APPLICATIONS

Development of a 2-step Liraglutide Purification Process on a Single Stationary Phase

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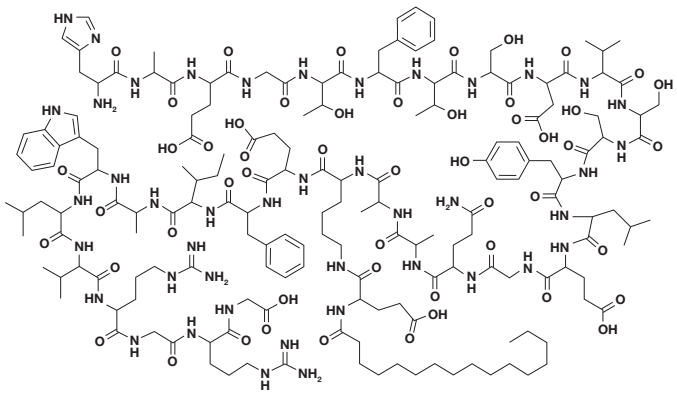
Introduction

Liraglutide is a human glucagon-like peptide-1 (GLP-1) analogue with a 31 amino acids sequence that is 97% similar to endogenous human GLP-1. See **Figure 1**. Liraglutide was approved in the EU in 2009, followed closely by approval in the U.S. in 2010. Currently, Liraglutide is commercially available in more than 95 countries and has been approved for the treatment of type 2 diabetes and obesity in adults with related comorbidity.

Manufacturing a commercially successful synthetic peptide API often needs a multistep purification process to achieve the necessary purity, yield and throughput. The first step will typically isolate the desired component from the crude mixture but not achieve the purity level required. A "polishing" step is needed to achieve the desired purity. In order to keep manufacturing costs down, the purification process needs to be optimized. In particular, the number of steps and chromatographic stationary phases used should be kept to a minimum.

Peptides are chains of amino acid monomers linked by amide bonds. Unlike proteins, their smaller size allows certain polarity and ionization properties to be predicted from its amino acid sequence. These properties can provide insight into the selection of chromatographic stationary phases and mobile phases used for the purification process development. A useful attribute of peptide chromatography is that selectivity can be altered by several means. The typical changing of the stationary phase is effective but can be costly for a preparative process. Changing chromatographic selectivity by adjusting the pH, buffer composition or organic modifier can be effective, relatively simple and inexpensive. By modifying these variables, a cost-effective multistep purification process can be developed for the purpose of achieving a high purity peptide product.

Figure 1.
Chemical Structure for Liraglutide



H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(γ-Glu-palmitoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH

Table 1.
Characterized Liraglutide Sequence

Positively charged residues (basic):	**Non-polar aliphatic residues:**	**Aromatic residues:**
• Arginine	• Glycine	• Phenylalanine
• Histidine	• Alanine	• Tyrosine
• Lysine	• Valine	• Tryptophan
	• Leucine	
	• Isoleucine	
Negatively charged residues (acidic):	**Polar non-charged residues:**	
• Glutamic acid	• Threonine	
• Aspartic acid	• Glutamine	
• Serine		

Materials and Methods

The crude material was provided from a major insulin and insulin analogs manufacturer. Sodium phosphate, sodium chloride, and 1-propanol were obtained from Fisher Scientific (Waltham, MA, USA); acetonitrile was obtained from Honeywell (Morris Plains, NJ, USA); and acetic acid, ammonium acetate and ammonium chloride were obtained from Sigma Chemical (St. Louis, MO, USA).

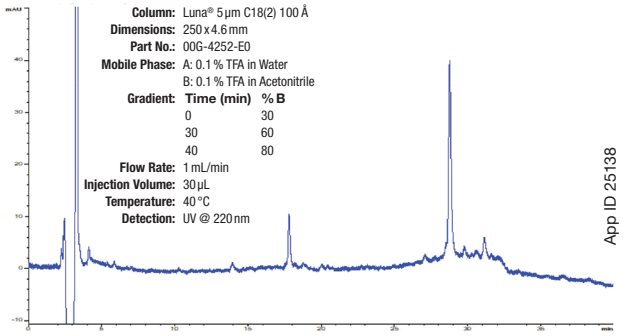
Table 2.
Liraglutide Sequence Table

	Isoelectric Point	Acidic Side Chains	Basic Side Chains	Non-Polar Side Chain	Aromatic Side Chains	Polar (Uncharged) Side Chains
Liraglutide	4.9	5	4	13	4	6
	Neutral pH	pH Sensitivity	Aliphatic Character		Interactions	Polar Interactions

APPLICATIONS

Figure 2.
Effect of pH on Post Impurity Separation

Low pH using TFA



Higher pH using Ammonium Bicarbonate

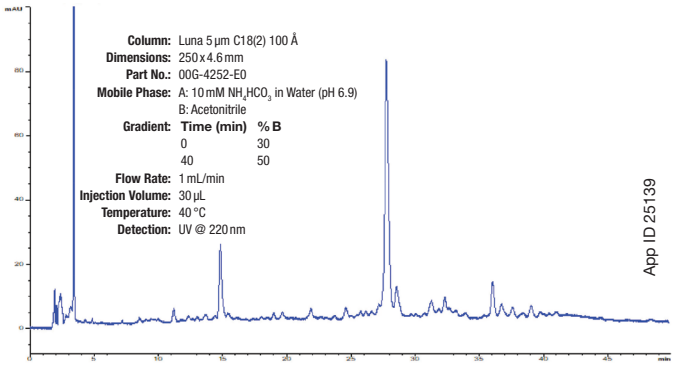
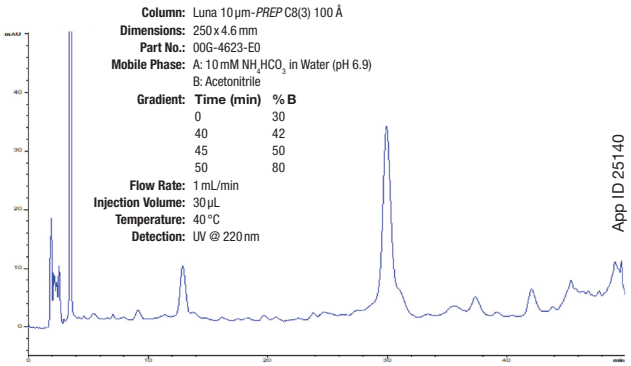
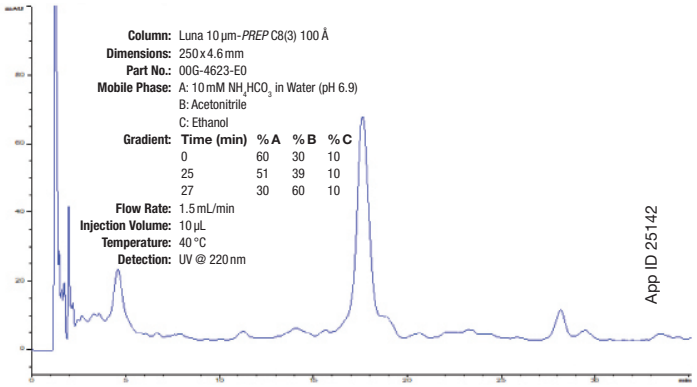


Figure 3.
Effects of Type of Organic Modifiers on Separation

Acetonitrile Only



Acetonitrile and Ethanol



Acetonitrile and Methanol

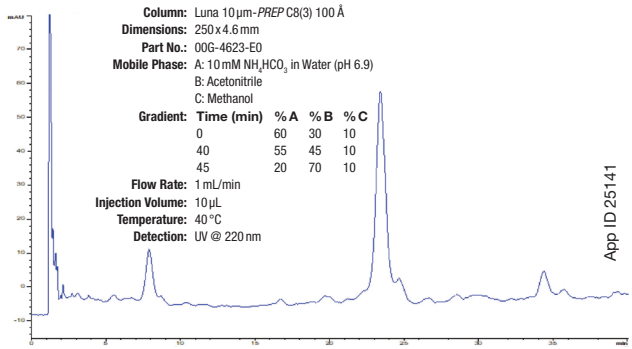


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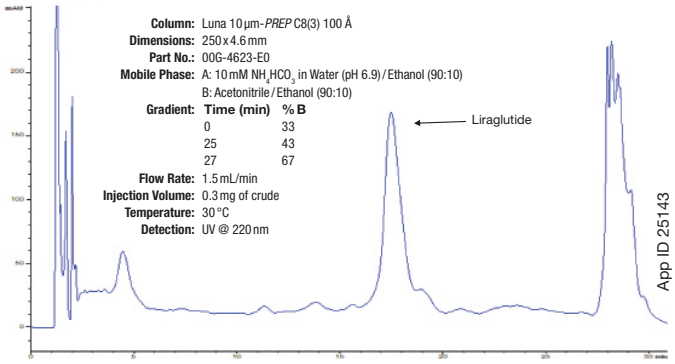
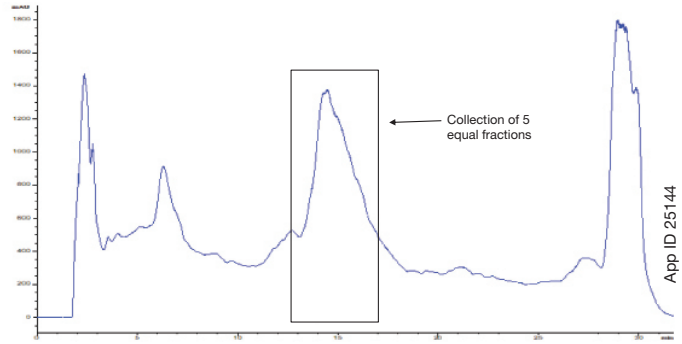
Figure 4.
Step 1 Methodology, Loading Chromatogram, Fraction Analysis and Pooled Results

Prep Methodology for Crude Liraglutide Sample

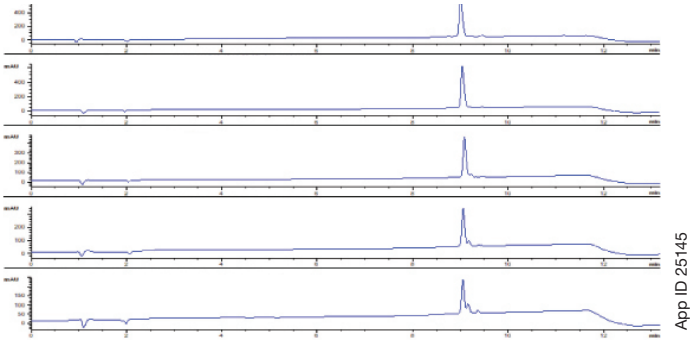


Loading Chromatogram: 0.5% loading



Fraction %	1	2	3	4	5
% Purity	94.7	95.9	91.6	81.3	74.4
Yield	28	25	18	12	7

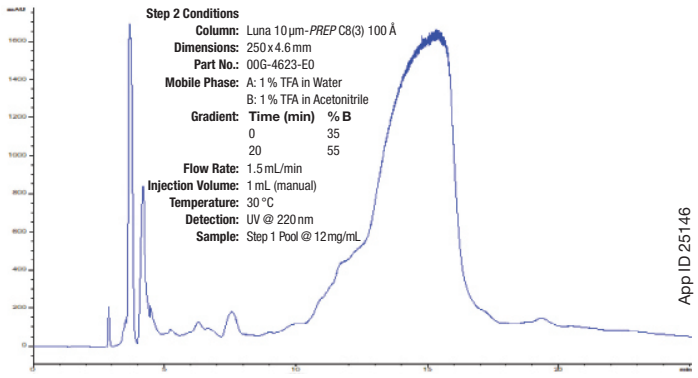
Collected Fractions 1-5



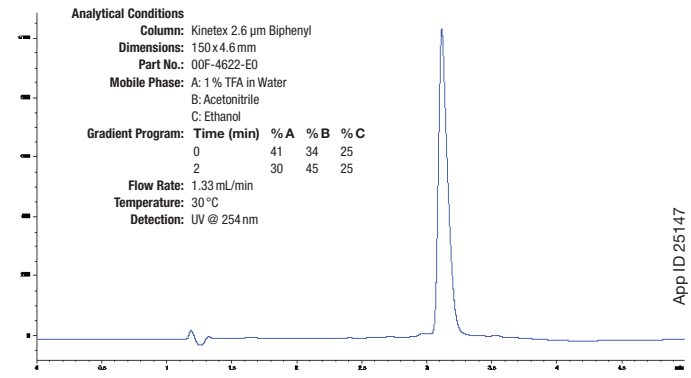
Column: Kinetex® 5 µm Biphenyl
Dimensions: 150 x 4.6 mm
Part No.: 00F-4627-E0
Mobile Phase: A: 1 % TFA in Water
B: 1 % TFA in Methanol
Gradient: Time (min) %B
0 50
10 60
Flow Rate: 1 mL/min
Injection Volume: 0.3 mg of crude
Temperature: 30 °C
Detection: UV @ 254 nm

Figure 5.

Polishing Step



Example Fraction



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Results and Discussion

Examination of the Liraglutide sequence of amino acids identified several chemical properties that are useful for chromatographic development. See **Table 1.** These properties include aliphatic, pi-pi and polar interactions. With over a third of the amino acids being nonpolar and aromatic, stationary phases such as phenyl or aliphatic hydrocarbon stationary phases are suitable for this peptide. The isoelectric point for Liraglutide is 4.9 and the amino acid sequence includes 4 acidic and 4 basic side chains. This would indicate that pH could have a significant effect on this chromatography. From the perspective of resolving power, this method development initially evaluated a C18 stationary phase. See **Figure 2**. With acidic eluent conditions, sufficient resolution was difficult to obtain between the main component and a significant impurity that eluted just after the main peak. The ionization state of this peptide and impurity was altered by a buffer of Ammonium Bicarbonate pH to 6.9 with Acetic Acid as the aqueous component. This change in pH altered the chromatographic selectivity and reversed the elution order so this impurity eluted before the main peak. There was also a change in selectivity when using acetonitrile versus a mixture of acetonitrile - alcohol as the organic component. The gradient conditions were for initial conditions and rate of change.

See **Figure 3**. Organic modifiers were evaluated for their impact for the separation. First, 10% of the Acetonitrile was replaced with Methanol, which provided different selectivity. The amount of Methanol was increased to 20% but this was still not enough to fully separate the impurity. Therefore a slightly higher polarity alcohol, Ethanol, was used in place of Methanol as the organic modifier. Good separation was achieved using the combination of Ethanol and Acetonitrile as the organic components, even with an increase in the flow rate.

The most optimal conditions were evaluated with a 0.5% crude load on a Luna® C8(3) column and Liraglutide was collected as a series of fractions. See **Figure 4**. Not a single fraction collected was able to meet the required 98% purity. It was determined at this time that a polishing step would be necessary to achieve the level of purity needed for the Liraglutide. The pooled fractions gave a purity of 91% with a yield of 91% and was taken forward to the polishing step. The final polishing step was performed on the same column, Luna C8(3). See **Figure 5**. The polishing step had different selectivity for the impurities of Liraglutide since acidic conditions were used with acetonitrile. Material isolated from the first step methodology was processed with the polishing step. Fractions were collected and a pool of these fractions provided material with a final purity of 98.2% with a yield of 80%.

Conclusion

A 2-step process was successfully developed for the purification of Liraglutide. Both steps used Luna 10µm *PREP* C8(3) as the stationary phase. The crude Liraglutide sample for this study had an initial purity of 30%. The first step upgraded the purity to 91%. A second polishing step was needed and elevated the purity to the desired 98.5%. During the development process, the use of a single stationary phase was a primary objective to minimize the overall cost of the methodology.

Luna 10µm *PREP* C8(3) was shown to be effective in the purification of Liraglutide as part of a mulit-step process. This phase was introduced by Phenomenex and is available in prepacked preparative HPLC columns and in large quantities for packing in dynamic axial compression columns. The viability of Gemini® C8(3) media was also confirmed to be suitable with this final optimized methodology, this stationary phase is suitable for high pH applications and has shown good stability for caustic washes*. This can be significant in a large scale purifications with synthetic processes that are susceptible to aggregation as well as other impurities which may remain on the column after the purification.

* Gemini C8(3) data not represented within this technical note, available upon request.

APPLICATIONS

Ordering Information

Bulk HPLC Media

Luna® (100 Å)				
Phases	**100 g**	**1 kg**	**5 kg**	**10 kg**
10 µm-*PREP*				
C18(3)	04G-4616	04K-4616	04L-4616	04M-4616
C18(2)	04G-4324	04K-4324	04L-4324	04M-4324
C8(3)	04G-4623	04K-4623	04L-4623	04M-4623
C8(2)	04G-4323	04K-4323	04L-4323	04M-4323
C4(2)	04G-4460	04K-4460	04L-4460	04M-4460
Phenyl-Hexyl	04G-4325	04K-4325	04L-4325	04M-4325
Polar-RP	04G-4757	04K-4757	04L-4757	04M-4757
Silica(3)	04G-4617	04K-4617	04L-4617	04M-4617
Silica(2)	04G-4322	04K-4322	04L-4322	04M-4322
10 µm				—
CN	04G-4300	04K-4300	04L-4300	—
NH_2	04G-4379	04K-4379	—	—
15 µm				
C18(2)	04G-4273	04K-4273	04L-4273	04M-4273
C8(2)	04G-4272	04K-4272	04L-4272	04M-4272
Phenyl-Hexyl	04G-4286	04K-4286	04L-4286	04M-4286
Silica(2)	04G-4271	04K-4271	04L-4271	04M-4271
20 µm				
Silica(2)	04G-4437	04K-4437	—	—

Gemini® (110 Å)				
Phases	**100 g**	**1 kg**	**5 kg**	**10 kg**
10 µm				
C8(3)	04G-4763	04K-4763	04L-4763	04M-4763

Scout Columns

Luna (100 Å)		
Phases	**250 x 4.6 mm**	**250 x 10 mm**
10 µm-*PREP*		
C18(3)	00G-4616-E0	00G-4616-N0
C18(2)	00G-4324-E0	—
C8(3)	00G-4623-E0	00G-4623-N0
C8(2)	00G-4323-E0	00G-4323-N0
C4(2)	00G-4460-E0	00G-4460-N0
Phenyl-Hexyl	00G-4325-E0	00G-4325-N0
Polar-RP	00G-4757-E0	00G-4757-N0
Silica(3)	00G-4617-E0	00G-4617-N0
Silica(2)	00G-4322-E0	00G-4322-N0
10 µm		
CN	00G-4300-E0	—
NH_2	00G-4379-E0	00G-4379-N0
15 µm		—
C18(2)	00G-4273-E0	00G-4273-N0
C8(2)	00G-4272-E0	00G-4272-N0
Phenyl-Hexyl	00G-4286-E0	00G-4286-N0
Silica(2)	00G-4271-E0	—
20 µm		—
Silica(2)	00G-4437-E0	—

Gemini (110 Å)		
Phases	**250 x 4.6 mm**	**250 x 10 mm**
10 µm		
C8(3)	00G-4763-E0	00G-4763-N0

Kinetex® Core-Shell LC Columns

5 µm Analytical Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Phases	**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
F5	00B-4724-E0	00D-4724-E0	00F-4724-E0	00G-4724-E0	AJ0-9320
Biphenyl	00B-4627-E0	00D-4627-E0	00F-4627-E0	00G-4627-E0	AJ0-9207
XB-C18	00B-4605-E0	00D-4605-E0	00F-4605-E0	00G-4605-E0	AJ0-8768
C18	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJ0-8768
C8	00B-4608-E0	00D-4608-E0	00F-4608-E0	00G-4608-E0	AJ0-8770
Phenyl-Hexyl	00B-4603-E0	00D-4603-E0	00F-4603-E0	00G-4603-E0	AJ0-8774
					per ID 4.6 mm

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

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