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Stationary Phases for the Process Scale Purification of Peptides and Insulin Analogs

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Introduction

The crude peptide analog used in this purification study is crude Insulin Glargine first brought to market by Sanofi-Aventis, Germany in 2000 mainly for the purpose of managing type 1 diabetes. Since coming off patent in 2015, there has been a large interest in the purification process for generic alternatives.

Well established methods have been used in industry for the purification of insulin while improvements are being made continuously. One of the limitations to high-throughput purification is the cost and time prohibitive nature of the media used. Here we examine two silicas, both C8 phases, for the purification of an insulin analog as well as evaluating their advantage for the removal of aggregate (build-up) post purification. While some insulin-like peptides such as Liraglutide have shown not to have issue with aggregate buildup, others do require significantly longer rewashes to remove build-up.

The crude material was obtained from a major insulin and insulin analogs manufacturer. Insulin Glargine is a 53-amino acid peptide and its chemical structure is represented in Figure 1. The analog has the base structure of A chain and B chain of human insulin but the asparagine residue at A21 on the A chain has been replaced with glycine. Additionally, two arginine were added to the C-terminus of the B-chain at B31 and B32.

The development of a multi-step purification process on two types of silicas is presented here for commercially significant crude insulin analog, Insulin Glargine. The focus of this tech note is to demonstrate the efficacy of both prep C8 phases on a well-established USP method that has been further optimized on the analytical format and then scaled to the prep format to produce material of a suitable purity. The investigative parameters include base silica and gradient conditions.

Materials and Methods

The crude material was provided from a major insulin and insulin analogs manufacturer. Sodium phosphate, sodium chloride, 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).

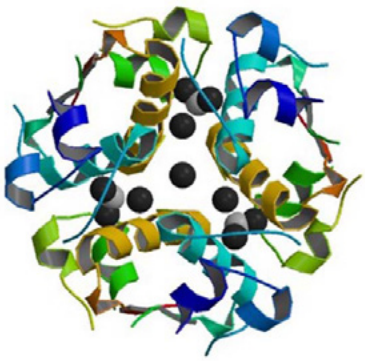
Results and Discussion

The purity of the crude insulin glargine material was obtained with a slight modification of the USP insulin methodology. The arginine modification of insulin Glargine shifts the PI of human insulin from 5.4 to 6.7. As such the pH of the mobile phase was modified from pH 2.3 to pH 4. This modification was made in the analytical format. Prep conditions were then used to show efficacy of both C8 media for purification. While the 20 minute gradient was sufficient to demonstrate purification, a longer run at 40 minutes, with a prolonged gradient, proved to be more effective for larger scale purification.

The main intent was to show the Gemini C8(3) hybrid silica has comparable selectivity to a typical C8 media found in industry. As proof of concept, we only took a representative single step purification approach. Fractions were collected every 30 secs and all fractions were assayed. The analytical results were used to calculate purities and yields for theoretical pools. On both Luna and Gemini media, the chromatographic profile were similar and the purity and yield results were comparable. The purity obtained from these one step purifications were at least 98%.

An additional study was conducted to verify the applicability of the Gemini C8(3) material for insulin purification. To remove aggregate buildup, a caustic wash is often employed. Figure 2 shows the sustainability of the silica material chromatographically even after 20 washes using 50:50 1 N NaOH/1-propanol (3.5 CV), then 55:45 0.02 M Acetic acid/1-propanol (12 CV).

Chemical Structure of Insulin Glargine



Peptide chemical formula
C267H404N72O78S6
Peptide average weight 6063.0 Da

Sequences

>A chain
GIVEQCCTSICSLYQLENYCG

>B chain
FVNQHLCGSHLVEALYLVCGERGFFYTPKTRR

https://www.drugbank.ca/drugs/DB00047

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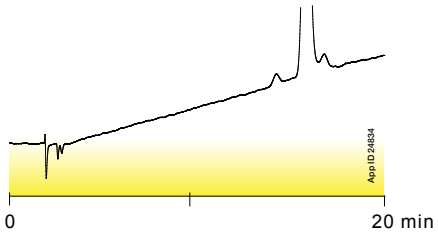
Figure 1.

Comparison of Luna C8(3) and Gemini C8(3) for Different Gradient Times and Formats

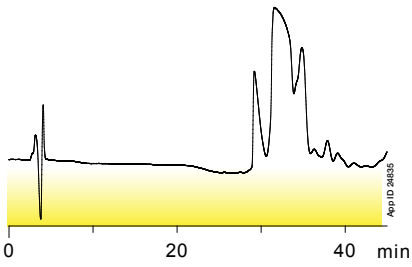
20 min. Run. Analytical Load
Luna® µm-PREP C8(3)



Conditions same for both columns:

Columns:	As described
Dimensions:	250 x 4.6 mm
Mobile Phase:	A: 20 mM Ammonium acetate, 200 mM Ammonium chloride, 10 % Acetonitrile, pH 4 B: 20 mM Ammonium acetate, 50 % Acetonitrile, pH 4
Gradient:	**Time (min) % B** 0 35 20 55
Flow Rate:	1.2 mL/min
Injection Volume:	20 µL
Temperature:	35 °C
Detection:	UV @ 214 nm (ambient)

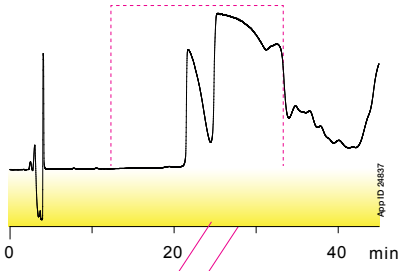
40 min. Run. 10 mg Load
Luna 10 µm-PREP C8(3)



Conditions same for both columns:

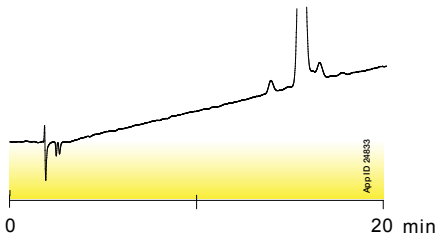
Columns:	As described
Dimensions:	250 x 4.6 mm
Mobile Phase:	A: 20 mM Ammonium acetate, 200 mM Ammonium chloride, 10 % Acetonitrile, pH 4 B: 20 mM Ammonium acetate, 50 % Acetonitrile, pH 4
Gradient:	**Time (min) % B** 0 35 40 50 45 50
Flow Rate:	1.2 mL/min
Injection Volume:	200 µL (10 mg)
Temperature:	35 °C
Detection:	UV @ 214 nm (ambient)

40 min. Run. 25 mg Load
Luna 10 µm-PREP C8(3)



Collected Fractions

Gemini10 µm-PREP C8(3)



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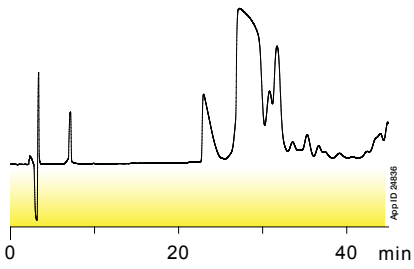
Mobile Phase:	A: 20 mM Ammonium acetate, 200 mM Ammonium chloride, 10 % Acetonitrile, pH 4 B: 20 mM Ammonium acetate, 50 % Acetonitrile, pH 4
Gradient:	**Time (min) % B** 0 35 20 55
Flow Rate:	1.2 mL/min
Injection Volume:	20 µL
Temperature:	35 °C
Detection:	UV @ 214 nm (ambient)

Gemini10 µm C8(3)



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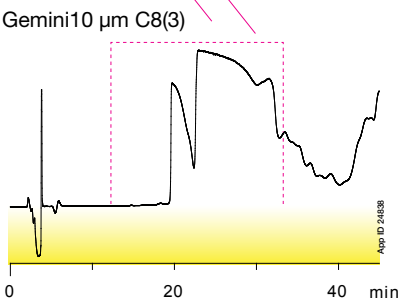
Mobile Phase:	A: 20 mM Ammonium acetate, 200 mM Ammonium chloride, 10 % Acetonitrile, pH 4 B: 20 mM Ammonium acetate, 50 % Acetonitrile, pH 4
Gradient:	**Time (min) % B** 0 35 40 50 45 50
Flow Rate:	1.2 mL/min
Injection Volume:	200 µL (10 mg)
Temperature:	35 °C
Detection:	UV @ 214 nm (ambient)

Gemini10 µm C8(3)



Conditions same for both columns:

Columns:	As described
Dimensions:	250 x 4.6 mm
Mobile Phase:	A: 20 mM Ammonium acetate, 200 mM Ammonium chloride, 10 % Acetonitrile, pH 4 B: 20 mM Ammonium acetate, 50 % Acetonitrile, pH 4
Gradient:	**Time (min) % B** 0 35 40 50 45 50
Flow Rate:	1.2 mL/min
Injection Volume:	500 µL (25 mg)
Temperature:	35 °C
Detection:	UV @ 214 nm (ambient)

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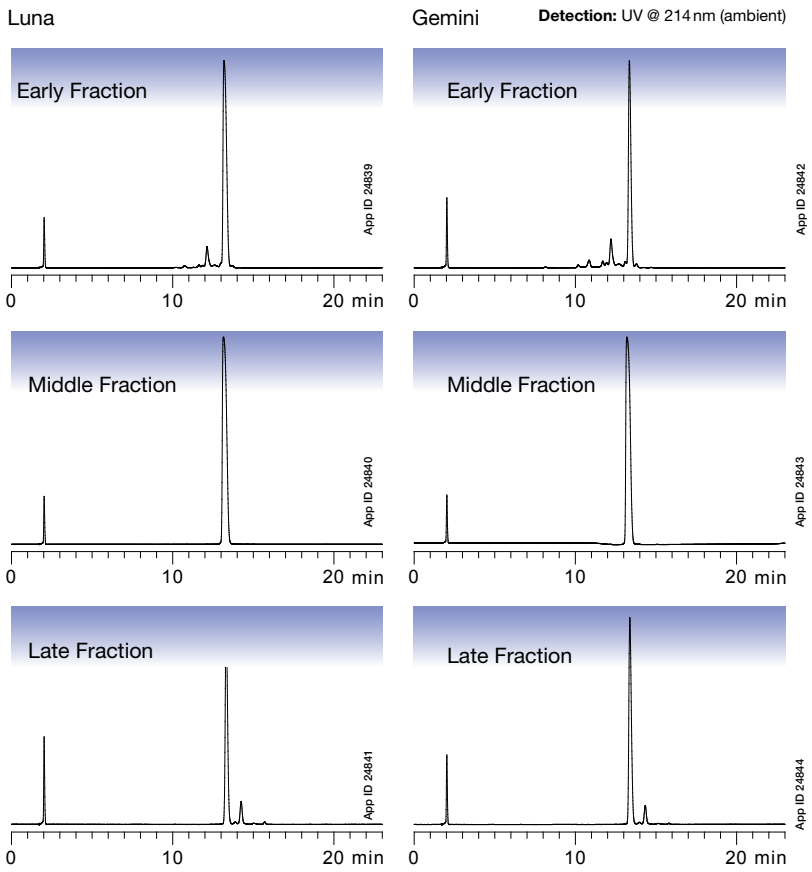
Figure 2.

Collected Fractions from Gemini C8(3) vs Luna C8(3)



Column: Kinetex® 5 µm C18
Dimensions: 250 x 4.6 mm
Mobile Phase: A: 58 mM Sodium phosphate, 417 mM Sodium chloride, 25 % Acetonitrile, pH 2.5
B: 125 mM Sodium phosphate, 893 mM Sodium chloride, 65 % Acetonitrile, pH 2.5

Gradient:

Time (min)	% B
0	4
20	17
30	37

Flow Rate: 1.2 mL/min
Injection Volume: 5 µL
Temperature: 35 °C
Detection: UV @ 214 nm (ambient)

Conclusion

Chromatographic media is often a significant portion of the cost for large scale peptide purification. The cost is not solely due to the media, but also the time spent regenerating the column back to initial conditions. Therefore, it is often more cost and time effective to use a durable silica media that can be used under caustic conditions.

This work used the analog Insulin Glargine to demonstrate the comparability of the high pH resistant silica to that of a typical silica used in industry chromatographically. Multiple fractions were assayed from both Luna C8(3) and high pH stable Gemini C8(3). These two media were found to be comparable and yield insulin glargine at greater than 98% purity.

Gemini C8(3) and Luna C8(3) were used as the stationary phase for the purification of Insulin Glargine. Luna C8(3) was introduced in 2014 and Gemini C8(3) in 2017.

For both columns, 25 mg of crude (1% load) was loaded and fractions were collected every 30 seconds from before the main peak eluted. These fractions were assayed by the analytical USP methodology using a Kinetex C18 column.

From the fraction analysis data, purity and yield results for different possible pools were calculated.

Luna 10 µm-*PREP* C8(3)*

Pool	% purity	% yield
1	98.41	90.9
2	98.91	87.4
3	99.30	82.2
4	99.60	75.4

Gemini 10 µm C8(3)*

Pool	% purity	% yield
1	98.24	92.8
2	98.91	84.3
3	99.44	71.9
4	99.71	61.5

* **Table note:**
% yield is the peak area in the pool divided by the total peak area available.
It is not pool divided by crude.

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Luna® Ordering Information

Luna 10 µm-*PREP*	Columns	Bulk Media				
Phases	250 x 4.6 mm	1 kg	5 kg	10 kg	50 kg	100 kg
C18(3)	00G-4616-E0	04K-4616	04L-4616	04M-4616	04N-4616	04P-4616
C8(3)	00G-4623-E0	04K-4623	04L-4623	04M-4623	04N-4623	04P-4623
Silica(3)	00G-4617-E0	04K-4617	04L-4617	04M-4617	04N-4617	04P-4617

Gemini® C8(3) Ordering Information

10 µm Analytical, Semi-Prep and Axia™ Packed Preparative Columns (mm)					
Phase	250 x 4.6	250 x 10	250 x 21.2	250 x 30	250 x 50
C8(3)	00G-4763-E0	00G-4763-N0	00G-4763-P0-AX	00G-4763-U0-AX	00G-4763-V0-AX

Bulk Media				
Phase	100 g	1 kg	5 kg	10 kg
C8(3)	04G-4763	04K-4763	04L-4763	04M-4763

Kinetex® Ordering Information

5 µm Analytical Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Fase	50 x 4,6	100 x 4,6	150 x 4,6	250 x 4,6	3/pz
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	A J0-8768
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

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