Analysis of PEGylated Proteins with Agilent AdvanceBio SEC Columns

Application Note

Bio-Pharmaceutical

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Abstract

PEGylation of therapeutic proteins has significantly improved their value by altering the physicochemical and biological properties such as increased solubility, decreased immunogenicity, increased half-life, and protection against proteases. Size Exclusion Chromatography (SEC) is the method of choice for determining impurities with molecular mass higher than that of PEGylated proteins. SEC of PEGylated proteins poses a significant challenge due to PEG-mediated interaction with silica stationary phases, leading to lower recovery, poor peak shape, and undue tailing. This application note describes a simple and sensitive SEC method for determining the purity of PEG Granulocyte Colony Stimulating Factor (PEG GCSF). Separation and quantification of PEG GCSF was achieved using an Agilent AdvanceBio SEC, 130Å, 7.8 × 300 mm, 2.7 µm column employing aqueous mobile phase. An excellent correlation coefficient was observed for the linearity curve in the range 12.5 to 2,000 µg/mL, indicating that the method is quantitative. Retention time and peak area precision of the method were excellent, demonstrating the suitability of the method. Furthermore, AdvanceBio SEC was able to separate and quantify aggregates obtained by forced-stress studies.

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Introduction

PEGylation is the process of covalent attachment of polyethylene glycol (PEG) polymer chains to another molecule, normally a drug or therapeutic protein. PEGylation is routinely achieved by incubation of a reactive derivative of PEG with the target macromolecule. The PEG moiety offers numerous advantages for increasing a protein's stability and circulating half-life in the body. PEG has been approved by the Food and Drug Administration (FDA) as generally recognized as safe [1]. There are PEGylated products currently approved by the FDA. PEG Granulocyte Colony Stimulating Factor (PEG GCSF) is a long-acting form of recombinant GCSF. It is composed of GCSF with a 20 KDa PEG molecule covalently bound to the N-terminal methionine residue. GCSF is a 175 amino acid protein with a molecular weight of 18,800 daltons. PEG GCSF has a total molecular weight of 39 KDa. The draft monograph method recommends Size Exclusion Chromatography (SEC) HPLC for determining the purity and higher aggregates [2]. Most published methods for PEG GCSF use aqueous mobile phases containing 100 mM NaCl, 85% orthophosphoric acid, and up to 10% ethanol to prevent nonspecific interactions and improve peak shape and resolution [3]. Nonideal adsorption of therapeutic proteins is a matter of concern during SEC, as aggregates sometimes show a greater tendency to bind to the stationary phase than the native form. Due to this preferential binding, SEC analysis of aggregates is inaccurate, and there is the danger that aggregates may not be detected. Mobile phases containing organic solvents or extremes of pH have been used to overcome this problem and have been shown to enhance resolution and recovery. However, in addition to possibly dissociating reversible aggregates, they may also dissociate aggregates that are irreversible in the formulation buffer [4].

Here we show the benefits of the Agilent AdvanceBio SEC, 130Å, 7.8 × 300 mm, 2.7 µm column, a breakthrough technology for SEC analysis. These columns contain an innovative silica particle and unique hydrophilic bonding chemistry to deliver resolution and size separations over a wide range of sample types, without the need to add organic modifiers to the mobile phase.

Materials and Methods

Instruments

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar consisting of the following modules was used:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (p/n G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (p/n G5667A)
- Agilent 1200 Infinity Series Thermostat (p/n G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing Bio-inert click-in heating elements (p/n G1316C, option 19)
- Agilent 1260 Infinity DAD VL (p/n G1315D with Bio-inert standard flow cell, 10 mm)
- Agilent AdvanceBio SEC, 130Å, 7.8 × 300 mm packed with 2.7 µm particles (p/n PL1180-5350)

Software

Agilent ChemStation B.04.03 (or higher)

SEC parameters

Table 1 shows chromatographic parameters for SEC using an Agilent 1260 Bio-inert LC System.

Table 1. Chromatographic parameters used for SEC HPLC.

Parameter	Condition
Mobile phase:	150 mM Sodium phosphate buffer, pH 6.8
TCC temperature:	Ambient
Isocratic run:	Mobile phase A
Injection volume:	10 µL
Flow rate:	0.8 mL/min
UV detection:	214 and 280 nm

Reagents, samples, and materials

Commercial PEG GCSF was purchased from a local pharmacy and stored according to manufacturer's instruction. Monobasic and dibasic sodium hydrogen phosphate and hydrochloric acid were purchased from Sigma-Aldrich. All chemicals and solvents used were HPLC grade. Highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used.

Procedure

A 10 µL volume of mobile phase was injected as blank, followed by individual linearity levels, in triplicate. The area and retention time (RT) of each level were used to calculate standard deviation (SD) and relative standard deviation (RSD %) values. Limit of detection (LOD) and limit of quantitation (LOQ) were established from the lower linearity level injections. The average area of each linearity level was plotted against the concentration of the analyte to determine the calibration curve for the monomers.

Linearity and range

The calibration curve was constructed with nine standard concentrations of PEG GCSF in the range of 7.8 to 2,000 µg/mL.

LOQ and LOD

The PEG GCSF concentration that provided a signal-to-noise ratio (S/N) of >3 was considered the LOD, and a S/N >10 was considered the LOQ.

Preparation of PEG GCSF aggregates

Aggregates of PEG GCSF were prepared following the draft monograph. Briefly, about 2 mg/mL of the drug product was incubated at 55 °C for 60, 120, and 180 minutes in a polypropylene tube. Samples were then cooled to room temperature, and immediately analyzed.

System Suitability

As per the draft monograph, the percentage of aggregate should not be more than 5%. In addition, the RSD % of the percent area of aggregate between the triplicate injections should not be more than 10%. The RT variation of the peak due to PEG GCSF monomer in triplicate injections is not more than 0.2 minutes.

Results and Discussion

Separation and detection

Figure 1 demonstrates the excellent separation of intact PEG GCSF as a single symmetrical peak at 5.989 minutes under the chromatographic conditions. From the figure, it is evident that the conjugate contains dimers and higher aggregates as indicated in the enlarged view. However, the sample does not contain free GCSF, as indicated by the absence of a late eluting peak.

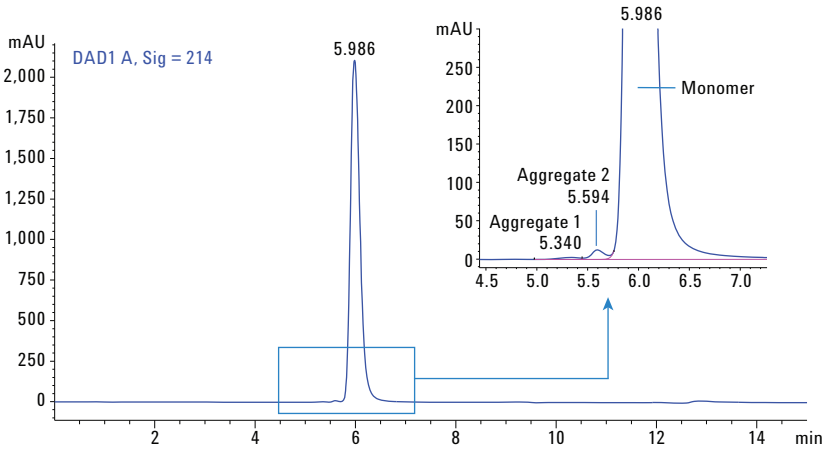

Figure1. SEC profile of intact therapeutic PEG GCSF on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

Precision of retention time and area

The precision of the procedure is expressed as the closeness of agreement between a series of measurements. These measurements can be obtained from multiple analyses of the homogeneous sample under the prescribed conditions, and is often expressed as relative standard deviation (RSD). Figure 2 demonstrates the overlays of six replicates showing excellent separation reproducibility. Table 2 shows the average RTs and peak area RSDs for monomer and aggregates from six replicates of PEG GCSF. The RT and peak area RSDs for the main peak were 0.023 and 0.081%, respectively, demonstrating excellent reproducibility of the analytical method and the precision of the system.

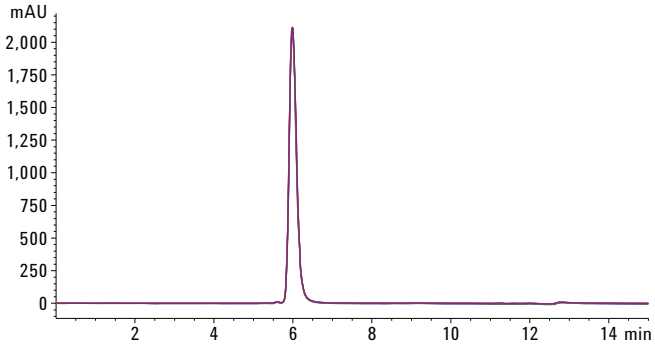

Figure 2. Overlay of six replicates of PEG GCSF separated on an Agilent AdvanceBio SEC, 130Å, 7.8 × 300 mm, 2.7 µm column.

Table 2. Retention time and peak area precision (n = 6).

	Retention time		Peak area	
Samples	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
PEG GCSF	5.987	0.023	99.39	0.081
PEG GCSF aggregate 1	5.594	0.01	0.413	4.91
PEG GCSF aggregate 2	5.340	0	0.155	5.1

This precision also conforms to the system suitability requirements:

- The percent aggregate is not more than 5%.
- The RSD for the percent area of aggregate between the triplicate injections is not more than 10%.
- The retention time of the peak due to PEG GCSF monomer in triplicate injections is not more than 0.2 minutes.

Thus, the content of high molecular weight aggregates in the PEG conjugate does not exceed 0.6%. In addition, the purity of PEG GCSF according to SEC HPLC is greater than 99%.

LOD and LOQ

The LOD and LOQ were found to be 3.125 µg/mL and 12.5 µg/mL, respectively, indicating that the method is sensitive. The observed LOD and LOQ values of PEG GCSF are tabulated in Table 3. Figure 3 shows an overlay of LOD and LOQ chromatograms of the conjugate with blank.

Table 3. LOD, LOQ and S/N results (n = 3).

Concentration (µg/mL)	S/N ratio	Average area
3.125 (LOD)	4.6	13.69
12.5 (LOQ)	17.7	27.16

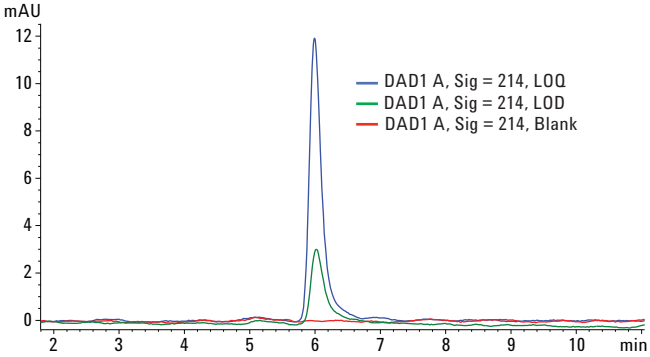

Figure 3. LOD and LOQ chromatograms of PEG GCSF separated on an Agilent AdvanceBio SEC, 130Å, 7.8 × 300 mm, 2.7 µm column, overlaid with blank.

Linearity

Linearity curves for PEG GCSF were constructed from the LOQ level to a highest concentration level in the study using area response and concentration of PEG GCSF. Figure 4 shows the linearity curve for PEG GCSF in the concentration range 12.5 to 2,000 µg.

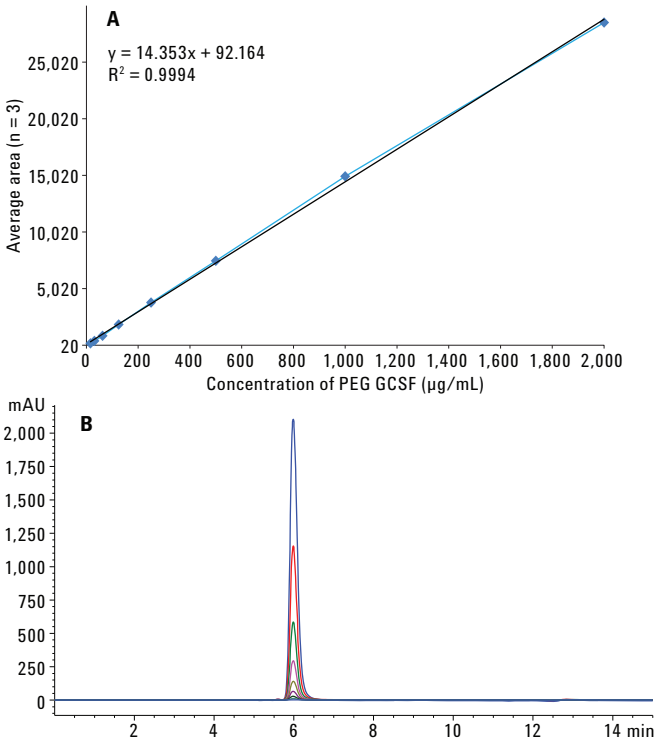

Figure 4. Linearity curve (A) with eight standard concentrations of PEG GCSF ranging from 12.5 to 2,000 µg/mL showing excellent coefficient value. The chromatogram overlay (B) for the linearity range is also shown.

Aggregation/degradation analysis and quantification

The SEC profiles of heat stressed PEG GCSF shown in Figure 5 indicates that the AdvanceBio SEC column was able to separate and detect aggregates. Intact and higher aggregates of PEG GCSF were distinctly separated from each other, as seen in the chromatogram. Table 4 summarizes the relative quantitation of monomer and aggregates of PEG GCSF based on the area percent.

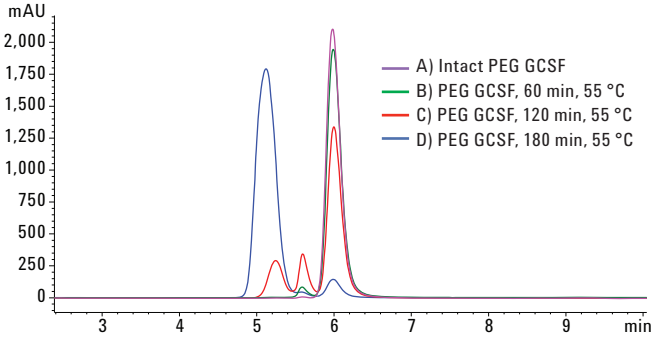

Figure 5. Trend of PEG GCSF aggregation determined by SEC HPLC on an Agilent AdvanceBio SEC column. (A) Intact PEG GCSF control, (B) 60 minutes at 55 °C (C), 120 minutes at 55 °C, and (D) 180 minutes at 55 °C.

It is evident from the data that there was an increase in the levels of aggregate when stressed at 55 °C, with a relative decrease in the amount of the monomeric form from 96% to 70% and 6.44%, respectively.

Table 4. Relative quantification of monomer and aggregates based on peak area.

Stressed PEG GCSF (60 min)		Stressed PEG GCSF (120 min)		Stressed PEG GCSF (180 min)	
Time	**Area %**	**Time**	**Area %**	**Time**	**Area %**
5.59	2.85	5.24	16.01	5.12	91.89
5.99 (monomer)	96	5.59	12.74	5.57	1.14
		5.99 (monomer)	70.29	5.98 (monomer)	6.44

Conclusion

This application note demonstrates several excellent solutions for the analysis of PEGylated proteins using PEG GCSF as a model protein. A simple method for SEC HPLC using an Agilent AdvanceBio SEC column was developed for monitoring the purity of PEG GCSF without the use of organic modifiers in the mobile phase. RT and area RSDs of replicates were excellent, and met the system suitability requirements of PEG GCSF. The LOD and LOQ for PEG GCSF were found to be 3.125 µg/mL and 12.5 µg/mL respectively, indicating the sensitivity of the analytical method. A linearity curve with eight standard concentrations of the conjugate 12.5 to 2,000 µg/mL showed an excellent coefficient of linearity value, illustrating that the method is quantitative and accurate. In addition, stress studies of therapeutic PEG protein demonstrated that the AdvanceBio SEC column was able to separate, detect, and quantify aggregates based on area percent. Such a simple and reproducible method, coupled with the bio-inertness and corrosion resistance of the instrument, makes this solution reliable and suitable for the QC of PEGylated proteins in Biopharma research.

References

1. Gaberc-Porekar, V.; Zore, I.; Podobnik, B.; Menart, V. Obstacles and pitfalls in the PEGylation of therapeutic proteins. *Current Opinion in Drug Discovery and Development* **2008**, *11*, 242–250.
2. ipc.nic.in/writereaddata/monoprepimages/Pefilgrastim-2961377726.pdf
3. Ratto, J. J.; O'Conner, S. R.; Distler, A. R.; Wu, G. M.; Hummel, D.; Treuheit M. J.; Herman, A. C.; Davis, J. M. Ethanol-sodium chloride-phosphate mobile phase for size-exclusion chromatography of poly (ethylene glycol) modified proteins. *J. of Chromatog. A* **1997**, *763*, 337–344.
4. Tsutomu Arakawa; *et al.* The Critical Role of Mobile Phase Composition in Size Exclusion Chromatography of Protein Pharmaceuticals. *J. of Pharm. Sci.* **2010**, *99*, 1674–1692.

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