ProPac™ 3R 3 µm IEX

Separation and analysis of adeno-associated virus vectors using a 3 µm monodisperse strong anion exchange chromatography column

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Abstract

Purpose: To demonstrate salt gradient method development and optimization for adeno-associated virus (AAV) empty and full capsids separation and characterization using a 2 × 50 mm strong anion exchange chromatography column packed with a 3 µm monodisperse SAX resin.

Methods: A Thermo Scientific™ Vanquish™ Flex UHPLC system coupled with Thermo Scientific™ Vanquish™ fluorescence detector is used for analyzing AAV empty and full capsids.

Results: Excellent separation of AAV empty and full capsids and other impurities using both a linear salt gradient and linear salt gradient with isocratic hold.

Introduction

Adeno-associated viruses (AAVs) are one of the most used viral vectors for delivering genes due to their low immunogenicity, safety, and long-term transient expression. Separation and quantitation of the empty and full capsids of a given AAV therapeutic are important for understanding their quality and potential efficacy. Anion exchange (AEX) chromatography is commonly used to characterize AAV particles as both empty and full capsids typically have a negative surface charge. [1-3] Strong anion exchange (SAX) chromatography is particularly well-suited for this application as the functional quaternary amine AEX group of the SAX phase is always charged. This enables adsorption and separation of AAV particles using a salt gradient with a buffer pH sufficiently greater than the AAV isoelectric point (pI).

In this study, we provide practical examples of method design using a Thermo Scientific™ ProPac™ 3R SAX column for the separation of AAV empty and full capsids. First, we demonstrate a straightforward approach for the separation of empty and full capsids and associated impurities for three AAV samples (AAV1, AAV6, and AAV8) with a simple linear salt gradient. Second, we show the development of a method to optimize the separation of these components for AAV6 by incorporating an isocratic hold during the linear gradient.

Materials and methods

Sample preparation

AAV1, 6, and 8 empty and full samples are used as received (2 × 10^{13} vg/mL).

For linear salt gradient methods, AAV empty and full samples are mixed with a 1:10 ratio.

For linear salt gradient with isocratic hold methods, AAV6 full sample is used directly without further modification.

Column

ProPac 3R SAX, 3 µm, 2 × 50 mm (P/N 43203-052068)

Data analysis

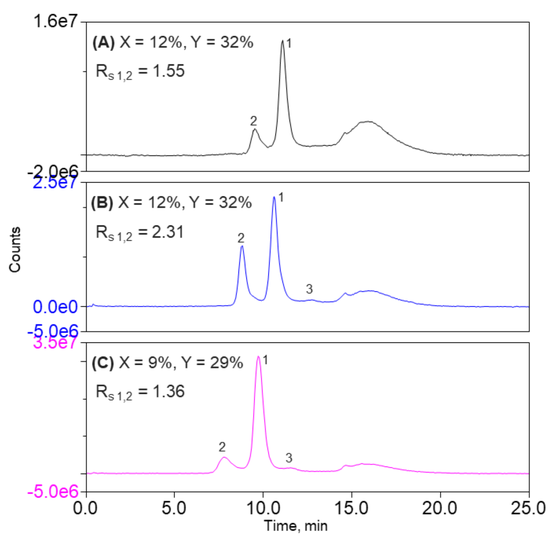
The Thermo Scientific™ Chromeleon™ 7.2.10 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results

Linear salt gradient results

Figure 1 shows the analysis of AAV1, AAV6, and AAV8 samples using a simple linear salt gradient. For each of the AAV samples tested, empty (peak 2) and full (peak 1) capsid peaks are baseline resolved using the linear gradient indicating that a simple linear salt gradient is often sufficient for separating these peaks for the purposes of quantitation. For AAV6 and AAV8 samples, an impurity peak (peak 3) is observed to elute after the full capsid peak. For the AAV separations, the relative peak areas for empty capsids (peak 2) compared to full capsids (peak 1) are visually greater than 10% as would be expected from the 1:10 mixing of empty:full standards. This is particularly obvious for AAV6 and may be due in part to the accuracy of sample concentrations from the supplier. However, we note that the fluorescence signal response for empty and full capsids is not equivalent, which will contribute to the differences in relative peak areas measured.[1] Calibration curves for both empty and full capsids would be needed to accurately measure the exact amounts of each capsid. For simplicity in this study, we report the relative peak areas for empty and full capsids in subsequent analyses.

Figure 1 Linear salt gradient separation of full capsid AAV samples spiked with empty capsid to give a 1: 10 Empty:Full ratio: (A): AAV1 sample, (B): AAV6 sample, and (C): AAV8 sample



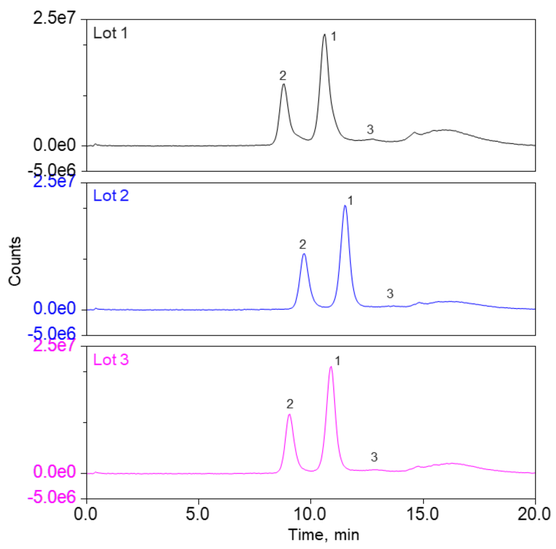
Column:	ProPac™ 3R SAX, 3µm
Format:	2 × 50 mm
Mobile phase A:	Water
Mobile phase B:	1 M Tetramethylammonium Chloride
Mobile phase C:	200 mM Bis-Tris Propane, pH 9.0
Gradient:	

Time (min)	%A	%B	%C
0.0	90-X	X	10
1.0	90-X	X	10
11.0	90-Y	Y	10
11.1	0	90	10
13.0	0	90	10
13.1	90-X	X	10
25.0	90-X	X	10

Temperature:	20 ºC
Flow rate:	0.2 mL/min
Inj. volume:	0.2 µL
Detection:	FLD (Ex: 280 nm, Em: 330 nm)
Sample:	A: AAV1, E:F 1:10 (2 X 10^{13} vg/mL)
	B: AAV6, E:F 1:10 (2 X 10^{13} vg/mL)
	C: AAV8, E:F 1:10 (2 X 10^{13} vg/mL)

Using the method for spiked AAV6 sample analysis in Figure 1, we have evaluated the lot-to-lot reproducibility for three different lots of ProPac 3R SAX media column using the linear gradient as shown in Figure 2. Good lot-to-lot reproducibility with baseline separation for the spiked AAV6 sample is observed for ProPac 3R SAX columns.

Figure 2 Separation of spiked AAV6 sample on 3 different lots of ProPac 3R SAX media column using a linear salt gradient



Column:	ProPac™ 3R SAX, 3µm
Format:	2 × 50 mm
Mobile phase A:	Water
Mobile phase B:	1 M Tetramethylammonium Chloride
Mobile phase C:	200 mM Bis-Tris Propane, pH 9.0
Gradient:	

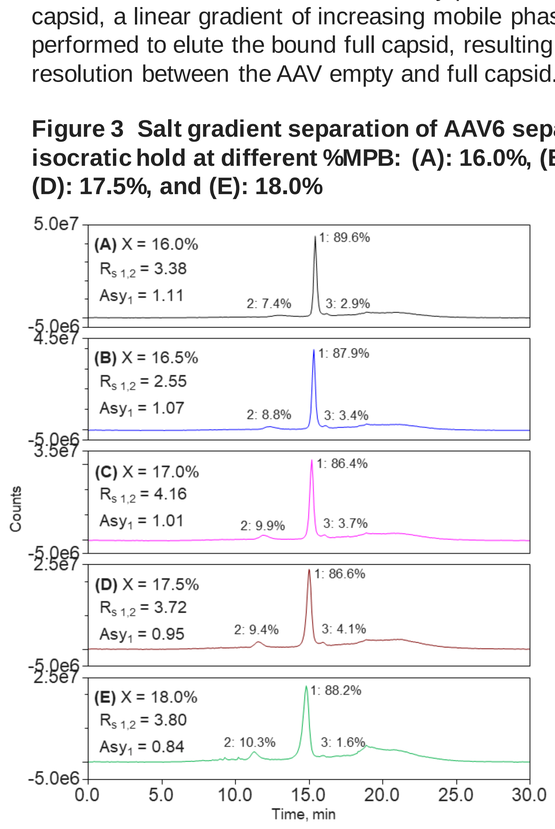
Time (min)	%A	%B	%C
0.0	78	12	10
1.0	78	12	10
11.0	58	32	10
11.1	0	90	10
13.0	0	90	10
13.1	78	12	10
25.0	78	12	10

Temperature: :	20 ºC
Flow rate:	0.2 mL/min
Inj. volume:	0.2 µL
Detection:	FLD (Ex: 280 nm, Em: 330 nm)
Sample:	Concentration: 2 X 10^{13} vg/mL
	AAV6, Empty:Full 1:10
Peaks:	1. Full capsid
	2. Empty capsid
	3. Impurity

Linear salt gradient with isocratic hold method development

The simple linear salt gradient method is straightforward to implement and provides good separation for each of the AAV samples evaluated; however, further improvements to the separation between the AAV empty and full capsid may be required for more difficult to separate samples to either increase resolution or improve detection sensitivity. In this section, we demonstrate a method to improve the separation of AAV6 using a linear gradient by incorporating an isocratic hold midway through the method to increase the separation of the empty capsid from the full capsid. The isocratic hold is used to elute the empty capsid at a constant salt concentration that is insufficient to promote elution of the full capsid, which remains bound to the column stationary phase. After elution of the empty capsid, a linear gradient of increasing mobile phase B concentration is performed to elute the bound full capsid, resulting in significantly improved resolution between the AAV empty and full capsid.[2,3]

Figure 3 Salt gradient separation of AAV6 separation using an isocratic hold at different %MPB: (A): 16.0%, (B): 16.5%, (C): 17.0%, (D): 17.5%, and (E): 18.0%



Column:	ProPac™ 3R SAX, 3µm
Format:	2 × 50 mm
Mobile phase A:	Water
Mobile phase B:	1 M Tetramethylammonium Chloride
Mobile phase C:	200 mM Bis-Tris Propane, pH 9.0
Gradient:	

Time (min)	%A	%B	%C
0.0	88	2	10
1.0	88	2	10
6.0	90-X	X	10
10.0	90-X	X	10
15.0	40	50	10
15.1	0	90	10
18.0	0	90	10
18.1	88	2	10
30.0	88	2	10

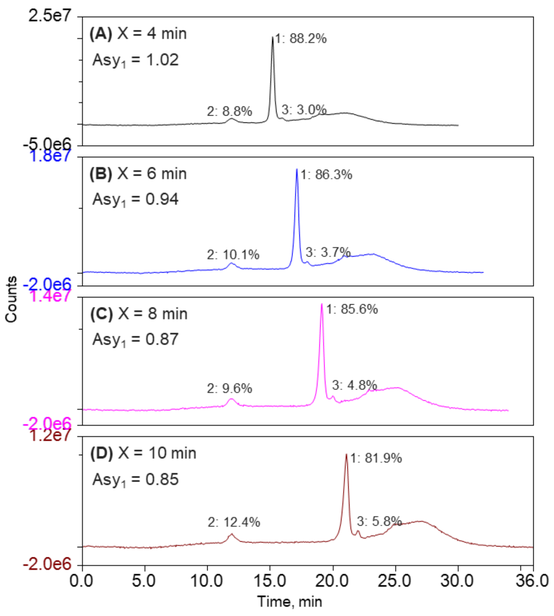
Temperature: :	20 ºC
Flow rate:	0.2 mL/min
Inj. volume:	0.2 µL
Detection:	FLD (Ex: 280 nm, Em: 330 nm)
Sample:	AAV6 (2 X 10^{13} vg/mL)
Peaks:	1. Full capsid
	2. Empty capsid
	3. Impurity

Figure 3 shows the results of different %MPB from 16.0% to 18.0% for the isocratic elution stage. Baseline separation of empty and full capsid are achieved for all the choices of isocratic %MPB tested; however, differences are observed in the quality of the separation. 17.0% MPB is considered for further optimization of this method since the empty and full capsid are baseline separated, and the full capsid peak asymmetry is close to 1 (Asy1 = 1.01).

Figure 4 shows the method used in Figure 3 with an isocratic hold at 17% MBP with holds ranging in time from 4 min to 10 min. This shows separation of the empty (peak 2) and full (peak 1) capsid peaks changing due to the later elution of the full capsid peak with increasing isocratic holding time. However, as the isocratic stage holding time prolongs, there is a decrease in the intensity and relative peak area of the full capsid peak due to peak broadening.

The user can employ orthogonal methods such as AUC or cryo-EM[3] in combination with AAV standards to determine which isocratic hold time method will provide the most accurate characterization of the empty/full ratio.

Figure 4 AAV6 analysis using a linear salt gradient with isocratic hold with different isocratic stage holding times: (A): 4 min, (B): 6 min, (C): 8 min, and (D): 10 min



Column:	ProPac™ 3R SAX, 3µm
Format:	2 × 50 mm
Mobile phase A:	Water
Mobile phase B:	1 M Tetramethylammonium Chloride
Mobile phase C:	200 mM Bis-Tris Propane, pH 9.0
Gradient:	

Time (min)	%A	%B	%C
0.0	88	2	10
1.0	88	2	10
6.0	73	17	10
6.0+X	73	17	10
11.0+X	40	50	10
11.1+X	0	90	10
14.0+X	0	90	10
14.1+X	88	2	10
26.0+X	88	2	10

Temperature:	20 ºC
Flow rate:	0.2 mL/min
Inj. volume:	0.2 µL
Detection:	FLD (Ex: 280 nm, Em: 330 nm)
Sample:	AAV6 (2 X 10^{13} vg/mL)
Peaks:	1. Full capsid
	2. Empty capsid
	3. Impurity

Conclusions

- The ProPac 3R SAX 3 µm column provides excellent separation of AAV empty and full capsids and other impurities using both a linear salt gradient and linear salt gradient with isocratic hold. The unique column design provides high resolution, robust performance, and lot-to-lot reproducibility needed for AAV analysis..
- Design of a linear salt gradient with an isocratic hold at an appropriate isocratic elution salt concentration and holding time can provide optimized methods for the separation and quantitation of AAV empty and full capsid.

References

1. Wang, C.; Mulagapati, S. H. S.; Chen, Z.; Du, J.; Zhao, X.; Xi, G.; Chen, L.; Linke, T.; Gao, C.; Schmelzer, A. E.; Liu, D., Developing an Anion Exchange Chromatography Assay for Determining Empty and Full Capsid Contents in AAV6.2. Molecular Therapy – Methods & Clinical Development 2019, 15, 257–263.

2. Aebischer, M. K.; Gizardin-Fredon, H.; Lardeux, H.; Kochardt, D.; Elger, C.; Haindl, M.; Ruppert, R.; Guillarme, D.; D'Atri V., Anion-Exchange Chromatography at the Service of Gene Therapy: Baseline Separation of Full/Empty Adeno-Associated Virus Capsid by Screening of Conditions and Step Gradient Elution Mode. International Journal of Molecular Sciences 2022, 23(20), 12332.

3. Khatwani, S.L.; Pavlova, A.; Pirot, Zhu., Anion-exchange HPLC assay for separation and quantification of empty and full capsid in multiple adeno-associated virus serotypes. Molecular Therapy – Methods & Clinical Development 2021, 21, 548–558.

4. Ma, K.; Bechler, S., Salt gradient separation and analysis of adeno-associated virus samples using a 3 µm monodisperse strong anion exchange chromatography column. Thermo Fisher Scientific AN001816-EN 0223S.

5. Mietzsch, M.; Liu, W.; Ma, K.; Bennett, A.; Nelson, A.R.; Gliwa, K.; Chipman, P.; Fu, X.; Bechler, S.; McKenna, R.; Viner, R. Production and Characterization of an AAV1-VP3 Only Capsid: An Analytical Benchmark Standard. Molecular Therapy – Methods & Clinical Development. Published: May 08, 2023.

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