High Throughput Sub-4 Minute Separation of Antibodies using Size Exclusion Chromatography

Introduction

Gel Filtration Chromatography (GFC) is a powerful analytical tool in the separation of antibodies. Traditionally, GFC columns with a dimension of 7.8 mm ID × 30 cm are used for analytical purposes. The longer column dimension, however, leads to longer run times and sample dilution, as well as substantial solvent waste. Alternatively, a column of smaller dimension provides high throughput separation with shorter run times, high resolution and minimal solvent waste when used on a conventional HPLC system. This Application Note demonstrates the use of a 4.6 mm ID × 15 cm TSKgel® SuperSW mAb HTP SEC column for the highly reproducible separation of antibodies in less than 3.5 minutes by using a moderate flow rate of 0.75 mL/min. The SuperSW mAb HTP column provides excellent stability for high speed, sub-4 minute separations of monoclonal antibodies.

Materials and Methods

Column: TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm
Instrument: Agilent 1100 HPLC system
Mobile phase: 100 mmol/L phosphate/100 mmol/L sulfate buffer, pH 6.7 + 0.05% NaN₃
Flow rate: 0.75 mL/min
Detection: UV @ 280 nm
Temperature: ambient
Injection vol.: 5 μL
Samples: PABA, 0.01 mg/mL
mAb 01, 4.6 mg/mL
mAb 02, 4.6 mg/mL
human IgG, 4.6 mg/mL
mouse IgG, 4.6 mg/mL

Results and Discussion

Figure 1 shows the separation of four different monoclonal antibodies in less than three minutes using the TSKgel SuperSW mAb HTP column at a flow rate of 0.75 mL/min. High resolution separation of the monomer, dimer, and fragment peaks of the mouse IgG sample are clearly shown under these conditions. The separation of these IgG-based proteins within 3 minutes using the TSKgel SuperSW mAb HTP column corresponds to a 3.75-fold decrease in analysis time relative to conventional (7.8 mm ID × 30 cm) SEC columns.

Sustained pressure from operating at elevated flow rates can lead to voids within the column, generating poor peak shapes and drifting retention time. As shown in Figure 2, 540 consecutive injections of mAb 02 and PABA separated on the TSKgel SuperSW mAb HTP column at 0.75 mL/min show good reproducibility with no discernible drift in retention. Additionally, no significant loss in resolution between the mAb monomer and dimer was observed on the TSKgel SuperSW mAb HTP column operated at 0.75 mL/min, yielding a %RSD < 3 (Figure 3).
Conclusions

Separations of IgG-based proteins using the TSKgel SuperSW mAb HTP column at 0.75 mL/min yielded highly reproducible results with high resolution and moderate back pressure within 3 minutes. This corresponds to a 3.75-fold decrease in analysis time relative to traditional SEC methodology. Additionally, due to the smaller dimension of the TSKgel SuperSW mAb HTP column, minimal solvent waste is observed even at increased flow rates, making this a cost effective and "green" method for protein separations when compared to that of traditional 7.8 mm ID × 30 cm SEC columns. The TSKgel SuperSW mAb HTP column operated at 0.75 mL/min for 540 injections of monoclonal antibody showed no drift in retention and good reproducibility. These results demonstrate that the TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm column clearly has a competitive advantage in fast assay and high throughput analysis of antibodies using a conventional HPLC system.

Tosoh Bioscience and TSKgel are registered trademarks of Tosoh Corporation.
Physical Stability of a Silica-Based Size Exclusion Column for Antibody Analysis

Atis Chakrabarti and Roy Eksteen
Tosoh Bioscience LLC, King of Prussia, PA 19406
Abstract

● There are many answers to the question of how long a column lasts. The most trivial reply is still the most appropriate - it all depends on what you do with the column. Particle structure, bonded phase composition, number of injections, mobile phase, pH, flow rate, sample type/mass/volume, temperature, pressure, storage, cleaning protocol, et cetera - all play a more or less important role in determining when to write the column’s inevitable obituary.

● When it comes to gel filtration chromatography, particle stability and thus column stability is even more of an issue than in reversed phase chromatography for the simple reason that the pore volume of SEC columns is maximized to provide optimal mass resolution, and the higher the pore volume the more fragile the particle.

● From a user’s perspective, it would be most desirable if no precautions would be required to obtain a long column lifetime. From a manufacturer’s perspective, such a column would be impossible to make and even if it could be made, such a column would be too costly to sell.

● In this initial report of our ongoing study about the stability of the TSKgel G3000SW\textsubscript{XL} column for quality control of monoclonal antibodies, we evaluated three columns each of three bonding lots. Preliminary indications are that retention times and efficiency did not change over at least 1,000 injections of a 10\textmu L mixture of protein standards into a 100\textmu L injection loop. During the lifetime studies the analytical column was neither protected by a guard column nor by inline filters other than the ones that are a standard fixture in the Agilent 1100 HPLC system.
Introduction

- Column lifetime is a critical factor in all modes of liquid chromatography but particularly so for silica-based SEC columns as the benefit of increased particle porosity has to be balanced with the requirement to maintain physical integrity of the particles.

- While the physical stability of reversed phase HPLC columns is taken for granted by its practitioners, the same cannot be said for the physical stability of polar bonded phase columns.

- Although polar-bonded phase columns can display comparable efficiency and peak symmetry as RPLC columns, polar bonded phase columns are sometimes plagued by a sudden loss of performance; usually from the development of a void due to resettling of the packing at the top of the column.
Factors playing a more or less important role in determining column lifetime

- Particle structure
- Bonded phase composition
- Number of injections
- Sample type, sample mass, and sample volume
- Mobile phase conditions (pH, buffer, salt)
- Pressure
- Temperature
- Column cleaning
- Storage

In this study we report on variations in column efficiency, peak symmetry, and retention when making 1,000 injections of a 10uL standard mixture of globular proteins on five (5) TSKgel G3000SW\textsubscript{XL} columns operated under standard operating conditions.
Objective

This study was conducted to determine the long term physical stability of TSKgel G3000SW\textsubscript{XL} columns as a function of operating conditions.

In this initial study we report on experiments performed using a common mobile phase composition, a standard flow rate, and a small injection volume of globular protein standards.

Characteristics of TSKgel SW columns

- Particle size: 5µm
- Pore size: 250Å
- Bonded phase: diol-containing ligands
- Protein calibration range: 10,000 – 500,000Da
Material and methods: Columns

- TSKgel G3000SW_{XL}, 5\mu m, 7.8mm ID x 30cm

  - Bonding Lot 08R
    - S1237
    - S1238
    - S1239
  - Bonding Lot 09R
    - S1261
    - S1262
    - S1263
  - Bonding Lot 30P
    - S6210
    - S6211
    - S6212

Silica Lot A

Silica Lot B

In this poster we report on the stability of the highlighted columns prepared from three bonding lots, made from two silica lots.
Chromatographic conditions stability study

- TSKgel G3000SW<sub>XL</sub>, 5µm, 7.8mm ID x 30cm
  - **Silica lot A**
    - Bonding lot 08R: columns S1237, S1238, S1239
    - Bonding lot 09R: Columns S1261, S1262, S1263
  - **Silica lot B**
    - Bonding lot 30P: columns S6210, S6211, S6212
- Mobile Phase: 0.1mol/L KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, pH 6.7, + 0.1mol/L Na<sub>2</sub>SO<sub>4</sub> + 0.05% NaN<sub>3</sub>
- Flow rate: 1.0mL/min
- Detection: UV@280nm
- Temperature: ambient
- Injection vol.: 10µL
- Samples
  - **TSKgel SW<sub>XL</sub> test mixture**: thyroglobulin, γ-globulin, ovalbumin, ribonuclease A, p-ABA (see next slide for more details)
  - **Monoclonal antibody**: BI-MAb-2 (Boehringer-Ingelheim), 4.5g/L in glycine/sodium phosphate, pH 6.0
Chemicals

- **Protein test mixture**

<table>
<thead>
<tr>
<th>Globular Proteins</th>
<th>Sigma PN</th>
<th>Conc. (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>thyroglobulin, <em>bovine</em> (670kDa)</td>
<td>T1001</td>
<td>0.50</td>
</tr>
<tr>
<td>γ-Globulin, <em>bovine</em> (150kDa)</td>
<td>G5009</td>
<td>1.00</td>
</tr>
<tr>
<td>ovalbumin, <em>chicken</em> (45kDa)</td>
<td>A5503</td>
<td>1.00</td>
</tr>
<tr>
<td>ribonuclease A, <em>bovine</em> (14.7kDa)</td>
<td>R4875</td>
<td>1.50</td>
</tr>
<tr>
<td>p-Amino benzoic acid (137g/mol)</td>
<td>A9878</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Notes:**

- Sample solvent: 100mmol/L phosphate buffer, pH 6.7, unless mentioned otherwise.
- The small molecular weight compound p-aminobenzoic acid is used to mark the mobile phase volume in the column, while the efficiency and peak symmetry of the pABA peak are indicators of the integrity of the packed bed.
- All chemicals and standards were obtained from Sigma-Aldrich and were of electrophoretic or analytical grade.
- High purity HPLC grade solvents were used for the preparation of stock standards, samples and mobile phases.
Column protection

- Although it is recommended practice to protect the column from potential sources of contamination no such precautions were made in this study.
  - Standards and mobile phases were not filtered through a 0.45μm syringe filter.
  - A frit filter was not used between injector and column.
  - TSKgel G3000SW_{XL} columns were not protected by a guard column in any of these studies.

- Note: the PTFE Purge Valve Frit on the high pressure side of the Agilent 1100 HPLC system was replaced after about 3,000 injections (~ 45L mobile phase).
Results

- Tables
  - (I) N, AF and $t_R$ at 1<sup>st</sup>, 500<sup>th</sup> and 1000<sup>th</sup> injection on S1261-09R
  - (II) %RSD values of N, AF and $t_R$ during 1000 injections

- Figures
  1. Precision: column S1261-09R
  2. Precision: columns from bonding lot 08R
  3. Precision: columns from bonding lots 08R + 09R
  4. Precision: between silica lots A and B
  5. Sample load – protein standards
  6. Sample load – monoclonal antibody
Table I: Retention, efficiency and peak symmetry at the 1\textsuperscript{st}, 500\textsuperscript{th} and 1000\textsuperscript{th} injection

<table>
<thead>
<tr>
<th></th>
<th>γ-globulin</th>
<th></th>
<th>p-aminobenzoic acid</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inj. 1</td>
<td>Inj. 500</td>
<td>Inj. 1000</td>
<td>Inj. 1</td>
</tr>
<tr>
<td>(t_R)</td>
<td>8.065</td>
<td>8.027</td>
<td>8.065</td>
<td>12.635</td>
</tr>
<tr>
<td>(N)</td>
<td>2021</td>
<td>2002</td>
<td>1847</td>
<td>32,483</td>
</tr>
<tr>
<td>(AF)</td>
<td>1.47</td>
<td>1.47</td>
<td>1.49</td>
<td>1.19</td>
</tr>
</tbody>
</table>

Table II: \%RSD (n=100) over 1000 injections

<table>
<thead>
<tr>
<th></th>
<th>thyroglobulin</th>
<th>γ-globulin</th>
<th>ovalbumin</th>
<th>rib. A</th>
<th>p-ABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(t_R)</td>
<td>0.16</td>
<td>0.21</td>
<td>1.05</td>
<td>0.20</td>
<td>0.28</td>
</tr>
<tr>
<td>(N)</td>
<td>5.48</td>
<td>5.27</td>
<td>2.40</td>
<td>2.87</td>
<td>1.70</td>
</tr>
<tr>
<td>(AF)</td>
<td>4.32</td>
<td>1.38</td>
<td>2.24</td>
<td>2.56</td>
<td>2.66</td>
</tr>
</tbody>
</table>
High degree of reproducibility from injection to injection is illustrated by superimposed chromatograms of the 1st and 1000th injection on column S1261-08R.
High degree of reproducibility within a bonding lot is illustrated by superimposed chromatograms of the 250th injection on three columns from bonding lot 08R.
Reproducibility between bonding lots

High degree of reproducibility between bonding lots is illustrated by superimposed chromatograms of the 1000<sup>th</sup> injection on one column each of bonding lots 08R and 09R.
High degree of reproducibility between silica lots is illustrated by superimposed chromatograms of the 1\textsuperscript{st} and 1000\textsuperscript{th} injection on one column each of silica lots A and B.
Efficiency of $\gamma$-globulin declined slightly, while peak shape and retention time were not affected when injecting 100$\mu$L sample containing 6mg protein.
Loading capacity of monoclonal antibody

Peak shape and efficiency were not affected when injecting 400µg of a monoclonal antibody preparation.
Preliminary conclusions 1

- Based on five TSKgel G3000SW$_{XL}$ columns tested, each column was stable for at least 1,000,10µL injections of a protein standard mixture in 0.1mol/L, pH 6.7 phosphate buffer containing 0.1mol/L sodium sulfate and 0.05% sodium azide, operated at 1mL/min at room temperature.

- Efficiency (N) and peak symmetry (AF) values showed minimal variation from the first to the last (1000$^{th}$) injection indicating that the integrity of the packed bed was maintained throughout the experiment.

- Retention times of globular protein standards did not change while pumping 15L mobile phase through the column, indicating that the integrity of the diol-bonded phase was maintained throughout the experiment.

- Plate numbers, peak symmetry values and retention times for each protein studied showed minimal variation for TSKgel G3000SW$_{XL}$ columns prepared from:
  - The same bonding lot – 3 columns tested.
  - Different bonding lots – one column each of 3 lots tested.
  - Different silica lots – one column each of two lots tested.
Preliminary conclusions 2

- Efficiency, peak symmetry and retention times did not change when injecting a 100µL of a standard protein mixture containing 6mg total protein.

- Chromatographic parameters were also not affected when injecting 0.4mg of a monoclonal antibody preparation.
Future studies

- When performing column stability studies one first needs to understand the effect of common chromatographic conditions on the integrity of the packed bed before one can study how ‘real’ samples influence column lifetime.

- In the next phase of this study we plan to investigate the role of injection volume and the role played by the nature of the injection solvent on column stability.

- Another topic of interest is the role of mobile phase additives, such as organic modifiers and detergents.