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Separation of free filgrastim and oxidized impurities from a pegfilgrastim sample using a Thermo Scientific BioBasic C18 300 Å column

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Keywords

Pegfilgrastim, pegylated protein, BioBasic C18, biopharma, UV detection, HPLC

Goal

Showcase the ability of the Thermo Scientific™ BioBasic™ reversed-phase C18 HPLC column to simultaneous analyze the oxidized and free filgrastim in a pegfilgrastim sample using reversed-phase HPLC chromatography.

Introduction

Pegfilgrastim is a PEGylated form of the recombinant human granulocyte colony-stimulating factor (G-CSF) analogue of filgrastim. Filgrastim is a human G-CSF that belongs to a family of cytokines. It helps in stimulating the production of neutrophils.

Active molecule filgrastim is a 175 amino acid polypeptide that is produced using R-DNA technology in recombinant *E. Coli* cells. Filgrastim molecules contain four methionine residues: Met1, Met122, Met127, and Met138, which are susceptible to oxidation either during storage of the product or during production of the product. Oxidized impurity forms major CQA for pegfilgrastim activity and it is critical to monitor and have a control during production and storage of product.

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Pegfilgrastim is synthesized with conjugation chemistry where the free filgrastim molecule is conjugated with 20 kDa of PEG (polyethylene glycol). The pegylation process increases circulatory half-life time of protein in the blood. However, the pegylation reaction is incomplete, and the remaining free filgrastims, which do not undergo complete pegylation, are critical to monitor as they form product variants.

Reversed-phase chromatography is a widely used technique to separate oxidized impurity from product. In this study, pegfilgrastim samples are analyzed using a 300 Å Thermo Scientific™ BioBasic™ reversed-phase C18 HPLC column. The reversed-phase HPLC profile for pegfilgrastim shows pre-peaks, main peak, and post-peaks. As per the available literature, the pre-peak impurity observed in the chromatographic profile of pegfilgrastim is assumed to be an oxidized impurity, whereas the post-peak impurity obtained could be free filgrastim or a reduced pegfilgrastim peak.

To identify the oxidation-related and suspected impurities in pre-peak, we investigated a peroxide treated pegfilgrastim sample with the same chromatographic conditions.

To identify free filgrastim in the post-peak impurity, we analyzed free filgrastim again with the same chromatographic conditions. To further confirm separation of free filgrastim from the main peak of pegfilgrastim, we spiked free filgrastim molecules into a pegfilgrastim sample and the spiked sample was analyzed with the same chromatographic condition. The recovery of free filgrastim in the spiked sample was also checked.

The data obtained shows that the BioBasic C18 column, under the developed conditions, not only has the capability to separate but also to quantitate the oxidized and unreacted free filgrastim.

Experimental

Recommended consumables

- Fisher Scientific™ Optima™ UHPLC-MS grade water (P/N W8-1)
- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Scientific™ Analytical grade trifloroacetic acid (P/N 139721000)
- BioBasic C18 column (P/N 72105-254630)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)
- Hydrogen peroxide, 30% w/v Qualigens™ (P/N 15465)

Sample pretreatment / sample preparation

Pegfilgrastim samples were diluted to 1.0 mg/mL using deionized water. A filgrastim concentration of 300 µg/mL was used for analysis. Then, 25 µg of pegfilgrastim sample and 2.5 µg of filgrastim sample were injected for analysis.

Oxidized samples of pegfilgrastim were prepared by adding 5 µL of hydrogen peroxide (H_2O_2) to 100 µL of 1 mg/mL pegfilgrastim sample. The samples were then incubated at room temperature in darkness for 140 minutes and immediately injected. Ten percent of the filgrastim sample was spiked into the pegfilgrastim sample.

Internal standards

1) Pegfilgrastim

2) Filgrastim

Separation conditions

Instrumentation:	Thermo Scientific™ UltiMate™ 3000 RSLC system equipped with: • SRD-3600 Solvent Racks with Degasser (P/N 5035.9230) • DGP-3600RS Rapid Separation Pump (P/N 5040.0066) • WPS-3000TRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020) • TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000) • DAD-3000RS Rapid Separation Diode Array Detector (P/N 5082.0020)
Column:	BioBasic C18, 4.6 mm i.d. × 250 mm, 5 µm particle size (P/N 72105-254630)
Mobile phase A:	Water + 0.1% TFA
Mobile phase B:	Acetonitrile + 0.1 % TFA
Gradient:	See Table 1
Flow rate:	0.6 mL/min
Column temp.:	55 °C
Injection details:	25 µL for pegfilgrastim and 8.3 µL for filgrastim
Injection wash solvent:	50% acetonitrile in water
Wavelength:	215 nm

Table 1. LC gradient conditions

Time (min)	A%	B%
0.0	45	55
2.0	45	55
28.0	37	63
36.0	20	80
38.0	20	80
38.1	45	55
48.0	45	55

Data processing

The Thermo Scientific™ Chromeleon™ 7.0 version 7.2.2.6394 Chromatography Data System was used for data acquisition and analysis.

Result and discussion

In the analysis of pegfilgrastim molecules with the BioBasic C18 column, the chromatogram shows nine different peaks (Figure 1). Of these, four peaks elute before the main peak and are termed pre-peaks, while four more peaks elute after the main peak and are termed post-peaks (Figure 1B). The chromatographic profile for filgrastim (Figure 2), shows a single major peak that differs in retention time compared to the main peak of pegfilgrastim. Differences in retention times for the peaks can be attributed to the presence of an additional PEG moiety in the pegylated filgrastim sample. Attachment of a PEG molecule to filgrastim makes the molecule more hydrophilic, and hence has less retentivity on a reversed-phase (RP) column. This makes the pegfilgrastim molecule elute before filgrastim.

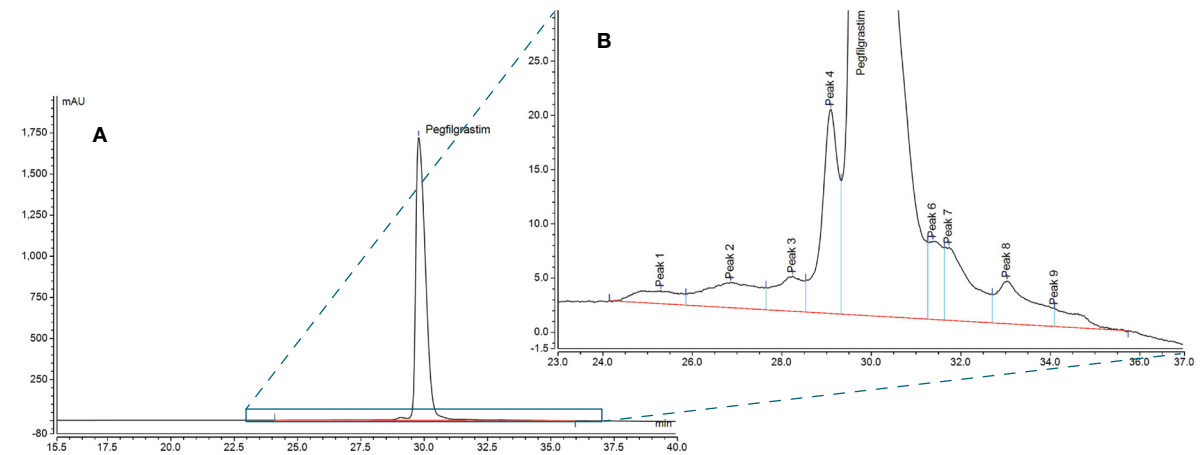

Figure 1. Reversed-phase chromatogram of pegfilgrastim sample (A) and chromatogram zoomed (B)

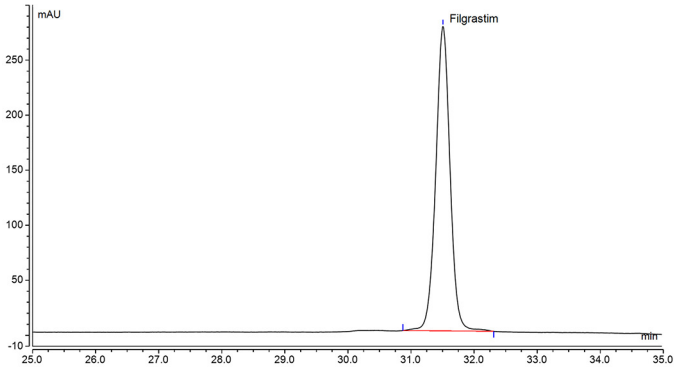

Figure 2. Reversed-phase chromatogram of filgrastim

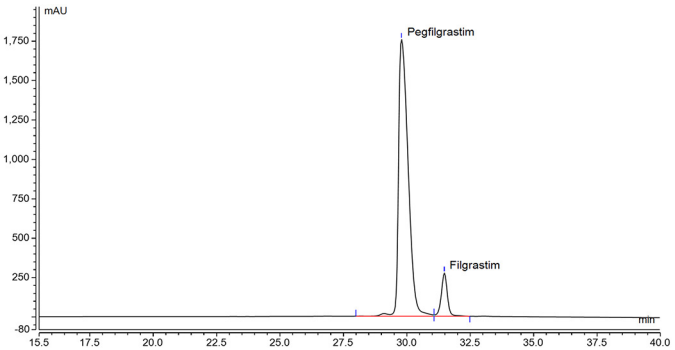

Figure 3. Reversed-phase chromatogram of pegfilgrastim sample spiked with filgrastim

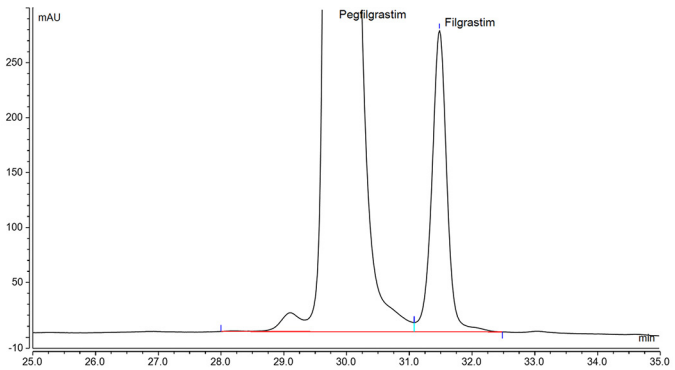

Figure 4. Reversed-phase chromatogram of pegfilgrastim sample spiked with filgrastim (zoomed)

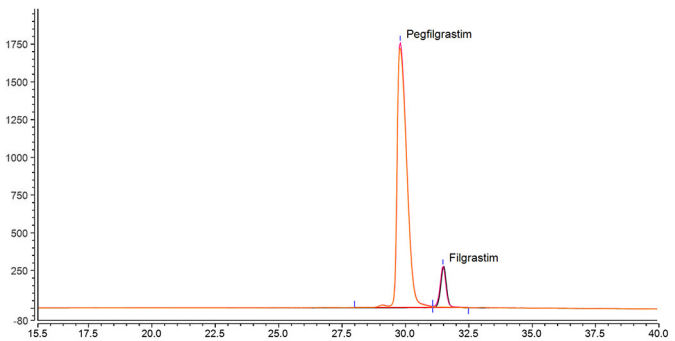

Figure 5. Overlay chromatogram of pegfilgrastim, filgrastim, and pegfilgrastim spiked with filgrastim molecules

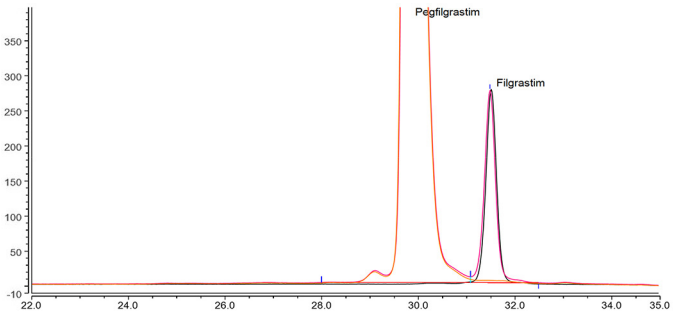

Figure 6. Overlay chromatogram of pegfilgrastim, filgrastim, and pegfilgrastim spiked with filgrastim molecules (zoomed)

Further comparison of both profiles (Figures 1 and 2) indicate that the peaks related to filgrastim are well separated. To further identify/confirm the behavior and separation of filgrastim in pegylated filgrastim sample (matrix impact), a known amount of filgrastim was spiked into the pegfilgrastim sample and was back-calculated. It was found that 96% of spiked filgrastim was recovered in the sample, indicating a simple RP approach method for efficiently separating free filgrastim on a BioBasic 300 Å C18 column.

Oxidized impurities have been confirmed by the oxidation of pegfilgrastim sample with hydrogen peroxide. This was confirmed in the chromatographic profile obtained for peroxide-oxidized pegfilgrastim molecule, which showed two additional peaks eluting before the unknown impurity (peak 4) and the major pegfilgrastim peak, in Figure 1. Oxidized impurity 3 in the H_2O_2-treated sample as shown in Figure 7 matches exactly with the retention time of peak 4, in a control pegfilgrastim sample, mentioned in Figure 1. This indicates that the peak corresponds to an oxidized impurity, and it is observed that the proportion of peak area response increased after treatment of hydrogen peroxide. The first and second peak in the peroxide-treated sample are also attributed to oxidized impurities, which are formed with interaction of peroxide with methionine residue present in pegfilgrastim. The retention time of the fourth peak in the peroxide-treated pegfilgrastim sample (Figure 7) matched exactly with the retention time (RT) of the pegfilgrastim peak in the pegfilgrastim-controlled sample as shown in Figure 8. This shows that the fourth peak in peroxide corresponds to unoxidized pegfilgrastim that is not oxidized in the process of peroxide treatment.

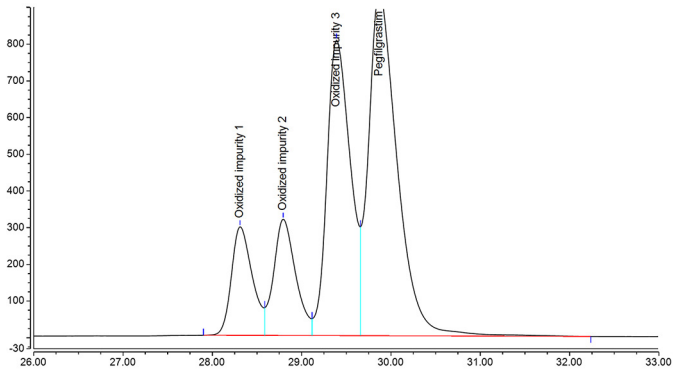

Figure 7. Reversed-phase profile of H_2O_2-treated pegfilgrastim molecule

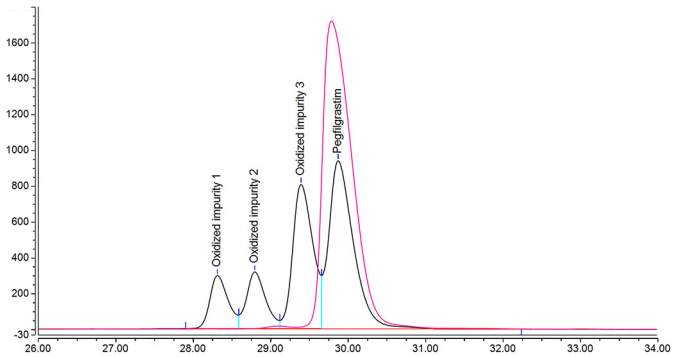

Figure 8. Overlay chromatogram of pegfilgrastim and H_2O_2-treated pegfilgrastim molecules

Conclusion

In this study, we have demonstrated an estimation of free filgrastim in the pegylated sample using a simple reverse phase approach. The method could also be used to characterize the oxidized impurities in the pegylated filgrastim sample. Though the degree of pegylation is usually determined using a SEC (size exclusion chromatography) technique, this simple RP approach could be orthogonal and, thus, a complementary technique to confirm the pegylation. The BioBasic C18 300 Å column is suitable for simultaneous analysis of both impurity, i.e., free filgrastim, and oxidized impurity at an intact level.

References

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