Optimizing Adeno-Associated Virus (AAV) Capsid Protein Analysis Using UPLC and UPLC-MS

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Abstract

This application note demonstrates the performance of the BioAccord System by developing LC-optical and LC-MS methods for the analysis of intact AAV capsid proteins and to apply these methods to the AAV8 serotype as a case study for the improved characterization of capsid proteins, including their identification, stoichiometry, and post-translational modifications. The developed methods were then applied to additional rAAV serotypes to demonstrate their general applicability for the intact protein analysis of AAV vectors.

Benefits

- An optimized LC-MS solution for the separation and intact mass analysis of viral proteins (VPs) from recombinant AAV vectors to support the characterization and development of gene therapy products
- A sensitive and quantitative LC-FLR method for stoichiometry measurement of AAV capsid proteins

Introduction

Recombinant adeno-associated viruses (rAAVs) are the most widely used vectors in gene therapy development due to their low toxicity and long-term expression ability.\(^1\) With different tissue tropism, a total of 13 common serotypes of AAVs were isolated, and many of them have been explored for the treatment towards multiple diseases.\(^2\) As the protector of the viral genome and mediator of cellular internalization, the AAV capsid (Figure 1) consists of proteins that share high sequence homology across serotypes,\(^3\) requiring reliable and specific methods for vector identification during gene therapy development and commercialization. In addition, the AAV capsid composition is reported to be critical to viral infectivity and gene transduction.\(^4,5\) To ensure the safety and quality of drug products, the structure and properties of the AAV capsid and its constituent proteins need to be well characterized and monitored throughout the gene therapy product development process. Conventional analytical techniques, such as ELISA, Western Blot, or SDS-PAGE, are frequently used to provide basic functional and compositional information, but these techniques are either laborious to deploy and validate or insensitive to AAV serotype.\(^6\) To this end, a robust, specific method that can provide reliable results in a timely fashion is highly desired for identification and characterization of AAV capsid proteins.
Mass spectrometry (MS) has been widely adopted for structural analysis of proteins for its sensitivity and specificity. However, extensive training has been traditionally required for users to operate MS instruments and develop methods, limiting the wider deployment of MS technology in the biopharmaceutical industry. Designed as a robust, easy-to-use, and small footprint LC-MS platform, the Waters BioAccord System was developed to deliver fit-for-purpose MS analysis of biotherapeutics accessible to organizations and operators not previously able to deploy LC-MS technologies. As demonstrated in previous publications, the BioAccord System renders a highly reproducible chromatographic separation and accurate mass measurement in an automated manner through the integration of the ACQUITY UPLC I-Class PLUS System and the ACQUITY RDa Mass Detector. The system, operating under the compliant-ready waters_connect informatics platform is ideally suited for gene therapy product development and commercialization teams to provide structure, composition, and identity information for rAAV capsids.

The objectives of this application note are as follows: To demonstrate the performance of the BioAccord System by developing LC-optical and LC-MS methods for the analysis of intact AAV capsid proteins and to apply these methods to the AAV8 serotype as a case study for the improved characterization of capsid proteins, including their identification, stoichiometry, and posttranslational modifications. The developed methods were then applied to additional rAAV serotypes to demonstrate their general applicability for the intact protein analysis of AAV vectors.
Experimental

Chemical and reagents

Multiple serotypes of AAV samples were donated by BioReliance (Rockville, MD, USA) or purchased from Vigene Bioscience (Rockville, MD, USA), including AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9. Acetic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS-grade water and acetonitrile were purchased from Honeywell (Charlotte, NC, USA) and used as received. Waters IonHance difluoroacetic acid (DFA) (p/n: 186009201) was used as the additive to prepare the mobile phase.

Sample preparation

AAV samples were diluted with Milli-Q water to a final physical titer of 1 × 10^13 GC/mL or used as is if the received sample concentration is lower than 1 × 10^13 GC/mL. As previously reported, AAV samples were treated with acetic acid at 10% (v/v) concentration for 15 min, then centrifuged at 12,000 rpm for 5 min. For LC-MS analysis, a 10-μL (~1 μg of proteins) AAV sample was used for each injection. For LC-fluorescence (FLR) analysis, a 1-μL (~0.1 μg of proteins) AAV sample was used, unless stated otherwise.

System Settings

Analytical conditions

Analytical system: BioAccord incorporating ACQUITY UPLC I-Class PLUS ACQUITY UPLC FLR Detector ACQUITY RDa MS Detector

LC column: ACQUITY UPLC Protein BEH C₄, 1.7 μm, 300 Å, 2.1 × 100 mm (p/n: 186004496); ACQUITY UPLC BEH C₈, 1.7 μm, 130 Å, 2.1 × 100 mm (p/n: 1860002878) ACQUITY UPLC Peptide BEH C₁₈, 1.7 μm, 300 Å, 2.1 × 100 mm (p/n: 186003686)

Column temp.: 80 °C

Sample vial: QuanRecovery with MaxPeak HPS 12 × 32 mm Screw Neck Vial, 300 μL (p/n: 186009186)
Mobile phase A: LC-MS-grade water with 0.1% DFA

Mobile phase B: LC-MS-grade acetonitrile with 0.1% DFA

Gradient Table:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (mL/min)</th>
<th>%A</th>
<th>%B</th>
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<tr>
<td>30.00</td>
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</tbody>
</table>

ACQUITY UPLC FLR Detector settings

λ<sub>excitation</sub>: 280 nm

λ<sub>emission</sub>: 350 nm

Sampling rate: 2 Hz

ACQUITY RDa Mass Detector settings

Mass range: 400–7000 m/z

Mode: ESI positive

Sampling rate: 2 Hz

Cone voltage: 65 V for full scan
Results and Discussion

RPLC-MS Method Optimization

AAV capsids are composed of three viral proteins (VP1, VP2, and VP3) at an approximately 1:1:10 ratio with the masses between 50 and 85 kDa. Due to limited sample availability and the difference in protein relative abundances, the characterization of AAV capsid proteins has been challenging through conventional LC-MS-based methods. When performing reversed-phase LC-MS analysis of AAV8 proteins using formic acid as mobile phase modifier, all VP proteins co-eluted as a single TIC peak (Figure 2A). The masses of VP1 and VP2 were not obtained upon the deconvolution of the summed MS spectra (Figure 2A inset), which we attribute to ion suppression from the more abundant VP3 ions during co-elution. To improve the separation of VP proteins, a new RPLC-MS method using difluoroacetic acid as the mobile phase additive was developed on an ACQUITY UPLC BEH C₈ Column. Compared to the conventional mobile phase modifiers, such as formic acid, chromatographic resolution among the capsid proteins was improved while maintaining sufficient MS sensitivity (Figure 2B). The previously co-eluting VP1 and VP2 species were now resolved from VP3, with a third additional peak observed at 12.2 min. This additional peak was assigned as a fragment of VP3 protein (labeled as "VP3 clip") based on intact mass results. To further optimize the separation of the VP proteins, columns packed with BEH particles bearing alternative bonded phases (e.g., C₄ and C₁₈ ligands) were evaluated. As shown in Figure 2C, VP1 and VP2 were further resolved on the C₄ column, which can be attributed to the increased selectivity provided by the C₄ bonded phase. The pore size of the packed particles, in addition to selectivity of the surface chemistry, contributed to the improved separation. Compared to the use of the ACQUITY UPLC BEH C₈ Column packaged with particles of 130 Å pore size, the...
peak width was reduced using the wide pore (300 Å) C₄ column (Figure 2C), resulting in additional enhancement in MS response.
Figure 2. Method development of AAV8 capsid protein analysis. (A) Separation using ACQUITY UPLC BEH C\textsubscript{8} Column and formic acid as mobile phase modifier; (B) separation using the same ACQUITY UPLC BEH C\textsubscript{8} Column with DFA as mobile phase modifier; (C) improved resolution by using an ACQUITY UPLC BEH C\textsubscript{4} Column with DFA as mobile phase modifier. Gradient: (A) 70–62\% A in 32 min; (B) 67–63\% A in 16 min; (C) 68–64\% A in 16 min. Flow rate: 0.2 mL/min.
With the optimized mobile phase and column, the improved chromatographic resolution facilitated detailed MS analysis of individual VP proteins (Figure 3A). Loading 0.5 μg of AAV8, MS data were obtained for all three capsid proteins and their variants (Figure 3B–E). The deconvoluted masses for peaks at 6.74 min, 7.10 min, and 8.32 min were observed to be 81,668 Da, 66,518 Da, and 59,805 Da, respectively. As shown in Table 1, the observed masses of these proteins were consistent with theoretical masses of VP1, VP2, and VP3, confirming that the developed method is suited for measuring viral protein masses. In addition, the accurate mass measurement of variants allows assignments of potential post-translational modifications (PTMs) on the VPs, including acetylation and phosphorylation, while confirming removal N-terminal methionine on VP1 and VP3. The later eluting peak at 9.38 min was found to have a mass of 50,592 Da, matching the MW of a VP3 fragment due to labile Asp659-Pro660 bond hydrolysis. Together, these results demonstrated the capability of the method for developmental characterization of AAV-related products.
While the theoretical ratio of VP1:VP2:VP3 is at 1:1:10, it has been reported that the production process can be skewed, leading to variations in the abundance of these proteins. Figure 4 shows the calculated relative abundance for each resolved chromatographic peak. The sensitivity of the developed methods is demonstrated in these analyses, allowing for the accurate determination of the stoichiometry of these viral capsid proteins.

To evaluate the broader applicability of the developed methods for intact mass and stoichiometry of other rAAV serotypes, the developed method was applied to a variety of serotypes. While the developed method is effective in the separation of most AAV serotypes in the study, the chromatograms presented illustrate the specificity and sensitivity of the method for each serotype.

In conclusion, an optimized RPLC method was developed using DFA as a mobile phase modifier for improved separation and detection of AAV capsid proteins. The application of this method to other rAAV serotypes demonstrates its potential for widespread use in the field of viral vector analysis.


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BioAccord LC-MS System for Biopharmaceuticals <https://www.waters.com/135005818>

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