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.

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 1. Characterized Liraglutide Sequence

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<tr>
<th>Positively charged residues (basic):</th>
<th>Non-polar aliphatic residues:</th>
<th>Aromatic residues:</th>
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<tr>
<td>Arginine</td>
<td>Glycine</td>
<td>Phenylalanine</td>
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<tr>
<td>Histidine</td>
<td>Alanine</td>
<td>Tryptophan</td>
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<td>Lysine</td>
<td>Valine</td>
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<td></td>
<td>Leucine</td>
<td></td>
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<td></td>
<td>Isoleucine</td>
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<table>
<thead>
<tr>
<th>Negatively charged residues (acidic):</th>
<th>Polar non-charged residues:</th>
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<td>Glutamic acid</td>
<td>Threonine</td>
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<td>Aspartic acid</td>
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<td>Serine</td>
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Table 2. Liraglutide Sequence Table

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<tr>
<th>Liraglutide</th>
<th>Isoelectric Point</th>
<th>Acidic Side Chains</th>
<th>Basic Side Chains</th>
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<th>Aromatic Side Chains</th>
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<td>5</td>
<td>4</td>
<td>13</td>
<td>4</td>
<td>6</td>
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Figure 1. Chemical Structure for Liraglutide

APPLICATIONS

Figure 2.
Effect of pH on Post Impurity Separation

Low pH using TFA

Column: Luna® Symp C18(2) 100 Å
Dimensions: 250 x 4.6 mm
Part No.: 00G-4252-60
Mobile Phase: A: 0.1 % TFA in Water
B: 0.1 % TFA in Acetonitrile
Gradient: Time (min) % B
0 30
30 80
80 80
Flow Rate: 1 mL/min
Injection Volume: 30 µL
Temperature: 40 °C
Detection: UV @ 220 nm

Higher pH using Ammonium Bicarbonate

Column: Luna® Symp C18(2) 100 Å
Dimensions: 250 x 4.6 mm
Part No.: 00G-4252-60
Mobile Phase: A: 10 mM NH₄HCO₃ in Water (pH 6.9)
B: Acetonitrile
Gradient: Time (min) % B
0 30
30 50
50 50
Flow Rate: 1 mL/min
Injection Volume: 30 µL
Temperature: 40 °C
Detection: UV @ 220 nm

Figure 3.
Effects of Type of Organic Modifiers on Separation

Acetonitrile Only

Column: Luna® Symp C18(2) 100 Å
Dimensions: 250 x 4.6 mm
Part No.: 00G-4252-60
Mobile Phase: A: 10 mM NH₄HCO₃ in Water (pH 6.9)
B: Acetonitrile
Gradient: Time (min) % B
0 30
30 40
40 40
45 50
50 80
Flow Rate: 1 mL/min
Injection Volume: 30 µL
Temperature: 40 °C
Detection: UV @ 220 nm

Acetonitrile and Ethanol

Column: Luna® Symp C18(2) 100 Å
Dimensions: 250 x 4.6 mm
Part No.: 00G-4252-60
Mobile Phase: A: 10 mM NH₄HCO₃ in Water (pH 6.9)
B: Acetonitrile
C: Ethanol
Gradient: Time (min) % A % B % C
0 60 30 10
25 51 39 10
50 27 30 60 10
Flow Rate: 1.5 mL/min
Injection Volume: 10 µL
Temperature: 40 °C
Detection: UV @ 220 nm

Acetonitrile and Methanol

Column: Luna® Symp C18(2) 100 Å
Dimensions: 250 x 4.6 mm
Part No.: 00G-4252-60
Mobile Phase: A: 10 mM NH₄HCO₃ in Water (pH 6.9)
B: Acetonitrile
C: Methanol
Gradient: Time (min) % A % B % C
0 60 30 10
40 50 40 10
45 50 20 10
Flow Rate: 1 mL/min
Injection Volume: 10 µL
Temperature: 40 °C
Detection: UV @ 220 nm
**Figure 4.**
Step 1 Methodology, Loading Chromatogram, Fraction Analysis and Pooled Results

**Prep Methodology for Crude Liraglutide Sample**

**Loading Chromatogram: 0.5% loading**

<table>
<thead>
<tr>
<th>Fraction %</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tr>
<td>% Purity</td>
<td>94.7</td>
<td>95.9</td>
<td>91.6</td>
<td>81.3</td>
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<tr>
<td>Yield</td>
<td>28</td>
<td>25</td>
<td>18</td>
<td>12</td>
<td>7</td>
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</table>

**Collected Fractions 1-5**

**Figure 5.**
Step 2 Methodology, Polishing Chromatogram, Analytical Conditions and Example Fraction

**Polishing Step**

**Example Fraction**

**App ID 25143**

**Column:** Luna 10 µm PREP C8(3) 100 Å
**Dimensions:** 250 x 4.6 mm
**Part No.:** 00G-4825-ED
**Mobile Phase:** A: 10 mM NH₄HCO₃ in Water (pH 6.9) / Ethanol (90:10)
B: Acetonitrile / Ethanol (90:10)
**Gradient:** Time (min) % B
0 | 0 23
25 | 27 87
**Flow Rate:** 1.5 mL/min
**Injection Volume:** 0.3 mg of crude
**Temperature:** 30 °C
**Detection:** UV @ 220 nm

**App ID 25144**

**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 Acetonitrile
**Gradient:** Time (min) % B
0 | 35
20 | 55
**Flow Rate:** 1.5 mL/min
**Injection Volume:** 0.6 mL (manual)
**Temperature:** 30 °C
**Detection:** UV @ 220 nm

**Sample:** Step 1 Pool @ 12 mg/mL

**App ID 25145**

**Column:** Kinetex 2.6 µm Biphenyl
**Dimensions:** 150 x 4.6 mm
**Part No.:** 00F-4622-E0
**Mobile Phase:** A: 1% TFA in Water
B: Acetonitrile
C: Ethanol
**Gradient Program:** Time (min) % A % B % C
0 | 41 34 25
2 | 41 45 25
**Flow Rate:** 1.33 mL/min
**Temperature:** 30 °C
**Detection:** UV @ 254 nm

**Figure 6.**
Analytical Conditions

**App ID 25146**

**Column:** Luna 10 µm PREP C8(3) 100 Å
**Dimensions:** 250 x 4.6 mm
**Part No.:** 00G-4825-ED
**Mobile Phase:** A: 10 mM NH₄HCO₃ in Water (pH 6.9) / Ethanol (90:10)
B: Acetonitrile / Ethanol (90:10)
**Gradient:** Time (min) % B
0 | 23
25 | 43
**Flow Rate:** 1.5 mL/min
**Injection Volume:** 0.3 mg of crude
**Temperature:** 30 °C
**Detection:** UV @ 220 nm

**App ID 25147**

**Column:** Kinetex® 5 µm Biphenyl
**Dimensions:** 150 x 4.6 mm
**Part No.:** 00F-4627-ED
**Mobile Phase:** A: 1% TFA in Water
B: Acetonitrile
**Gradient:** Time (min) % B
0 | 50
10 | 60
**Flow Rate:** 1.5 mL/min
**Injection Volume:** 0.3 mg of crude
**Temperature:** 30 °C
**Detection:** UV @ 220 nm
Applications

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® 10μm PREP 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 mult-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

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| TN-1246 | TN-1250 |

**Ordering Information**

**Bulk HPLC Media**

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

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

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-8000

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-8000

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-8000

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-8000

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