD separation profile and intact mass deconvoluted results for the detected viral protein and associated truncated peaks from the AAV8 sample. The observed average masses for the viral proteins with and without PTMs were summarized in the top table on the top of the figure. The mass accuracy was less than 10 ppm. Significant phosphorylation was observed with VP1 and VP2.

VP1				VP2				VP3			
AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)
2 (Ac)-738	81666.9	81667.31	5.0	139-738	66518.50	66518.57	0.1	204 - 736	59763.07	59763.07	0
2 (Ac)-738 +1xPhosphorylation	81747.32	81747.29	3.7	139-738 + 1xPhosphorylation	66598.30	66598.55	3.7	205 (Ac) - 738	59804.82	59805.11	4.9

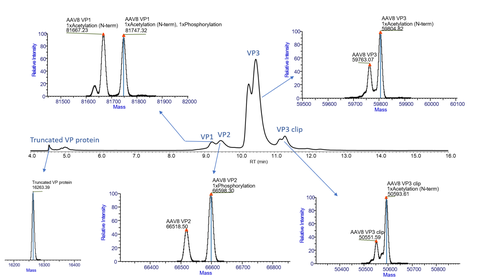


Figure 5. The FLD separation profile and intact mass deconvoluted results for the detected viral protein and associated truncated peaks from the AAV6 sample. The observed average masses for the viral proteins with and without PTMs were summarized in the table on the top of the figure. The mass accuracy was less than 10 ppm.

VP1				VP2				VP3			
AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)
2 (Ac)-736	81321.92	81322.21	3.6	139-737	66095.12	66095.41		204 (Ac) - 736	59518.79	59519.13	
2 (Ac)-738 +1xPhosphorylation	81402.02	81402.19	2.1								

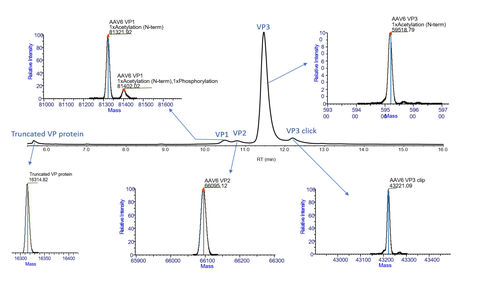
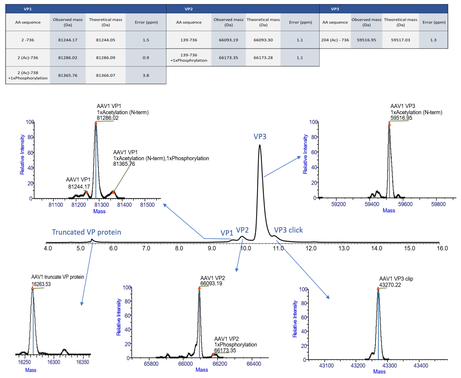


Figure 6. The FLD separation profile and intact mass deconvoluted results for the detected viral protein and associated truncated peaks from the AAV1 sample. The observed average masses for the viral proteins with and without PTMs were summarized in the table on the top of the figure. The mass accuracy was less than 10 ppm.

VP1				VP2				VP3			
AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)
2 -736	81244.17	81244.05	1.5	139-736	66093.19	66093.30	1.1	204 (Ac) - 736	59516.95	59517.03	1.3
2 (Ac)-736	81286.02	81286.09	0.9	139-736 +1xPhosphorylation	66173.35	66173.28	1.1				
2 (Ac)-738 +1xPhosphorylation	81365.76	81366.07	3.8								



CONCLUSIONS

- An UHPLC-FLD-HRAM Orbitrap MS method was developed for rapid critical quality attributes monitoring.
- Excellent spectral quality enabled by the Orbitrap Exploris MX mass spectrometer enabled great mass accuracy (<10 ppm) for the AAV capsid protein intact mass analysis.
- The accurate intact mass measurement allowed rapid AAV serotype confirmation.
- The accurate intact mass measurement allowed rapid AAV viral protein conformation and their associated PTM identification.
- The sensitive fluorescence detection allowed rapid assessment of the ratio of the AAV structural proteins VP1:VP2:VP3.
- The UHPLC-FLD-HRAM Orbitrap MS method enabled rapid monitoring of the AAV product quality attributes across different batches, manufacturing processes and over different AAV serotypes.

TRADEMARKS/LICENSING

PO66151-EN0422S