Anion-exchange HPLC assay for separation and quantification of empty and full capsids in multiple adeno-associated virus serotypes

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Gene therapy has entered a new era where numerous therapies for severe and rare diseases are generating robust and compelling clinical results. The rapid improvements in gene therapies over the past few years can be attributed to better scientific understanding of the critical quality attributes that contribute to a safe and efficacious product, as well as a better understanding of the manufacturing processes that are required to yield consistent products, which routinely meet the quality standards required for clinical studies. Of particular concern is the need for an effective, quality control (QC)-compatible, and versatile test method for the quantification of empty and full capsids in recombinant adeno-associated virus (rAAV) samples from multiple serotypes. In that regard, we describe the development of a QC-compatible anion-exchange chromatography method consisting of a modular discontinuous gradient to achieve full baseline peak separation and quantification of empty and full AAV capsids. Using an rAAV6 vector, our assay was shown to be precise, linear, robust, and accurate—correlating well with orthogonal methods such as analytical ultracentrifugation (AUC) and cryogenic transmission electron microscopy (Cryo-TEM). Additionally, we demonstrate the versatility of our approach by adapting the method to separate and quantify empty/full capsids in samples from several rAAV serotypes.

INTRODUCTION

Recombinant adeno-associated viruses (rAAVs) define a promising gene-delivery system for genomic therapies addressing a wide variety of diseases. Recent approvals of Luxturna and Zolgensma by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have demonstrated the promise of these systems as one-time cures for a diverse set of diseases.[1] The analytical characterization of viral vectors with respect to process- or product-related impurities is an essential component of the overall product quality control (QC) strategy. One type of rAAV product-related impurity that is of particular concern is the assembled capsid that fails to package a genome.[2,3] Although the full impact of empty capsids on clinical effect is not clear, the presence of a large proportion of empty capsids adversely impacts the volume of vector necessary to achieve the patient dose.[3,4] This in turn results in higher concentrations of product- and process-related impurities per dose and may underpin patient

safety issues that contribute to the generation of severe adverse events. For a consistent manufacturing process, it is important to characterize each batch of viral vector product with respect to its empty-to-full particle ratio.[5] This ratio is a critical quality attribute of gene therapy products,[6] and monitoring this ratio throughout the manufacturing process is strongly recommended by regulatory agencies,[7] despite there being very few robust or reliable methods to accurately quantify empty and full AAV capsids.

To date, a variety of methods have been used to quantify empty and full AAV capsids, which include UV/Vis spectroscopy and capsid (cp)-to-vector genome (vg) titer ratio,[8] transmission electron microscopy (TEM),[9] analytical ultracentrifugation (AUC),[10] size exclusion chromatography coupled to multiangle light scattering (SEC-MALS),[11] capillary electrophoresis,[12] mass photometry,[13] and mass spectrometry (MS).[14–16] Each method utilizes a unique approach toward addressing the quantification of the empty-to-full ratio of an AAV capsid. Each method, however, also has shortcomings.[5] In general, these methods have low throughput, are time consuming, require high product concentrations and purity,[8] and are less amenable for in-process or release testing in a QC environment.[17] Over the last several years, various attempts have been made to use ion-exchange (IEX) chromatography for removal of empty capsids to allow the enrichment of full capsids with various serotypes by use of adsorptive IEX membranes,[18] resins,[19–21] or monoliths.[22–24] More recently, Gagnon et al.[24] demonstrated in-line use of anion-exchange (AEX) chromatography with linear salt gradient and simultaneous monitoring by UV absorbance, light scattering, and intrinsic fluorescence. In addition, Dickerson et al.[23] used a novel approach where an isocratic wash was used to enrich full AAV2 capsids. The separation of empty and full capsids was achieved on the basis of the overall surface charge of the capsid, where very small charge differences (∼0.4) in isoelectric point (pI) arise from the presence or absence of negatively charged encapsidated genomic DNA.[25] Recently, however, AEX-high-performance liquid chromatography (HPLC) using analytical scale
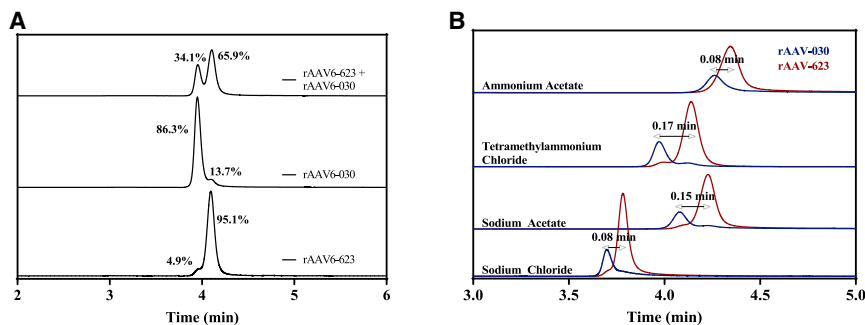
Figure 1. Selection of the mobile phase buffer for optimal separation of empty and full capsids

(A) Stacked AEX-HPLC chromatograms representing fluorescence signal versus time trace of rAAV6-623 and rAAV6-030 along with their mixture over a gradient of 0%–100% NaCl (pH 9.0) are shown. Each chromatogram shows two overlapping peaks (R_s < 2.0), one for empty AAV (earlier eluting peak) and another for full AAV (later eluting peak) capsids, along with their respective relative quantitation. (B) Representative stacked AEX-HPLC chromatograms representing fluorescence signal versus time trace of rAAV6-623 and rAAV6-030 over 0%–100% of various salts (pH 9.0) as eluants are shown. The retention time of major peak in each sample was compared to calculate net retention time (ΔRT).

quaternary amine (QA)-based strong AEX monolith columns has been garnering attention as a rapid and QC-friendly method for quantifying rAAV empty-to-full capsid ratios.[22,26,27] Fu et al.[27] utilized an analytical AEX monolith (CIMac AAV full/empty-0.1) column to demonstrate the separation of empty and full rAAV2 capsids with improved sensitivity using native tryptophan fluorescence from capsid proteins. Wang et al.[26] used a similar approach for separation of empty/full rAAV6.2 capsids as a QC-friendly method with excellent linearity, precision, and accuracy. Despite these improvements, these methods displayed partial overlap (resolution, R_s < 2.0) of the chromatographic peaks of AAV empty and full capsids. According to United States Pharmacopeia (USP) guidelines, it is desirable to attain a full baseline separation (USP resolution, R_s > 2.0) between chromatographic peaks for a quantitative HPLC assay to be amenable to validation and implementation in a QC environment.[28] In addition, all these methods were shown to perform well with the selected AAV serotypes and may or may not be suitable for other serotypes.

In this manuscript, we describe a unique approach for the analytical separation and quantitation of empty and fully packaged rAAV capsids of multiple serotypes that utilizes AEX-HPLC. Our methodology allows for full baseline resolution of chromatographic peaks with excellent peak asymmetry/tailing, signal-to-noise (S/N) ratio, and theoretical plate counts (column efficiency) as recommended by Center for Drug Evaluation and Research (CDER)[28] and USP guidelines (USP < 621 >). The method described here employs a discontinuous salt gradient comprising an isocratic hold step flanked by modular linear salt gradients. The method was developed and optimized with respect to peak resolution (R_s >> 2.0), tailing (T_f < 2.0), and asymmetry (A_s < 2.0) and shows excellent precision, linearity, and accuracy, indicating suitability for method validation and QC applications. The robustness of this method was evaluated by varying the mobile phase flow rate, pH, and column temperature while observing the effect of each manipulation on peak resolution and tailing. The relative quantitation of empty and full capsids by AEX-HPLC for rAAV6 samples showed an excellent correlation with orthogonal methods such as AUC and cryogenic transmission electron microscopy (Cryo-TEM), supporting the validity and accuracy of the HPLC results. Furthermore, we demonstrate that the method is easily adapted to evaluate the separation and quantification of empty and

full capsids of different AAV serotypes including AAV1, AAV2, AAV3, AAV8, and AAV9, suggesting broad applicability and versatility of the approach. It is envisioned that this analytical methodology can be extended to engineered, hybrid, and novel AAV serotypes. In the process development arena, it may be worth evaluating the method described here for "at-scale" removal of empty capsids from an rAAV production run as a means of improving drug product purity.

RESULTS

Screening of mobile phase buffer and salt combinations

Initial development attempts were aimed at obtaining a method that produced rAAV empty/full (E/F) partial peak separation, as previously described in the literature.[22,26,27] A CIMac AAV full/empty analytical column was selected for these experiments since it has previously demonstrated the ability to achieve partial separation of AAV capsids.[22,26,27] As a proof of principle, 20 mM bis-tris propane (BTP, pH 9.0) was used as buffer A in conjunction with 20 mM BTP (pH 9.0) containing 1 M NaCl as buffer B, and the chromatographic separation was performed over a linear gradient of 0%–100% buffer B. All samples tested exhibited two distinct overlapping peaks (Figure 1A). The first peak corresponds to empty capsids, while the second peak represents full capsids. Significant spectral overlap between both peaks and lack of baseline resolution was consistent with previous findings.[22,26,27] It has been previously demonstrated that a molecule's charge (cationic/anionic) and hydrodynamic radius as well as pH, chaotropic properties, and ionic strength of eluate buffers affect peak separation in IEX chromatography.[26,29] As such, four separate mobile phase combinations with different properties were screened in order to further improve the separation of empty and full capsid peaks. Previous studies have demonstrated that BTP was an optimal buffering agent for separating empty and full capsids.[26] The base/binding buffer (buffer A) was selected as 20 mM BTP (pH 9.0), while the elution buffer (buffer B) was composed of 20 mM BTP (pH 9.0) plus one of a variety of 1 M salts. Specifically, either sodium chloride (NaCl), tetramethylammonium chloride (TMAC), sodium acetate (NaOAc), or ammonium acetate (NH$_4$OAc) was used in the elution buffer. Each mobile phase system was evaluated with respect to its ability to improve peak resolution of empty and full capsid peaks for two rAAV6 samples, namely rAAV6-030 and rAAV6-623. Each
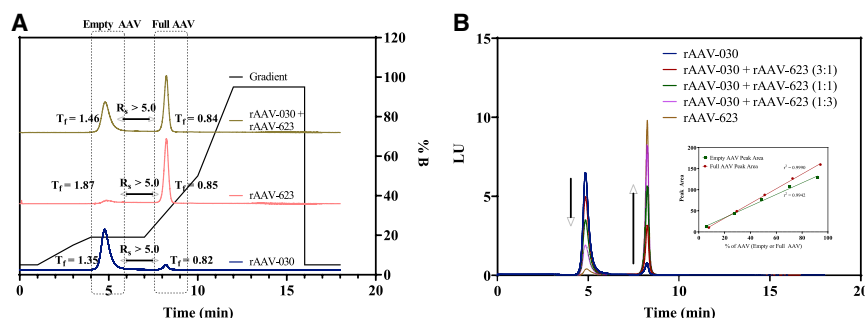
(A) The fluorescence signals of two different rAAV6 samples, empty (rAAV6-030) and full (rAAV6-623) capsids, along with a mixture containing both are shown. The discontinuous gradient containing an isocratic hold flanked by a linear gradient on each side that was used to separate empty and full AAV6 capsids is also shown. The overall gradient indicates binding, separating, cleaning, and re-equilibration phases. (B) Fluorescence signal versus time trace chromatograms of a full (rAAV6-623), an emp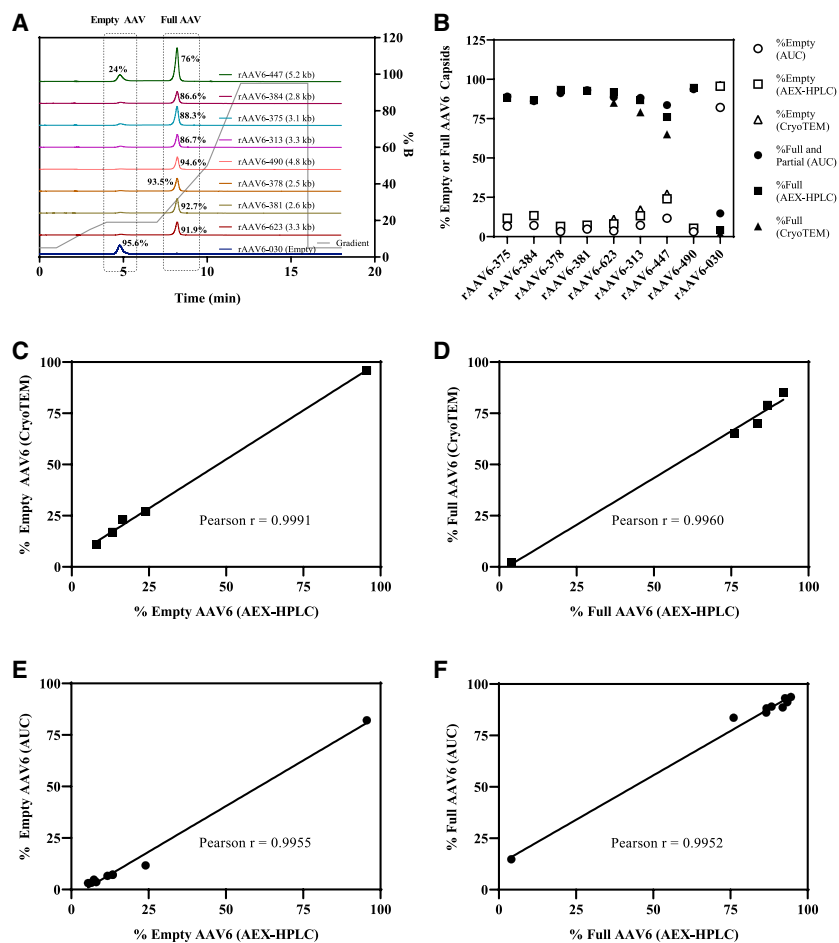ty (rAAV6-030), and their three different AAV mixtures are shown. The peak areas of both peaks within each chromatogram of rAAV are plotted against the nominal % empty or full capsid AAV content. The linear relationship ($r^2 >$ 0.99) in each case indicates that the peak area responses for either empty or full capsid AAV6 peak are additive and consistent with increasing concentrations of peak areas. (A) and (B) show consistency of peak retention times ($< \pm 2\%$) for both empty and full capsid peaks in each chromatogram, indicating consistency of the separation method.

sample was bound to the column in the base buffer A, elution was performed with a linear gradient (0%–100%) of buffer B, and elution profiles of both samples were compared. As expected, samples eluted with each of the four buffer systems showed two chromatographic peaks, one corresponding to empty capsids and the other to full AAV6 capsids (Figure 1A). The retention times (RTs) of major peaks in rAAV6-623 (full peak) and rAAV6-030 (empty peak) sample chromatograms were compared and used to calculate net difference in retention times (ΔRT) of major peaks among both samples in each mobile phase system. For both samples, the lowest RT was achieved with NaCl as eluant, while ammonium acetate showed highest RT among the buffer systems tested (Figure 1B). Overall, TMAC-based elution buffer was found to yield largest ΔRT and selected as a preferred eluant for further optimization. Sodium acetate showed the next best ΔRT and can be considered as an equivalent alternative for future development. The improvement in resolution between empty and full AAV peaks with TMAC may be due to its anti-chaotropic property resulting in improved hydrophobic interactions with empty and full capsids, as seen with previous studies.[26,29] It is important to note here that rAAV exhibited optimal binding to the monolith column at pH 9.0, while at lower pH the loss of binding along with loss of peak resolution was observed (data not shown).

Development of step gradient method for baseline separation of empty and full capsids

The next phase of method development focused on the optimization of the gradient profile with the goal of achieving baseline separation of the empty and full chromatographic peaks. In previous experiments, both empty and full AAV6 peaks eluted at an ionic strength of ∼150–300 mM TMAC (15%–30% buffer B). Step gradients with isocratic holds are commonly used in chromatographic separations. Thus, it was envisioned that despite a relatively small difference in the pIs of the empty and full capsids, it might be possible to keep full AAV6 capsids bound to the column while empty AAV6 capsids are eluted at a lower but constant ionic strength. Further attempts to improve the method focused on the addition of an isocratic hold within the gradient (15%–30% buffer B) at a preselected ionic strength. For rAAV6 samples,

different isocratic holds were tried (e.g., 17%, 18%, 19%, 20% buffer B) within a linear gradient of 15%–30% buffer B (Figure S1). An isocratic hold at 19% TMAC proved optimal for complete isolation of the empty AAV6 peak. After elution of the empty capsid, increasing concentrations of TMAC were used to elute the full AAV6 peak, resulting in a full baseline separation between both peaks. In the resultant separation profile, various chromatographic parameters such as peak tailing, asymmetry, and resolution were assessed. The peak tailing observed with the empty AAV6 peak was, however, still higher than desired ($T_f > 2.0$) per USP/ICH guidelines. A high peak tailing can lead to inaccurate quantitation of the peak area and could result in partial or full overlap with adjacent peaks, resulting in loss of resolution and inaccurate quantitation. To address this, a step gradient of TMAC (5%–15% buffer B) was added just before the start of the isocratic hold (19% B), which significantly decreased the peak tailing (T_f) to <2.0 for empty capsid peaks. Raising the post-isocratic hold gradient from 19% to 50% resulted in the elution of full AAV6 peak with excellent full peak symmetry and low peak tailing ($T_f < 2.0$). Overall, these modifications resulted in a final optimized AEX-HPLC method in which successful baseline separation of AAV6 empty and full capsids was achieved. The resulting method showed high resolution (>>2.0) as well as excellent asymmetry and tailing (<2.0) for both peaks in rAAV6-030 and rAAV6-623 (Figure 2A). These peak performance criteria are in alignment with the USP and CDER recommendations for the validation of an HPLC method. The calculated percentages of empty and full AAV6 capsids in lot rAAV6-623 were ∼8.1% and ∼91.9%, respectively, and in lot rAAV6-030, they were ∼95.6% and ∼4.0%, respectively. In addition, three mixtures, where both rAAV6-623 and rAAV6-030 samples were co-mixed in different percentages, were also analyzed. The analysis of resultant chromatograms of all three co-mixtures along with pure samples exhibited a linear change in the peak area of both peaks with corresponding changes in percent peak areas of empty or full AAV6 capsids ($r^2 > 0.99$) (Figure 2B). These observations indicated that the area response for each peak was additive, linear, and specific to either empty or full AAV6 capsid.

Figure 3. Correlation of % empty and full capsids using orthogonal methods

(A) Stacked chromatograms representing fluorescence signal versus time trace for different rAAV6 samples by optimized AEX-HPLC assay are shown. (B) AAV6 % empty and full capsids of these samples obtained by AUC and Cryo-TEM analysis are shown. (C–F) The correlation of % empty and full AAV6 capsids obtained by AEX-HPLC with Cryo-TEM (C and D) and AUC (E and F) are also shown. A linear correlation with an R > 0.90 was obtained across all comparisons. The rAAV6 samples tested contained AAV genomic size ranging from 2.5 to 5.2 kb.

Correlation of quantification of % empty and full AAV6 capsids by AEX-HPLC to AUC and Cryo-TEM

In order to demonstrate the validity and accuracy of our AEX-HPLC method for determining the percentage of AAV6 empty and full capsids, we compared the values obtained for several rAAV6 samples with AEX-HPLC to those obtained for the same samples with two orthogonal methods, namely, Cryo-TEM and AUC. Although each method has its advantages and disadvantages, both of these methods are widely accepted for quantification of empty and full capsids.[5,9,10] A set of test samples comprising 10 different rAAV6 vectors, each with unique transgenes and genomes of different sizes (2.5–5.2 kb), were evaluated. All samples were analyzed with the optimized AEX-HPLC and the results are presented here (Figure 3A). Each sample showed two distinct baseline separated peaks for empty and full capsids. The retention times of both empty and full peaks for all samples were constituent (<5% RSD), indicating that the method performance was independent of the size of the encapsidated AAV genome. The excessive negative surface charge contributed by DNA within the capsid did not impact the assay performance significantly. It should be noted that the peak labeled as "full capsid" in AEX-HPLC may also contain AAV capsids that are only partially filled with transgene DNA and/or host cell nucleic acids, since the difference in pI for the partially filled versus full capsids is insufficient to resolve them from each other.[26,27] All samples were additionally analyzed by AUC (KBI Biopharma, San Diego, CA), and 5 of the samples were selected for analysis by Cryo-TEM (Vironova, Stockholm, Sweden). The results from all three methods were compared (Figure 3B) to demonstrate the validity of the AEX-HPLC method. A linear regression coupled with Pearson correlation coefficient (r) is a commonly used statistical analysis tool for demonstrating correlation between two continuous variables. This coefficient (r) can have a value between −1 and 1, where −1 indicates negative correlation, 0 indicates no correlation, and 1 indicates positive correlation between two continuous variables. It is important to note that, unlike AEX-HPLC, AUC can resolve partials from full capsids successfully. In our case, % empty capsids obtained by AEX-HPLC was correlated to those obtained by either AUC or Cryo-TEM, while % full capsids obtained by AEX-HPLC was correlated with full capsids by Cryo-TEM and (partial + full) capsids as determined by AUC. It is evident that the peak area values obtained by the AEX-HPLC method for empty and full AAV6 capsids show a highly positive correlation with the result values obtained with Cryo-TEM (Figures 3C and 3D) and AUC (Figures 3E and 3F), with a Pearson correlation coefficient r > 0.99 in each case. These findings suggested that the AEX-HPLC method can be used to accurately quantify the relative percentage of the empty and full AAV6 capsids in an rAAV6 sample.

Robustness of the AEX-HPLC method for empty/full AAV6 analysis

To evaluate the robustness of the optimized AEX-HPLC method, the flow rate and pH of the mobile phase and column temperature were varied and the effects on peak retention times, resolution, and tailing were assessed with a sample containing a mixture of rAAV6-030 and rAAV6-623. Column temperature was varied from 15°C to 50°C, in 5°C increments. The performance of the method remained comparable with the column temperature ranging from 15°C to 25°C.

However, at higher temperatures (>30°C), reduced peak areas of the major peaks were observed, and the appearance of additional minor peaks eluting after the full AAV6 peak was witnessed (Figure 4A). These additional minor peaks have also been observed in previous studies[26] and could represent AAV capsids containing larger-than-full sized genomes or damaged capsids or may be a result of secondary interaction of the sample with the column under the test conditions or thermal degradation products.[26] Future work is necessary to understand the nature and identity of the extra peaks. In light of these findings, all subsequent experiments were performed at column temperature of 20°C, with the added knowledge that the method performance will remain consistent within ±5°C of this temperature, thus resulting in an overall acceptable range of 15°C–25°C for the column temperature. To investigate how slight changes in the flow rate affect method performance, three different flow rates, 1, 0.8, and 1.2 mL/min, were evaluated. The performance of the method remained comparable as flow rates were still significantly higher than column volume (Figure 4B). Finally, the effect of variations in the pH of the mobile phase was explored by evaluating method performance when mobile phase buffers with pH values ranging from 8.0 to 9.5 were used (Figure 4C). It was observed that acceptable peak resolution (R_s) was achieved (>4.0) when the pH of the mobile phase was maintained between 8.8 and 9.2. Buffers with a pH lower than 8.8 or higher than 9.1 resulted in significant shifts in peak retention times coupled with the loss of peak resolution and increased peak tailing. Previous studies have shown similar pH dependence impact on the separation of empty/full capsids with AAV2 serotype.[19,23] It is possible that the peak resolution can be still improved at these suboptimal pH values by adjusting the salt gradient parameters. These results suggest that the method is robust and resistant to small but deliberate changes in mobile phase flow rate (0.8–1.2 mL/min), mobile phase pH (8.8–9.1), and column temperature (15°C–25°C). However, it must be noted that pH of the mobile phase and starting gradient (%B) are extremely important in binding of the AAV capsids to the column before they can be separated by the discontinuous gradient.

Characterization of the optimized AEX-HPLC method

The optimized AEX-HPLC was evaluated with respect to injection volume linearity, sample dilution linearity, and precision of multiple injections, using rAAV6-030 and rAAV6-623 as representative samples. Different injection volumes of rAAV6-030 and rAAV6-623 were analyzed by the AEX-HPLC method, and the peak areas of both empty and full AAV6 peaks in each chromatogram were plotted against the injection volume of the test sample (Figures 5A and 5B). Both samples showed excellent injection volume linearity up to 10 μL (10% of column volume). The injection volumes above 10 μL resulted in appearance of a peak in the void volume region of the chromatogram as a non-bound fraction (data not shown). In addition, saturation of the resultant peak areas of both empty and full AAV6 peaks resulted in diminished injection linearity at higher injection volumes. The presence of a non-binding fraction in the chromatogram could be attributed to overloading the column binding capacity, higher ionic strength, low residence time of sample on the column, and/or the presence of excessive sample matrix additives
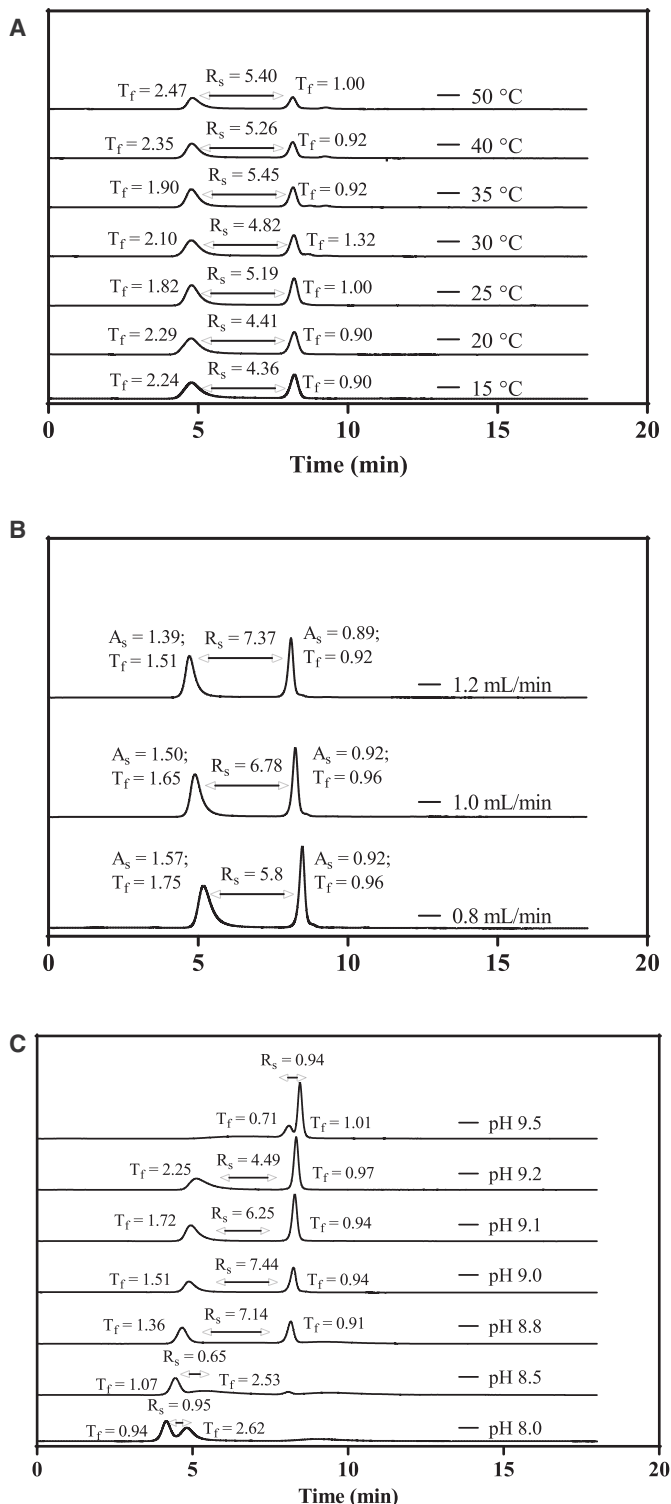
or impurities when higher volumes were injected. Based on these data, the injection volume was kept at 10 μL for all future experiments. Next, the dilutional linearity of the AEX-HPLC assay was determined for both rAAV6-623 and rAAV6-030. Both samples, rAAV6-623 (3.18E+12 capsids/mL) and rAAV6-030 (1.73E+13 capsids/mL), were serially (2×) diluted in formulation buffer, and 10 μL of each of seven dilutions was analyzed with the AEX-HPLC method. The chromatograms for each injection were analyzed for peak areas of empty and full AAV6 peaks (Figures 5C and 5D). The peak area response of major peak showed excellent linearity ($r^2 > 0.99$) over the tested ranges of 3.18E+12 to 4.97E+10 capsids/mL for rAAV6-623 and 1.73E+13 to 2.70E+11 capsids/mL for rAAV6-030. The peak area responses of minor peak for each sample were also linear as long as they remained detectable. These results suggested that the AEX-HPLC method is very sensitive and can be used to quantify empty and full AAV6 capsids in samples with capsid concentrations as low as ∼5E+10 capsids/mL (S/N > 100; column efficiency USP plates > 5,000 for main peak). In all experiments, excellent precision of retention times (<1.0%) and peak asymmetries (<2.0) was observed while maintaining high resolution (>5.0) between peaks.

Applications of the empty-full HPLC assay

A reliable and robust assay for quantifying empty AAV capsids is crucial to understanding product quality and lot-to-lot or product-to-product variability. To that end, we evaluated the suitability of the method for testing a variety of process intermediates including post-cesium chloride (CsCl), post-affinity column purification, post-IEX column, drug substance ultra/diafiltration, and filled drug product (data not shown). In addition to analyzing different in-process rAAV6 samples, the method can be used to identify rAAV6 samples with a high percentage of empty capsids. The AEX-HPLC analysis of a rAAV6 drug product manufactured by the same process on two different occasions is shown in Figure 6A. It is evident that the quality of rAAV6 drug products differed considerably between two runs, with rAAV6-071 containing >70% empty AAV6 capsids in the product, with desired full product being only a minor component (<25%), whereas rAAV6-069 was shown to contain full AAV6 capsids as the major product (>90%). The comparative analysis of A260/280 chromatograms for rAAV6-071 showed that A260-to-280 ratios for empty and full AAV6 peaks were 0.67 and 1.32, respectively (Figure 6B). Similar ratios for empty and full capsids have been seen in previous studies.[8,24,27] These observations confirmed that rAAV6-071 indeed contained excessive amounts of empty capsids.

Extension of the empty-full HPLC assay to other serotypes

We next applied our strategy for separating empty and full AAV6 capsids to other AAV serotypes. The calculated net pI for empty AAV for different serotypes is quite similar (∼6.3),[25] but each serotype may behave differently in AEX chromatography. This may be due to overall differences in amino acid composition, resulting in varying capsid surface charge profile and overall structural characteristics of different AAV serotypes.[30,31] As a proof-of-concept study, commercially available or in-house produced samples or mixtures corresponding to five different AAV serotypes (AAV1, AAV2,

AAV3, AAV8, and AAV9) were selected. When analyzed by AEX-HPLC using linear separation gradient of 0%–50% buffer B, all samples from different serotypes showed two distinct peaks with considerable overlap as seen with AAV6 (Figure S2). Next, these samples were tested with the optimized method for AAV6 as described above. Throughout this study, it was seen that the optimized AEX-HPLC method for rAAV6 yielded partial separation of empty and full capsids for rAAV samples of serotypes other than AAV6 when the method was used unchanged (Figure S3). Therefore, like most platform methods, it was necessary to optimize the conditions for each serotype separately to achieve baseline peak separation while using the same core principles (mobile phase, column, and isocratic hold flanked by two linear gradients) of the original method with AAV6. For complete binding of samples to the column, different initial starting salt concentrations (%B) were needed for each serotype, which once again speaks for striking differences in overall capsid surface charge profile for different serotypes and the method's exquisite sensitivity to distinguish these differences. In addition, for each serotype, different isocratic holds (%B) were needed until peak for empty capsids separated from peak for full capsids. Finally, both linear gradients on either side of the isocratic hold were fine-tuned to obtain optimal symmetry and tailing for each peak. The optimized method parameters for each serotype are presented in Table S1. To further demonstrate the suitability of each of the serotype-specific methods, numerous samples were analyzed using optimized method parameters for the corresponding serotypes, and the resultant chromatograms for each serotype are shown in Figure 7. For instance, for AAV8, a commercially obtained empty rAAV8 (14-361), AAV8 reference standard material (VR-1816, American Type Culture Collection [ATCC]) and four in-house produced rAAV8 samples were tested with the AEX-HPLC method optimized for AAV8. The assay results confirmed the presence of a significantly large proportion of empty AAV8 capsids in the test article named 14-361, while remaining samples including the ATCC reference standard were shown to contain mostly full AAV8 capsids, which confirmed earlier findings with AAV8 reference standard material.[32] Similar results were obtained for samples of other serotypes, namely AAV1, AAV2, AAV3, and AAV9 as shown in Figure 7. Similarly, AUC analysis of some of rAAV8 and rAAV9 samples (Figure S4) confirmed that the AEX-HPLC analysis produced comparable quantitative results for empty and full capsids for different serotypes. It is important to note that despite the ability of each of these AEX-HPLC methods to successfully separate empty and full AAV for multiple serotypes, each method may require further optimization to obtain a robust assay that can be adaptable to the QC environment. This would include selecting appropriate initial gradient (%B) for binding the AAV capsid to the column along with optimization of discontinuous gradient, mobile phase pH, flow rate, and column temperature.
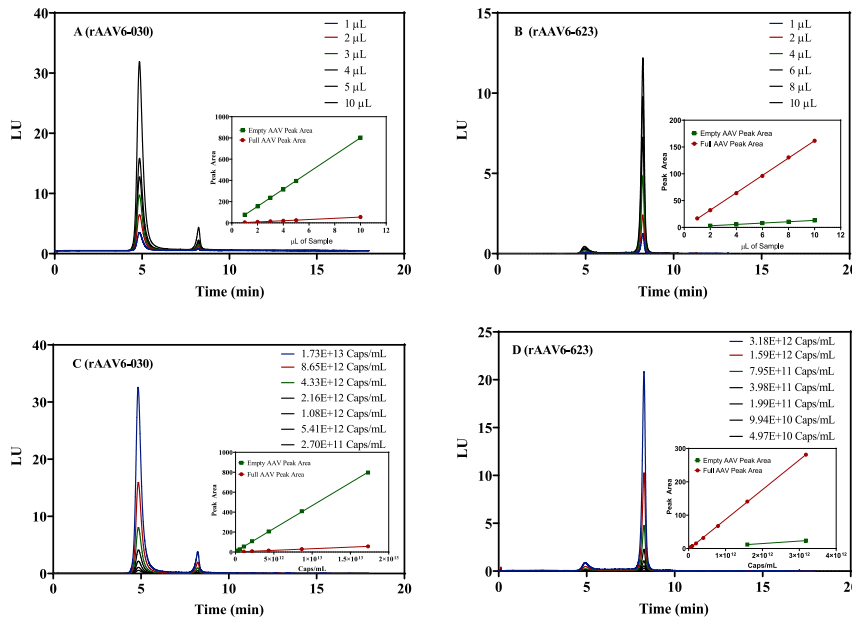
Figure 4. Robustness of the optimized AEX-HPLC assay

(A–C) Robustness of the AEX-HPLC method was evaluated on a mixture of rAAV6-030 and rAAV6-623 by implementing deliberate changes in the column temperature (A; 15°C–50°C), flow rate (B; 0.8–1.2 mL/min), and pH (C; 8.0–9.5) of the mobile phase, and their subsequent effect on peak resolution (between empty and full AAV6 peaks) and peak tailing was monitored. Each chromatogram represents fluorescence versus time trace and indicates the peak resolution (R_s) and tailing factor (T_f) for both empty and full AAV6 peaks.

(A and B) AEX-HPLC assay was used to demonstrate the injection volume linearity of rAAV6-030 (A) and rAAV6-623 (B). (C and D) In addition, dilutional linearity of rAAV6-030 (C) and rAAV6-623 (D) with respect to capsid titer concentration was demonstrated. In (A)–(D), main graph contains overlaid chromatograms representing fluorescence signal versus time trace of the sample or its dilutions against injected capsid titer or volume of the sample. The inset in each graph shows a plot of AAV6 empty and full capsid peak area versus the parameter being evaluated. The regression analysis for peak area versus either injected capsid titer or volume in each graph showed $r^2 > 0.99$, indicating linearity of the response.

DISCUSSION

In the context of rAAV gene therapies, product-related impurities encompass a wide variety of defective interfering particles, with a significant percentage of these particles comprising empty AAV capsids that are incapable of delivering any therapeutic benefit and yet may act as functional decoys that neutralize AAV-specific antibodies.[2,4,33] Quantifying the empty-to-full capsid ratio deserves special attention, as increasing percentages of empty capsids have the ability to reduce the overall potency of the gene therapy product.[34] Although there are several analytical methods available to measure this quality attribute, shortcomings such as low throughput and sample purity have limited their use in routine analysis. For product characterization, Cryo-TEM and AUC are increasingly common but are less suitable to use in a QC environment for drug product release. In that respect, AEX-HPLC technique is well suited for high-throughput characterization and is amenable to use in a GMP QC environment. Prior studies have shown that analytical AEX-HPLC can separate and quantify empty and full AAV capsids for different AAV serotypes.[26,27] While these earlier approaches have resulted in only partial resolution of empty and full AAV capsid peaks, we demonstrate here a QC-amenable method for quantitation of empty and full capsids in rAAV6 samples by achieving full baseline separation along with excellent peak symmetry and tailing. The method was optimized to allow for full baseline resolution and quantification of empty and full AAV capsids with excellent precision and peak performance criteria that conform to existing guidelines for purity method validation.[28] The AEX-HPLC analytical approach described in this manuscript utilizes two short linear gradients flanking either side of an isocratic elution step using an optimally selected eluant, TMAC. The use of an isocratic hold was critical for a full separation of empty capsids while full capsids were still bound to the column. Previous studies used a similar approach in which they were able

to use isocratic wash and subsequent elution steps for enrichment of full capsids for AAV2 serotype.[19,23] In addition to isocratic hold, optimization of linear gradients on either side of the isocratic hold resulted in optimal peak asymmetry and tailing per USP/ICH guidelines and suitable for a QC environment. The assay presented here demonstrates excellent injection and dilutional linearity for rAAV samples and is thus applicable for analysis of rAAV samples spanning a broad range of empty to full capsids. Overall, our assay showed improved dilution linearity range (∼100-fold) and the ability to analyze much lower sample concentrations (∼5E+10 capsids/mL) compared with an earlier report.[26] Assay performance was found to be robust and to be largely unaffected by small but deliberate changes in mobile phase flow rate, mobile phase pH, and column temperatures. Moreover, our assay was shown to be accurate by comparison of the empty and full capsids obtained with our AEX-HPLC method for various rAAV6 samples to those obtained with Cryo-TEM and AUC. Despite the limitation that the AEX-HPLC method cannot separate partial from full capsids, our method offers a high-throughput approach with full resolving power to separate and accurately quantify empty from full capsids, which is a significant advancement from earlier studies.[22,24,26,27] We also show that AEX-HPLC assay can be a valuable control system tool to monitor product quality by identifying and potentially rejecting drug product lots that have higher than desired % empty capsids, thereby supporting the in-process control strategies.

The AEX-HPLC method described here has been shown to be "suitable for its intended purpose" and can serve as a valuable tool for the analysis of in-process samples as a means to monitor the removal of empty capsids from the manufacturing process. Moreover, the approach used for AAV6 has been further assessed as a "platform method" for use with five additional rAAV serotypes—requiring minimal optimization of method principle and design. The broad utility of such an approach can also be extended to other natural or artificially designed serotypes. Since decreasing the overall percentage of the empty capsids has been one of the key goals of a successful manufacturing process for AAV products, we firmly believe that
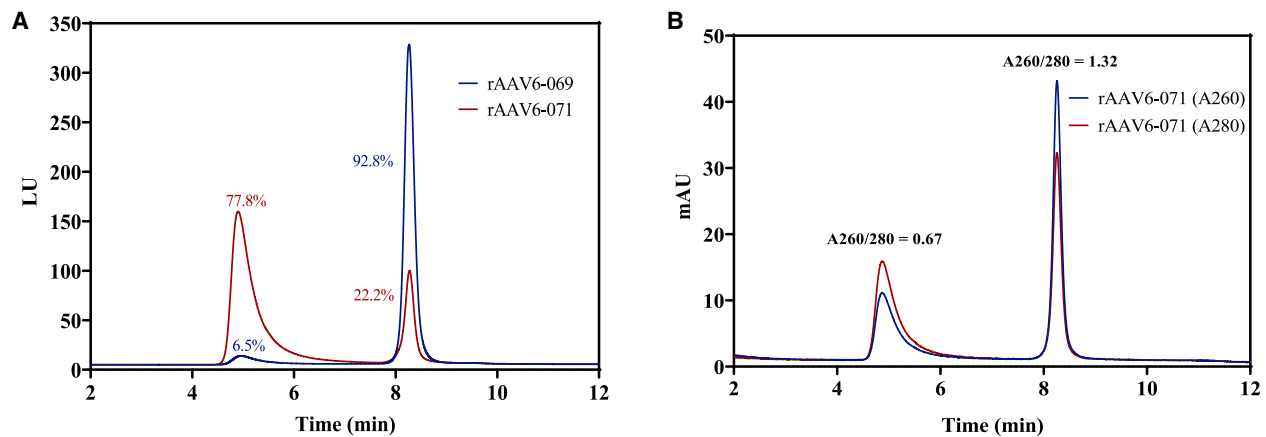
Figure 6. Applications of the optimized AEX-HPLC assay

(A) The overlaid AEX-HPLC chromatograms representing fluorescence signal versus time trace of two rAAV6 samples (lots 069 and 071) purified from two different production runs using the same process are shown. Lot 071 was shown to contain significantly high % empty capsid compared with lot 069, which was attributed to a defect in starting materials that may have caused impairment in the optimal packaging of the AAV cassette. (B) The overlaid chromatograms for A260 nm and A280 nm versus time trace for lot 071 are shown, which exhibit peak maxima for AAV6 empty and full capsid peak at 280 nm and 260 nm, respectively. The A260-to-280 ratios, 0.67 and 1.32, for these peaks indicated that they correspond to AAV6 empty and full capsids, respectively.[24]

our approach can find applications in the removal of empty AAV from full AAV capsids, thereby improving product quality.

MATERIALS AND METHODS

Chemicals and reagents

BTP (≥99%) and all solvents (2-propanol, methanol, and water; all liquid chromatography-mass spectrometry [LC-MS] grade) were purchased from VWR. Sodium chloride, TMAC, sodium acetate, and ammonium acetate (all chromatographic grade) were purchased from Sigma (Milwaukee, WI, USA). The buffers were prepared by dissolving appropriate chemicals in LC-MS-grade water and adjusted to appropriate pH with 1 N HCl (VWR). The solutions were filtered through a 0.2 μm membrane filter and transferred into amber HPLC bottles to avoid exposure to light. As an HPLC pump seal wash, 10% IPA solution was used, while 50% methanol was used as an injection needle wash solution. For analysis by AUC and Cryo-TEM, rAAV samples were sent to KBI Biopharma (San Diego, CA, USA) and Vironova (Stockholm, Sweden), respectively.

Production and purification of rAAV vectors and empty rAAV control

AAV8 reference standard (VR-1816) was purchased from ATCC. Other commercially available rAAVs (empty AAV1, 14-061; AAV1-CMV-GFP, 20-113 and 19-037; AAV3-CMV-GFP, 20-189; empty AAV3, 20-165; empty AAV2, 18-133; empty AAV8, 14-361) were purchased from Virovek (Hayward, CA, USA) and used either as received or diluted. These rAAVs were formulated in phosphate-buffered saline (1× PBS) containing 0.001% Pluronic F68 with or without 100 mM sodium citrate. The remainder of the rAAV samples were manufactured by Sangamo Therapeutics using either triple transfection of HEK293 cells or by baculovirus-based infection of Sf9 cells using procedures similar to those described in the litera-

ture.[35–38] The rAAV produced by triple-transfection method in the Sangamo Vector Core used an in-house platform production method. Briefly, HEK293 cells were plated in ten-layer CellSTACK chambers (Corning, Acton, MA, USA) and grown for 3 days to a density of 80%. Three plasmids, an AAV helper plasmid containing the Rep and Cap genes, an adenovirus helper plasmid containing the adenovirus helper genes, and a transgene plasmid containing the sequence to be packaged flanked by AAV2 inverted terminal repeats (ITRs), were transfected into the cells with calcium phosphate.[35] After 3 days the cells were harvested. The cells were lysed by three rounds of freeze/thaw, and cell debris was removed by centrifugation. The rAAV was precipitated with polyethylene glycol. After resuspension, the virus was purified by ultracentrifugation overnight on a CsCl gradient. The virus was formulated by dialysis and then filter sterilized.

The Sf9 baculovirus-based production of rAAV was executed in the Sangamo Process Development Department with Sangamo's platform baculovirus system. Briefly, Sf9 cells were seeded at target cell density in the production vessel. No later than the day after seeding, the Sf9 cells were infected with two recombinant baculoviruses encoding the AAV6 Rep and Cap proteins and the transgene cassette, respectively (e.g., rAAV6-623). The empty rAAV control (rAAV6-030) was manufactured with the Sf9 production system without using transgene cassette to produce primarily empty AAV6 capsids. However, this product may still contain small amounts of capsids containing host cell nucleic acids as a result of low-fidelity packaging of the capsids.[4] About 6–7 days after infection, the cell culture was harvested with a series of filtration steps. The filtrate, which contains rAAV, was purified by immune-affinity chromatography. The purified rAAV was then buffer exchanged into formulation buffer for long-term storage by either ultrafiltration or dialysis. For some products, immune-affinity
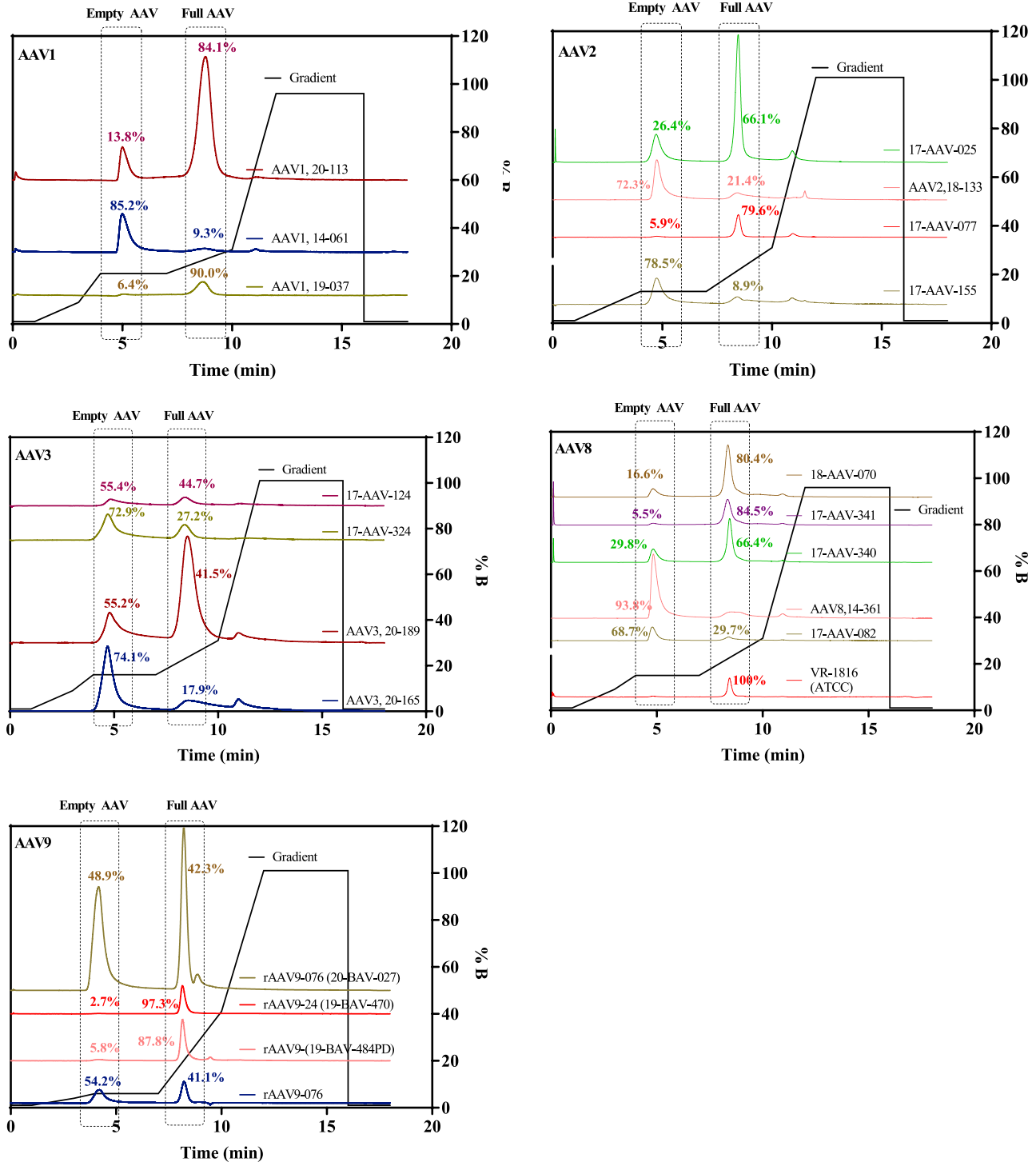
Figure 7. AEX-HPLC chromatograms representing fluorescence signals versus time trace of rAAV samples for different serotypes including AAV1, AAV2, AAV3, AAV8 and AAV9

Top left: AAV1. Top right: AAV2. Middle left: AAV3. Middle right: AAV8. Bottom: AAV9. The separation method (gradient profile) for each serotype is also shown. For each serotype, binding of test sample to the column, isocratic hold, and both linear gradients were specifically optimized to allow for optimal separation of empty from full rAAV capsids. Within the results for each serotype, the retention times for AAV peaks were consistent (<2%) among the samples tested.

chromatography was followed by an IEX polishing step before ultra-filtration. For the development, optimization, and characterization of the AEX-HPLC assay, rAAV6-030 and rAAV6-623 were used as controls for primarily containing empty (>95%) and fully packaged (> 94%) AAV6 capsids, respectively. All in-house rAAVs were formulated with 1× PBS containing 35 mM NaCl, with or without 1% sucrose, and 0.001% or 0.05% of a non-ionic surfactant, Pluronic F-68. All rAAV products were assessed for vector genome titer by Droplet Digital PCR targeting an amplicon within the ITR region with the QX200 Droplet Digital PCR system (Bio-Rad Laboratories, USA). In addition, capsid titers were measured with the AAV6 Titration ELISA kit (Progen, Germany) as per manufacturer's instructions. For samples received from Virovek, the titers were measured by SDS-PAGE as provided by the manufacturer.

AEX-HPLC method for separation of empty and full AAV capsids

The AEX-HPLC experiments were performed on an Agilent 1260 Infinity II series HPLC equipped with a multisampler, multi-column thermostat along with a diode-array detector (DAD) and fluorescence detectors. The separations were performed on a monolith CIMac AAV full/empty-0.1 Analytical Column (1.3 μm, 100 μL column volume) (BIA Separations, Slovenia) at a constant column temperature of 20°C and flow rate of 1 mL/min unless stated otherwise. Before the start of any experiment, the column was cleaned with high-salt (NaCl, TMAC, sodium acetate, or ammonium acetate) buffer for 10–15 min or until the baseline was steady, followed by re-equilibration of the column with initial conditions of the method for 10–15 min or until a steady baseline was achieved (see Table S1). The UV signal was monitored at 260 (±20 nm) and 280 (±20 nm) with a fixed slit width (4 nm), while fluorescence signal was monitored at Ex./Em. = 280 nm/348 nm. The response time for both UV and fluorescence signals was >0.25 s. Unless stated otherwise, mobile phase A contained 20 mM BTP buffer (pH 9.0) while mobile phase B contained 20 mM BTP buffer with 1 M salt (pH 9.0). Sample injection volume was kept at 10 μL unless otherwise specified. For quantitation purposes, only fluorescence signal in the chromatogram was used. In each experiment, formulation buffer (two injections) was run as a blank and chromatograms obtained for samples were blank subtracted. The chromatographic data were processed with preset processing method parameters, and % of peak areas corresponding to each peak (empty and full AAV) was calculated by dividing the individual peak area by the total peak area in the chromatogram. The entire runtime for a single sample analysis was 18 min. The individual steps of the separation method and their runtimes for rAAV6 are shown in Table S1. For other serotypes, modified method parameters with respect to initial equilibration, initial gradient, isocratic hold, and final gradient and column cleanup steps are shown in Table S1.

Statistical analysis

Statistical analyses by linear regression correlation and Pearson correlation coefficient were performed with GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA). The statistical significance of difference was considered as p <0.05.

AUTHOR CONTRIBUTIONS

S.L.K. conceptualized the idea and developed the approach under supervision of Z.P. A.P. performed experimental optimizations and supported development studies. S.L.K. and A.P. co-wrote the manuscript.

DECLARATION OF INTERESTS

All authors are either current or past employees of Sangamo Therapeutics Inc. S.L.K. and Z.P. declare competing financial interests: they are named inventors on a pending US patent application (publication no. US20210009964). The patent application publication covers the underlying concept and results described in this manuscript. The remaining authors declare no competing interests.

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