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Cation-exchange high-performance liquid chromatography of recombinant adeno-associated virus type 2

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Abstract

There has been much interest recently in the development of recombinant viruses as vectors for gene therapy applications. We have constructed a recombinant adeno-associated viral (AAV) vector containing the gene encoding CFTR (cystic fibrosis transmembrane chloride regulator). This vector is currently being used in clinical trials as a treatment for cystic fibrosis. In the course of scale-up and process optimization efforts, a variety of analyses have been developed to characterize yield and quality. Although these methods produce quantitative and highly reproducible results, most are very time intensive. For example, a standard bioassay requires a 72-h incubation period followed by an additional day of analysis. Other tests such as UV spectrophotometry are fast, but unable to distinguish between whole virus, free protein, and DNA. Here, we describe an analytical cation-exchange high-performance liquid chromatographic method utilizing a TSKgel SP-NPR strong cation-exchange column. Unlike the bioassay which requires a 96-h wait for information, this method yields data in less than 20 min. In addition to the quick assay turn-around, the material eluting in the single peak was found to be intact, infectious, nuclease resistant AAV particles. This offers a significant advantage over the limited information one gains from UV spectrophotometry. This demonstrates the utility of chromatography for analysis and purification of viral vectors.

Keywords: Recombinant adeno-associated virus

1. Introduction

Adeno-associated virus (AAV) was first identified as a small defective viral contaminant of adenovirus [1]. AAV has unique features that makes it attractive as a vehicle for gene therapy applications [2]. AAV infects a wide range of cells, can transduce non-dividing cells [3] and is capable of long-term persistence [4]. Our work utilizes a recombinant AAV CFTR (rAAVCF) vector [5] currently in clinical trials as a treatment for cystic fibrosis. We have been able to produce large amounts of vector, but lacked a quick assay for quantification. Our standard bioassay [6] requires a 72-h infection period followed by another day of analysis. This lag has developed into a major hindrance to rapid process optimization. We have developed a cation-exchange HPLC method for AAV that reduces assay time to less than 20 min and can be applied to a broad range of sample conditions. Column-based chromatography as a method for

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purification of recombinant viral vectors has been described for a variety of systems: the purification of HIV-1 for the manufacturing of inactivated gp120 depleted HIV-1 [7]; the recovery of recombinant adenovirus encoding the human p53 tumor suppressor protein [8]; a heparin resin [9] for the purification of AAV; and several authors have reported the use of anion-exchange chromatography as a method for viral analysis [10,11].

After screening various chromatographic supports, we found cation-exchange resins useful for an analytical AAV HPLC assay. We show that cation-exchange HPLC allows characterization of AAV with only minimal sample preparation. The particle-to-infectivity ratio (P/I) of the rAAV contained in the peak elution vs. that of the injected sample remains constant, indicating that this method may serve as a means to track active material without having to wait for the results of a bioassay. The column chosen for the analysis was the TSKgel SP-NPR, a 3.5 cm×4.6 mm I.D, strong cation-exchange column (TosoHaas, Montgomeryville, PA, USA), which contained 2.5 μm, methacrylate resin beads functionalized with sulfopropyl groups with a pK_a value of 2.3 [12,13]. The SP-NPR was selected due to its nonporous nature, particle size, and methacrylate backbone. Nonporous resins provide excellent recoveries for peptides, proteins, oligonucleotides, and DNA fragments with rapid analysis [13,14]. Sample loading provides a linear response down to nanogram levels of these biomolecules. Therefore, the projected recoveries and mass responses for larger biomolecules, such as viruses, should also be quite good. The size of the resin beads allowed for high resolution of peptides and proteins with reasonably high flow-rates which led to short analysis times [13,14]. Finally, the polymeric methacrylate resin is stable within a pH range of 2–12 which permitted the use of sodium hydroxide for cleaning purposes [15].

2. Experimental

For the purposes of the HPLC assay development, we used the AAVCFTR [5] derived from adeno associated virus type 2. AAVCF was designed to deliver the correct copy of CFTR (cystic fibrosis transmembrane chloride regulator) to the lungs of cystic fibrosis patients who possess a defective copy.

2.1. AAVCFTR production

AAVCFTR was produced according to published methods [16]. Briefly, producer cells containing rep, cap, and the AAVCF construct were infected with adenovirus type 5 at an MOI of 10. At 72 h, AAVCF vector was harvested and purified by cesium chloride density gradients or various ion-exchange chromatography resins. Purified vector was used for cation-exchange HPLC analysis. The level of infectious AAVCF in each fraction was determined by microtiter infectivity assay [6].

2.2. wtAAV production

Wild type AAV2 was made according to methods previously described [17]. Briefly, wt AAV was generated by a co-infection of 293A cells with wtAAV and Ad5. At 72 h post-infection, cells were harvested and lysed by sonication. The cell lysate was double banded on two successive CsCl gradients. The material was dialyzed into Ringer's balanced salt solution (Biowhittaker,Walkersville, MD, USA)+5% glycerol and assayed by A_{260} for particle number and $TCID_{50}$ end-point dilution for infectious titer [6].

2.3. Slot blot assay

To determine the number of DNase-resistant particles, 50-μl aliquots of each sample were taken and treated with Benzonase (Sigma, St. Louis, MO, USA) to a final concentration of 25 U/ml. Samples were then incubated in a 37°C water bath for 60 min. After digestion the reaction was stopped with 50 μl of EDTA to a final concentration of 10 m*M*. Log dilutions of the samples were made in 1 μg/ml salmon sperm DNA and denatured for 60 min in NaOH at 70°C. pAAVCF plasmid dilutions in the range of $1\cdot10^7$–$2.5\cdot10^5$ copies denatured in the same way were used as standards. After denaturation, standards and sample dilutions were loaded onto a slot blot (Schleicher & Schuell, Keene, NH, USA) apparatus holding a piece of nylon Genescreen membrane (NEN, Boston, MA, USA). A vacuum was applied and the material transferred to the membrane. This was followed by a rinse with 0.4 *M* NaOH.

The apparatus was disassembled and the blot rinsed with 2×SSC. The blot was then UV cross-linked using a Stratalinker (Stratagene, La Jolla, CA, USA) and probed with a ^{32}P-labeled 1488 bp EcoRI fragment, representing pAAVCF plasmid sequences, by standard techniques.

The blot was then encased in plastic wrap and exposed to a phosphorimager cassette (Molecular Dynamics, Sunnyvale, CA, USA) for 14–20 h. The resulting image was gridded and the volume of each well recorded on an excel spreadsheet. A macro was used to generate a standard curve and analyze the data. This analytical technique will detect only vector particles with intact DNA, and will not quantitate empty or non-specific DNA containing vector capsids.

2.4. UV spectral determination

Material from CsCl purified AAVCF or wtAAV pools was diluted 1:10 in phosphate-buffered saline (Biowhittaker)+0.1% SDS and analyzed on an HP model G1103A spectrophotometer (Hewlett-Packard, Waldbron, Germany). Samples were placed in cuvettes and scanned from 235 to 310 nm. The spectrum was recorded to serve as a comparison to the spectra of material eluting from the column.

2.5. Western blot analysis

Samples were mixed twice with SDS–PAGE reducing buffer (125 m*M* Tris–HCl pH 7, 20% glycerol, 4% w/v SDS, 0.005% bromphenol blue, 0.5% β-mercaptoethanol) to 30 μl, boiled for 5 min, and loaded onto a 1 mm×10 well Bio-Rad 10% Tris–HCl polyacrylamide minigel. Samples were electrophoresed for 1 h at 150 V.

The bands were then transferred to a membrane prewetted in 100% methanol and soaked in transfer buffer (39 m*M* glycine, 0.037% SDS, 20% methanol, 48 m*M* Tris base, pH 8.3). The gel was also equilibrated in transfer buffer. The proteins were then transferred to the membrane using a Bio-Rad semidry transfer apparatus at 12 V for 60 min. The membrane was then blocked for 20 min with 5% (w/v) non-fat dry milk at room temperature. The membrane was rinsed twice with TBS–T (Tris-buffered saline with 0.1% Tween 20. The membrane was then incubated with a monoclonal mouse anti-VP1,VP2,VP3 antibody (ARP, Belmont, MA, USA) for 1 h at room temperature. Following primary incubation, the membrane was washed three times with TBS–T and incubated with a goat anti-mouse horseradish peroxidase conjugated secondary antibody (Sigma) for 1 h. Three more washes were performed with the TBS–T buffer and then the membrane was incubated with Amersham ECL reagents for 1–20 min. To visualize the blot, the membrane was exposed to film (Kodak, Rochester, NY, USA) and developed in an X-ray film developer.

2.6. Quantitative HPLC assay

A 0.58-ml TSKgel SP-NPR (Tosohaas) cation-exchange column was used for HPLC analysis of the samples. Purified material was first dialyzed against the equilibration buffer (50 m*M* HEPES, 1 m*M* EDTA, 5 m*M* MgCl, 100 m*M* NaCl pH 7.5) for 2 h prior to injection.

The column was equilibrated at a flow-rate of 1 ml/min on an LC626 chromatographic system (Waters, Milford, MA, USA) equipped with a 717 plus autosampler and a model 996 photodiode array detector (PDA). The chromatography was monitored on the PDA detector scanning from 240 to 320 nm. Chromatograms were extracted at 280 nm for quantitation. MILLENNIUM Software (Waters) was used to integrate peak areas.

After sample loading, the column was washed with two column volumes of equilibration buffer followed by a linear gradient from 100 to 500 m*M* NaCl in 50 m*M* HEPES, 1 m*M* EDTA, 5 m*M* MgCl, pH 7.5. The eluted peak was quantitated at 280 nm against a standard curve run in the same sample set. Standard curves were comprised of five injections of purified virus ranging in concentration from $5\cdot10^{9}$ to $1\cdot10^{11}$ DRP. The column was periodically cleaned between sample sets with a 0.25-ml injection of 0.5 *M* NaOH.

3. Results

For this HPLC analytical method to be an effective replacement for standard assays, several criteria

had to be met: the spectral profile of the vector peak must be consistent with that of the AAV standard, the presence of AAV proteins in the eluted peak must be confirmed, and the vector eluted in the peak and the injected sample must have a similar particle-to-infectivity ratio. After characterizing the physical aspects of the eluted material, the linear range and volumetric constraints of the load were established. The results of these analyses provided the information necessary to establish cation-exchange HPLC as a valid addition to current analytical methods.

3.1. Identification of peak using purified AAVCF

Purified AAVCF was quantitated by slot blot against a set of plasmid standards. The sample was treated with nuclease to assure that any signal on the blot was coming from packaged DNA and not free, unencapsidated template. Quantitation of DNase resistant particles (DRP) was performed and this material was used for the first series of injections. Vector sample containing $1 \cdot 10^{11}$ DRP was injected on the TSKgel SP-NPR column and eluted in a single peak with a retention time ≈6 min (Fig. 1). To confirm the identity of the material in the peak as AAV the spectrum was determined from 235 to 310 nm (Fig. 2b). The spectral signatures of wtAAV and AAVCF within the same range were determined separately on a spectrophotometer to serve as a comparison (Fig. 2a). All three exhibit the same spectral pattern with a maximum absorbance at 275 nm and a small shoulder or inflection at 290 nm. Laughlin et al. [18] have reported a spectrum with a UV maximum closer to 260 nm. The viral stocks used for these studies may contain a mixture of full and empty capsids not resolved by HPLC. Western Blot analysis (Fig. 3) confirmed the presence of AAV VP1, VP2, VP3 proteins [19] in the eluted peak.

3.2. Recovery analysis

After confirming that the material eluting in the peak was indeed AAV, the next step was to confirm that the AAV particles retain activity after elution. Although no flow-through or other elution peaks were visible, it was possible that vector may be inactivated in the course of the assay. In order to assure that the eluted peak is representative of the loaded sample material, confirmation that the eluted material had the same particle-to-infectivity (P/I) ratio was necessary. AAVCF with a P/I ratio of 1300 was loaded on the column and the peak fraction collected. Three separate injections were made using the same input material to assess reproducibility. Slot blot and infectivity analysis analysis showed no significant difference in the particle-to-infectivity ratio of the peak material (1200 ± 200) over that of the injected sample (Table 1). The conclusion from

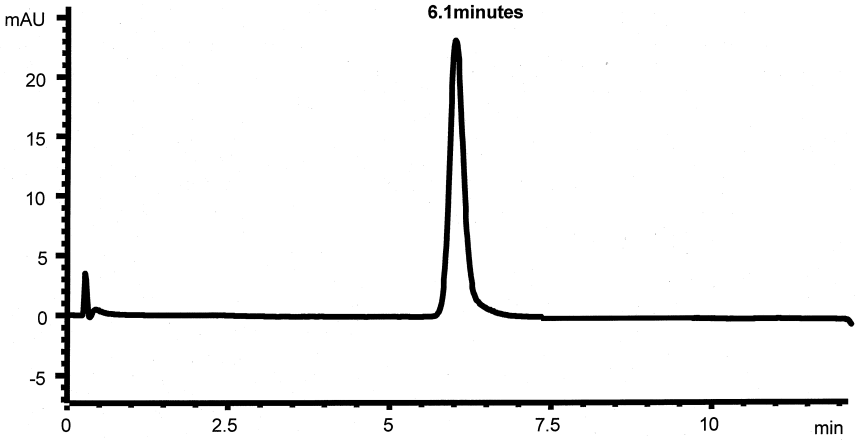

Fig. 1. Analysis of purified AAVCFTR with TSKgel SP-NPR. AAVCF was injected onto a TSKgel SP-NPR cation-exchange column. The column was washed with two column volumes of equilibration buffer followed by a 10 column volume linear gradient from 100 to 500 m*M* NaCl. Detection was UV at 280 nm. A single peak was observed with an elution time of 6.1 min. The unbound material and peak were collected for analysis.

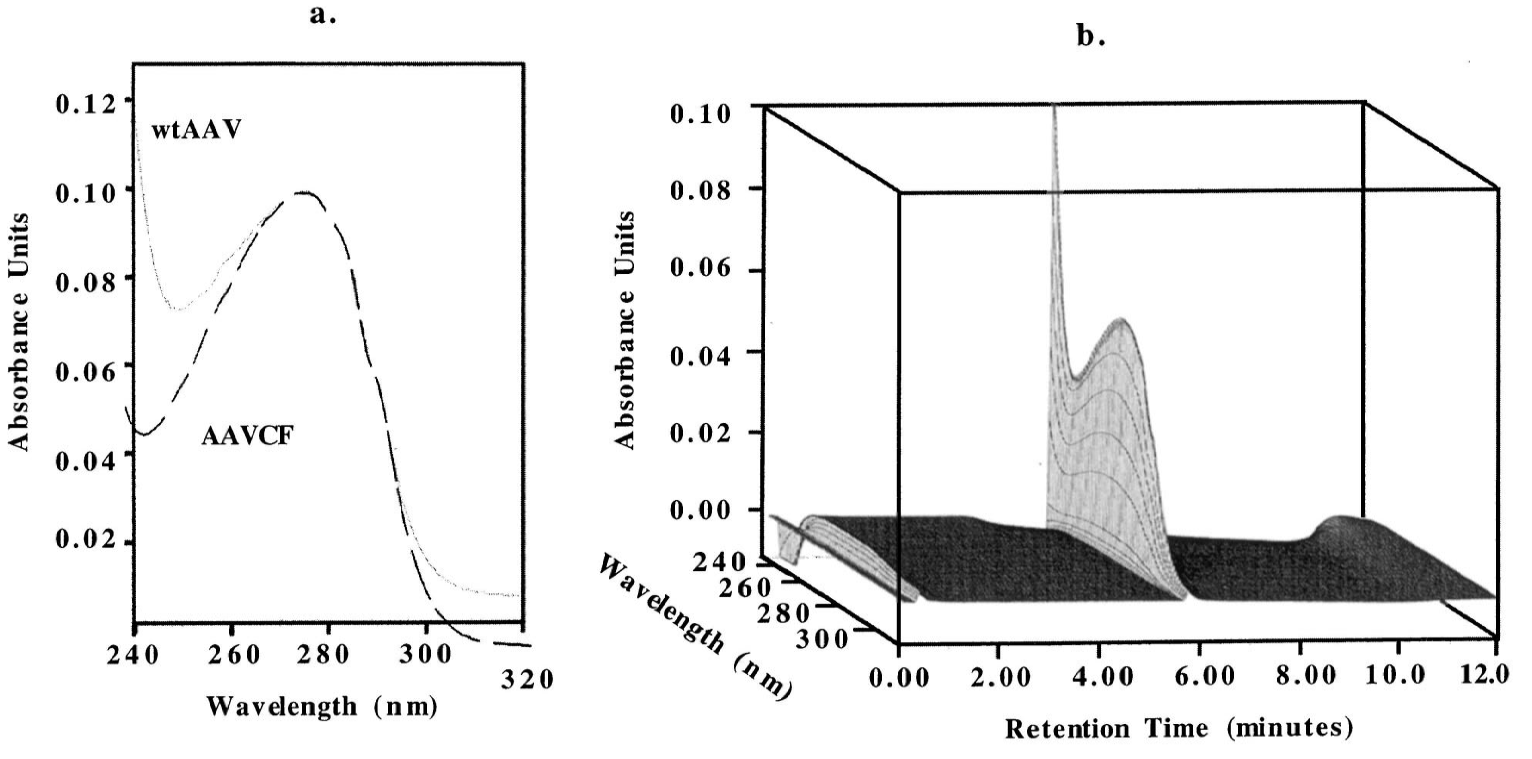

Fig. 2. (a) AAVCF or wtAAV purified by CsCl density centrifugation were mixed with 0.1% SDS and the absorbance scanned from 240 to 320 nm. The wtAAV and the recombinant AAV vector exhibit the same spectral profile: a UV maximum at 275 nm and a small inflection at 290 nm. (b) Three-dimensional plot of time vs. wavelength vs. absorbance of purified AAVCF injected onto the SP-NPR resin and scanned from 235 to 310 nm shows the same spectral pattern for the 6-min peak as that of AAV.

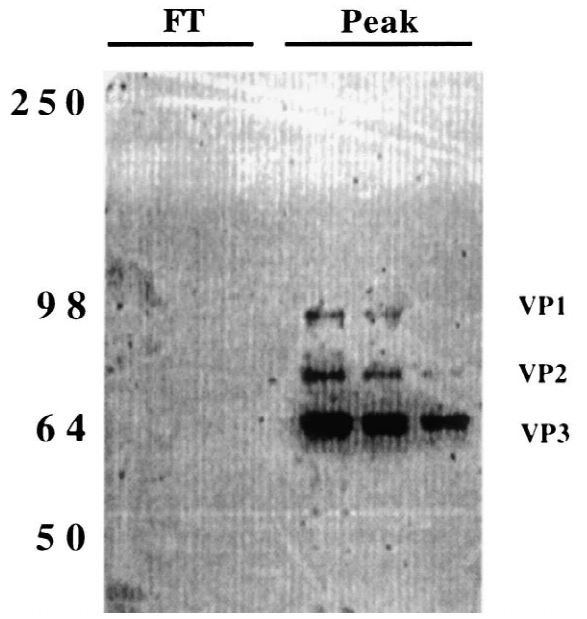

Fig. 3. Western blot analysis of column flow-through and peak fractions. Purified AAVCF was injected and fractions collected. Three 250-μl aliquots of the flow-through and three 250-μl aliquots of the 6.1-min peak were collected. The AAV2 capsid consists of three proteins (VP1, VP2, and VP3) with molecular masses of 87, 73 and 62 kD, respectively. Western blot analysis on the samples taken confirmed that the material eluting in the 6-min peak was indeed AAV. In addition, no AAV was seen in the unbound fraction.

Table 1
Recovery analysis of the 6-min peak[a]

	P/*I* ratio		
Load	1300		
Peak elution run 1	1300		
Peak elution run 2	1000		
Peak elution run 3	1400		
	Mean=1200	SD=210	C.V.=17%

[a] AAVCF with a particle-to-infectivity ratio (*P*/*I*) of 1300 was injected in three separate experiments and the peak fractions collected and pooled. The mean particle-to-infectivity ratio of the recovered material determined by slot blot and infectivity assays was 1200±210. Since the vector is not being inactivated in the course of the method, it follows that this would be a good way to track infectious vector as well as total particles. Infectivity in this case is measured using a vector-specific replication assay and is therefore expressed as replication units (RU).

this study was that the eluted peak is representative of the injected material in that the *P*/*I* remains the same.

3.3. Assay characterization

To establish the linearity and limit of detection of the assay, increasing amounts of purified AAVCF were loaded on the column. An AAVCF vector peak was observed in the range of $3\cdot10^{9}$–$5\cdot10^{11}$ DRP. Below $3\cdot10^{9}$, the peak was indistinguishable from baseline. The peak area vs. load was linear in the range of $1\cdot10^{10}$–$5\cdot10^{11}$ DRP with an R^2 of 0.997 (Fig. 4). The comparison of the spectra of the peaks used to establish the linear range served to confirm the identity of the eluted material (data not shown).

Having established the linear range of the assay, it was next necessary to determine if load volume would affect the peak area as samples typically come in a variety of volumes and concentrations, and changes in processing may yield product with a volume ten-fold greater than that of an earlier step. To address this, $1\cdot10^{11}$ DRP were loaded in a range of volumes from 10 to 100 μl. The area of each resulting peak was determined and graphed (Fig. 5). The peak area is independent of the concentration of virus injected and the C.V. of the peak areas is 9%. Volumes tested greater than 100 μl gave increased C.V.s (data not shown).

3.4. Summary of results

An analytical method for the quantitation of recombinant AAV vectors has been developed using cation-exchange HPLC. Purified AAVCF loaded onto a TSKgel SP-NPR cation-exchange column eluted as a single peak. The eluted material was infectious, DNase resistant, and demonstrated a UV spectrum in agreement with that of the injected material. AAVCF recovered in the peak demonstrated no change in the particle-to-infectivity ratio. This showed that the vector was not being inactivated by the resin. In addition to this, the spectrum of the material recovered in the peak corresponded exactly to that seen for wtAAV. Increasing amounts of AAVCF were loaded and the area of the resulting peaks were

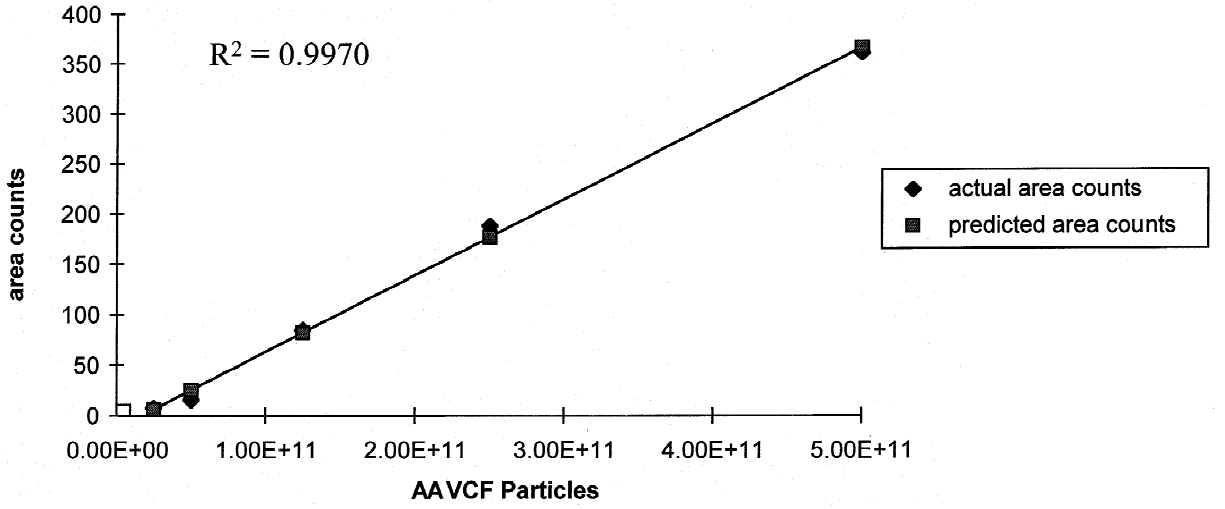

Fig. 4. AAVCF particles vs. area counts. Injections of purified AAVCF were made to establish the range and degree of linearity of the assay. Peaks were visible in the range of $5\cdot10^{9}$–$5\cdot10^{11}$ DRP. Linear regression gave a line with an R^2=0.997 in the range of $1\cdot10^{10}$–$5\cdot10^{11}$ DRP.

plotted against the load. The assay proved to be linear over a wide range and thus have utility for dilute and concentrated samples. This method should have wide utility for process development, and the purification and analysis of recombinant adeno-associated viruses as gene therapy vectors.

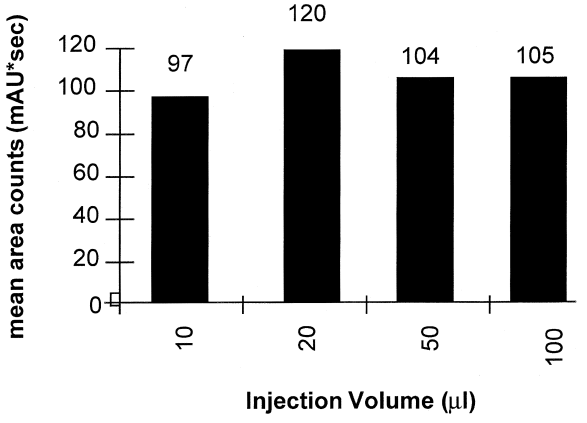

Fig. 5. Constant particle load with variable volume; $1\cdot10^{11}$ DRP of AAVCFTR in volumes ranging from 10 to 100 μl were injected to assess if the area (mAU s) of the peak would be independent of the concentration. In this range, the resulting area counts showed good consistency with a mean of 107±10 and a C.V. of 9%. This indicates the peak area is not a function of concentration and can be used to assess a range of sample sizes.

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