APPLICATIONS

Development of a Multi-Step Purification Process for the Purification of Exenatide

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The work presented here demonstrates the development of a multi-step purification process on a single stationary phase for the crude synthetic peptide mixture of Exenatide. The focus of this technical note is on the initial development work, including the screening of multiple conditions to evaluate which steps will produce material of suitable purity. The investigated parameters include eluent pH, buffer components, and organic solvent composition.

Introduction

Purification of crude synthetic peptide mixtures often employs a multi-step chromatographic purification process. The first step removes most of the undesired components, followed by another step to "polish" the material to the desired purity level. If applicable, a single step process can produce significant time and cost savings provided the single step can achieve the necessary purity while maintaining a desirable yield and throughput. A multi-step process using the same stationary phase, can provide considerable savings of time and costs compared to a process utilizing multiple stationary phases.

Selectivity is the ability of a chromatographic method to separate different components. When multiple chromatographic steps are employed, the conventional idea is to use two or more complementary modes of chromatography such as ion-exchange, gel permeation, affinity, and reversed phase to take advantage of their different selectivities. Changing the stationary phase is an effective way to obtain different selectivity, but this can be expensive and time consuming in a large scale purification process. Fortunately, the very nature of peptides allow for changes in selectivity based on changes in pH or the choice of organic solvent. These changes are relatively inexpensive and easily implemented. Amino acids are categorized by their side chains as non-polar, aromatic, polar non-charged, positively charged, or negatively charged. See **Table 1**.

By knowing the amino acid sequence of a peptide, its ionization and polarity properties are predictable. There are a few key properties that are significant for chromatographic applications. Probably the most significant property is ionization. There are amino acids with acidic and basic ionizable side chains. The pH of the eluent will determine if these side chains are charged or neutral. If the stationary phase is silica-based, the pH will also determine if the surface of the stationary phase is neutral or negatively charged. Also, the peptide will interact differently with the stationary phase depending on the different types of organic solvents and additives used in the eluent. This is related to the polarity of the peptide and is mainly due to the non-ionizable side chains.

The crude synthetic peptide used in this purification development study is crude Exenatide. The crude was obtained from a major peptide manufacturer located in the United States. Exenatide is a glucagon-like peptide-1 agonist and was approved in April 2005 for the treatment of diabetes mellitus type 2. Exenatide is a synthetic version of exendin-4, a hormone found in the saliva of the Gila monster. Exenatide is a 39 amino acid peptide, its chemical structure is represented in **Figure 1**.

Table 1.
Natural Amino Acids Classification

Non-polar aliphatic residues
- Glycine
- Alanine
- Valine
- Leucine
- Isoleucine
- Proline

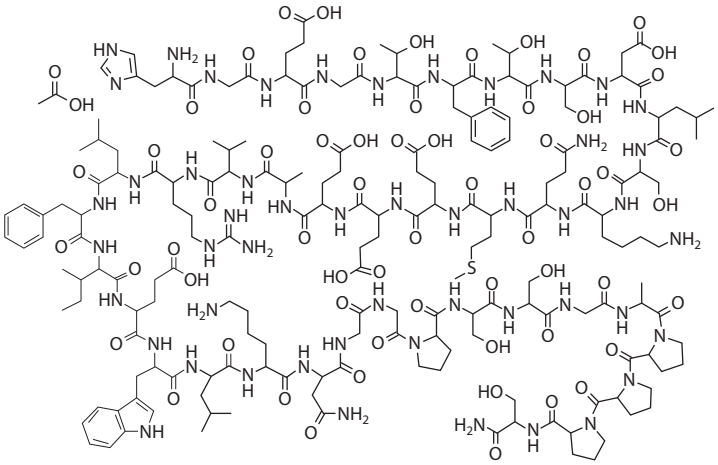
Polar non-charged residues
- Serine
- Threonine
- Cysteine
- Methionine
- Asparagine
- Glutamine

Aromatic residues
- Phenylalanine
- Tyrosine
- Tryptophan

Positively charged residues (basic)
- Lysine
- Arginine
- Histidine

Negatively charged residues (acidic)
- Aspartic acid
- Glutamic acid

Figure 1.
Chemical Structures for Exenatide



His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2

Results and Discussion

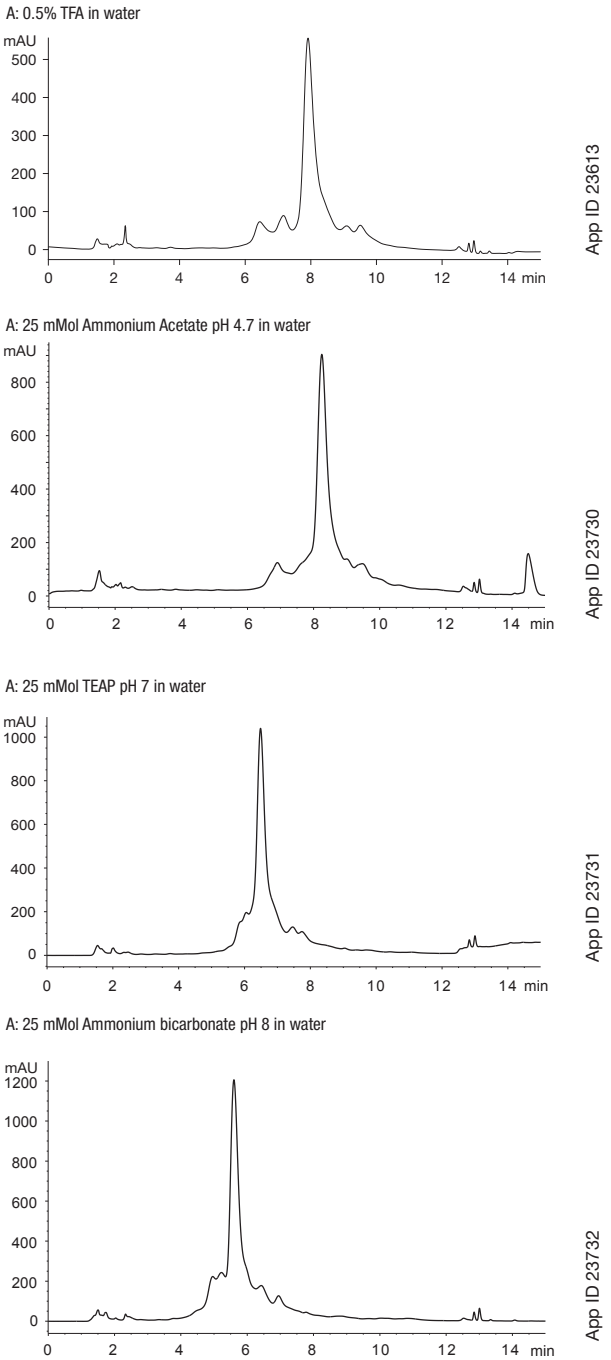
Mobile Phase Screening on Luna® 10 µm-PREP C8(3)

The peptide used in this study contains 16 non-polar and 3 aromatic side chains (**Table 2**.). Exenatide is relatively non-polar and the use of a Luna C8 stationary phase is therefore an appropriate stationary phase. Acetonitrile was initially chosen for the strong solvent eluent component and because the final product is a lyophilized solid. Also from **Table 2**, it can be deduced that pH could be an effective tool for altering the chromatographic selectivity between Exenatide and its related impurities. The compound of interest has an ionization point of 4.38 and there are 6 acidic side chains and 4 basic side chains. The initial screening of possible PREP conditions evaluated 4 different aqueous eluent components covering a pH range of 2 – 8 (**Figure 2**.). The isolated material from the optimized PREP screening experiments (**Figure 3**.) were evaluated with analytical HPLC methodology on Kinetex® EVO (**Figure 4**.). The first assessment was to determine if a single step purification process was feasible. There was not a single step that could meet the purity requirement of 98.5% with a suitable yield (**Figure 4**.). A multi-step purification process was required. The desired final product was an acetate salt, so the use of acetate in the final step had significant processing advantages. The TFA screening material and the acetate screening material did show complementary selectivity with different early and late eluting components. The first step was selected to be a TFA - Acetonitrile gradient and the second step was an acetate - Acetonitrile gradient.

Table 2.
Amino Acids Distribution in Exenatide

	Isoelectric point	Acidic side chains	Basic side chains	Non-polar side chains	Aromatic side chains	Polar uncharged side chains
Exenatide	4.38	6	4	16	3	10

Figure 2.
Preliminary Screening Result at Various pH

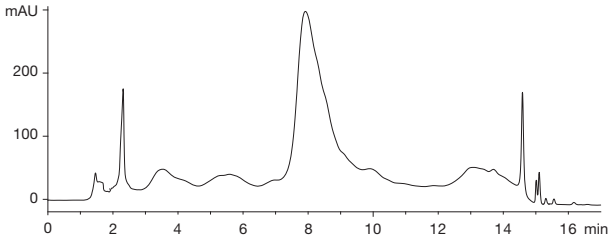


Conditions

Column: Luna® 10 µm-*PREP* C8(3) 100A
Dimensions: 250 x 4.6 mm
Part No.: 00G-4623-E0
Mobile Phase: A: As noted in figure
B: 100 % Acetonitrile
Gradient: 25-45 % B in 10 min, 45-70 % B in 1 min and hold 1 min at 70 % B
Flow Rate: 1.5 mL/min
Injection: 10 µL @ 40 mg/mL
Temperature: Ambient
Detection: UV @ 220 nm

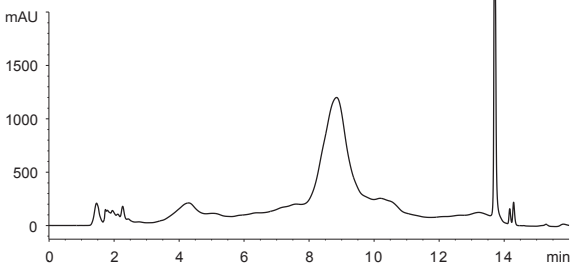
Figure 3.
Optimized Gradient for the Different pH Eluents

A: 0.5% TFA in water
Gradient: 31-36% B in 10 min, 36-75% B in 1 min and hold 1 min at 75% B



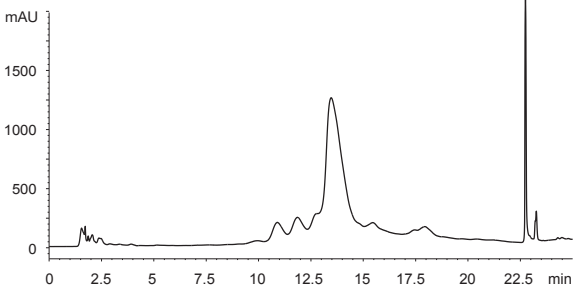
App ID 23615

A: 25 mMol Ammonium Acetate pH 4.7 in water
Gradient: 30-40% B in 15 min, 40-80% B in 1 min and hold 1 min at 80% B



App ID 23733

A: 25 mMol TEAP pH 7 in water
Gradient: 25-35% B in 10 min, 35-80% B in 1 min and hold 1 min at 80% B



App ID 23734

A: 25 mMol Ammonium bicarbonate pH 8 in water
Gradient: 25-31% B in 12 min, 31-80% B in 1 min and hold 1 min at 80% B

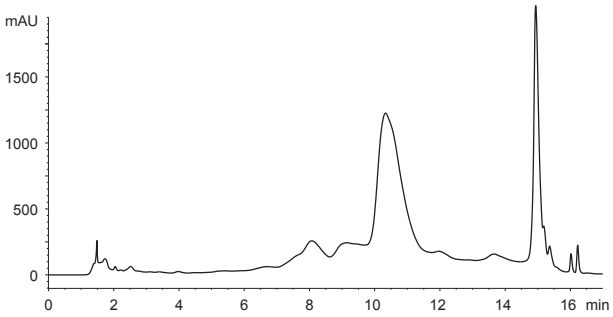


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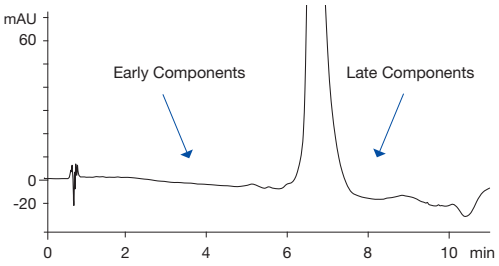
Figure 4.
Pool Result for Each Mobile Phase Screened

TFA - Acetonitrile Gradient Isolated Material



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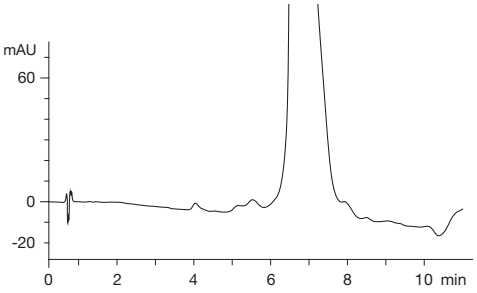
Acetic pH 4.7 - Acetonitrile Gradient Isolated Material



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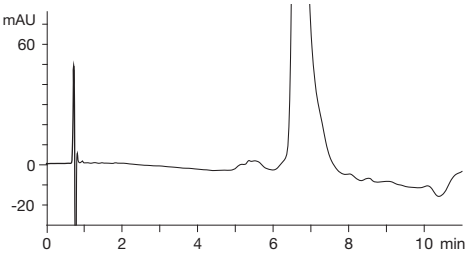
TEAP pH 7 - Acetonitrile Gradient Isolated Material



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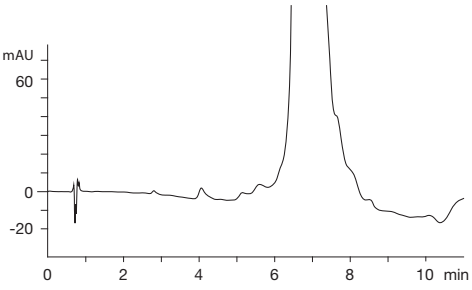
Ammonium Bicarb pH 8 - Acetonitrile Gradient Isolated Material



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Conditions

Column: Luna® 10 µm-*PREP* C8(3) 100A
Dimensions: 250 x 4.6 mm
Part No.: 00G-4623-E0
Mobile Phase: A: As noted in figure
B: 100 % Acetonitrile
Gradient: As noted in figure
Flow Rate: 1.5 mL/min
Injection: 40 µL @ 40 mg/mL
Temperature: Ambient
Detection: UV @ 220 nm

Conditions

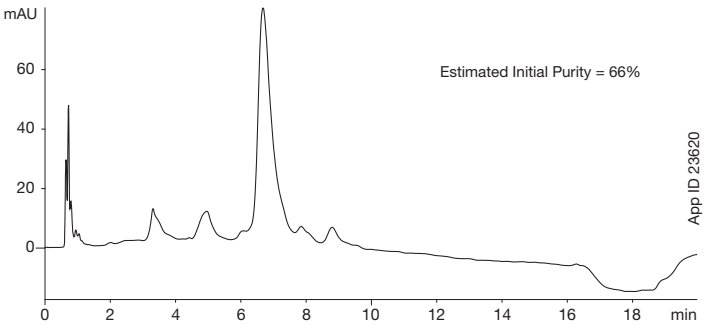
Column: Kinetex® 5 µm EVO C18
Dimensions: 150 x 4.6 mm
Part No.: 00F-4633-E0
Mobile Phase: A: 0.1 % TFA in Water
B: 100 % Acetonitrile
Gradient: 15-30 % B in 5 min, 30-50 % B in 0.5 min and hold 2 min at 50 % B
Flow Rate: 2.0 mL/min
Injection: 5 µL
Temperature: Ambient
Detection: UV @ 220 nm

Results and Discussion (continued)

Two Step Purification Methodology to Achieve Purity >98.5%

The initial crude material had an estimated purity of 66% using Kinetex EVO as analytical column (**Figure 5**.). A simple loading study was conducted for the first step conditions and it was determined that the crude material could be loaded at 1% of the column bed mass (**Figure 6**.). The main component for the first step was collected as a series of fractions. These fractions were evaluated by analytical HPLC and the appropriate fractions were pooled together. The obtained material had an estimated purity of 96.5%. The pooled material was processed with the second step acetate methodology. Again the main component was collected as a series of fractions. These fractions were evaluated by analytical HPLC and the appropriate fractions were pooled together. The second step pooled material was dried by lyophilization and evaluated by analytical HPLC. The purity of the final material was 99.3%.

Figure 5.
Initial Crude Purity on Kinetex EVO



Conditions for Figure 5.

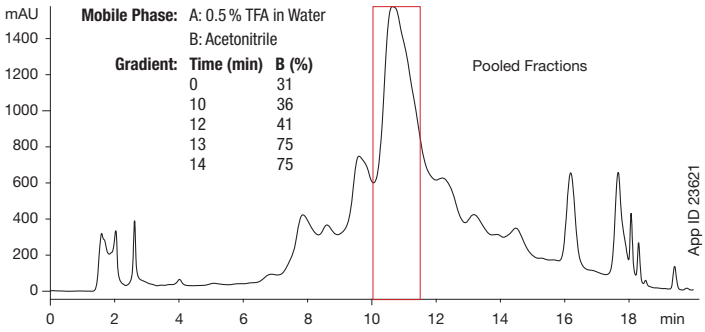
Column: Kinetex® 5 µm EVO C18
Dimensions: 150 x 4.6 mm
Part No.: 00F-4633-E0
Mobile Phase: A: 0.1 % TFA in Water
B: 100 % Acetonitrile
Gradient: 15-30 % B in 5 min, 30-50 % B in 0.5 min and hold 2 min at 50 % B
Flow Rate: 2.0 mL/min
Injection: 5 µL
Temperature: Ambient
Detection: UV @ 220 nm

Conditions for Figure 6.

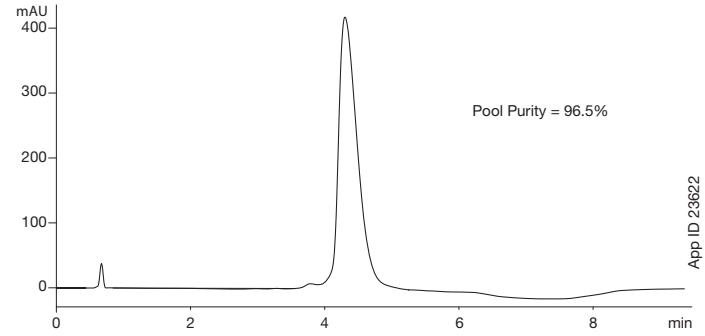
Column: Luna® 10 µm-PREP C8(3) 100A
Dimensions: 250 x 4.6 mm
Part No.: 00G-4623-E0
Mobile Phase: As noted in figure
Gradient: As noted in figure
Flow Rate: 1.5 mL/min
Temperature: Ambient
Detection: UV @ 220 nm

Figure 6.
Multi-Step Purification Process of Crude Exenatide

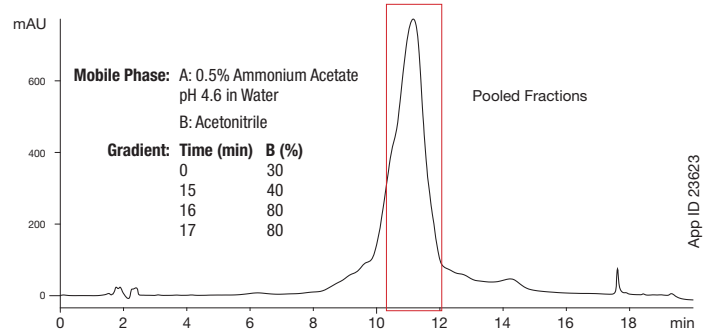
Initial Crude 1% On-Column Load First Step: TFA - Acetonitrile Gradient



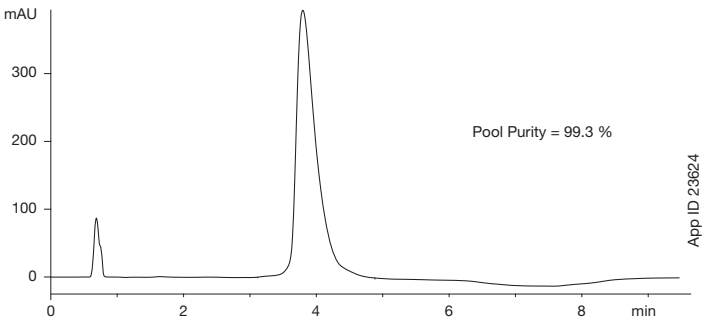
Pool from TFA - Acetonitrile First Step on QC Analytical Methodology



Isolated Pool from First Step Second Step: Acetic - Acetonitrile Gradient



Pool From Acetic - Acetonitrile Second Step on QC Analytical Methodology



APPLICATIONS

Conclusions

The column used for large scale chromatography can be a significant part of the cost for a large scale purification process. This cost is not solely based on the actual stationary phase inside the column, but needs to include the time and hardware needed to pack, unpack and switch between columns. A considerable amount of time and expense can be saved by using the same column packed with a single stationary phase for each step in a multi-step purification.

This work used Exenatide to demonstrate the development of a 2 - step purification methodology for a synthetic peptide. The 66% pure initial crude mixture was upgraded to 96.5% after the first step. The second step polished the final material to a purity of 99.3%.

Luna 10 µm-*PREP* C8(3) was used as the single stationary phase for the purification of Exenatide. This phase was introduced by Phenomenex in 2013 and is available in large quantity for packing in dynamic axial compression columns.

Ordering Information

Luna® 10 µm -*PREP* Scout Columns (mm)			SecurityGuard™ Cartridges (mm)	
Phases	**250 x 4.6**	**250 x 10**	**4 x 3.0***	**10 x 10*****
C18(3)	00G-4616-E0	00G-4616-N0	AJ0-4287	AJ0-7221
C8(3)	00G-4623-E0	00G-4623-N0	AJ0-4290	AJ0-7222
			for ID: 3.2-8.0 mm	9-16 mm

Luna 10 µm -*PREP* Preparative Columns (mm)				SecurityGuard Cartridges (mm)	
Phases	**250 x 21.2**	**250 x 30**	**250 x 50**	**15 x 21.2****	**15 x 30♦**
C18(3)	00G-4616-P0-AX	00G-4616-U0-AX	00G-4616-V0-AX	AJ0-7839	AJ0-8301
C8(3)	00G-4623-P0-AX	00G-4623-U0-AX	00G-4623-V0-AX	AJ0-7840	AJ0-8302
				for ID: 18-29 mm	30-49 mm

Luna 10 µm -*PREP* Axia™ Packed Bulk Media (mm)					
Phases	**1kg**	**5kg**	**10kg**	**50kg**	**100kg**
C18(3)	04K-4616	04L-4616	04M-4616	04N-4616	04P-4616
C8(3)	04K-4623	04L-4623	04M-4623	04N-4623	04P-4623

Kinetex® 5 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phase	**50 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**3/pk**
EVO C18	00B-4633-E0	00D-4633-E0	00F-4633-E0	00G-4633-E0	AJ0-9296
					for 4.6 mm ID

* SecurityGuard™ Analytical Cartridges require holder, Part No.: KJ0-4282

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

*** SemiPrep SecurityGuard Cartridges require holder, Part No.: AJ0-9281

** PREP SecurityGuard Cartridges require holder, Part No.: AJ0-8223

♦ PREP SecurityGuard Cartridges require holder, Part No.: AJ0-8277

APPLICATIONS

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