Separation of Semaglutide and Liraglutide by Analytical NanoPak-C All Carbon HPLC Columns

Introduction

Separation and purification are crucial steps in peptide drug manufacturing workflows. Analytical methods that can separate peptide drugs from impurities are essential for quality control testing. These methods can also be used in preparative chromatography to purify the peptide drugs during manufacturing. Choosing suitable chromatography media is crucial for cost-effective production, as it allows for the seamless development and transfer of methods from analytical to preparative chromatography.

NanoPak-C All carbon spherical microbead media is stable across the entire pH range, providing flexibility in developing reverse-phase HPLC methods [1]. They are available in analytical (5-7 µm diameter), preparative (10-15 µm), and solid-phase extraction (SPE)/ flash (40-60 µm) grades, applicable across the entire drug discovery and manufacturing workflows (**Figure 1**).

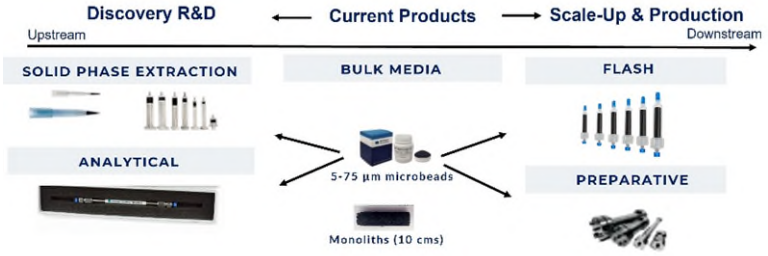

Figure 1. Image illustrating one media for the entire chromatography workflow.

This white paper presents a method for the analytical HPLC of Semaglutide and Liraglutide using analytical-grade NanoPak-C All carbon microbeads under alkaline conditions. To add context, we also show the HPLC separation results under identical conditions using two commercially available silica C18 media.

Materials & Methods

Probe Analytes
Semaglutide & Liraglutide
1mg/mL in DI water
Alkaline pH 8.5

HPLC Column
150 x 4.6 mm
NanoPack-C All Carbon Media
6 um diameter, 22% RSD

Method
Mobile Phase A
20 mM Ammonium Acetate Buffer (Alkaline pH 8.5)

Mobile Phase B
Acetonitrile

Gradient: Time	%B
0	20
10	65
12	65
13	20

Total Run Time:17 min
UV: 220 nm
Temp: 25 C
Injection: 20 uL

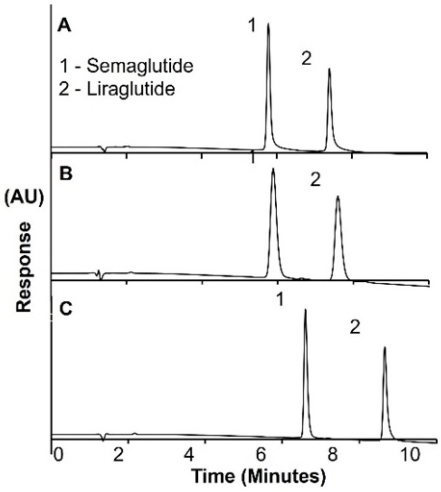

Figure 2. Representative chromatogram of (1) Semaglutide and (2) Liraglutide obtained using HPLC columns packed with (A) NanoPak-C (6 µm media), (B) Si-C18 (9 µm media), and (C) Si-C18 (4.2 µm media).

Table 1. Analyte

	Semaglutide			Liraglutide		
	NanoPak-C 6 µm Dia. 22% RSD	Si-C18 9 µm Dia. 29% RSD	Si-C18 4.2 µm Dia. 25% RSD	NanoPak-C 6 µm Dia. 22% RSD	Si-C18 9 µm Dia. 29% RSD	Si-C18 4.2 µm Dia. 25% RSD
Retention Time (t_R) (min)	5.7	5.89	6.59	7.4	7.6	8.66
Resolution (R)	1.4	1.52	2.99	9.97	3.21	9.01
Selectivity (α)	7.9	6.98	1.15	1.38	1.19	1.26
Retention Factor (k)	2.86	2.93	3.41	3.95	4.09	4.8
Plates Number (N)	21715	6457	25542	29215	8944	44080
Area (mAU*s)	14164	9163	8737	8080	7660	7365
Height (mAU)	1189	782	1302	781	610	950
Width (50%)	0.09	0.18	0.09	0.1	0.18	0.1
Symmetry (S)	0.45	0.72	0.67	0.46	0.73	0.55

Dia. = Diameter; RSD = Relative Standard Deviation

Results and Discussions

Figure 1 shows representative chromatograms of Semaglutide and liraglutide for NanoPak-C (6 µm media), Si-C18 (9 µm media), and Si-C18 (4.2 µm media) HPLC columns. **Table 1** presents the key chromatography parameters of each chromatogram.

The retention times of NanoPak-C media for Semaglutide and Liraglutide are marginally lower than the Silica C18 (9 µm media), and significantly lower than Si-C18 (4.2 µm media).

In HPLC, achieving the best possible separation in the shortest time is essential. A resolution of 1.5 or higher between two peaks guarantees that the sample components are sufficiently well separated, allowing for accurate measurement of the area or height of each peak.

The resolution between Semaglutide and Liraglutide peaks for NanoPak-C media was 9.97. It was 3.21 for Silica C18 - Si-C18 - 4.2 µm media. The 9 µm media and 9.01 for

resolution values of NanoPak-C media and Si-C18 4.2 µm media meet the US Pharmacopeia assay specification (R ≥ 5)[2].

According to the fundamental resolution equation (eqn, 1), selectivity (separation factor α), efficiency (N), and retentivity (retention factor k) are the three critical parameters that affect resolution.

R = ¼ √N x ((α-1)/ α) x (k/(k+1)) ……. Eqn. 1.
Efficiency Selectivity Retention

The retention factor (k) is a measure used to assess how well an analyte is retained on a chromatographic media. It is calculated as the ratio of the retention time of the analyte on the column to the retention time of a non-retained compound using the formula $k = (t_{R2}-t_0)/t$. where t_R ($t_{R2} > t_{R1}$) is the retention time of the analyte and t_0 is the retention time of the non-retained compound. The retention factor k of NanoPak-C and Si-C18 media is 2.86 and 3.95 for Semaglutide and Liraglutide, respectively. These values are within the k = 1-10 range, which provides good separations.

The selectivity (or separation) factor (α) calculated using the equation α = α = k2/k1 = $(t_{R2}-t_0)/ (t_{R1}-t_0)$, measures the ability of the chromatographic media to distinguish between analytes in a mixture. It is calculated as the ratio of the retention factors of the two analyte peaks (k). The selectivity value should be greater than one. If the α value equals one, the two analyte peaks are co-eluting (i.e., their retention factor k values are identical). The higher the selectivity value, the further apart the two peaks. The selectivity values of NanoPak-C and Si-C18 media are above one.

Efficiency, a measure of chromatography peak dispersion, depends on the plate number (N), which is calculated using the equation $N = 5.54 (t_R/ (\text{width } (50\%))^2$ The plate numbers for NanoPak-C media were 21,715 for Semaglutide and 29,215 for Liraglutide. These plate numbers were higher than Silica C18 9 µm media and lower than Si-C18 4.2 µm media. However, the higher plate number of Si-C18 4.2 µm is due to higher retention times with this media. Both media have the same width (50%) at 0.09, indicating that peak dispersions and efficiencies are similar.

Conclusions

The combined results show that NanoPak-C analytical grade media can effectively separate Semaglutide and Liraglutide with good chromatography resolution. The key chromatography parameters of NanoPak-C align with the expected values for 4-7 µm media. These analytical columns can be used for quality testing of impurities or method development. Alternatively, during peptide manufacturing, the 40 µm NanoPak-C media can be used as a cleanup method for Semaglutide and Liraglutide or as a pre-purification step to initially reduce sample complexity and remove a significant portion of impurities. This cleanup can be followed by final purification using 5 µm analytical or preparative 10 µm grade media. The workflow would allow straightforward scale-up procedures, ensuring consistent results at the manufacturing scale.

References

[1] M.J. Parente, B. Sitharaman, Synthesis and Characterization of Carbon Microbeads, ACS Omega 8, 34034–34043 (2023) .
[2] USP, Harmonized Standards Supplement USP Stage 4 Harmonization (December 1, 2022 (latest revision in April 2023).).