

Fast Separation of Recombinant Human Erythropoietin Using Reversed Phased Agilent ZORBAX RRHD 300SB-C18, 1.8 μm

Application Note

BioPharma

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Abstract

Fast separation of proteins with increased sensitivity, reduced separation time and run-to-run reproducibility is in great demand by biotechnology and pharmaceutical companies. Highly sensitive analytical techniques are necessary for monitoring the purity and stability of proteins during manufacturing, purification, formulation, and storage. The Agilent ZORBAX 300SB-C18 1.8 μm Rapid Resolution High Definition (RRHD) column is designed to address these challenges. The C18 StableBond coating technology provides increased sensitivity, exceptional pH, and thermal stability. The use of a 1.8- μm column designed for UHPLC systems significantly reduces analysis time, critical for increasing the efficiency of QC for protein primary structure analysis. The data presented here focuses on the fast separation of various recombinant human erythropoietin isoforms. Like many other biomolecules, recombinant EPO protein exhibits heterogeneity due to modifications that occur during manufacturing. Methods are optimized for flow rates, gradient and reproducibility under acidic conditions that contain trifluoroacetic acid.



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Introduction

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 antiapoptotic 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. Recombinant human EPO protein is one of the most widely produced by many bio- and pharmaceutical companies throughout the world for therapeutic agents. The HPLC separation of EPO protein from its impurities can be achieved by using a variety of chemistries, including reversed-phase chromatography. The data presented here focuses on the use of analytical reversed phase for separation of varying hydrophobicities of a CHO derived-EPO protein. Methods were developed on the Agilent ZORBAX 300SB-C18 1.8- μ m column using the Agilent 1290 Infinity LC system.

Material and Methods

HPLC system

The Agilent 1290 Infinity LC system operates at a maximum pressure of 1,200 bar. The Agilent 1290 Infinity LC is designed to provide the highest speed, resolution, and ultra-sensitivity. A new power range lets you deploy any particle type, any column dimensions, or any mobile, and stationary phases. Innovative technology components offer the next level of performance for UHPLC, RRLLC, and HPLC applications. With revolutionary Intelligent System Emulation Technology, the 1290 Infinity is the world's first universal LC system, as it can execute other HPLC and UHPLC methods and deliver the same chromatographic results without any change of the instrument or the original method.

HPLC column

The column used was an Agilent ZORBAX 300 SB-C18 RRHD, 2.1 \times 50 mm, 1.8 μ m (p/n 857750-902). These columns are used for protein primary structure analysis to confirm protein identity, quantify post translational modifications and provide impurity profiles for biotherapeutic protein discovery and development and production applications. This new wide-pore (300 Å) RRHD column brings UHPLC performance to the reversed-phase separation of intact proteins and protein digests. The ZORBAX

StableBond stationary phase provides high stability in low pH that allows mobile phase eluents with trifluoroacetic acid (TFA) or formic acid (FA) to be used routinely without compromising the columns' life time. The columns are stable up to 90 °C.

Chemicals and reagents

CHO-cell derived, humanized EPO protein from Creative BioLabs, Shirley, NY
Trifluoroacetic acid from Sigma-Aldrich, St. Louis, MO
Acetonitrile from Honeywell-Burdick & Jackson, Muskegon, MI

LC methods

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

Table 1. Standardized Chromatographic Conditions

Parameter	Item
Column	Agilent ZORBAX RRHD 300 SB-C18, 2.1 \times 50 mm, 1.8 μ m
Sample	Recombinant human EPO protein (rEPO)
Sample concentration	1.0 mg/mL
Injection volume	3 μ
Flow rate	1.0 mL/min
Pressure	650 bar
Mobile phase A	0.1% TFA in deionized water
Mobile phase B	0.01% TFA in 100% ACN
Detector	UV, 280 nm
System	Agilent 1290 Infinity HPLC

Results and Discussion

Optimization of flow rate and gradient for rapid separation and increased resolution

Three flow rates were selected, 0.5, 1.0, and 1.5 mL/min, with gradient started at 5.0% mobile phase B and ended at 100% mobile phase B from 0 to 2.5 min, respectively. The column was then equilibrated at 5.0% mobile phase B for another 2.5 min. The column separated the test mixture very quickly, distinguishing rEPO protein from its impurities in less than five minutes. The integrity of peak shape and asymmetry of 1.0 and 1.5 mL/min indicated that fast flow rates had a major advantage when using ZORBAX 300 SB-C18, 1.8 μ m for protein separation (Figure 1, Table 2), a feature of 1.8- μ m particles that facilitates rapid separation.

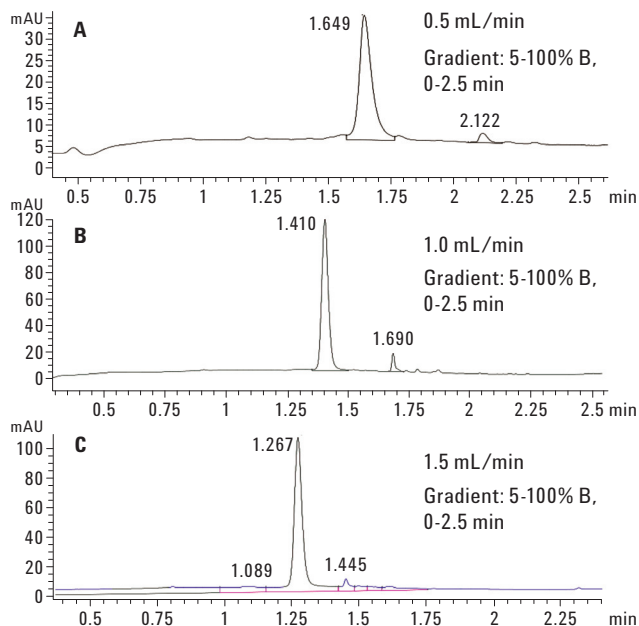


Figure 1. Different flow rates can be selected to separate rEPO protein on the Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 50 mm, 1.8 μm column.

Table 2. Effect of Flow Rate on Retention Time, Asymmetry, and Peak Width in the Separation of rEPO Protein

Flow rate (mL)	Pressure (bar)	Retention time (min)	Asymmetry	Peak width
0.5	350	1.64	0.6	0.047
1.0	650	1.41	0.85	0.030
1.5	890	1.26	0.84	0.030

Different gradient systems were also selected to achieve the same fast analysis time. Figure 2 shows the separation of rEPO protein at 1.0 mL/min with gradient set at 10 to 50 to 70% mobile phase B from 0, 2.5 and 3.0 min, respectively. The column was then equilibrated for another 2 min at 10% mobile phase B before the next injection. Figure 2 indicates that the column was able to separate EPO protein from its impurities in a similar manner to the gradient shown in Figure 1, panel B. The same number of peaks was obtained, just with different retention times. In addition, rapid equilibration was evident, demonstrating that the column was suitable for use with a wide range of organic content.

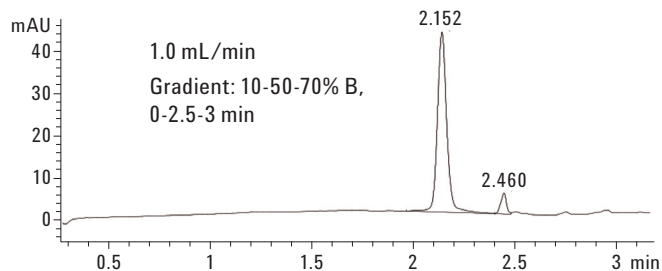


Figure 2. Different gradients can be selected to separate EPO protein from its impurities in less than 5 min using the Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 50 mm, 1.8 μm column.

Separation of heat-degraded recombinant human EPO protein

Recombinant human EPO protein was heated at 60 °C overnight (16 hours) at neutral pH (pH 7.0) and acidic pH (pH 4.0). Due to its nature, rEPO protein will be degraded or form other isoforms when heated at high temperatures such as 60 °C at different pH. At neutral pH, rEPO forms limited isoforms, but when acidic pH conditions are used, the structure of rEPO protein will be altered significantly. The lower the pH, the more changes¹. Figure 3 shows both conditions of heating. Panel A shows data from a sample was heated at neutral pH. The column resolved the main peak of rEPO from its degraded products or isoforms very well. Note that the conformation of rEPO protein was not much changed. The retention of the main peak remained at 1.4 min, as seen in Figure, panel A (unheated sample). However, the impurity peak was substantially increased and well separated by the column (compared to Figure 1, panel B). Figure 3, panel B shows the separation of heat-treated rEPO at pH 6.0. The separation conditions were the same with neutral pH but the chromatogram was drastically changed. As indicated earlier, heating with acidic conditions can highly alter and degrade the conformation of the rEPO protein. The retention time of the major peak changed from 1.4 min to 1.68 min and the peak also contained a shoulder peak on the right. In addition, more impurity peaks also were observed. This clearly indicated that the ZORBAX RRHD 300 SB-C18, 1.8 μm could also very well resolve rEPO impurities and degradation products.

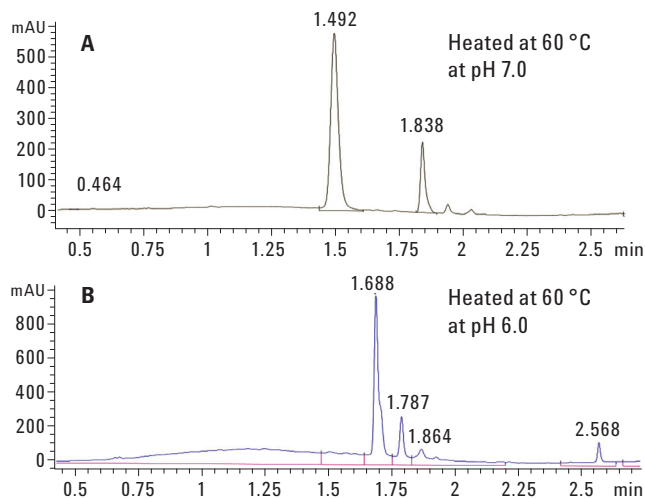


Figure 3. Heat-treated rEPO protein well resolved by the Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 50 mm, 1.8 μm column. The column separated these heat-treated rEPO proteins at 1.0 mL/min, 5 to 100% B solvent from 0 to 2.5 min.

Reference

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

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