A Simple Method for Analyzing Aggregates of EPO (Erythropoietin) Using a BioSep™-SEC-s2000 GFC Column

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Gel filtration chromatography is the primary method used to analyze the amount of aggregate and dimer present in a therapeutic protein sample. A rugged yet simple method using a BioSep-SEC-s2000 column is presented for analyzing EPO samples to determine difference in the amounts of aggregate present in both fresh and long-term stored EPO samples.

Introduction

With recent patent expirations, erythropoietin (EPO) is rapidly becoming the most widely manufactured recombinant biosimilar protein behind insulin with several companies throughout the world currently developing their version of the recombinant protein. Methods for analyzing EPO and quantitating post-translational modifications were mostly stagnant for the last 15 years since major manufacturers were reluctant to re-validate existing methods. However, with patent expirations, several new groups are free to use advances in instrument and column technologies to develop better analytical methods. Since protein aggregation is a major concern in the manufacture of any recombinant therapeutic, many groups are currently developing new methods for quantitating dimer and aggregate for EPO and other biosimilar proteins using higher-performing gel filtration media manufactured using modern technologies.

Materials and Methods

All mobile phase solvents were purchased from EMD (San Diego CA) and reagents used in buffer preparation were obtained from Sigma Chemicals (St. Louis, MO). Recombinant Human EPO was purchased from either Cell Sciences (Canton, MA) or Sigma Chemicals. BioSep-SEC-s2000 columns (300 × 4.6 mm dimension) were used for all GFC (gel filtration chromatography) separations (Phenomenex, Torrance, CA). All samples were analyzed on an Agilent 1100 HPLC (Palo Alto, CA) with an autosampler and variable wavelength detector set at 214 or 220 nm; data was collected using ChemStation software (Agilent). The mobile phase was 50 mM sodium phosphate pH 6.8 with 300 mM sodium chloride in water running at a flow rate of 0.35 mL/min.

Results and Discussion

While ion-exchange and reversed phase chromatography are typically used for identifying many of the post-translational modifications of a protein, GFC is exclusively used for identifying the aggregation state of most recombinant proteins. GFC specifically separates proteins by size-based differences in exclusion from a porous media which is directly related to the molecular weight of a species in solution. Recombinant EPO is approximately 30 kDa molecular weight in its glycosylated form (approximately 18 kDa for proteins), and any dimer of EPO would be expected to be around 60 kDa in size. A BioSep-SEC-s2000 series column was used for all separations, as it provides the largest separation window for proteins below 100 kDa molecular weight. This is in contrary to other methods in the past which often have used “3000-series” GFC columns. **Figures 1** and **2** show GFC chromatography on the BioSep-SEC-s2000 column for two different samples of EPO. **Figure 1** is a chromatogram of a freshly frozen EPO sample and **Figure 2** is a chromatogram of an EPO sample that had been frozen for over a year. While one can see peaks for aggregate, monomer EPO, and buffer salts for both samples, note the increase in a dimer peak for EPO for the sample frozen for more than a year. This increase in EPO dimer suggests that the formulation used was less than ideal for this sample.

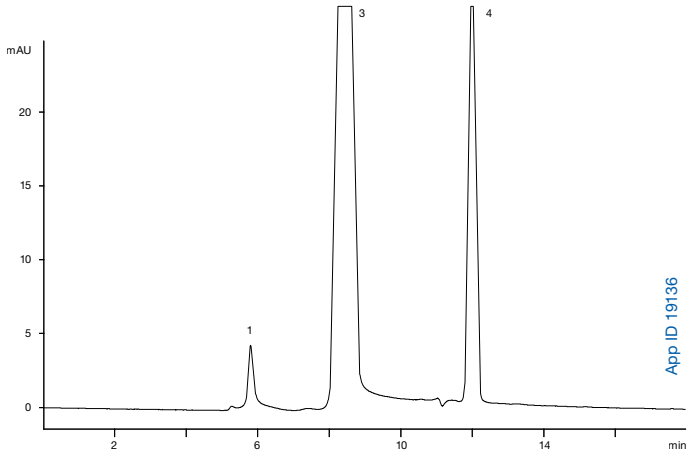

Figure 1. A freshly frozen EPO sample run on a BioSep-SEC-s2000 column. Note the early eluting high molecular weight protein (assumed to be EPO aggregate), the monomer EPO peak at 9 minutes RT, and the low molecular weight peak at the void of the column (assumed to be buffer salts in the diluent). Little or no dimer appears to be present.

Column: BioSep-SEC-s2000
Dimensions: 300 x 4.6 mm
Part No.: 00H-2145-E0
Mobile Phase: 50 mM Sodium phosphate, 300 mM Sodium chloride, pH 6.8
Flow Rate: 0.35 mL/min
Detection: UV @ 220 nm
Sample: 1. HMW impurity (aggregate)
2. EPO dimer (not present in Figure 1)
3. EPO monomer
4. LMW impurity

Also note that the chromatography for the EPO sample run on the BioSep-SEC-s2000 shows good resolution between the monomer and dimer peaks of the EPO sample (in **Figure 2**) despite the sample being heavily overloaded to visualize the dimer peak. The assigned aggregate peak in both chromatograms is also very low level, but is well recovered and somewhat included into the pores

of the BioSep™-SEC-s2000 suggesting that aggregate has a finite size or that the early eluting peak is a different high molecular weight protein impurity in the sample.

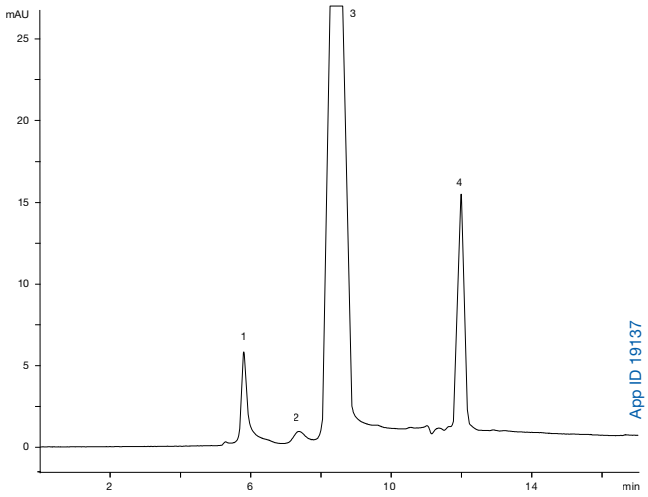

Figure 2. An EPO sample stored at -20 °C for more than a year run on a BioSep-SEC-s2000 column. The good resolution between peaks makes the BioSep-SEC-s2000 column an excellent choice for aggregate analysis of EPO and other biosimilar proteins.

Conclusions

In this technical note, an example method showing the separation of EPO and its dimer is used to demonstrate the utility of using a BioSep-SEC-s2000 column for aggregate analysis. The good peak efficiency, wide resolution window for low molecular weight proteins, and inertness make the BioSep-SEC-s2000 column an excellent GFC solution for aggregate analysis of EPO and other low molecular weight biosimilar proteins.

Ordering Information

Stainless Steel Columns (mm):	Narrow Bore	Analytical		SecurityGuard™ Cartridges (mm)
Phases	300 x 4.6	300 x 7.8	600 x 7.8	4 x 3.0*
BioSep-SEC-s2000	00H-2145-E0	00H-2145-K0	00K-2145-K0	AJ0-4487
BioSep-SEC-s3000	00H-2146-E0	00H-2146-K0	00K-2146-K0	AJ0-4488
BioSep-SEC-s4000	00H-2147-E0	00H-2147-K0	00K-2147-K0	AJ0-4489
				for ID: 4.6-7.8 mm

Stainless Steel Columns (mm):	Preparative	SecurityGuard™ Cartridges (mm)
Phases	300 x 21.2	15 x 21.2**
BioSep-SEC-s2000	00H-2145-P0	AJ0-8588
BioSep-SEC-s3000	00H-2146-P0	AJ0-8589
BioSep-SEC-s4000	00H-2147-P0	AJ0-8590
		for ID: 21.2 mm

*SecurityGuard Analytical cartridges require holder, Part No.: KJ0-4282
** PREP SecurityGuard Cartridges require holder, Part No.: AJ0-8223

guarantee

If BioSep analytical columns do not provide at least an equivalent separation as any other GFC column of similar porosity, type, and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

Terms and Conditions
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Trademarks
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SecurityGuard is patented by Phenomenex. U.S. Patent No. 5,863,428

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