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Optimized Liquid Chromatography-Mass Spectrometry Methods for Intact Protein Analysis and Peptide Mapping of Adeno-Associated Virus Capsid Proteins

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Introduction

- Recombinant adeno-associated viruses (AAV) have emerged as one of the most preferred platforms for gene therapy strategies and vaccine development. The characterization and analysis of AAV-based gene therapy products represent significant challenges due to their extremely large molecular sizes, structural complexity, and heterogeneity. AAV capsid protein characterization has important implications in AAV serotype confirmation and quality control in pharma/biopharmaceutical applications. Here, we present a rapid and robust Ultra High-Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS) method for intact capsid protein analysis and peptide mapping of different AAV serotypes.

Materials and Methods

- Sample preparation: rAAV serotype samples were purchased from Virovek and Vigene Biosciences. Each vial contained ~1x10^13 vg/ul genome copies. For intact analysis, the samples were treated with acetic acid for 5 minutes to dissociate the viral proteins and directly analyzed by LC-MS. For peptide mapping, the sample was denatured by Rapigest, reduced with dithiothreitol, and alkylated using iodoacetamide, followed by enzymatic digestion (2 hr, 37ºC) using trypsin and chymotrypsin (Promega).

- Chromatography: LC separation was carried out using an Elute UHPLC system (Bruker Daltonics). The separation of the intact viral proteins was performed using an ACQUITY UPLC protein BEH C4 column (2.1 x 50 mm, 1.7 µm, 300 Å, Waters) held at 70 ºC using a 10-minute gradient. MP A was water +0.1%DFA and MP B was acetonitrile + 0.1% DFA. Tryptic peptides were separated using an ACQUITY Premier C18 column (2.1x100 mm, 1.7 µm, 130 Å, Waters) held at 50 ºC using a 60-minute gradient. MP A was water +0.1% FA and MP B was acetonitrile + 0.1% FA.

- Mass Spectrometry: Data was acquired on a maXis II UHR QTOF (Bruker Daltonics, Billerica, MA). Intact protein data was acquired in full scan MS mode from 300 – 3000m/z with a scan rate of 1 Hz. Peptide mapping experiments were carried out in DDA mode and variable MSMS acquisition rate (4-16 Hz). The data was processed with Data Analysis and BioPharma Compass® 2021 for data visualization and automated deconvolution (Bruker Daltonics, Billerica, MA).

Rapid analysis of intact AAV6 viral proteins on C4 column

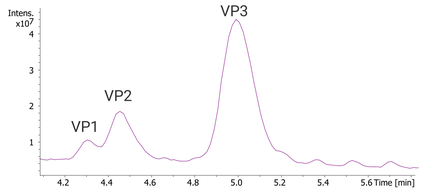

Fig. 1 A. Separation of AAV2 viral proteins in less than 10 min.

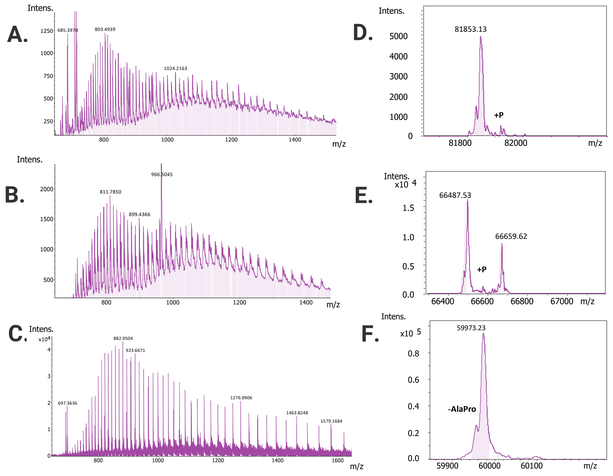

Fig. 2 Deconvoluted mass of the viral proteins and modifications. A) Viral protein 1 B) Viral protein 2 and C) Viral protein 3

- Intact viral proteins of AAV 2, 5, 6, and 8 were well separated by RPLC on a C4 column with high chromatographic resolution values of 1.5 - 3 (Figure 1).
- High-accuracy MS data were obtained for all VP proteins from different serotypes with a good correlation between the theoretical and observed mass (Table 1).
- Accurate mass determination of the VP proteins allowed the assignment of potential post-translational modifications such as phosphorylation (Figure 2).

Rapid separation of viral proteins VP1, VP2, VP3 on several AAV serotypes

Serotypes	AAV8		AAV6		AAV2		AAV5	
Mass (Da)/Viral protein	Observed mass	Theoretical mass	Observed mass	Theoretical mass	Observed mass	Theoretical mass	Observed mass	Theoretical mass
VP1	81,668	81,667	81,320	81,320	81,856	81,853	80,336	80,332
VP2	66,518	66,517	66,096	66,092	66,488	66,486	65,283	65,281
VP3	59,805	59,803	59,519	59,517	59,974	59,973	59,463	59,461

Table 1. Observed mass and theoretical mass of the AAV serotypes on Maxis II ETD

Peptide mapping of the AAVs showed 94.5% peptide coverage on the C18 column with optimized gradient

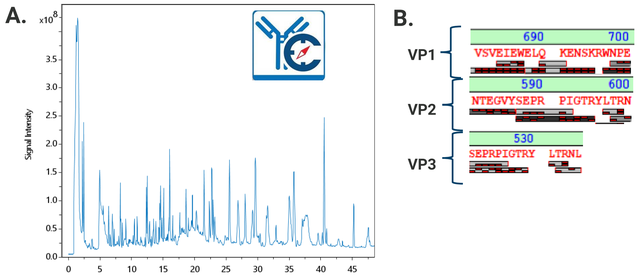

Fig. 3. A. AAV8 peptides are well–resolved on the C18 column and show high sequence coverage. B. The full sequence coverage of the unique peptide sequence of the VP1 (685-701 aa), VP2 (585-601 aa), and the VP3 common region (525-536 aa).

- Peptide mapping of the AAV serotypes was successfully achieved in 60 minutes by reversed-phase LC-MS/MS with a capsid protein sequence coverage of 94% or higher (Figure 3).
- Chymotrypsin and trypsin were used in combination for digestion. As there are limited Arg and Lys residues on the AAV proteins, a combination of the two enzymes gave ~15% more coverage than using only trypsin.
- Several PTMs were identified including deamidation, oxidation, phosphorylation, and acetylation on VP3 of both serotypes AAV8 and AAV6.

High sequence coverage achieved on the peptide mapping workflow

Serotypes	AAV8	AAV6
Coverage/Viral protein	Coverage (%)	Coverage (%)
VP1	92.2%	%90.2
VP2	95.5%	%91.5
VP3	96.2%	%95.2

Table 2. Peptide coverage of the serotypes AAV6 and AAV8. Showing an average of 94% peptide sequence coverage for the 3 viral proteins.

Conclusion

- The high resolution of Bruker maXis II allowed the development of a robust platform for intact protein analysis of AAV viral capsid proteins and identification of modifications such as phosphorylation and sequence variants.
- Intact viral proteins (VP1, VP2, VP3) of AAV serotypes 2,5,6,8 were well-separated on a reverse phase C4 column in less than 10 minutes with highly accurate mass determination for all VP proteins.
- A robust LC-MS/MS peptide mapping workflow was developed using chymotrypsin and trypsin.
- Sequence coverage of 94% or higher was achieved for the 3 VP proteins as well as a complete sequence coverage of the unique peptides for VP 1, VP2, and the common region for VP3
- Several post-translational modifications including deamidation, oxidation, phosphorylation, and acetylation were identified on VP3 of the serotype AAV8 and AAV6

References

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