

Application Note

Unique Selectivity of BioResolve Peptide C₁₈+ and Phenyl-Hexyl+ Columns for Separation of Deamidation Impurities of a GLP-1 Receptor Agonist

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Abstract

The BioResolve™ Peptide C₁₈+ and BioResolve Peptide Phenyl-Hexyl+ reversed-phase (RP) Columns were compared to three columns from alternative vendors for the separation of tirzepatide, a glucagon-like peptide-1 (GLP-1) therapeutic, from its impurities. Unique selectivity of two deamidation impurities was observed on the BioResolve Columns, resulting in superior separation of the two species relative to the alternative columns.

Benefits

- The BioResolve Peptide C₁₈+ and Phenyl-Hexyl+ Columns exhibited unique selectivity compared to competing columns in separating two deamidated impurities of tirzepatide
- The superior selectivity of the BioResolve cColumns enable effective separation of the two deamidated impurities even under high-throughput conditions with short gradients and high flow rates, where competing

columns are more prone to impurity co-elution

Introduction

Deamidation of proteins and peptides can occur during production and storage, converting an amide functional group to a carboxylic acid. At neutral pH, this change introduces a negative charge that alters the structure of the biopharmaceuticals, impacting their chemical and biological properties. Because deamidation can significantly compromise drug purity, stability, and shelf life, separating and characterizing these specific impurities is essential to ensure therapeutic safety and efficacy.¹

GLP-1 therapeutics are among the fastest growing biopharmaceuticals on the market. Waters BioResolve Peptide C₁₈+ and Phenyl-Hexyl+ RP Columns, designed specifically for analytes like insulins and GLP-1 receptor agonists, feature superficially porous particles with an average pore size of 230 Å, allowing improved accessibility and efficiency for larger peptides. A controlled positive surface charge enhances peak shape and loading capacity for basic analytes, particularly with formic acid mobile phases.² Furthermore, integrated MaxPeak™ Premier Columns hardware minimizes non-specific adsorption (NSA), ensuring superior recovery, consistency, and robustness.³

It has been shown that Waters BioResolve Peptide C₁₈+ and Phenyl-Hexyl+ RP Columns provide complementary selectivity in resolving structurally similar GLP-1 peptide variants.⁴ In this application note, the unique selectivity of these reversed phase columns resulting in superior separation of two deamidation impurities of tirzepatide compared to columns from alternative vendors are described.

Experimental

Sample Preparation

Tirzepatide was reconstituted in modified DPBS to make a 2 mg/mL solution. Tirzepatide impurities were spiked into the tirzepatide sample to achieve a final concentration of 0.02 mg/mL.

LC Conditions

LC system:	ACQUITY™ Premier UPLC™ System with Quaternary Solvent Manager (QSM), Sample Manager FTN, Column Heater and TUV Detector
Columns:	Prototype BioResolve Peptide C₁₈+ Column, 2.7 μm, 2.1 x 150 mm BioResolve Peptide Phenyl-Hexyl+ Column, 2.7 μm, 2.1 x 150 mm (p/n: 186011730)
Column temperature:	50 °C
Sample temperature:	6 °C
Injection volume:	0.5 μL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Detection λ:	214 nm
Sample vials:	Polypropylene 12 x 32 mm Screw Neck Vial with Cap and Preslit PTFE/Silicone Septum, 300 μL Volume

Gradient Table

30-Minute Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.15	99	1	
0.66	0.15	60 for C ₁₈ +; 65 for PH+	40 for C ₁₈ +; 35 for PH+	6
30.66	0.15	50 for C ₁₈ +; 55 for PH+	50 for C ₁₈ +; 45 for PH+	6
31.33	0.15	5	95	6
32.00	0.15	99	1	6
45.00	0.15	99	1	11

10-Minute Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.45	99	1	
0.22	0.45	60 for C ₁₈ +; 65 for PH+	40 for C ₁₈ +; 35 for PH+	6
10.22	0.45	50 for C ₁₈ +; 55 for PH+	50 for C ₁₈ +; 45 for PH+	6
10.44	0.45	5	95	6
10.67	0.45	99	1	6
17.00	0.45	99	1	11

Results and Discussion

A sample containing unmodified tirzepatide, Glutamine 19 deamidated tirzepatide (Impurity 1), and C-terminal deamidated tirzepatide (Impurity 2) was separated on Waters BioResolve Peptide C₁₈+ and Phenyl-Hexyl+ Columns and three columns from other manufacturers. All evaluated columns feature 2.7 µm superficially porous particles with positive surface charges. Figure 1 displays the chromatographic separations for C₁₈ Columns, while Figure 2 illustrates the performance of Phenyl-Hexyl Columns.

The unique selectivity of the BioResolve Peptide C₁₈+ Column is highlighted in Figure 1. On the BioResolve Column, Impurity 1 eluted before Impurity 2 whereas other columns (Column A and Column B) display the opposite elution order under the same gradient conditions. Additionally, the BioResolve Column delivered superior, baseline-resolved separation of the two deamidated impurities, which the other columns fail to achieve. This unique selectivity offers a distinct analytical advantage under high-throughput conditions featuring short gradient times and high flow rates. As shown on the right side of Figure 1, the BioResolve Column displays high resolution of tirzepatide's two deamidated impurities. Column A and Column B, on the other hand, suffer from co-elution of these two species under the same conditions.

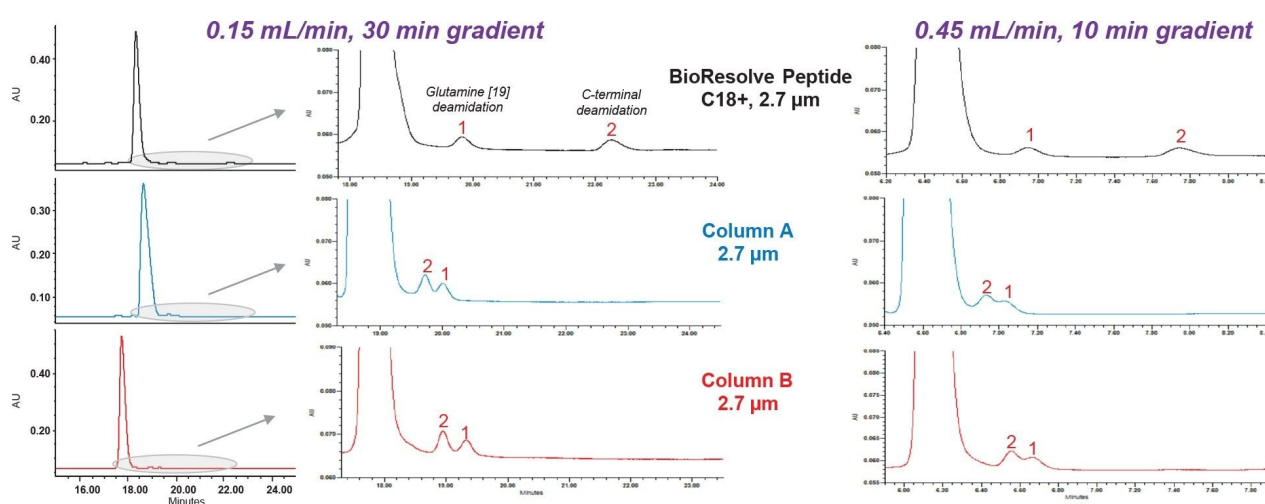


Figure 1. Separation of tirzepatide and two spiked-in impurities (1: Glutamine 19 deamidation, 2: C-terminal deamidation) on a BioResolve Peptide C₁₈+ Column and two competitor columns with C₁₈ ligands. Left: full chromatogram; Middle: zoomed-in view of the impurities; Right: high-throughput separation. All three columns have 2.7 μm superficially porous particles with positive surface charges, and all are 2.1 x 150 mm in column dimensions. The BioResolve Column, Column A and Column B have an average pore size of 230 Å, 160 Å and 100 Å, respectively. The elution order of the two deamidated impurities on the BioResolve Peptide C₁₈+ Column is opposite to that on the other two columns. Mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile. Gradient for Left and Middle: 40–50%B in 30 minutes, 0.15 mL/min. Gradient for Right: 40–50%B in 10 minutes, 0.45 mL/min. Column temperature: 50 °C. UV detection: 214 nm.

As demonstrated in Figure 2, the BioResolve Peptide Phenyl-Hexyl+ Column also displays an inverted elution

order for the two deamidated impurities compared to the phenyl-hexyl column from an alternative vendor (Column C). Additionally, the BioResolve Peptide Phenyl-Hexyl+ Column achieves baseline-resolved separation of these species that is maintained under high-throughput conditions, unlike Column C. While the C₁₈+ and Phenyl-Hexyl+ Columns display comparable selectivity for the two deamidated impurities, they offer complementary selectivity for other species. Specifically, an unknown impurity (marked with an asterisk in Figure 2) is resolved from the main peak on the Phenyl-Hexyl+ Column, whereas this unknown impurity co-elutes with the main peak on the C₁₈+ Column (Figure 1).

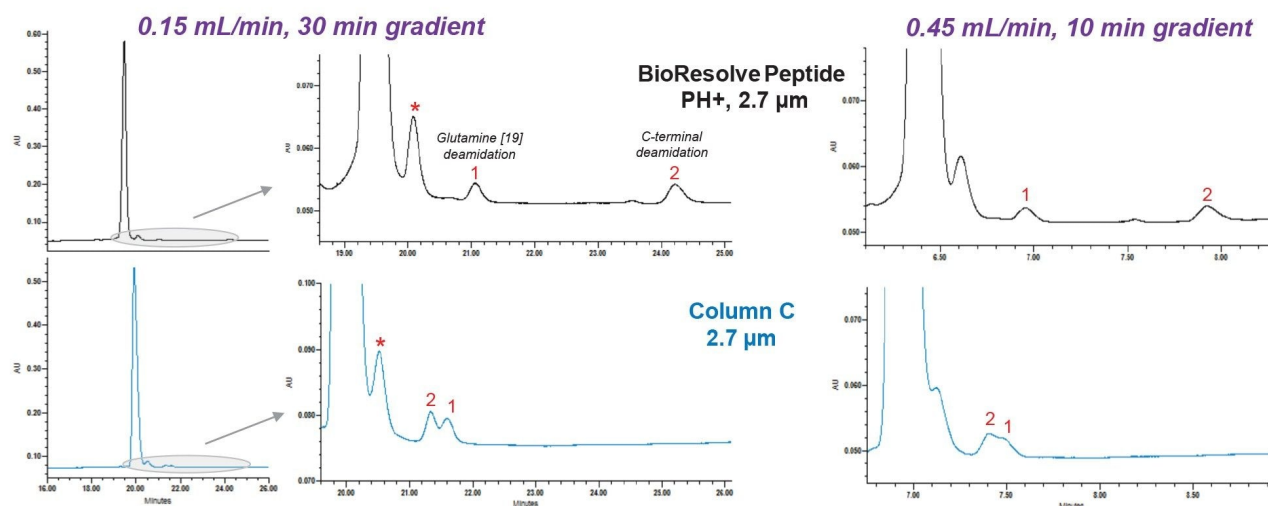


Figure 2. Separation of tirzepatide and two spiked-in impurities (1: Glutamine 19 deamidation, 2: C-terminal deamidation, *: unknown impurity in the unmodified tirzepatide sample) on a BioResolve Peptide Phenyl-Hexyl+ Column and a competitor column with phenyl-hexyl ligands. Left: full chromatogram; Middle: zoomed-in view of the impurities; Right: high-throughput separation. Both columns have 2.7 μ m superficially porous particles with positive surface charges and are 2.1 x 150 mm in column dimensions. The BioResolve Column and Column C have an average pore size of 230 Å and 90 Å, respectively. The elution order of the two deamidated impurities on the BioResolve Peptide Phenyl-Hexyl+ Column is opposite to that on Column C. Mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile. Gradient for Left and Middle: 35–45%B in 30 minutes, 0.15 mL/min. Gradient for Right: 35–45%B in 10 minutes, 0.45 mL/min. Column temperature: 50 °C. UV detection: 214 nm.

The fundamental resolution equation shown below outlines how three core parameters govern chromatographic

separation: efficiency (N), selectivity (α), and retentivity (k). The chromatographic examples detailed in this application note clearly demonstrate that selectivity is the primary driving force for optimizing and improving resolution.

$$Rs = \frac{1}{4} \sqrt{N} \left(\frac{\alpha-1}{\alpha} \right) \left(\frac{k}{1+k} \right)$$

In a previous study, the elution order of the two deamidated tirzepatide impurities reversed when switching between formic acid and trifluoroacetic acid (TFA) mobile phases on the BioResolve Peptide Phenyl-Hexyl+ Column.⁵ This change in elution order was driven by substantially different selectivity of the C-terminally deamidated species under the two mobile phase conditions. The differing selectivity observed in this application note between the BioResolve Columns and columns from other vendors likely arises from differences in stationary phase properties such as ligand density, surface charge, and average pore size. Further investigations are needed to clarify the underlying mechanism.

Conclusion

BioResolve Peptide C₁₈+ and Phenyl-Hexyl+ Columns offer unique selectivity, featuring a reversed elution order in separating two of the deamidated impurities of tirzepatide, compared to columns from alternative vendors. In addition, the BioResolve Columns provide superior, baseline-resolved separation of the two impurities, which leads to maintained high-resolution performance even under high-throughput conditions.

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