Application Note

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HILIC Analysis of GLP-1 Receptor Agonists

Using an Agilent 1290 Infinity III Bio LC with DAD and ELSD

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Abstract

GLP-1 receptor agonists are widely applied in managing diabetes and obesity. This application note describes HILIC analysis of these therapeutics on a low-adsorption and biocompatible LC flow path. Using DAD and ELSD, the active ingredient, product-related impurities, and formulation constituents can be simultaneously measured. The benefit of eliminating or deactivating all stainless-steel components in the LC flow path is demonstrated.

Introduction

Glucagon-like peptide-1 (GLP-1) receptor agonists represent a class of peptide-based therapeutics used to treat type 2 diabetes mellitus (T2DM) and, more recently, obesity. They mimic the action of the naturally occurring GLP-1 hormone that stimulates insulin release, reduces glucagon secretion, slows gastric emptying, and lowers appetite.[1] As native GLP-1 has limited clinical utility due to rapid clearance, marketed products have been engineered in several manners to extend half-life (e.g. amino acid substitution, fatty acid conjugation) (Figure 1). By way of example, Semaglutide, the active ingredient in the top-selling drug Ozempic, deviates from natural GLP-1 by two amino acid substitutions, in addition to the presence of stearic di-acid conjugated to lysine via a short spacer. Optimal glucose control in T2DM often requires multifaceted therapeutic strategies. Consequently, pharmaceutical companies have developed combination products such as Xultophy, containing the GLP-1 receptor agonist Liraglutide and Insulin degludec, or co-agonistic peptides such as Tirzepatide (the active ingredient of Mounjaro) simultaneously exerting the functions of GLP-1 and gastric-inhibitory polypeptide (GIP). Given the global rise in T2DM and obesity, there is a high demand for synthetic peptides targeting GLP-1 and other incretin receptors. There is also the need for proper analytical methodologies to assess the quality of these medicines. Reversed-phase liquid chromatography (RPLC), size exclusion chromatography (SEC), and mass spectrometry (MS) are commonly used to determine different attributes associated with the active pharmaceutical ingredient (identity, purity, impurities, content). In the present study, hydrophilic interaction liquid chromatography (HILIC) is introduced as a complementary methodology to study various features of the drug product (DP). In combination with diode array (DAD) and evaporative light scattering detection (ELSD), the simultaneous measurement of the therapeutic peptide and various formulation constituents in the DP is enabled. These include inorganic ions, sugars and amino acids added as buffering or tonicity agents, stabilizers, and antioxidants. To prevent nonspecific interaction of metal-sensitive constituents with metal surfaces and metal-contaminated flow paths, low adsorption and biocompatible LC hardware is implemented by means of an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology and an Agilent 1290 Infinity III Bio LC System.

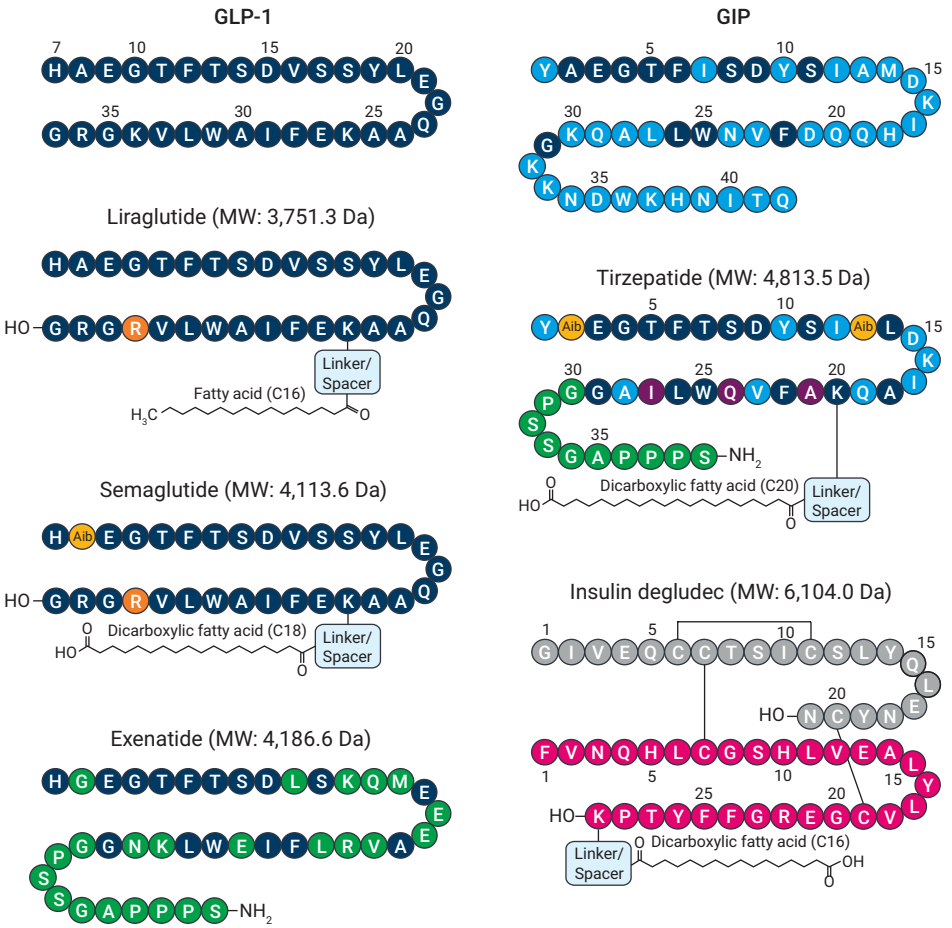

Figure 1. Molecular structures of native GLP-1 and GIP, GLP-1 receptor agonists Liraglutide, Semaglutide, and Exenatide, GLP-1 and GIP receptor co-agonist Tirzepatide, and Insulin degludec. Molecular weight of therapeutic peptides is added.

Experimental

Materials

Water (ULC/MS) and acetonitrile (HPLC-S) were supplied by Biosolve. Ammonium formate (LiChropur), dimethyl sulfoxide (DMSO, Suprasolv), hydrogen peroxide (≥ 30%, for trace analysis), D-mannitol, sucrose, L-methionine, sodium chloride, sodium phosphate monobasic, and zinc acetate dihydrate were sourced from Merck. Glycerol was purchased from Thermo Fisher Scientific. Semaglutide acetate (≥ 95%), Exendin-4 (48 to 86) amide acetate (exenatide acetate, ≥ 95%), Liraglutide acetate (≥ 95%) and Tirzepatide sodium salt (≥ 95%) were obtained from Cayman Chemical. Ozempic and Xultophy were received from Novo Nordisk and Byetta from Astra Zeneca (Table 1).

Sample preparation

Semaglutide and Liraglutide were prepared in DMSO at 1 mg/mL and heated to dissolve. Exenatide and Tirzepatide were dissolved at 1 mg/mL in 50/50 (v/v) acetonitrile/water. Further dilutions were done in water prior to injection. Byetta, Ozempic, and Xultophy were applied to the column. The excipient solutions (glycerol, mannitol, sucrose, NaCl, NaH_2PO_4, and zinc acetate) were prepared in water.

To induce methionine oxidation, Exenatide was incubated in 0.004% hydrogen peroxide for three hours at room temperature. The reaction was quenched by adding 1 mM L-methionine.

Instrumentation and method

The Agilent 1290 Infinity III LC System (stainless-steel (SST)) and the 1290 Infinity III Bio LC System (BIO) were used in this study. Details of both configurations can be found in Table 2 and method parameters are summarized in Table 3. Data were acquired and processed in Agilent OpenLab CDS, version 2.7.

Table 1. GLP-1 receptor agonist DPs used in the current study.

DP	Active Ingredient	Excipients
Byetta	Exenatide (300 µg in 1.2 mL)	Mannitol (43 mg/mL), metacresol (2.2 mg/mL), glacial acetic acid, and sodium acetate trihydrate (pH 4.5)
Ozempic	Semaglutide (2 mg in 1.5 mL)	Propylene glycol (14 mg/mL), phenol (5.5 mg/mL), disodium phosphate dihydrate (1.42 mg/mL), hydrochloric acid, and sodium hydroxide (pH 7.4)
Xultophy	Liraglutide (10.8 mg in 3 mL) and Insulin degludec (300 units in 3 mL)	Glycerol (19.7 mg/mL), phenol (5.7 mg/mL), zinc (55 µg/mL), hydrochloric acid, and sodium hydroxide (pH 8.15)

Table 2. Details of the instrument configurations used.

	Agilent 1290 Infinity III LC System	Agilent 1290 Infinity III Bio LC System
Pump	Agilent 1290 Infinity III High-Speed Pump (G7120A)	Agilent 1290 Infinity III Bio High-Speed Pump (G7132A)
Autosampler	Agilent 1290 Infinity III Multisampler (G7167B) with integrated Sample Thermostat	Agilent 1290 Infinity III Bio Multisampler (G7137A) with integrated Sample Thermostat
Column Compartment	Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect Heat Exchanger, standard flow (G7116-60015)	Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect Bio Heat Exchanger, standard flow (G7116-60071)
Detector (DAD)	Agilent 1290 Infinity III Diode Array Detector (G7117B)	
Flow Cell	Agilent Max-Light Cartridge Cell, standard, 10 mm (G4212-60008)	Agilent Max-Light Cartridge Cell, LSS, 10 mm (G7117-60020), aperture not attached
Detector (ELSD)	Agilent 1290 Infinity III Evaporative Light Scattering Detector (G4261B)	

Table 3. LC method parameters.

Parameter	Value
Columns	Agilent InfinityLab Poroshell HILIC-Z, 2.1 × 150 mm, 2.7 µm (p/n 683775-924) Agilent Altura Poroshell HILIC-Z with Ultra Inert technology, 2.1 × 150 mm, 2.7 µm (p/n 227215-924)
Flow Rate	0.4 mL/min
Mobile Phase	A) 100 mM ammonium formate pH 3 B) ACN
Gradient	Time (min) %B 0 to 1 90 1 to 19 90 to 45 19 to 20 45 to 90 20 to 30 90
Column Temperature	40 °C
Autosampler Temperature	10 °C
Injection	1 µL
Needle Wash	10 s 50/50 (v/v) acetonitrile:water
Detection DAD	280/4 nm, reference 360/100 nm, 2.5 Hz
Detection ELSD	Evaporator temperature: 50 °C Nebulizer temperature: 50 °C Gas flow rate: 1.0 SLM Data rate: 80 Hz Smoothing: 30 PMT Gain: 1.0

Results and discussion

HILIC retains and resolves polar and ionic solutes and offers a different selectivity in comparison to RPLC. Separation is based on the hydrophilic partitioning of solutes between the organic mobile phase and an aqueous layer immobilized on the stationary phase superimposed with other interactions such as electrostatic or hydrogen bonding (depending on the stationary phase chemistry and mobile phase conditions).[2] Since its inception 35 years ago, HILIC has successfully been applied for the analysis of a wide range of substances including amino acids, peptides, (glyco)proteins, sugars, (oligo)nucleotides, organic acids, and inorganic ions, among others.

The analysis of HILIC-amenable solutes commonly found as excipients in GLP-1 receptor agonist DPs is presented in Figure 2. Chromatographic conditions are based on earlier work describing the use of a HILIC-Z column and ELSD for the measurement of inorganic anions and cations.[3] The same method allows for the separation of the synthetic GLP-1 receptor agonist peptides Exenatide, Semaglutide, Liraglutide, and Tirzepatide, as illustrated in Figure 3. Peptide elution is based on the amino acid composition and conjugated fatty acids.

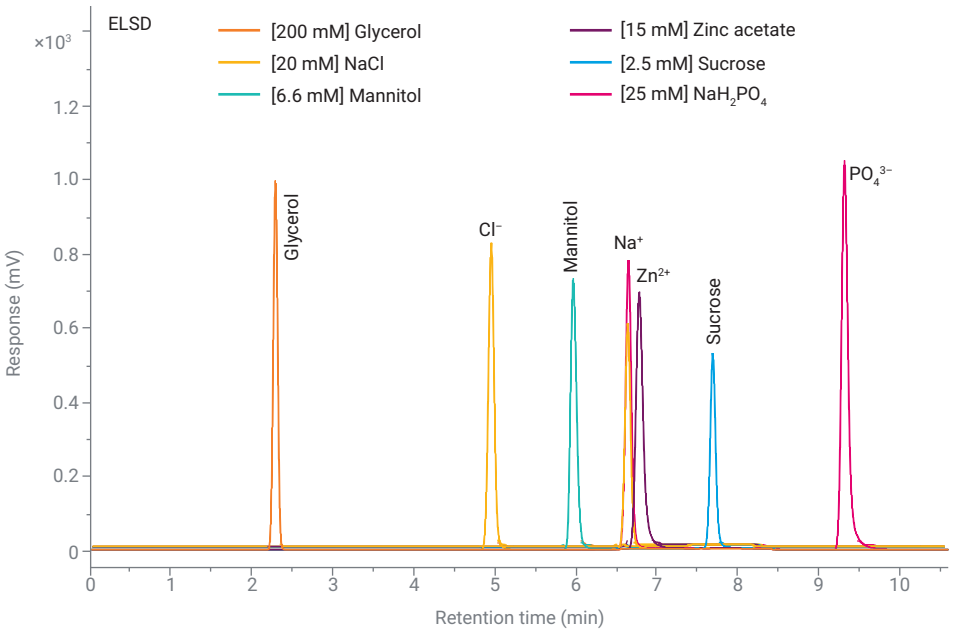

Figure 2. Overlaid HILIC-ELSD traces of various formulation constituents. Concentration has been varied to reach similar detector intensities. Acetate is not measurable by ELSD due to its volatility. Experiments were performed using an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology installed on an Agilent 1290 Infinity III Bio LC System.

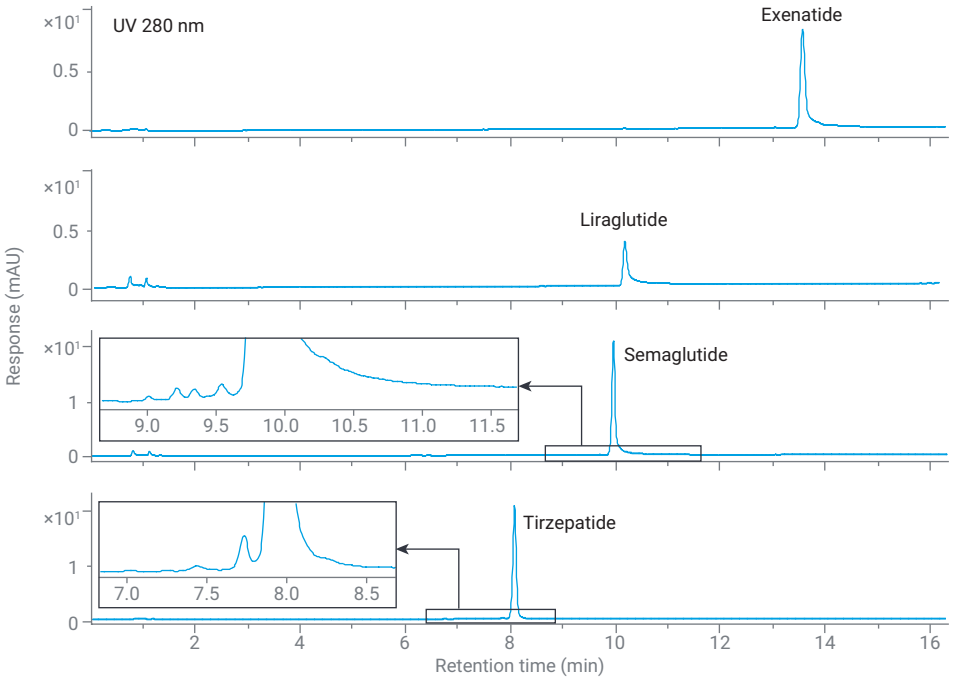

Figure 3. HILIC-UV 280 nm chromatograms of GLP-1 receptor agonists Exenatide, Liraglutide, Semaglutide, and Tirzepatide at 0.5 mg/mL. Experiments were performed using an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology installed on an Agilent 1290 Infinity III Bio LC System.

The HILIC-Z method with sequential DAD and ELSD detection opens possibilities to simultaneously assess the active ingredients and formulation constituents in GLP-1 receptor agonist DPs, as shown in Figure 4. The ELSD data provide a read-out of cosolvents (glycerol), tonicity agents (mannitol), and buffer/pH adjustment substances (Na-phosphate, HCl, NaOH) while the UV data allow detection of the synthetic peptide(s) and its impurities, as well as the unretained preservative (phenol, metacresol). The unretained preservative is not detectable by ELSD given its volatility. Note the saturating signal for mannitol, which is present at 43 mg/mL in Byetta.

Data shown thus far have been generated on a low-adsorption flow path using an Altura Poroshell HILIC-Z column with Ultra Inert technology and biocompatible 1290 Infinity III Bio LC. The rationale is that phosphate- and carboxylate-containing analytes tend to adsorb to metal surfaces or interact with leached metals, resulting in peak broadening and diminished recovery.[4] This phenomenon can, to a certain extent, be mitigated by passivating the flow path or adding metal chelators to the mobile phase. A superior alternative is the elimination or deactivation of stainless-steel surfaces.

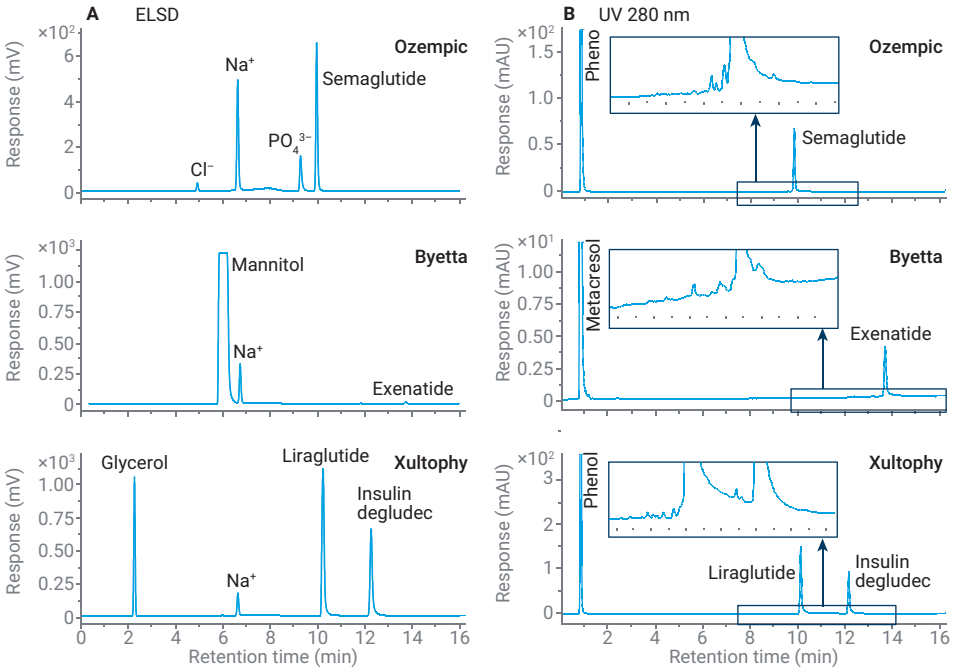

Figure 4. (A) HILIC-ELSD and (B) UV 280 nm chromatograms of Ozempic, Byetta, and Xultophy. Experiments performed using an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology installed on an Agilent 1290 Infinity III Bio LC System.

The impact of the latter strategy on the current study's samples is presented in Figures 5 and 6. Peak shape and recovery of inorganic phosphate, as observed in an NaH_2PO_4 standard and Ozempic, are drastically affected when evolving from a stainless-steel to a low-adsorption flow path (1290 Infinity III LC and stainless-steel HILIC-Z column versus the 1290 Infinity III Bio LC and Altura HILIC-Z column with Ultra Inert technology). Furthermore, peak area precision is substantially improved when opting for the 1290 Infinity III Bio LC and Ultra Inert HILIC-Z column (data not shown). Tirzepatide and Semaglutide elute equally well on both configurations, while Liraglutide and Exenatide have a more distorted peak shape on the stainless-steel setup. Despite the latter, near complete recovery is obtained for the active ingredient. The detection of product-related impurities is considerably better using the low-adsorption flow path, as illustrated for oxidized Exenatide eluting as a postpeak. This species remains undetectable in the tail of the main peak in DP and displays a deformed retention in H_2O_2-stressed Exenatide on a stainless-steel flow path. A partially resolved peak doublet is observed on HILIC as a result of the formation of methionine sulfoxide S- and R-diastereomers.

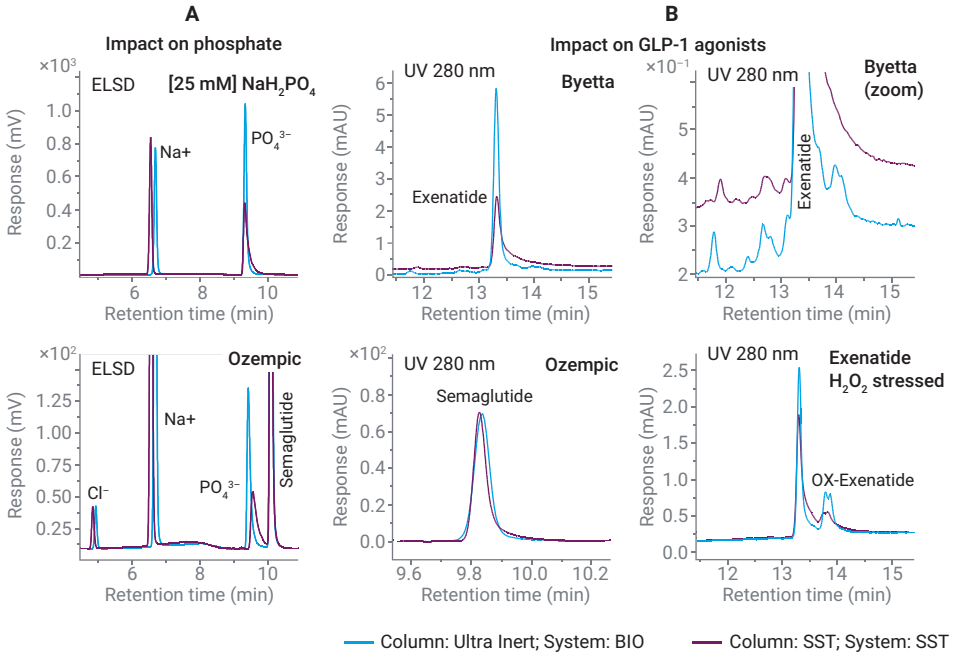

Figure 5. (A) HILIC-ELSD chromatograms of a 25 mM NaH_2PO_4 standard and Ozempic obtained on a low-adsorption and stainless-steel flow path. (B) HILIC-UV 280 nm chromatograms focusing on Semaglutide (Ozempic), Exenatide (Byetta), and oxidatively stressed Exenatide (0.25 mg/mL) elution on a low-adsorption and stainless-steel flow path. Chromatograms have been aligned (retention time) on the main peak to facilitate interpretation. BIO: Agilent 1290 Infinity III Bio LC, SST: Agilent 1290 Infinity III LC (Stainless-steel (SST)). Ultra Inert column: Agilent Altura Poroshell HILIC-Z with Ultra Inert technology, SST column: Agilent InfinityLab Poroshell HILIC-Z with stainless-steel column hardware.

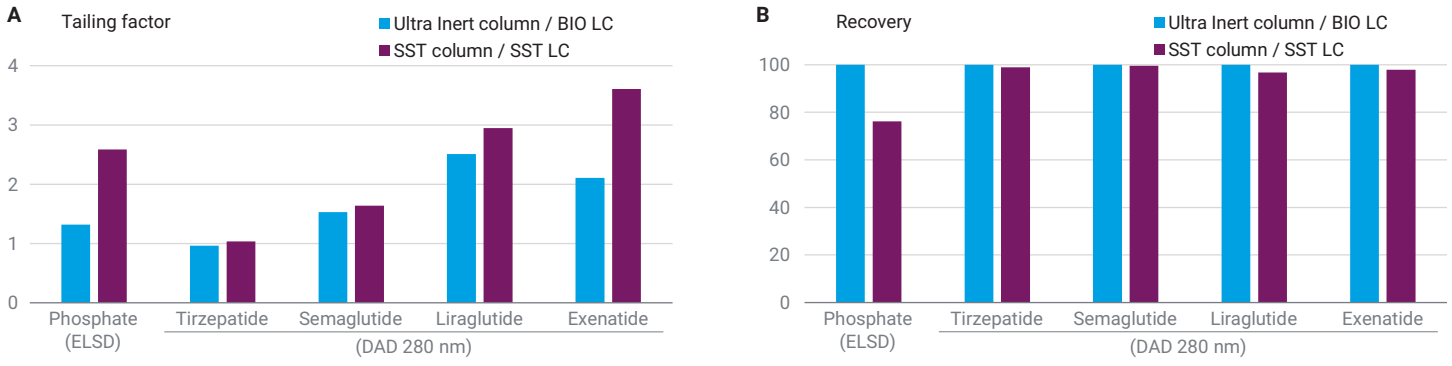

Figure 6. (A) Peak tailing factor and (B) recovery (peak area relative to low adsorption flow path) for 25 mM NaH_2PO_4 (see Figure 5) and Tirzepatide, Semaglutide, Liraglutide, and Exenatide at 0.5 mg/mL. BIO: Agilent 1290 Infinity III Bio LC, SST: Agilent 1290 Infinity III LC (Stainless-steel (SST)). Ultra Inert column: Agilent Altura Poroshell HILIC-Z with Ultra Inert technology, SST column: Agilent InfinityLab Poroshell HILIC-Z with stainless-steel column hardware.

Conclusion

This study demonstrates the simultaneous measurement of GLP-1 receptor agonist active ingredient, product-related impurities, and excipients by HILIC in combination with DAD and ELSD. To prevent nonspecific interactions of solutes with metal components in the flow path, an Agilent Altura HILIC-Z column with Ultra Inert technology and Agilent 1290 Infinity III Bio LC System were used. A generic method was presented here which can demonstrably be fine-tuned to further optimize resolution of specific species.

References

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