Separation of Recombinant Human Erythropoietin (rEPO) Using Agilent Bio SEC-3

Application Note

BioPharma

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Abstract

Monitoring the purity of recombinant protein during formulation, purification, storage, and clinical research use calls for sensitive and analytical techniques. In addition, run-to-run reproducibility is in great demand by biotechnology and pharmaceutical companies. Agilent Bio SEC columns are designed to address these challenges. The columns are coated with a hydrophilic polymeric layer that decreases the secondary interactions for maximum efficiency and stability. They are compatible with organic modifier denaturants and optimized for use at low salt concentrations. These conditions are very critical for increasing the efficiency of QC for protein primary structure analysis. The data presented here focus on the separation of various recombinant human erythropoietin sizes. Like many other biomolecules, recombinant EPO protein exhibits heterogeneity (aggregates, dimers and monomers) due to modifications that occur during manufacturing. Methods are optimized for flow rates, gradient, and reproducibility under neutral conditions that contain just sodium phosphate buffer.

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Introduction

Recombinant human EPO protein is one of the most widely produced by many bio and pharmaceutical companies throughout the world for therapeutic agents. Erythropoietin protein (EPO) is a glycoprotein hormone found in plasma. It is a cytokine for erythrocyte (red blood cell) precursors in the bone marrow. EPO controls red blood cell production. It also has neuroprotective activity against a variety of potential brain injuries, and anti-apoptotic functions in several tissue types. Recombinant human EPO protein (rEPO) is produced by Chinese hamster ovary (CHO) cells using recombinant DNA technology. The rEPO single polypeptide chain contains 165 amino acids and has a predicted molecular mass of 24,000 Dalton, and apparent glycosylated molecular mass of 30,400 Dalton. The HPLC separation of EPO protein from its impurities can be achieved by using a variety of chemistries, including size exclusion chromatography. The data presented here focus on the use of analytical size exclusion for separation of varying sizes of a CHO-derived EPO protein. Methods were developed on the Agilent Bio SEC-3, 3 µm, 100Å column using the Agilent 1260 Infinity Bio-inert Quaternary LC system.

Material and Methods

HPLC system

The 1260 Infinity Bio-inert Quaternary LC system is a dedicated solution for large biomolecule analysis. The design of new metal-free components in the sample flow path and the absence of iron and steel in solvent delivery ensure biomolecule integrity, minimize unwanted surface interactions, and increase column lifetime. This is ideal when working under harsh solvent or pH conditions. The power ranges from lowest pressure for traditional biopurification columns up to high pressure STM analytical biocolumns. Together with the new Bio-HPLC column portfolio for SEC and IEX, highest resolution per time is achieved for protein and NBE characterization.

HPLC column

Agilent Bio SEC-3 (p/n 5190-250) HPLC columns are a breakthrough technology for size exclusion chromatography (SEC). They are packed with spherical, narrowly dispersed 3-µm silica particles coated with a proprietary hydrophilic layer. This thin polymeric layer is chemically bonded to pure, mechanically stable silica under controlled conditions, ensuring a highly efficient size exclusion particle. Agilent Bio SEC-3 HPLC columns are available in 100Å, 150Å, and 300Å pore sizes to accommodate most peptide and protein size exclusion separations.

Chemicals and reagents

CHO-cell derived, humanized EPO protein from Creative BioLabs, Shirley, NY, USA. Sodium monobasic phosphate and sodium dibasic phosphate were purchased from Sigma-Adrich, St. Louis, MO, USA.

LC methods

The conditions in Table 1 were used throughout the investigation with variations as noted in the relevant chromatograms.

Table1. Standardized Chromatographic Conditions

Parameter	Item
Column	Agilent Bio SEC-3, 100Å, 4.6 × 300 mm, 3 µm
Sample	Recombinant human EPO protein (rEPO)
Sample concentration	1.0 mg/mL
Injection volume	5 µL
Flow rate	0.35 mL/min
Mobile phase	150 mM sodium phosphate buffer, pH 7.0
Detector	UV, 225 nm
System	Agilent 1260 Infinity Bio-inert Quaternary HPLC

Results and Discussion

Optimization for separation of intact rEPO

The optimized flow rate was 0.35 mL/min, with isocratic gradient from 0 to 30 minutes. The column separated the test mixture very quickly, distinguishing rEPO protein from its impurities in less than 30 minutes. The integrity of peak shapes and resolution between peaks were a major advantage when using small pore size and small particle for small protein separation (Figure 1). A feature of 100Å pore size particles is to facilitate high resolution for separation of small proteins. The data demonstrate that the Agilent Bio SEC columns perform very effectively without salt additive (sodium chloride) in 150 mM sodium phosphate buffer, pH 7.0. The separation of aggregates, dimers and moner are very well defined.

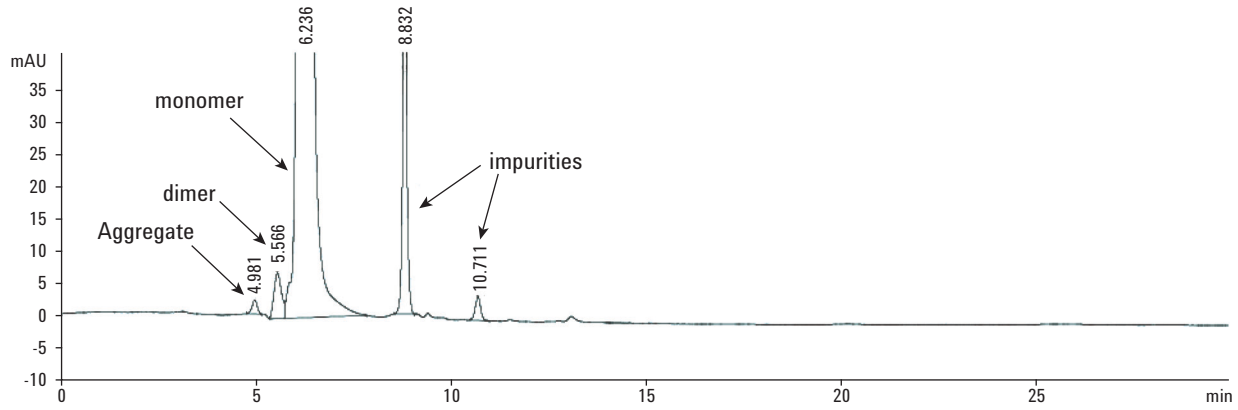
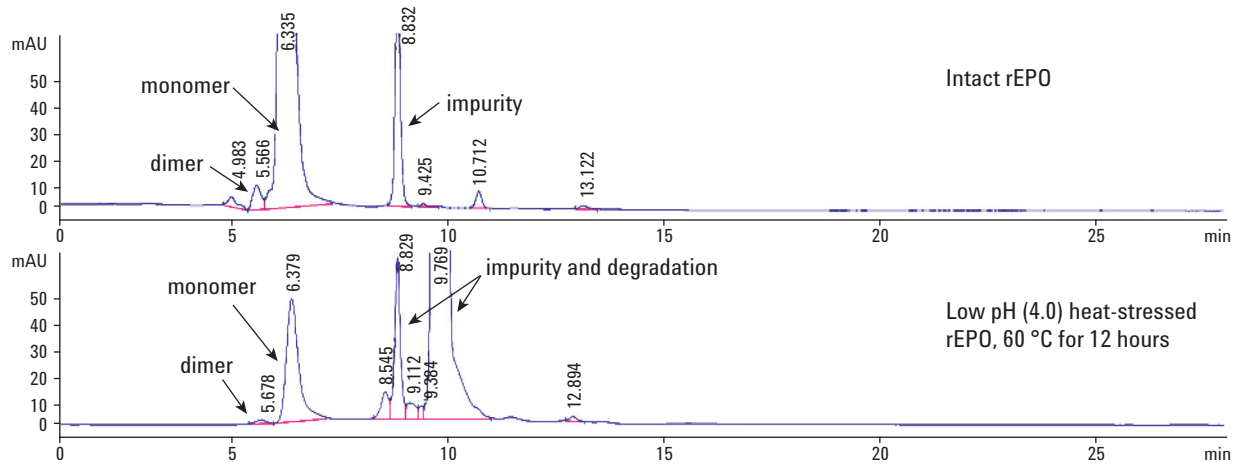


Figure 1. Separation of intact rEPO on the Agilent Bio SEC-3, 100Å, 4.6 × 300 mm, 3 µm column.

Separation of heat-stressed rEPO at low pH and its comparison to intact rEPO

Degradation of therapeutic proteins during drug manufacturing and formulation is considered a critical attribute and therefore needs to be closely monitored to prevent loss of drug efficacy or to prevent adverse immunogenic effects. To demonstrate the separation ability of the small pore size particle, rEPO was heat-stressed at low pH 4.0 with acetic acid for 12 hours at 60 °C and separated by the Bio SEC-3,100Å column. Separation conditions were exactly the same as for the separation of intact rEPO (Figure 1). Figure 2 (bottom panel) shows superior peak resolution of heat-degraded rEPO and how much the separation profile was different from the intact rEPO. The significant reduction of monomer, dimers and aggregates demonstrate that the rEPO was effectively degraded by low pH buffer at high temperature [1], and effectively separated by the Bio SEC-3, 100Å column (compare Figure 2 top panel (intact rEPO) to bottom panel (heat-reduce rEPO with low pH buffer)) (Table 2).

Top panel



Bottom panel

Figure 2. Separation of heat-stressed rEPO with low pH 4.0 (acetic acid) at high temperature by the Agilent Bio SEC-3, 100Å, 4.6 × 300 mm, 3 µm column.

Table 2. Calculation of Percentage Heterogeneity of Intact rEPO and Heat-Stressed rEPO (by Low pH at High Temperature)

Conditions of rEPO	% aggregates	% dimers	% monomer	% impurities
Intact	0.6	1.6	85.6	10.3
Heat-stressed at low pH	not detected	0.2	6.1	92.2

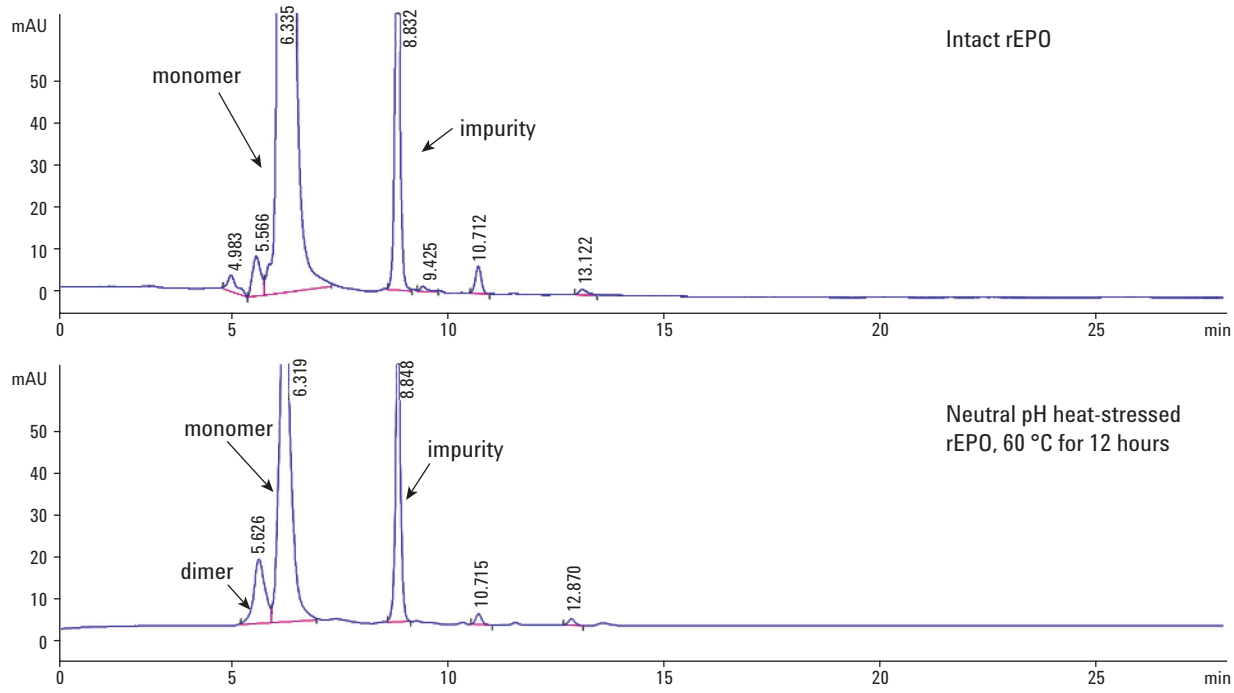
Separation of heat-stressed rEPO at neutral pH and high temperature

Recombinant human EPO was heated at 60 °C for 12 hours at neutral pH (pH 7.0). At neutral pH, the separation data show changes in the separation. However, this is not as significant as heat-stress at low pH (compare Table 2 to Table 3, and Figure 2 to Figure 3, bottom panels). The dimer peak increased to 11 %, whereas the monomer peak decreased to 62.5 %. Even though the reduction was not as severe as for low pH heat-stressed rEPO, the data clearly demonstrate that the Bio SEC-3 column well resolved the different sizes of rEPO impurities and degradation products with high resolution.

Table 3. Calculation of Percentage Heterogeneity of Intact rEPO and Heat-Stressed rEPO (by Neutral pH at High Temperature)

Conditions of rEPO	% aggregates	% dimers	% monomer	% impurities
Intact	0.6	1.6	85.6	10.3
Heat-stressed at low pH	not detected	11.7	62.5	24

Top panel



Bottom panel

Figure 3. Neutral pH heat-stressed rEPO protein is well resolved by the Agilent Bio SEC-3, 100Å, 4.6 × 300 mm, 3 µm column.

Conclusion

Agilent Bio-SEC columns in combination with the Agilent 1260 Infinity Bio-inert Quaternary HPLC demonstrated superior performance for the aggregation analysis of rEPO. This solution is an excellent tool for method development and monitoring the purity and stability of therapeutic recombinant proteins.

Reference

1. Yoshiyuki Endo *et al*., *J. Biochem*. (1992) 112 (5): 700-706.

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