INTRODUCTION

Size exclusion chromatography (SEC) is typically used to measure protein aggregates and other size variants present in biopharmaceuticals. New packing materials and instrumentation have allowed faster and more reproducible separations to be achieved. However, the recovery of proteins and higher order aggregates still remains a critical part of the success of an SEC method.

In this presentation, we will discuss the factors that can influence quantitation of biomolecules on size exclusion packing materials. Evaluation of an SEC method typically includes analysis of the resolution and aggregate quantitation. In the following discussion, we will outline the considerations in developing a SEC method. The effect of variables such as flow rate, mobile phase composition, and pH will be measured.

METHODS

UPLC-SEC Chromatographic Conditions:

Unless otherwise specified
LC System: ACQUITY UPLC® H-Class Bio System with PDA detector with Titanium Flow Cell
Wavelength: 280 nm
Mobile Phase: 25 mM Sodium Phosphate, pH 6.8, 0.15 M NaCl
Wash and Purge Needle Washes: Mobile Phase
Seal Wash: 80/20 H₂O/Methanol
Temperature: 35 °C
Flow rate: 0.4 mL/min

Columns, unless otherwise noted:
Waters: ACQUITY BEH200 SEC, 1.7 µm, 4.6 x 300 mm (p/n 186005226), 0.4 mL/min, 10 µL injection
Vendor A: Diol-coated silica 150Å, 3µm, 4.6 x 300mm, 0.4 mL/min, 10 µL injection
Vendor D: Diol-coated silica 250Å, 4µm, 4.6 x 300mm,0.4 mL/min, 10 µL injection

Protein Recovery
Colorimetric Assay: Pierce(R) BCA Assay Kit
Spectrometer: Perkin Elmer X35
Samples were collected in 2mL volumetric flasks. Protocol for colorimetric assay reproducibility was followed enhanced test tube protocol as described in Pierce BCA Assay Kit instructions.

RESULTS AND DISCUSSION

PROTEIN RECOVERY

The recovery of proteins and higher order aggregates remains a critical part of the success of an SEC method. However, protein adsorption has been an area of concern. In order to ensure accurate quantification, a variety of approaches have been used. These techniques may include pre-treatment of the column with high mass loads of either the sample of interest or another protein, such as BSA. However these methods do not assess total protein recovery. In order to evaluate protein recovery of UPLC-SEC packing materials, protein samples were injected. The samples were subsequently collected and measured by a colorimetric assay for total protein quantitation. Mass load was based on sensitivity and colorimetric assay reproducibility.

EVALUATION OF SIZE EXCLUSION CHROMATOGRAPHY PACKING MATERIALS FOR THE ANALYSIS OF PROTEINS AND HIGHER ORDER AGGREGATES

Paula Hong, Edouard S. P. Bouvier and Kenneth J. Fountain
Waters Corporation, 34 Maple Street, Milford, MA, USA

Protein Recovery on an Unconditioned Column

To determine the effects of column conditioning on protein recovery, a new BEH200 SEC 1.7 µm column was tested. The first ten injections of a human IgG (2 mg/mL, 40 µL) were individually collected and measured for protein content. The protein collected was compared to a standard and to injected samples collected from the system with no column in line. The studies were repeated for diol-coated silica columns designed for monoclonal antibody analysis.

Effect of Mobile Phase Salt Concentration

Figure 3. Effect of salt concentration on murine mAb peak shape and aggregate recovery. At low buffer concentration, secondary interactions affect peak shape, i.e. tailing. Higher buffer concentration improves peak shape and resolution as well as recovery of mAb (inset). Note: Orthogonal analysis may be required to determine effect of salt concentration on protein aggregation. Buffer: 100M Sodium Chloride, pH 6.8.

Effect of Flow Rate

Figure 5. Effect of flow rate on murine mAb aggregate recovery. For this sample, at pH 6.4 secondary interactions affect peak shape (asymmetry). Increasing the pH improves peak shape and affects aggregate recovery. Note: Orthogonal analysis may be required to determine effect of pH on protein aggregation. Buffer: 100M Sodium Phosphate, pH listed above.

CONCLUSION

- UPLC-SEC technology does not result in measurable loss of proteins other than due to the UPLC H Class Bio system or BEH200 SEC 1.7 µm column
- UPLC-SEC can provide improved resolution of monoclonal antibody aggregates as compared to traditional silica diol-coated SEC columns
- SEC method development strategies (mobile phase concentration, pH and flow rate) can be used to evaluate and/or minimize secondary interactions

References