Application Note

Pharmaceutical and Biopharmaceutical

Altura Size Exclusion 130 Å Allows Comprehensive Analysis of GLP-1 Analogues Over a Broad pH Range

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Abstract

Glucagon-like peptide-1 receptor agonists (GLP-1s) represent an emerging class of therapeutic peptides with a large number of chemically related impurities, resulting in challenging separations. With patents nearing completion for several GLP-1s in the next few years, it is critical that high-performance separation methods exist to support biosimilar manufacturers to ensure drug quality.

The non-specific adsorption of peptides to charged surfaces can lead to significant peak tailing. Silica supports often suffer from peak tailing due to exposed surface silanol groups. Replacing stainless-steel components with Agilent Altura Ultra Inert hardware can significantly improve the separation performance of GLP-1s without any additional method development, improving the applicability of SEC with downstream workflows. This application note outlines a systematic study on the impact of pH and organic fraction on the chromatographic performance of GLP-1s to support method development workflows. It also examines the significant separation performance that can be gained from upgrading to the new Altura SEC 130 Å column.

Introduction

Size exclusion chromatography (SEC) is the gold standard approach to monitor biotherapeutic size, including protein aggregation, conformational change, and polymerization. Biomolecules travel through the column with smaller molecules, penetrating further into the particle pore volume, which causes them to be retained longer. However, as biomolecules contain numerous reactive sites with differing pKa values, it is common for SEC to suffer from significant non-specific interactions with charged surfaces. The most common of these are exposed silanol groups as well as the metal surface of the column housing. For this study, the Agilent AdvanceBio size exclusion medium was coated with a chemically inert polymer to minimize silica binding. Additionally, Altura HPLC columns featuring Ultra Inert technology prevented metal binding, minimizing peak tailing in comparison to conventional stainless-steel column casings.

Here, we report a systematic study on the impact of pH and organic fraction on the aggregation and peak shape of GLP-1 analogues, including the significant benefits of the new Agilent Altura SEC 130 Å column in comparison to the same SEC media in stainless-steel. Peak tailing was reduced by over 60% for liraglutide, 60% for exenatide, and 30% for semaglutide under most conditions when compared to stainless-steel columns; it was also reduced by up to 75% in some cases versus competitor inert columns.

Experimental

Reagents and chemicals

Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). All HPLC-grade buffer salts, 35% ammonium solution and lyophilized proteins were purchased from Sigma-Aldrich. Semaglutide (HY-114118CP) was purchased from Medchem Express. Liraglutide (SML3925-25MG) and exenatide acetate (AT27767-25MG) were purchased from Sigma-Aldrich.

Sample preparation

All samples were diluted to 1 mg/mL with 1x PBS pH 7.4.

Mobile phase preparation

Eluent C was prepared by mixing 1 mL of trifluoroacetate with 1.1 mL of a 35% v/v ammonia solution up to 1 L with water to prepare 0.1% ammonium trifluoroacetate, pH 8.5.

Instrumentation

- Agilent Infinity III 1260 Multiple wavelength detector (G7165A)
- Agilent Infinity III 1260 Multicolumn thermostat (G7116A)
- Agilent Infinity III 1260 Bio multisampler (G5668A)
- Agilent Infinity III 1260 Bio-Inert Pump (G5654A)

Software and data processing

- Agilent OpenLab CDS Acquisition version 2.8
- Agilent OpenLab CDS Analysis version 2.8

Method conditions

Table 1. LC-UV conditions.

Parameter	Value
Column	– Agilent AdvanceBio SEC 130 Å, 4.6 × 300 mm, 2.7 µm (p/n PL1580-5350) – Agilent Altura SEC 130 Å, 4.6 × 300 mm, 2.7 µm with Ultra Inert technology (p/n PL1580-5350A) – Agilent Altura SEC 300 Å, 4.6 × 300 mm, 2.7 µm with Ultra Inert technology (p/n PL1580-5301A) – Competitor inert SEC column, 125 Å, 4.6 × 300 mm, 2.5 µm
Mobile Phase	Eluent A: 1x PBS pH 7.4 Eluent B: 25:65 acetic acid:1 g/L arginine in water, pH 2 Eluent C: 0.1% Ammonium TFA, pH 8.5 Eluent D: ACN
Flow Rate	0.35 mL/min
A:B:C:D	See Table 2
Column Temperature	25 °C
Injection Volume	1 µL
Total Run Time	20 min
UV	220 nm
Response Time	10 Hz

Table 2. Method conditions for pH/ACN conditions.

Method	Eluent A%	Eluent B%	Eluent C%	Eluent D%
1	90	0	0	10
2	80	0	0	20
3	50	0	0	50
4	0	90	0	10
5	0	80	0	20
6	0	50	0	50
7	0	0	90	10
8	0	0	80	20
9	0	0	50	50

Results and discussion

Altura SEC 300Å screening highlights significant pH dependency on GLP aggregation

Numerous journals have reported the tendency of GLP-1 analogues to aggregate under differing pH conditions, including liraglutide[2], semaglutide[3] and exenatide, although to a lesser degree.[4] Subsequently, to prevent initial condition screening from being convoluted with potential complete exclusion of higher-order aggregates, the new Altura SEC 300 Å column was used.

It was evident that the need for organic solvents is critical for improving peak shape for liraglutide and semaglutide. This is because they contain a conjugated fatty acid, either palmitic acid or a stearic diacid at Lys26, respectively. Exenatide, which does not contain an additional fatty acid, is far less sensitive to organic solvent. Liraglutide can be seen to wrap around into subsequent injections at 10% ACN, while semaglutide shows an extremely wide peak at 10% although not as deleterious. It is worth noting that these wide peaks at low ACN were found regardless of pH.

When investigating the impact of pH (Figure 2), all GLP-1s investigated exhibited a decrease in elution volume as pH was increased from pH 2 to pH 8.5. This coincided with literature that suggest that aggregation into higher-order oligomers occurs roughly between pH 6-9. While liragutide and exenatide presented similarly, albeit with improved peak width when going from pH 2 to pH 8.5, semaglutide demonstrated a significant gain in peak fronting or peak tailing at pH 2 and 7.4, respectively, most likely driven by the ionization state of the stearic diacid.

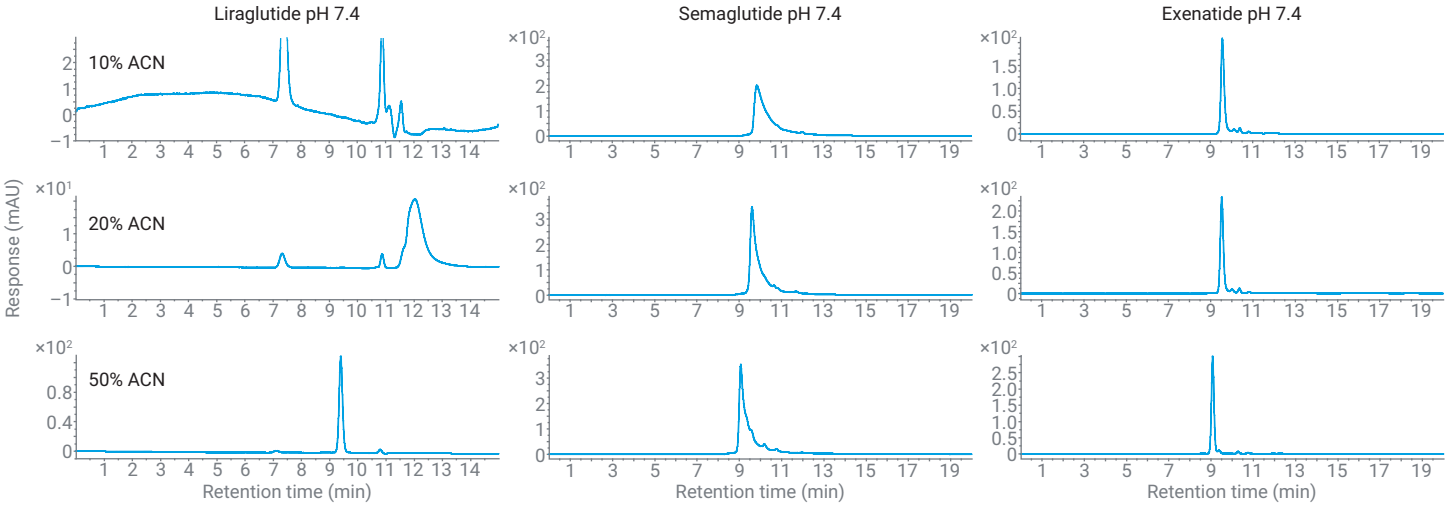

Figure 1. Impact of ACN fraction on the peak shape of GLP-1s at pH 7.4.

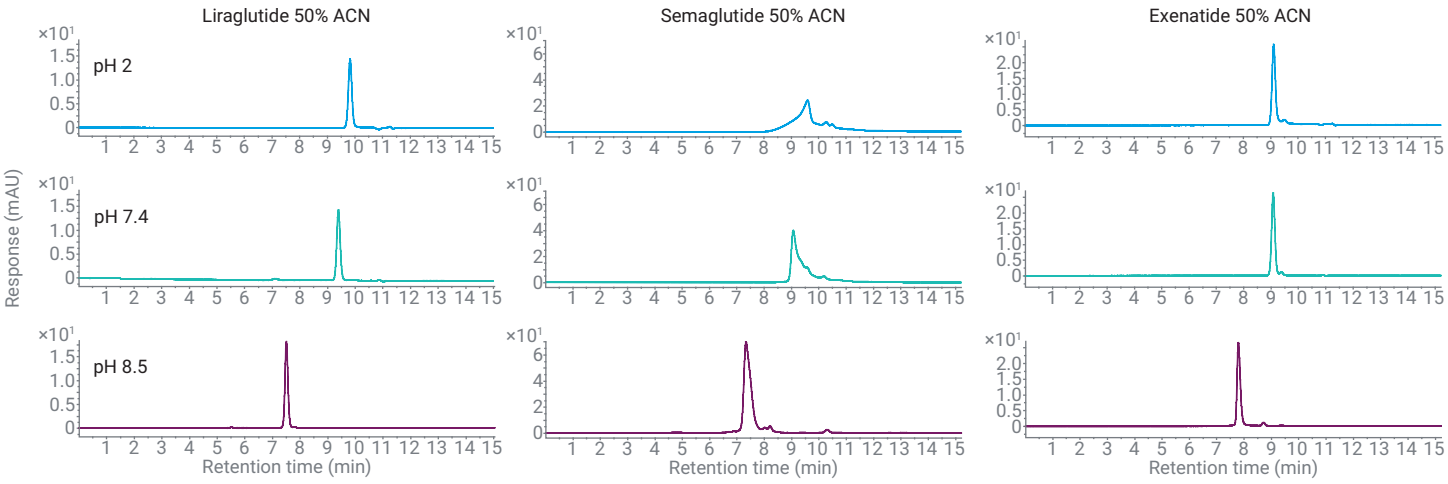

Figure 2. Impact of pH on the peak shape and elution volume of GLP-1s containing 50% ACN.

These data indicated a significant sensitivity in the peak shape and elution volume of GLP-1s, which seems to be largely related to the chemistry of the K26 side chain. This suggests that many other GLP-1s containing various modifications to this site will need method screening to ensure optimal peak shape.

Altura Ultra Inert SEC 130 Å columns enable superior separation and recovery of GLP-1 agonists versus its stainless-steel counterpart and an inert competitor column

The previous data suggest a significant impact of the K26 side chain modification on the chromatographic properties of GLP-1 analogues. However, to monitor the ability of the Altura Ultra Inert hardware to reduce non-specific interactions due to metal binding, the same batch of SEC material was packed into both stainless-steel and Altura hardware.

Figure 3 highlights how the Altura Ultra Inert hardware results in a significant gain in peak symmetry under the majority of conditions tested. Interestingly, pH 7.4 tends to result in less metal binding versus the more extreme conditions of pH 2 and pH 8.5. Subsequently, the Ultra Inert hardware allows a more versatile range of pH conditions to be employed during method development, which looks to be highly impactful with the next generation of GLP-1 analogues, which all feature modifications to K26.

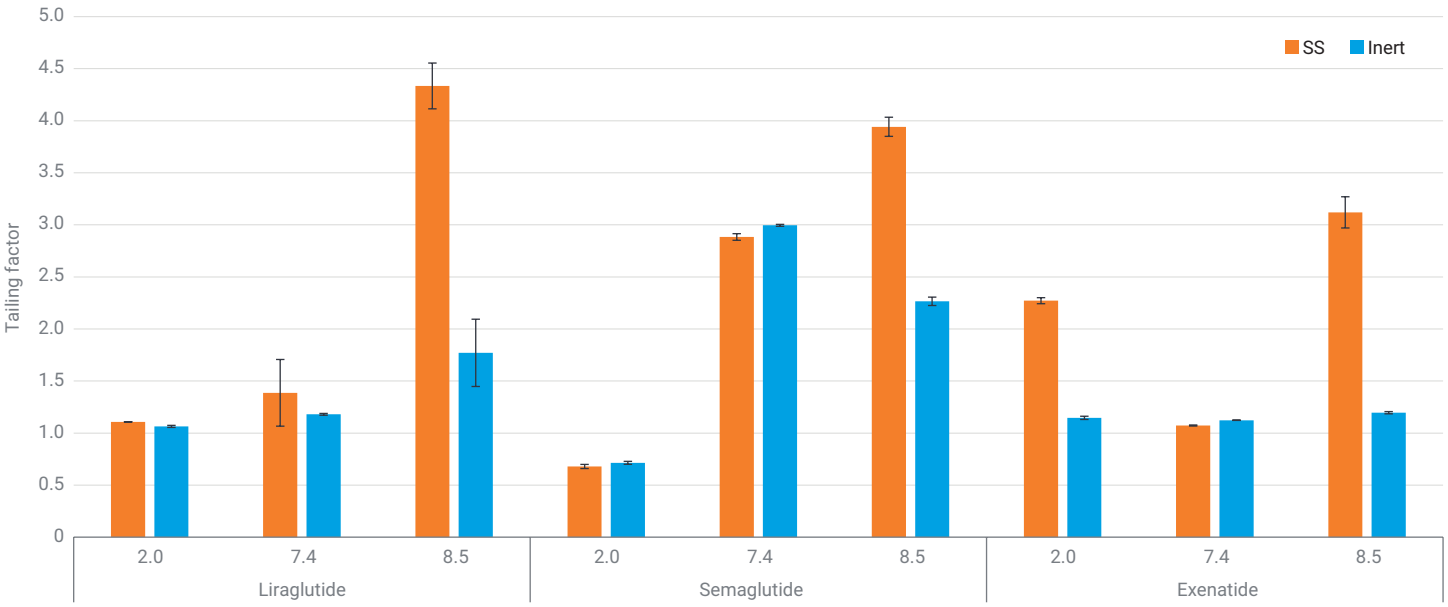

Figure 3. Impact of Agilent Ultra Inert hardware on the peak symmetry of GLP-1 analogues under different pH conditions at 50% ACN.

Figure 4 highlights representative gains in peak symmetry for the three GLP-1 analogues used in this study. In particular, higher pH conditions resulted in significant non-specific adsorption for semaglutide and liraglutide, while both low and high pH in exenatide resulted in adsorption in stainless-steel. Interestingly, in the case of semaglutide (Figure 4B), the column efficiency remained largely unchanged as seen by the comparable half-heigh peak widths while the tailing led to reduced efficiency for fragment impurities and the loss of aggregate peaks.

The impact of pH on the aggregation behavior of these GLP-1 analogues is critical in achieving optimal chromatography. Semaglutide in particular seems to exhibit a number of coeluting components when run at high pH using a narrower pore column. Interestingly, when run on the Altura SEC 300 Å column, the peak shape is largely improved (Figure 5) and the separation of the fragments is much better. This suggests that column pore size should be screened in parallel to pH to ensure that no confounding exclusion effects are observed.

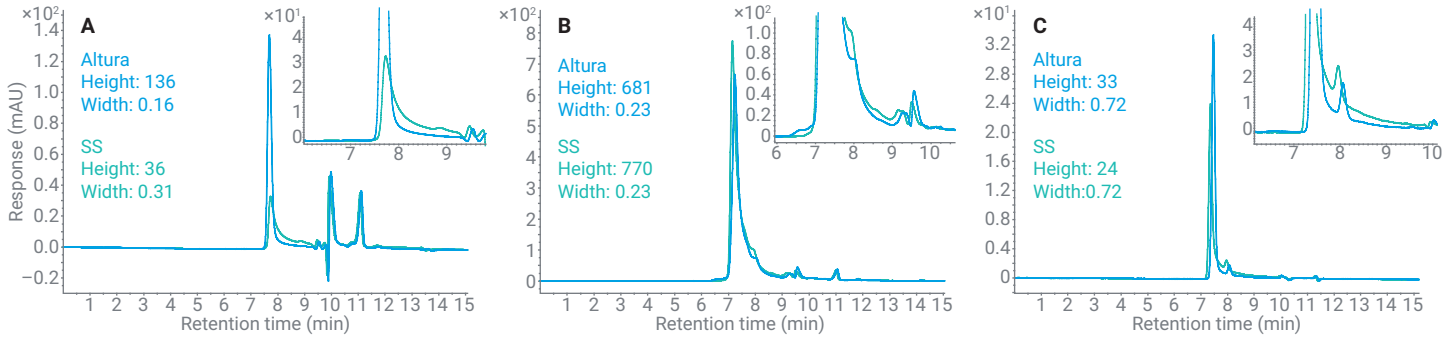

Figure 4. Representative chromatograms of the gain in performance with the Agilent Altura SEC 130 Å (blue chromatograms) versus stainless-steel (green chromatograms) for liraglutide (A), semaglutide (B), and exenatide (C) under pH 8.5 (A, B) and pH 2 (C) with 50% ACN. Peak width given as FWHM.

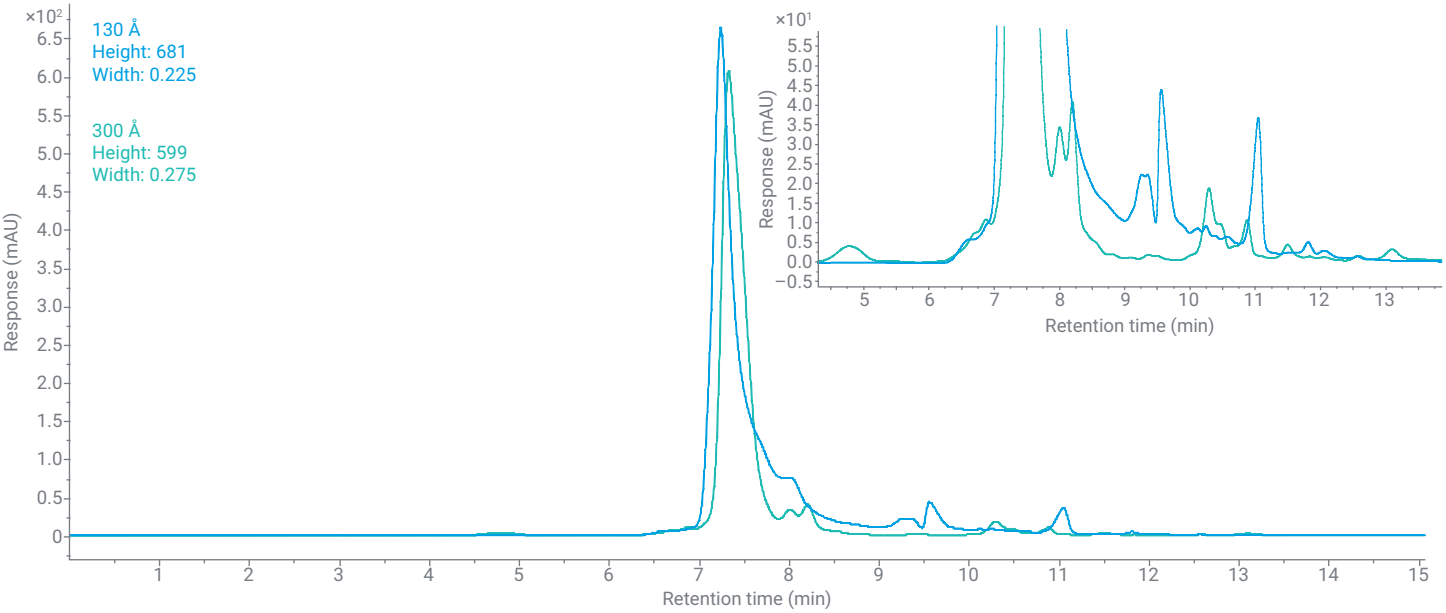

Figure 5. Representative chromatogram of the difference in peak shape and resolution for semaglutide when run on a 130 Å versus a 300 Å Agilent Altura SEC at pH 8.5 with 50% ACN. Peak width given as FWHM.

When comparing the Ultra Inert column with an inert competitor (Figure 6), the Altura SEC 130 Å showed large gains in performance again at the extreme ranges of pH, further demonstrating the improved range of conditions that can be employed with Altura Ultra Inert technology.

When looking at representative chromatograms for all three GLP-1 analogues (Figure 7), the peak tailing of the competitor column demonstrated worse reproducibility, which in the case of exenatide (Figure 7C) resulted in improved resolution for the fragment peak in the tail region of the main peak when run on the Altura SEC (Rs 2.42 versus 1.88).

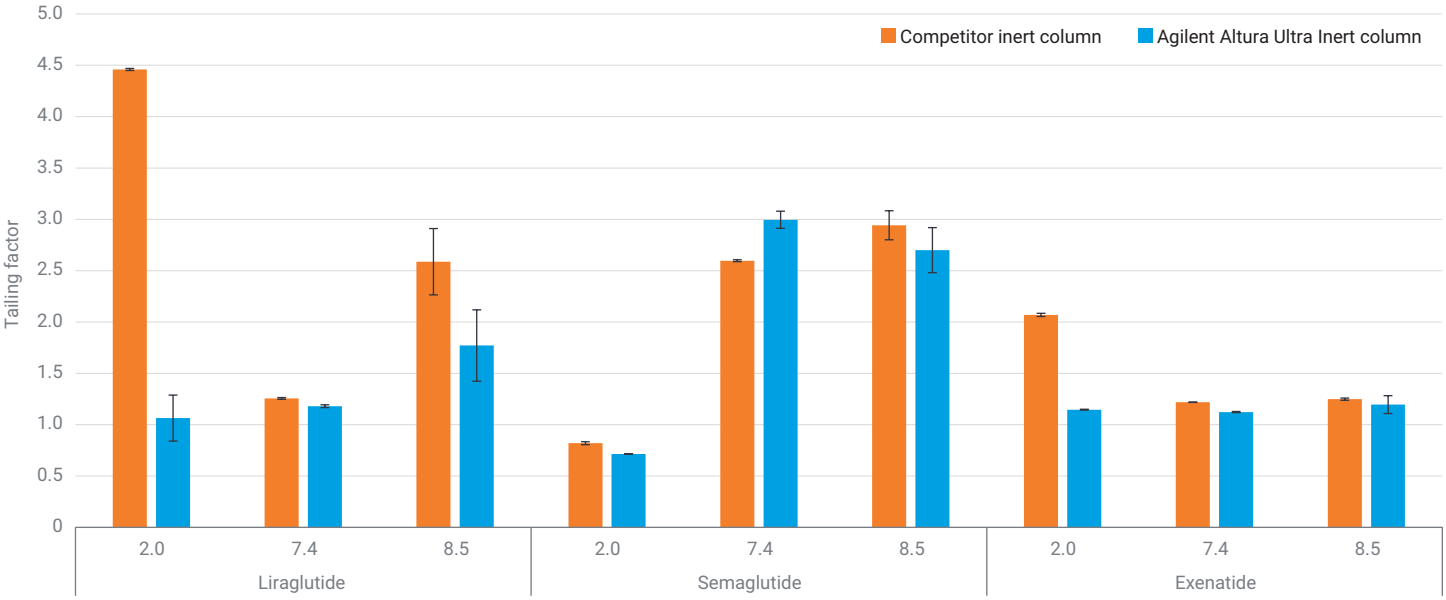

Figure 6. Comparison of peak symmetry of GLP-1 analogues under different pH conditions at 50% ACN using an inert competitor column and an Agilent Altura Ultra Inert column.

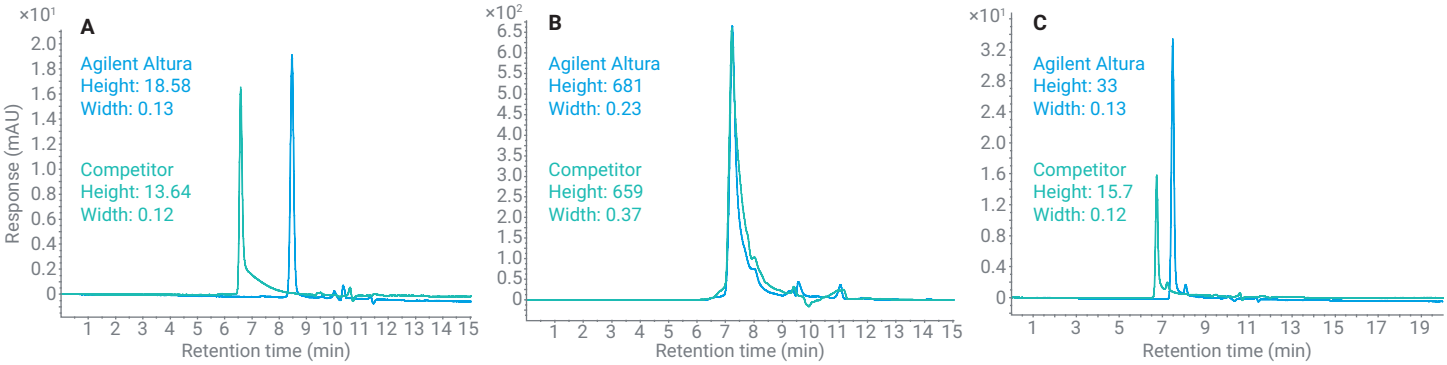

Figure 7. Representative chromatograms of the gain in performance with the Agilent Altura SEC 130 Å (blue chromatograms) versus an inert competitor column (green chromatograms) for liraglutide (A), semaglutide (B), and exenatide (C) under pH 2 (A, C) and pH 8.5 (B) with 50% ACN. Peak width given as FWHM.

Conclusion

GLP-1 analogues represent a significant analytical challenge due to the propensity to aggregate under conventional analysis conditions. Additionally, conjugation of various spacers and fatty acid chains to K26 to extend peptide half-life imparts significant hydrophobicity and peak tailing. The Agilent Altura SEC 130 Å and SEC 300 Å have demonstrated superior peak symmetry at a broad range of pH values, allowing for more method design space as well as the ability to more effectively screen pH values for optimal separation performance than with stainless-steel or versus competitor inert columns.

References

1. Giannetti, M.; *et al.* A Spectroscopic and Molecular Dynamics Study on the Aggregation Properties of a Lipopeptide Analogue of Liraglutide, a Therapeutic Peptide Against Diabetes Type 2, *Molecules* **2023**, *28(22)*, 7536. doi:10.3390/molecules28227536.
2. Castelletto, V.; *et al.* Fibrillar and Micellar Aggregation of Semaglutide and Formation of a Chiral-Imprinted Glass, *Biomacromolecules* **2026**, *27(4)*, 2818–2827. doi:10.1021/acs.biomac.5c02669.
3. Benet, A.; *et al.* The Effects of pH and Excipients on Exenatide Stability in Solution, *Pharmaceutics* **2021**, *13(8)*, 1263. doi:10.3390/pharmaceutics13081263.

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