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应用纪要

Using a 2.1 mm ID Narrow Bore GTxResolve™ Premier™ SEC Column to Reduce Sample Consumption During Size Exclusion Analyses

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Abstract

To demonstrate the utility of a narrow bore GTxResolve Premier BEH SEC 450 Å 2.5 µm Column for the biophysical characterization and purity testing of cell and gene therapy drug substances.

Introduction

In the cell and gene therapy industry, it is size-exclusion chromatography (SEC) that has become an essential method for detecting both product-related and process-related impurities. Product-related impurities include aggregates or degraded forms of the therapeutic molecules that can impact efficacy or safety. Process-related impurities, such as residual host cell proteins, DNA, or buffer components, may also compromise product quality if not thoroughly removed. SEC efficiently separates these impurities from the desired product, allowing an analyst to collect a precise purity profile. One of the

important impurities to check for is molecular aggregates. SEC, when operated under native conditions, is one of the most suitable techniques for aggregate quantitation. Beyond impurity detection, SEC is valuable for concentration determination, as it can quantify the therapeutic biomolecule based on its peak area, offering a multiattribute measurement to the purity assessment. This combined functionality makes SEC an indispensable tool for rigorous process development and quality control testing of cell and gene therapy drugs. The availability of new column technology in 2.1 mm ID narrow bore hardware means that more SEC runs can be applied to facilitate the development of drug substances. Narrow bore SEC columns make it possible to complete SEC-UV/FLR characterization without consuming as much precious lead candidate material.

Experimental

The Solution

Size exclusion chromatography separates biomolecules according to their hydrodynamic size and shape in solution. This technique is an entropy driven process, but it can be impacted by non-specific secondary interactions between analytes and the column packing material. Most SEC column manufacturers have relied on metal column hardware that requires significant method optimization through use of mobile phase additives including the addition of increasing concentrations of salt. To address this problem, Waters has developed MaxPeak Premier hydrophilic high-performance surface (HPS) column hardware to minimize non-specific electrostatic interactions. This technology makes use of vapor deposited surfaces comprised of an organic and inorganic (carbon and silica) composition that shield analytes from interacting with the surfaces of metallic flow path components.

Traditionally, SEC columns are used in 4.6 or 7.8 mm ID dimensions to overcome the negative impact of large extra-column volume and dispersion effects imposed by HPLC instruments. However, narrower bore SEC columns continue to be requested to help reduce sample consumption, especially when drug substances are limited during discovery and pre-clinical project work. Fortunately, Waters can provide optimal packing into 2.1 mm columns constructed with MaxPeak HPS hardware. GTxResolve Premier SEC columns can be purchased as custom manufactured parts using Waters p/n: 186011284 by reaching out to a Waters customer representative. Use of narrower bore columns reduce sample consumption while maintaining high data quality of wider bore columns, though it should be noted that 2.1 mm ID

should only be performed on a low dispersion UHPLC instrument. Moreover, care should be given to optimize pre- and post-column dispersion effects as much as possible. Upon request, MaxPeak Premier 2.1 mm ID Columns can be prepared with both diol bonded 2.5 µm BEH™ 450 Å particles and bridged ethylene polyethylene oxide (BE-PEO) modified 3 µm SEC 1000 Å particles. This application note demonstrates the performance of a 2.1 x 150 mm GTxResolve Premier BEH SEC 450 Å 2.5 µm Column compared to a commercially available 2.1 x 150 mm SEC titanium hardware column containing 3 µm 700 Å particles.

Results and Discussion

Experimental Design

The analytes tested in this work include Waters BEH 450 Protein Mix Standard (p/n: 186006842 < https://www.waters.com/nextgen/global/shop/standards--reagents/186006842-beh450-sec-protein-standard-mix.html>), NISTmAb Reference Material (RM8671), and AAV2-Empty serotype Virovek Cat#449B000-2-100, 2E+13 vp/mL. Lliquid chromatography was performed using an ACQUITY Premier Quarternary LC System with high pH flowpath kit and a CH-A 15 cm column heater and TUV detector. Flow rate was set to 0.052 mL/min with a mobile phase comprised of 20 mM Phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4 (2x dPBS). Column temperature was set to 30 °C. Extra column tubing considerations are important to the optimization of 2.1 mm ID SEC chromatography. As such, Figure 1 is provided to give guidance on some pre and post-column tubing options.

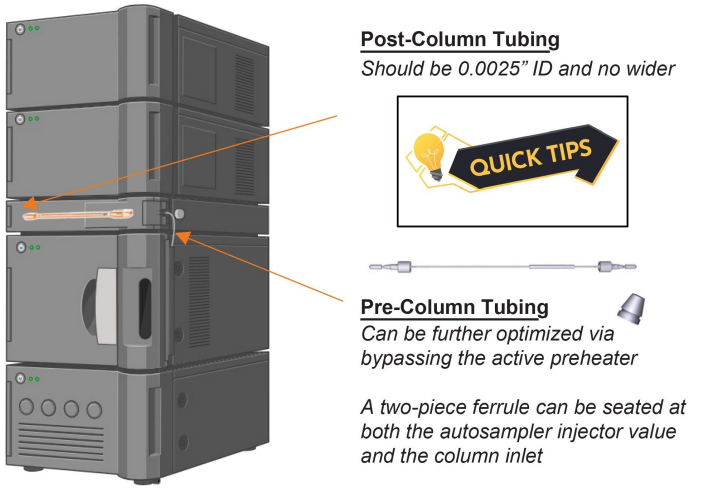

Figure 1. Schematic providing guidance on optimizing an instrument for SEC-UV analyses with a 2.1 mm ID GTxResolve Premier SEC Column. Created with biorender.com.

Examining the Performance of a Narrow Bore SEC Column

The utility of a GTxResolve Premier BEH SEC 450 Å 2.5 µm Column 2.1 x 150 mm column was compared to a commercially available 700 Å 2.1 x 150 mm titanium hardware column, starting with the analysis of a common protein system suitability mixture. Figure 2 demonstrates that the narrow bore GTxResolve Premier BEH SEC 450 Å Column was able to resolve each component of the mixture without issue. USP half-height (HH) resolution was easily obtained for all the analytes indicating the separation power of the custom packed GTxResolve Premier BEH SEC 450 Å 2.5 µm 2.1 x 150 mm Column (Figure 2A). In contrast, USP HH resolution values could not be computed for several peak pairs when the separation was performed with an alternative, commercially available option (Column P; Figure 2B). The 700 Å pore size of Column P particles would suggest that it would have improved fractionation for the thyroglobulin monomer and dimer species in the test mixture. However, the GTxResolve SEC Column ultimately produced higher resolution. These effects may be attributable to differences in packing efficiency.

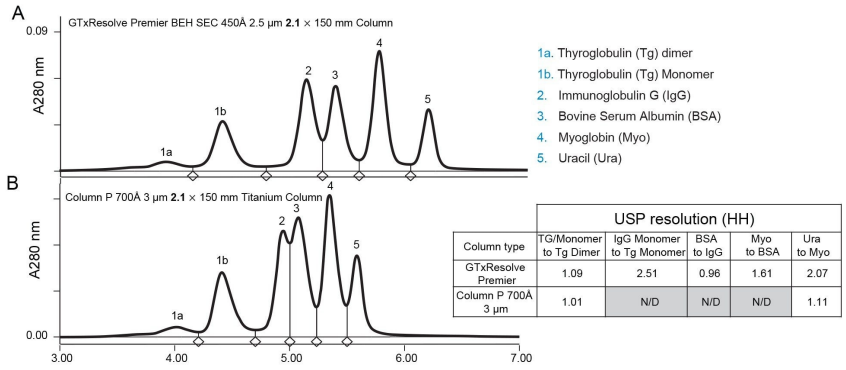

Figure 2. Comparison of 280 nm chromatograms acquired with a GTxResolve Premier BEH SEC 450 Å 2.5 µm 2.1 x 150 mm Column (A) versus an alternative commercially available 2.1 mm ID titanium hardware column(B). A 2 µL injection was made of a 1 mL reconstituted volume of Waters BEH 450 Protein Mix Standard.

Next, we examined the secondary interactions and separation performance as observed for NISTmAb and an AAV sample. Figure 3A illustrates the salt dependence of the GTxResolve Premier BEH SEC 450 Å 2.1 x 150 mm column and the commercially available 700 Å 2.1 x 150 mm column. A NISTmAb RM8671 sample was used for this testing. Overlaid chromatograms are provided that correspond to mobile phases starting with no NaCl (red trace), followed by 50 mM (blue trace), 100 mM (purple trace), and 200 mM (black trace) NaCl. Figure 3A (top panel) shows the chromatograms collected from the GTxResolve SEC column and that little to no change in peak tailing nor recovery was observed with changing amounts of mobile phase NaCl. Conversely, notable effects were observed with the 700 Å 2.1 x 150 mm column. Figure 3A (bottom panel) shows the corresponding chromatograms and the apparent salt dependence of this column (Column P). There was an observable decrease in peak tailing with increasing salt, which is indicative of metal adsorptive interactions.

Differences in secondary interactions are also observed when applying these columns to the analysis of an AAV sample (Figure 3B). Herein, an empty AAV2 sample has been applied to investigate the utility of these narrow bore columns. AAV2 peak shape and recovery were unaffected by application of 1x or 2x strength dPBS mobile phase when the GTxResolve Premier BEH SEC 450 Å 2.5 µm 2.1 x 150 mm Column was applied (Figure 3A top panel). In contrast, when Column P was used under the same conditions,

poor recovery of AAV2 with was observed for 1x dPBS mobile phase (Figure 3B bottom panel). The column was confirmed to be dependent on mobile phase ionic strengths corresponding to 2x strength dPBS. As with 4.6 mm studies that have come before, these results suggest that comparatively minimal method development is needed to optimize a mobile phase for a GTxResolve Premier BEH SEC 450 Å 2.5 µm Column, even when it is packed as a specialized 2.1 x 150 mm configuration.[2]

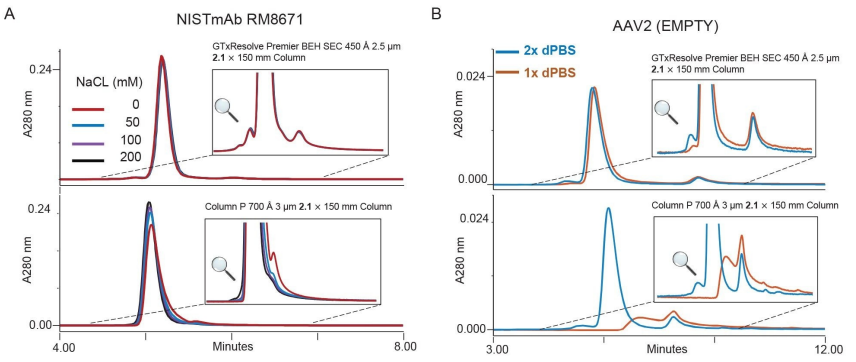

Figure 3A. Comparison of electrostatic secondary interactions using NISTmAb (RM8671) evaluated at 0.073 mL/min with 100 mM sodium phosphate pH 6.8 mobile phase containing varying amounts of sodium chloride (NaCl) from 0 mM to 200 mM.

Figure 3B. Illustrates the dependence on ionic strength of mobile phase for analysis of AAV2-Empty samples when evaluated at 0.052 mL/min using 10 mM Phosphate, 138 mM NaCl, 2.7 mM KCl pH 7.4 (1x dPBS) versus 20 mM Phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4 (2x dPBS).

Conclusion

In this application, we demonstrate the generation of high quality chromatography from a GTxResolve Premier BEH SEC 450 Å 2.5 µm 2.1 x 150 mm Column. With small considerations made to pre- and post-column accessories, the fidelity of peaks can be effectively maintained. Although the performance of the separation is not as precisely optimized as a 4.6 x 150 mm column but the slight loss in resolving power is compensated for by a sizable reduction in sample consumption and a corresponding increase in

senstivity. A Waters custom made, narrow bore column showed higher resolution of biomolecules when compared to a commercially available alternative (a 2.1 x 150 mm 700 Å 3 µm titanium hardware column). The Waters custom made column also exhibited little or no salt dependency while characterizing biotherapeutic moleclules. That feature was further confirmed upon use of the column to analyze a AAV2 sample where high quality information was obtained with a 1x strength dPBS buffer. In contrast, the alternative column (Column P) showed decreased resolution and high salt dependence. Although the use of narrow bore (2.1 mm ID) columns requires extra system considerations, it is our hope that their availability will hasten the development of gene therapies by giving project teams a new option to preserve more drug material for critical *in vitro* and *in vivo* studies.

References

1. "Heightened Characterization of AAVs by SEC-MALS with XBridge™ Premier GTx BEH™ SEC 450 Å 2.5 µm Columns" Lavelay Kizekai, Balasubrahmanyam Addepalli, Matthew A. Lauber, Waters Application Note. 720007969. June 2023.
2. "Fast and Efficient Size Exclusion Chromatography of Adeno Associated Viral Vectors With 2.5 Micrometer Particle Low Adsorption Columns" Mateusz Imiołek, Szabolcs Fekete, Lavelay Kizekai, Balasubrahmanyam Addepalli, Matthew Lauber, *Journal of Chromatography* A 1714 (2024) 464587.

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