Characterization and Comparison of Neulasta and its Biosimilar at Intact Level on a Quadrupole-**Orbitrap Mass Spectrometer**

ABSTRACT

Purpose: Assessment of the similarity and differences between Neulasta and its biosimilar product.

Methods: Apply high resolution LC/MS analysis to measure the intact mass and profile PEG distribution.

Results: Successfully revealed difference in PEG distribution of two products

INTRODUCTION

Pegfilgrastim is marketed by Amgen under the trade name Neulasta[™]. It is a prescription medicine used to help reduce the chance of infection due to a low white blood cell count, in people with certain types of cancer (non-myeloid), who receive anti-cancer treatment (chemotherapy) that can cause fever and low white blood cell count.

In China, there are several biosimilars of Neulasta approved to the market. To meet regulatory guidelines, the manufacturers of biosimilars need to prove the similarity of their products to the innovator. In this study, we used a Thermo Scientific™ Q Exactive HF-X™ Biopharma Mass Spectrometer to measure the molecular weight and PEG polydispersity of both innovator and biosimilar to evaluate potential differences.

MATERIALS AND METHODS

Sample Preparation

The innovator and biosimilar were diluted to 1 mg/mL with pure water respectively for LC/MS analysis

Liquid Chromatography

The innovator and biosimilar samples were separated on a Thermo Scientific[™] UltiMate3000[™] HPLC System (A: 50 % ACN + 0.1 % TFA, B: 95 % ACN + 0.1 % TFA). Post column addition: 400 mM TEA in water, 5 µL/min; a Thermo Scientific ™ Hypersil GOLD™ C18 column $(150 \text{ mm} \times 2.1 \text{ mm}, 1.9 \mu \text{m})$ was employed.

Mass Spectrometry

A Q Exactive HF-X Biopharma Mass Spectrometer was used for all analysis

Data Analysis

Data analysis was performed using Thermo Scientific[™] BioPharma Finder[™] Software. Parameters shows in Figure 1.

Figure 1. Data processing parameters used in BioPharma Finder.

Component Detection	🗾 🗹 Identificat											
Chromatogram & Source	e Spectra Dec	onvolution Algorithm										
hromatogram Parameter	ers			Source Spectra Method			RT Range	1.297 🔹 to 1.592 🔹				
Use Restricted Time		Sliding Windows					2	LUCO LUCO				
Time Limits	0	0.029 🚍 to	Generate the source spectra by using the			e sliding windows algorithm.						
	0	hannel	Average Over Selected Retention Time									
Scan Range		1 📩 to	708 💌	Generate the source spect across multiple scans) on t		an (or averaging by dragging						
m/z Range	1,40	0.0000 to	6,000,0000									
Chromatogram Trace Type	ric .											
Real Time Optimization												
Component Detection												
hromatogram & Source !		nvolution Algorithm										
	Bas	sic Advanced onvolution Results Filter –								Choice of Peak Model		
convolution Algorithm -		Output Mass Range		30,000 to	50.000	Charge State Distribution Deconvolution Mass		,	20.00 ppm ~	Choice of Peak Model Choice of Peak Model	Intact Protein	
ReSpect™ (Isotopically L Xtract (Isotopically Reso				30,000 to 50,000		Tolerance		4	0.00 ppm *	Resolution at 400 m/z		
Ander (isotopically heso	olived)	Deconvoluted Spectra Display Mode	Isotopic Profile (new) ~							 Raw File Specific Method Specific 		10607.00
	Gen	Generate XIC for Each Component Calculate XIC										10007.00
Real Time Optimization	ı											
Component Detection	📝 Identificat											
hromatogram & Source !	Spectra Decor	nvolution Algorithm										
2		ic Advanced										
convolution Algorithm -	Cha	rge State Distribution				Choice of Peak Model				Specialized Parameters		
ReSpect™ (Isotopically U	Unresolved)	Model Mass Range		30,000.00 to	50,000.00	Target Mass		4	0,000.0000 Da	Peak Model Width Factor		1.00
Xtract (Isotopically Reso	olved)	Charge State Range		5 🔹 to	50 🜲	Peak Model Parameters				Intensity Threshold Scale		0.01000
		nimum Adjacent Charges		3 💌 to	5 单	Number of Peak Models			1	Deconvolution Parameters		
	£	ow & high model mass)				Left/Right Peak Shape	Left	2.00 Right	2.00	Noise Compensation	\checkmark	
		se Parameters								Charge Carrier	H+ (1.00727663)	
		el. Abundance Threshold %)				Peak Filter Parameters					 2H+ (2.013553) Na+ (22.9892213) 	
						Peak Detection Minimum Significance Measure	2.00 Standard Deviations			Negative Charge	O Custom	
	Dec	Deconvolution Quality Quality Score Threshold 0.00			Peak Detection Quality	68%	68% *					
		Quality score inreshold			0.00	Measure	0070					

RESULTS

distributions.

For PEGylated proteins, a multiplicity of signals is generated because of the presence of ladders of multiply charged protein ions and the overlaying polydispersity of the attached PEG. This results in spectra containing overlapping signals that are difficult to deconvolute and interpret. We chose TFA as solvent addition for charge reduction and TEA as post column addition to avoid dePEGylation.

Hundreds of components can be baseline separated and detected with great S/N ratio in this extremely complex mixture, benefiting from the high resolution and sensitivity of the Orbitrap analyzer (Figure 2). MS parameters are shown in Table1.

Table 1. MS parameters

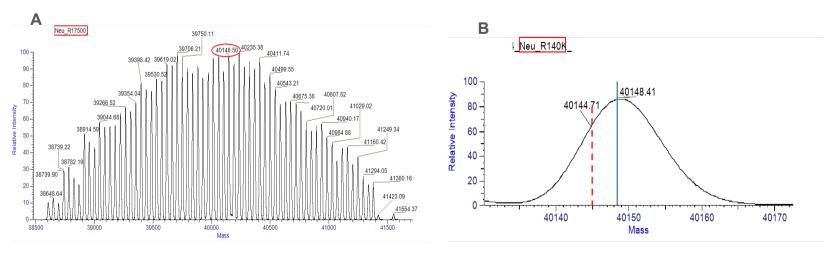
Runtime Polarity In-source CID Microscans Resolution AGC target **Maximum IT** Scan range Spray Voltage **Probe Heater Temp.** S-Lens RF Level

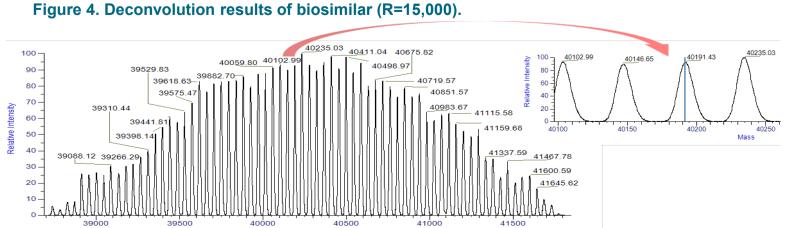
Deconvolution results

The PEG heterogeneity makes molecular weight measurement a real challenge. On the Orbitrap platform, we could use high resolution to separate adjacent peaks in MS spectrum without sensitivity loss.

As an example, we determined the intact molecular mass of the molecule that contains a PEG modification having a degree of polymerization of n=483 (theoretical average molecular mass of 40,148.7594 Da). For the innovator, Neulasta, as the resolution changes from 15,000 to 120,000, the delta mass between theoretical and experimental masses was reduced from 2.26 Da to 0.35 Da, showcasing the power of high resolution (Figure 3). Figure 4 shows the deconvolution results of biosimilar using 15,000 resolution.

Figure 3. Deconvolution results of Neulasta. A, R=15,000. B, R=120,000, enlarged view of component nPEG=483.



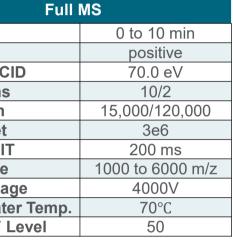


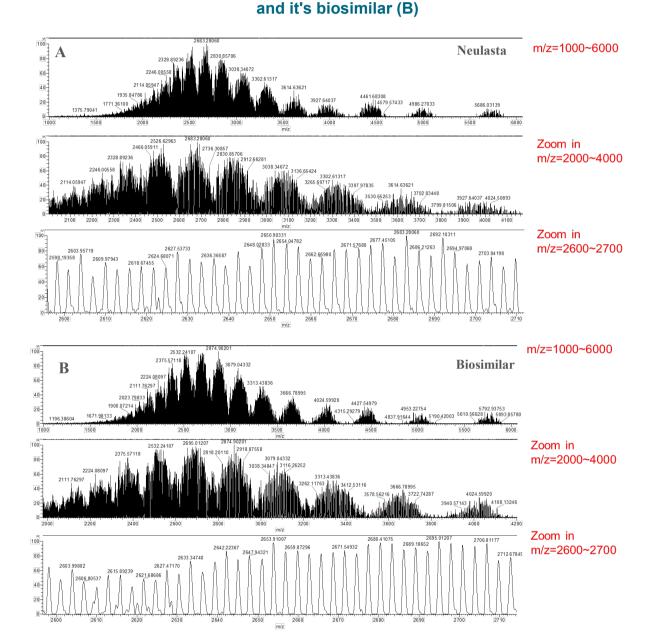
Xiaoxi Zhang¹, Haichuan Liu², Hao Yang², Min Du³. 1 Thermo Fisher Scientific, Shanghai, China; 2 Thermo Fisher Scientific, San Jose, CA, US; 3 Thermo Fisher Scientific, Massachusetts, US

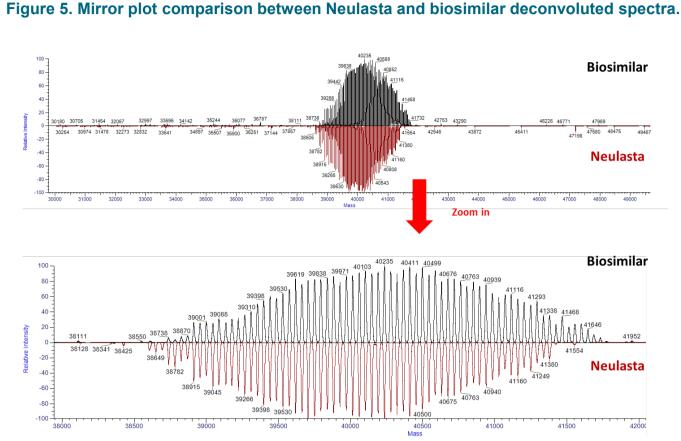
Molecular weight determination of Neulasta and its biosimilar

In this study, we measured the molecular weights of Neulasta and a domestic biosimilar. Neulasta is a PEGylated recombinant human granulocyte colony-stimulating factor, containing 174 amino acids (18-20 kDa) and around 20 kDa PEG chains in continuous

Figure 2. Comparison of combined raw spectra of Neulasta (A)







PEG polydispersity calculation

The covalent attachment of PEG (PEGylation) is a well-established approach to improve protein stability and solubility, to reduce renal clearance and proteolytic degradation, and to decrease immunogenicity and antigenicity, all of which contribute to an improved clinical efficiency and safety profile. To provide a safe and efficient drug and to meet the regulatory criteria for human use, however, the PEGylated protein needs to be thoroughly characterized.

Using mirror plot comparison, we found that the distributions of PEG modification are different between the innovator and biosimilar (Figure 5) – the distribution of biosimilar shifted to higher m/z range.

Based on the deconvolution results, number-average molecular mass (Mn), mass-average molecular mass (Mm) and PEG polydispersity (PD) were calculated using the formulas below:

Table 2. Mm, Mn and PD of both samples

Sample	Mm	Mn	PD	$\sum M M$	$\sum M_i^2 N_i$	М
Neulasta	39939.32174	39896.98880	1.0011	$M_n = \frac{\sum M_i N_i}{\sum N_i}$	$M_m = \frac{\sum M_i N_i}{\sum M_i N_i}$	$PD = \frac{M_m}{M_n}$
Biosimilar	40226.91232	40202.11802	1.0006			п

in table 2.

It's easy to calculate the PD for Neulasta and its biosimilar. While we observe the difference in intact mass measurement, the calculated PD also showed difference between Neulasta and its biosimilar, 1.0011 and 1.0006 respectively, which might lead to potential safety and efficacy differences.

CONCLUSIONS

- biosimilar Neulasta.

REFERENCES

- 2475-84.

TRADEMARKS/LICENSING

© 2020 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others.

PO65773-EN 0422S

The calculated number-average molecular mass (Mn), mass-average molecular mass (Mm) and PEG polydispersity (PD) are shown

Successfully apply the UHPLC-HRAM technology to profile the intact molecular weight and calculate PD of both innovator and

• The high resolution and sensitivity of the Orbitrap mass analyzer benefits in complex mixture separation with great S/N ratio.

PEG distribution difference was observed between these two samples.

The subsequent peptide mapping analysis could provide more insights and details on PEG modification.

1. Lyman GH, Dale DC, Culakova E, Poniewierski MS, Wolff DA, Kuderer NM, Huang M, Crawford J. Annals of Oncology. 24 (10):

2. Harris, J. M.; Chess, R. B. Nat. Rev. Drug Discovery 2003, 2, 214-21.

3. Mann, M.; Meng, C. K.; Fenn, J. B. Anal. Chem. 1989, 61, 1702–1708.

4. Trimpin, S.; Plasencia, M.; Isailovic, D.; Clemmer, D. E. Anal.Chem. 2007, 79, 7965–74.

5. Huang, L. H.; Gough, P. C.; DeFelippis, M. R. Anal. Chem. 2009, 81, 567–577.

