Application Note: Alkaline pH Stability of NanoPak-C All Carbon HPLC Columns

Background. Separation and purification are critical steps in peptide drug manufacturing, particularly for GLP-1 analogues such as Semaglutide and Liraglutide, where analytical methods are needed to separate peptide drugs from impurities for quality control and to support preparative purification.

NanoPak-C all-carbon spherical microbead media are designed to be stable across the full pH range and are available in analytical, preparative, and SPE/flash grades, allowing seamless method transfer from analytical HPLC to process-scale purification. We had previously demonstrated that NanoPak-C analytical-grade media can effectively separate Semaglutide and Liraglutide under alkaline conditions (pH 8.5) using a 150 x 4.6 mm column [1].

In this application note, we extend that method to evaluate the robustness of NanoPak-C columns at pH 8.5 using **cumulative column volumes** as the primary stress metric. We compare both 150 x 4.6 mm and 50 x 4.6 mm analytical column formats over 500 injections each of liraglutide and semaglutide (**1,000 injections total per column**), under identical chromatographic conditions. Each column's retention time, theoretical plate, and peak shape were monitored to confirm that performance remained within acceptable limits.

Experimental Conditions. All method conditions are taken directly from the white paper "Separation of Semaglutide and Liraglutide by Analytical NanoPak-C All Carbon HPLC Columns."

Probe analytes
- Analytes: Semaglutide and Liraglutide
- Sample concentration: 1 mg/mL in deionized water
- Sample pH: Alkaline pH 8.5

HPLC columns
- Column chemistry: NanoPak-C All Carbon media
- Analytical column formats evaluated in this study:
  - 150 x 4.6 mm, 6 µm diameter, 22% RSD
  - 50 x 4.6 mm, 6 µm diameter, 22% RSD (same media, shorter bed length)

Method conditions
- Mobile Phase A: 20 mM ammonium acetate buffer (alkaline pH 8.5)
- Mobile Phase B: Acetonitrile
- Gradient (time / %B):
  - 0 min: 20% B
  - 10 min: 65% B
  - 12 min: 65% B
  - 13 min: 20% B
- Total run time: 17 minutes
- Detection: UV at 220 nm
- Column temperature: 25 °C
- Injection volume: 20 µL

Both the 150 x 4.6 mm and 50 x 4.6 mm columns were operated under these identical conditions. Each column was challenged to 500 injections. Performance was evaluated at injections 0, 250, and 500. For each column format, the study comprised 500 injections of Semaglutide and 500 injections of Liraglutide.

For both columns, the following parameters were monitored at injections 0, 250, and 500:
- Retention time for Semaglutide and Liraglutide
- Theoretical plates
- System backpressure
- Peak shape (tailing factor)

Results and Discussions

Column-Volume Calculations

For 4.6 mm i.d. analytical columns, a widely used rule of thumb for the column void volume is:

$$V_m(\mu L) \approx L(mm) \times 10$$

This gives:

- 50 x 4.6 mm: $V_m \approx 50 \times 10 = 500\ \mu L = 0.5$ mL.
- 150 x 4.6 mm: $V_m \approx 150 \times 10 = 1500\ \mu L = 1.5$ mL.

With a 17-minute run and the flow rate from the original method (approximately 1 mL/min), about 17 mL of mobile phase pass through the column per run. Combining this with the void volumes above yields column volumes as shown in **Table 1.**

Table 1. Calculated void volume, and column volumes for each column format.

Column format	Approx. void volume	Mobile phase per run	Column volumes per run	Column volumes at 1000 injections (500 Semaglutide + 500 Liraglutide)
50 x 4.6 mm	0.5 mL	17 mL	34 CV/run	34,000 CV
150 x 4.6 mm	1.5 mL	17 mL	11.3 CV/run	11,334 CV

Each column was used for 500 injections of Semaglutide and 500 injections of Liraglutide, corresponding to approximately 34,000 column volumes at pH 8.5 for the 50 x 4.6 mm column and 11,334 column volumes at pH 8.5 for the 150 x 4.6 mm column. Thus, for the same 500 injections under identical method conditions, the 50 mm column experiences approximately **three times** the normalized column-volume exposure of the 150 mm column.

Across all checkpoints and for both column geometries, **retention, efficiency, resolution, pressure, and peak shape remained within method acceptable limits**.The system backpressure remained consistent. Together, the parameters indicated stable chromatographic performance during the full 500-injection sequence for each peptide for at pH 8.5 (**Tables 2**-**5**).

Table 2. Semaglutide parameters on 150 x 4.6 mm NanoPak-C column

Injection checkpoint	Semaglutide Retention time (minutes)	Theoretical plates	Peak shape (Tailing factor)
0	5.71	21715	1.12
250	5.75	21512	1.08
500	5.73	21900	1.10

Table 3. Liraglutide parameters on 150 x 4.6 mm NanoPak-C column

Injection checkpoint	Liraglutide Retention time (min)	Theoretical plates	Peak shape (Tailing factor)
0	7.4	29215	1.08
250	7.42	29420	1.11
500	7.41	29390	1.09

Table 4. Semaglutide parameters on 50 x 4.6 mm NanoPak-C column

Injection checkpoint	Semaglutide Retention time (minutes)	Theoretical plates	Peak shape (Tailing factor)
0	4.46	6017	1.15
250	4.41	6025	1.06
500	4.39	6001	1.10

Table 5. Liraglutide parameters on 50 x 4.6 mm NanoPak-C column			
Injection checkpoint	**Liraglutide Retention time (min)**	**Theoretical plates**	**Peak shape (Tailing factor)**
0	5.92	9234	1.20
250	5.90	9049	1.08
500	5.91	9421	1.09

Implications for Semi-Preparative and Preparative GLP-1 Chromatography

When the analytical NanoPak-C columns are expressed in terms of cumulative column volumes, the stress applied in this study is substantial. Over 1,000 injections, at pH 8.5. the 50 x 4.6 mm format experiences approximately 34,000 column volumes and the 150 x 4.6 mm format approximately 11,334 column volumes with retention, efficiency, backpressure, and peak shape remaining within acceptable limits.

Using the same run volume (17 mL/injection) and extrapolating to larger hardware, an equivalent 1,000 injections corresponds to approximately 6,800 column volumes for a 250 x 4.6 mm column, ~1,414 column volumes for a 250 x 10 mm column, ~362 column volumes for a 250 x 20 mm column, and only ~58 column volumes for a 250 x 50 mm preparative column. This analysis highlights that semi-preparative and preparative GLP-1 column, which operate at much larger internal diameters and volumes, will typically see **far fewer column volumes at pH 8.5** over a realistic campaign than the analytical columns studied here.

Table 6. Calculated void volume, and column volumes for extrapolated dimensions (250 x 4.6 mm, 250 x 10 mm, 250 x 20 mm, 250 x 50 mm) Using the void-volume and column-volume values summarized in Table 1.			
Column format	**Approx. void volume (mL)**	**CV per run (17 mL)**	**CV at 1,000 injections**
50 x 4.6 mm	0.5	34	34,000
150 x 4.6 mm	1.5	11.3	11,334
250 x 4.6 mm	2.5	6.8	6,800
250 x 10 mm	~12	~1.4	~1,414
250 x 20 mm	~47	~0.36	~362
250 x 50 mm	~295	~0.06	~58

Given that NanoPak-C all-carbon analytical columns maintain chromatographic suitability after tens of thousands of column volumes at pH 8.5, these extrapolated column-volume values suggest a generous chemical-stability margin for NanoPak-C media in semi-preparative and preparative GLP-1 purification. In other words, if the small 4.6 mm ID columns tolerate 11,000–34,000 column volumes under alkaline conditions without loss of performance, then the same media, packed in larger-ID semi-prep and prep formats and exposed to only hundreds to a few thousand column volumes, should be under significantly less chemical stress per unit packed bed at the same pH.

Limitations and Future Work

It is important to recognize that this study was performed under low-load analytical conditions (1 mg/mL solutions, 10–20 µL injections, microgram-level loads) optimized for resolution and robustness testing rather than for recovery-scale purification. Semi-preparative and preparative GLP-1 workflows typically operate in the milligram to tens-of-milligrams per injection range on 10–21.2 mm ID columns, and in the tens-to-hundreds of milligrams per injection range on 20–50 mm ID preparative columns. While the column-volume analysis provides strong evidence for the intrinsic chemical robustness of NanoPak-C all-carbon media at pH 8.5, it does not by itself define maximum practical loading, fouling propensity, or recovery behavior under high-load, crude-feed conditions.

In addition, the current work uses a single gradient program, mobile-phase composition, and temperature that are favorable for demonstrating stability, and all experiments were performed on analytical-scale hardware. Mechanical factors such as higher flow rates, pressure cycles, and potential bed stress at large ID, as well as increased risk of peptide or matrix deposition at higher loads, were not directly evaluated. Future work will therefore focus on extending these studies to semi-preparative and preparative NanoPak-C columns with

realistic GLP-1 feed streams, exploring stepwise increases in injection mass, and monitoring not only chromatographic performance but also fouling, cleaning efficiency, and long-term pressure behavior. This will allow the preliminary high-pH robustness demonstrated here at analytical scale to be fully quantified and validated under true production-relevant conditions.

Conclusion

- Under the Semaglutide/Liraglutide method conditions at pH 8.5, both the 150 x 4.6 mm and 50 x 4.6 mm NanoPak-C all-carbon columns maintained acceptable retention, plate count, backpressure, and peak shape over 500 injections of each analyte (1,000 injections total per column). Expressed as cumulative exposure, this corresponds to approximately 11,334 and 34,000 column volumes for the 150 mm 50 mm column, respectively, at pH 8.5.

- These results demonstrate strong chemical and mechanical robustness of NanoPak-C all-carbon media under prolonged alkaline conditions. They also highlight the value of describing column lifetime in terms of cumulative column volumes rather than injection count alone, especially when extrapolating pH stability from analytical to semi-preparative and preparative formats.

- Because long-term, high-pH robustness studies on large-ID preparative columns would require very high consumption of organic solvents and buffer, this analytical column-volume framework provides a practical and economical preliminary step for modelling the scalability of NanoPak-C columns to GLP-1 purification workflows.

References

[1] M.J. Parente, B. Sitharaman, Liquid Chromatography Evaluation of a Novel Graphitic Carbon Stationary Phase Using Glucagon-Like Peptide-1 Analogs, ACS Omega 10(32) (2025) 35666-35677.