A Suitable Size Exclusion Column for Efficient Adeno-Associated Virus Aggregate Analysis

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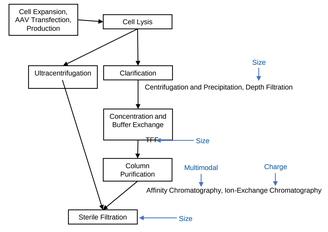
Introduction

To support the continued advancement in the use of the Adeno-Associated Viruses (AAVs) in gene and cell therapy, fit for purpose analytical liquid chromatographic (LC) methods are needed. Various non-LC based analytical methods exist (e.g., qPCR, ddPCR, NGS, Western blot, ELISA, Flow cytometry, dynamic light scattering-DLS and AUC, EM, SDS-page gels, and differential scanning fluorimetry-DSF) and they are commonly utilized in the development of the manufacturing process. However, each analytical method has its limitations. Many of these methods have limited accuracy and precision and require extensive method development or re-qualification whenever the matrix of the sample is changed due to limited specificity. Complementary or orthogonal analytical methods are advantageous to have, particularly during the early phases of process or analytical development, since the property of the biological target is not fully known and therefore multiple methods may be used to verify the CQAs to increase the confidence in steering critical decisions.

Analytical LC methods are developed and optimized to combine reproducible, highly resolving separation with accurate and precise detection. Separation based on size, charge, or hydrophobicity is commonly used in mid-stream and downstream purification (**Figure 1**). The knowledge obtained on how the target or impurities interacts with purification medium determined during process development aids greatly in the development of the analytical method and vice versa.

Figure 1. Typical Steps Used in AAV Production and the Modes of Separation Used.



Within the USP guidelines – Analytical Methods for DS and DP, LC methods are recommended for the following Quality-Attributes:

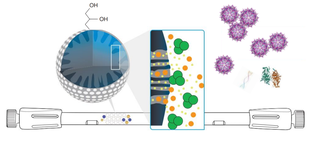
- Identity: Viral Capsid (RP-LC/MS)
- Quantity: Total viral capsid particles (SEC-MALS, AEX-LC)
- Purity: Capsid content – empty vs full (AEX-LC)

Combining size exclusion chromatography (SEC) with a suitable detector has been shown to further increase the analytical power of the LC based method to additionally determine:

- Purity: Capsid Ratio (empty: full)
- Purity: Aggregation
- Purity: Free DNA
- Identity: MW of capsid and encapsulated DNA
- Purity: Capsid size distribution
- Potency: Genome titer

Herein, a new prototype SEC column is presented. Its analytical capabilities will be demonstrated, and several applications will be discussed.

SEC Column Attributes for AAVs



The desirable attributes of a SEC column for AAVs are:

1. A suitable **pore size** to allow high resolution separation of AAVs (~25 nm diameter) from hydrodynamically larger and smaller analytes.
2. An **inert particle chemistry** to facilitate separation by size.
3. A suitable **particle size** to minimize sample dispersion through the column and maintain suitable back pressures on standard LC systems (e.g., 300 bar / 4350 psi limit).

Results

Study 1: Determining the Performance of a Prototype SEC Column for AAVs

Sample Preparation

AAV 1, 2, 3, 4, 5, 6, 8, 9 and rh10 were purchased from Virovek (Houston, TX). For AAV8-CMV-GFP, five samples of different concentrations (from $2x10^{11}$ vg/mL to $2x10^{12}$ vg/mL) were prepared in 0.2 µm filtered 1X Phophate Buffered Saline with 0.001 % Pluronics F68 into HPLC vials. For serotype AAV studies on the 300 x 4.6 mm column format, separate dilutions of $4x10^{11}$ vg/mL were prepared and centrifuged at 10000 rcf for 5 min prior to injection on the LC system.

LC Conditions

Column: Prototype SEC Column
Dimensions: 150 x 4.6 mm
300 x 4.6 mm
Mobile Phase: 20 mM Sodium Phosphate + 5 mM Potassium Chloride + 250 mM Sodium Chloride + 0.001 % Pluronics F68 at pH 7.4, conductivity 27 mS/cm (equivalent to 1.8X Phosphate Buffered Saline + 0.001 % Pluronics F68)
Flow Rate: 0.5 mL/min
0.25 mL/min
Injection Volume: 5 µL
Temperature: 30 °C
LC System: Agilent® 1260 Infinity
Detection: FLD 280/340 nm
UV @ 280 nm, 260 nm

Figure 2. Chromatogram of AAV8-CMV-GFP on a Prototype SEC, 150 x 4.6 mm Column of 5 Different Loads.

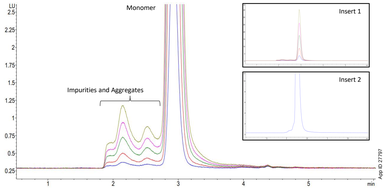
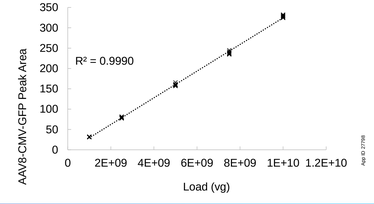


Table 1. Peak Area, Retention Time, Resolution and % Monomer of AAV8-CMV-GFP at $1x10^{12}$ vg/mL.

	Peak Area	Retention Time (min)	Resolution	% Monomer
Average	157.89	2.980	1.510	94.100
Standard Deviation	1.25	0.001	0.162	0.047
%RSD	0.79	0.030	10.760	0.050
N=3 Injections				

Figure 3. AAV8-CMV-GFP Load (vg) Versus FLD 280/340 nm Monomer Peak Area. (N=45 for 3 Repeats per Level per Column for 3 Columns).



Results

Study 2: Performance of a Prototype SEC Column for Various AAV Serotypes

Figure 4. Chromatograms of AAV Serotypes on a Prototype SEC Column to Examine the Effect of Plug Size with 2 Injection Volumes.

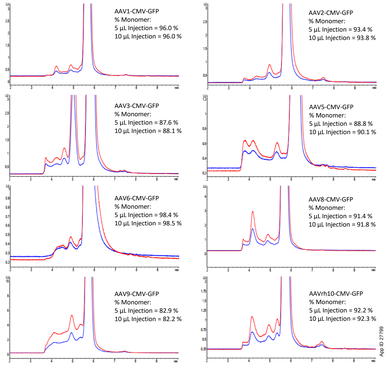


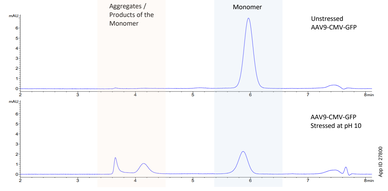
Table 2. Monomer Peak Retention Time for Each Serotype Observed on the Prototype SEC Column.

Serotype	Retention Time (min)
AAV1-CMV-GFP	5.633
AAV6-CMV-GFP	5.651
AAV8-CMV-GFP	5.655
AAV2-CMV-GFP	5.708
AAVrh10-CMV-GFP	5.722
AAV9-CMV-GFP	5.737
AAV3-CMV-GFP	5.827
AAV4-CMV-GFP	6.008
AAV5-CMV-GFP	6.190

Results

Study 3: AAV9 Stressed by High pH

Figure 4. Chromatograms of AAV9-CMV-GFP on a Prototype SEC Column (UV @ 280 nm).



Results

Study 4: Utilizing the Prototype SEC Column to Develop RP and IEX Methods

Figure 6. Chromatograms of AAV6 Full and Empty on the Prototype SEC Column with an AEX Chromatogram Inset.

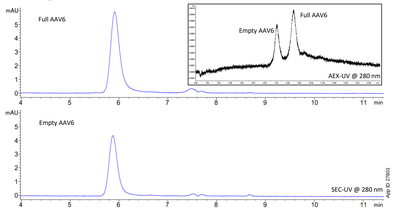
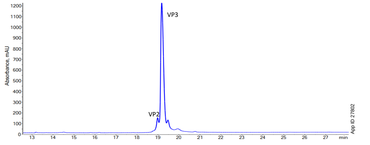


Figure 7. RP-LC Chromatogram (UV @ 215 nm) of AAV8 on a Biozen™ 3.6 µm Intact XB-C8 Column.



Discussion

Study 1: Inert particle chemistry and suitable hardware is demonstrated by the highly reproducible peak areas, retention time, and % monomer as shown in **Table 1**, and **Figures 2** and **3**. The suitability in the pore size is shown in **Figure 2, Insert 2** where a 200 Å column is unable to resolve the AAV monomer from its aggregates and impurities. The running pressures for the 150 x 4.6 mm and 300 x 4.6 mm columns at a flow rate of 0.5 mL/min are 78 and 155 bars, respectively. These running pressures combined with the resolution achievable in **Figure 2** demonstrates that a suitable particle size was chosen.

Study 2: The prototype SEC column is found to be suitable for separating the monomer from its aggregates and impurities for serotypes 1, 2, 3, 5, 6, 8, 9 and rh10. 10 µL can be loaded onto the 300 x 4.6 mm column without affecting % monomer determination. Different serotypes display different retention times (**Figure 4**). Compared to another SEC column for AAV (referred to as column-x), the greatest retention time difference was between AAV1 and AAV5. The retention time difference between AAV1 and AAV5 for the prototype SEC column and column-x are 0.557 min and 0.467 min, respectively. The greater retention time difference for the prototype SEC column suggests that it is a higher resolution column for AAVs.

Study 3: The stress produced additional species which appear to be hydrodynamically larger than the AAV monomer and the monomer peak area is also reduced (**Figure 5**).

Study 4: Anion exchange chromatography (AEX) has been shown to allow the separation of empty versus full capsids (**Figure 6**). Method development of an AEX requires the optimization of the LC conditions to bind and elute the analyte of interest in a reproducible and quantitative manner while achieving separation of the product (all charge variants) from product related impurities and other impurities. As the sample/impurity degrades, new species may form, and the observed peak areas may change making it difficult to evaluate assay performance. Additionally, AEX methods typically use high pH buffers to facilitate binding. An AEX chromatogram (in development) is shown in the insert of **Figure 6**, and AEX peak area recoveries were >93 % relative to peak areas obtained on SEC.

Intact VP methods enable the ability to determine the VP1, 2 and 3 ratios in AAVs. An example chromatogram is shown in **Figure 7** for AAV8 on a Biozen 3.6 µm Intact XB-C8, 2.1 x 150 mm column. Prior to analysis, SEC may be used to help develop a reproducible and high yielding denaturation method. The prototype SEC column may be used to monitor the degradation of the AAV, and dSEC-2 may be used to monitor the generation of VP1, VP2 and VP3 (data to be collected).

Conclusion

The analytical capabilities of a prototype SEC column designed for AAVs was demonstrated. Reproducible separations and recoveries for various AAV serotypes 1, 2, 3, 5, 6, 8, 9, and rh10 are shown. The SEC method utilizing a 150 x 4.6 mm column can be completed in 8 mins at a flow rate of 0.5 mL/min on a normal HPLC system. For higher resolution separations, the 300 x 4.6 mm format is recommended and a flow rate of 0.25 mL/min (supporting data not shown).

Due to the reproducible analytical performance of this column, it may be used in stability studies to determine the purity and quantity of the monomer left in the solution and it may also be used to drive the development of other analytical methods. It also enables the determination of the critical quality attributes in drug substances and drug products.

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