ANALYSIS OF BIOMOLECULES BY SIZE-EXCLUSION AND ION-EXCHANGE CHROMATOGRAPHY IN COMBINATION WITH ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

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INTRODUCTION

The complete analysis and characterization of biopharmaceuticals is most efficient when orthogonal analytical techniques are applied to the samples. Each technique should be based on different physical properties of the protein, and can include chromatographic methods such as size-exclusion (SEC), ion-exchange (IEX) and reversed-phase chromatography. In this presentation, we will demonstrate how the ACQUITY UPLC H-Class Bio System in combination with Auto•Blend™ Plus Technology can now be used in conjunction with new ion-exchange and size-exclusion packing materials for improved chromatographic separations. Size exclusion method development will include the effects of salt concentration and column length. Ion-exchange method development will also be outlined, including the use of pH and buffer composition. These factors will be utilized to demonstrate the improved impurity detection and/or faster analysis of biomolecules achievable with the combination of UPLC Technology and newer ion-exchange and size-exclusion packing materials.

METHODS

Chromatographic Conditions
LC System: ACQUITY UPLC® H-Class Bio System with PDA detector
Detection: PDA @ 280 nm
Flow Cell: Titanium

Size-Exclusion Chromatography
Column: ACQUITY BEH200 SEC, 1.7µm, 4.6 x 150 mm
Injection Volume: 5.0 µL
Flow Rate: 0.4 mL/min
Mobile Phase: 25mM Sodium Phosphate, pH 6.8, 0.15M NaCl
Wash and Purge Needle Washes: Mobile Phase
Seal Wash: 80/20 H_2O/MeOH
Temperature: 30˚C
Sample Diluent: 25mM Sodium Phosphate, pH 6.8, 0.15M NaCl (unless otherwise noted)

Ion-Exchange Chromatography
Column: Protein-Pak™ Hi Res CM, 7µm, 4.6 x 100 mm
Wash and Purge Needle Washes: Same as sample diluent
Seal Wash: 80/20 H_2O/MeOH
Temperature: 30˚C
Sample Diluent: 20mM Sodium Phosphate or MES Buffer pH 6

Additional conditions in figure headings.

SIZE-EXCLUSION CHROMATOGRAPHY

In a series of experiments, protein standards and monoclonal antibody biotherapeutics were analyzed with UPLC SEC. The reproducibility of the calibration was tested by analysis of proteins standards over the molecular weight range of 10,000- 450,000 Da at regular intervals over a two day period. The elution volume for each protein standard was found to be within 0.2 % RSD (Figure 1). The consistency of the calibration curve is indicative of both the column life and instrument control of flow rate and injection volume. To test the reliability of quantitation, a humanized IgG was analyzed and found to have an average aggregate quantitation of 6.82 % +/- 0.3 % of the monomeric species over the time period (Figure 2).

RELIABILITY OF CALIBRATION MEASUREMENTS

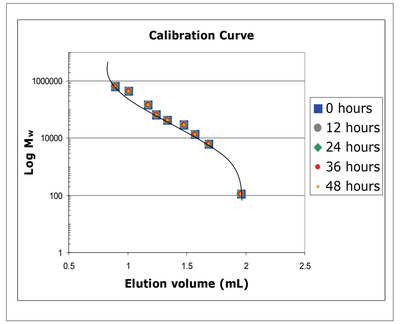
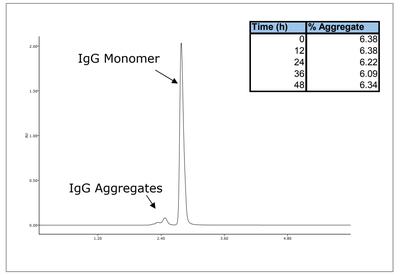


Figure 1 Protein calibration curve, ACQUITY BEH200, SEC, 1.7 µm, 4.6 x 150mm. Recommended molecular weight range is 10,000– 450,000. Overlay of 5 calibration curves over 48 hours.

REPRODUCIBILITY OF SEC AGGREGATE MEASUREMENT



Time (h)	% Aggregate
0	6.38
12	6.38
24	6.22
36	6.09
48	6.34

Figure 2. SEC separation of humanized IgG. Injection of undiluted humanized IgG over 48 hours showed aggregate quantitation relative to the monomer of 6.09-6.38% with a RSD of 0.3%.

EFFECT OF BUFFER CONCENTRATION

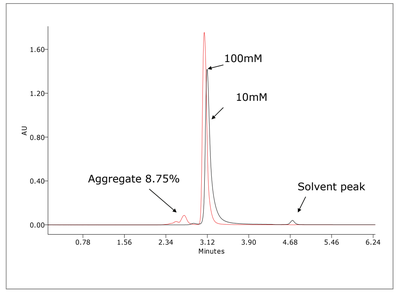


Figure 3. Effect of buffer concentration on humanized IgG aggregate measurement. At low buffer concentration, secondary interactions affect peak shape, i.e. tailing. Higher buffer concentration improves peak shape and allows for quantitation of aggregates. Buffer: XXM Sodium Phosphate, pH 6. 8.

EFFECT OF COLUMN LENGTH

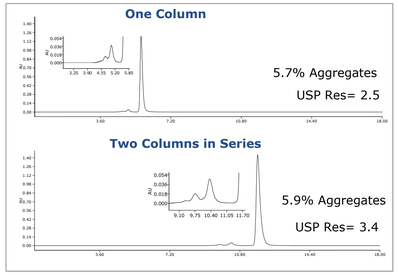
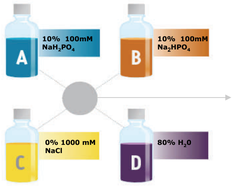


Figure 4. Effect of column length on SEC aggregate measurement. Coupling two 4.6 x 150mm columns provides a 50% improvement in USP resolution at identical flow rate (0.2 mL/min) with increased backpressure (~2300 psi for 4.6 x 150mm column ;~6000 psi for 2- 4.6 x 150 mm columns).

ION-EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography is often utilized to assess the distribution of proteins or the presence of protein variants formed by post-transitional modifications (*e.g.*, deamidation) that can be recognized by charge differences. For these analyses, adjustments in mobile phase pH or ionic strength are the most useful parameter for method development. Using Auto•Blend Plus Technology (schematic below), the effect of pH and buffer composition (Figure 5 and 6) was demonstrated for a mixture of proteins as well as C-terminal lysine variants of a monoclonal antibody (Figure 7). Variants of a chimeric mAb were confirmed by treatment with carboxypeptidase B to cleave the C-terminal lysines (Figure 8).

Auto•Blend Plus Technology



20mM Sodium Phosphate, 0 mM NaCl pH 6.8

EFFECT OF pH

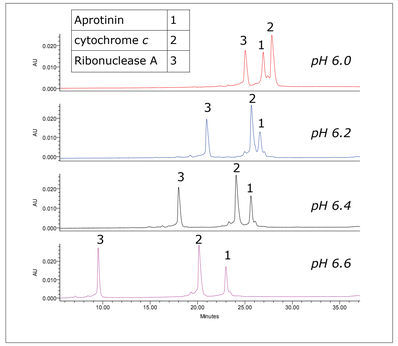


Figure 5. Effect of pH on IEX separation of protein mixture. Aprotinin elutes before cytochrome c at pH 6.0. Selectivity is reversed at pH 6.2. Conditions: 20mM Buffer Sodium Phosphate pH 6, 1 mL/min, 0 - 0.2 M NaCl in 34 min at 30 °C.

EFFECT OF BUFFER TYPE

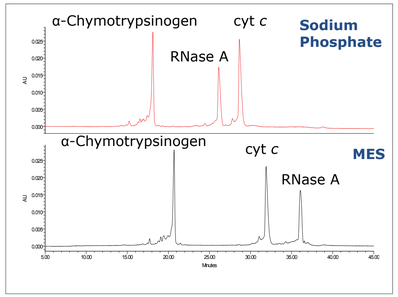


Figure 6. Effect of buffer type on the IEX separation of a protein standard mixture. Two common cation-exchange buffers, sodium phosphate and MES ((N-Morpholino)ethanesulfonic acid), were compared. At a pH of 6, different selectivity was observed for the most basic proteins. Conditions: 20mM Buffer (MES or Sodium Phosphate) pH 6, 1 mL/min, 0 - 0.2 M NaCl in 34 min at 30 °C.

IEX SEPARATION AND CONFIRMATION OF mAb C-TERMINAL LYSINE VARIANTS

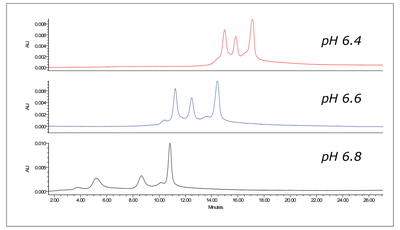


Figure 7. Effect of pH on mAb separation. IEX of C-terminal lysine variants of a monoclonal antibody was manipulated with pH. Conditions: 20mM Sodium Phosphate, 0 - 0.1 M NaCl, in 40 min, 0.5 mL/min.

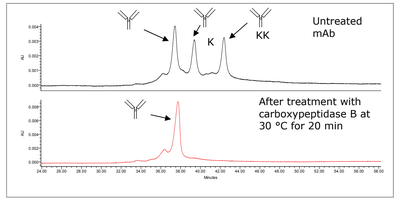


Figure 8. Confirmation of C-terminal lysine variants by cation–exchange chromatography. Analysis of chimeric mAb before and after treatment with carboxypeptidase B. Conditions. 20mM MES pH6.6, 0.0-0.1M NaCl in 60 min, 0.5 mL/min.

CONCLUSIONS

- **New packing materials for protein separations enable the benefits of UPLC Technology to be realized for both ion-exchange and size-exclusion chromatography.**
- **The ACQUITY UPLC H-Class Bio System with Auto•Blend™ Plus Technology can be used with new ion-exchange and size-exclusion packing materials for simplified methods development.**
- **This work extends UPLC technology to a wider range of bioseparations.**

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