Development of a Robust Process for Preparative Semaglutide Purification by MCSGP Using a Hybrid Silica C8 Stationary Phase

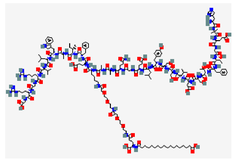
J Preston, PhD; Zeshan Aqeel

Introduction

The preparative purification of therapeutic peptides, such as semaglutide, presents unique challenges due to their size, complexity, and sensitivity to mobile-phase conditions. To develop a robust and scalable purification strategy, we investigate the performance of C8, C18 and butyl-phenyl stationary phases on a pH stable hybrid silica. The purification process is demonstrated on the ContiCUBE preparative HPLC system equipped with multi-column countercurrent solvent gradient purification (MCSGP). Crude semaglutide is used as a model GLP-1 compound to evaluate stationary phase selectivity and system performance under preparative loading conditions.

Semaglutide Overview

Semaglutide is a medication used to manage type 2 diabetes, promote weight loss in obese or overweight adults, and reduce the risk of major cardiovascular events (heart attack, stroke) in high-risk patients. It is a GLP-1 receptor agonist that works by mimicking a hormone to increase insulin, lower blood sugar, decrease appetite, and slow digestion.



Semaglutide is a modified form of human GLP-1. It consists of a 31-amino acid chain with two key modifications. There is an α-aminoisobutyric acid (AIB) at position 8 and a C18 fatty diacid attached via a linker to the Lys26 residue.

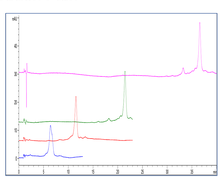
Crude Semaglutide from a was obtained from YMC India Pvt Ltd. The produced by solid phase peptide synthesis (SPPS).

The isoelectric point (pI) of semaglutide is 5.4. At this pH, the molecule has a net surface charge of zero, causing it to exhibit low aqueous solubility and increased risk of precipitation from aqueous solutions. It is a peptide with poor solubility between pH 2.5 and 6.0, often requiring pH adjustment for formulation stability. It is highly soluble in acidic conditions (pH 1–2) and shows increased solubility above pH 6.8. To maintain solubility and stability, formulations are often adjusted away from this pI, with a preference for neutral to slightly basic conditions.

Peptide Separation and MCSGP Method Development Plans

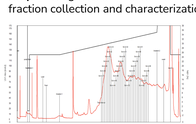
Peptide Separation Plan

- Column Screening
  - YMC pH stable Triart columns
  - 10um and 20um media available in bulk
  - 120Å C8, C18 and Butyl-Phenyl
- Mobile Phase Screening
  - Low – below pH 2.5
  - High – above pH 6.8
  - Use Acetonitrile
  - Avoid alcohols (if possible)
- Gradient Optimization
- Loading
  - Low, Medium, High

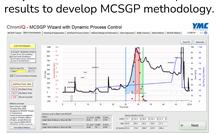


MCSGP 3-Step Development Plan

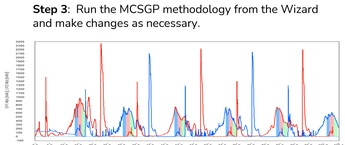
Step 1: Single column batch run with fraction collection and characterization



Step 2: MCSGP Wizard uses Step 1 results to develop MCSGP methodology.



Step 3: Run the MCSGP methodology from the Wizard and make changes as necessary.



Column Screening High and Low pH

YMC Triart Prep C18-S, Triart Prep C8-S and Triart Prep Phenyl-S were chosen as to be screened because they are commonly used for peptide purification processes.

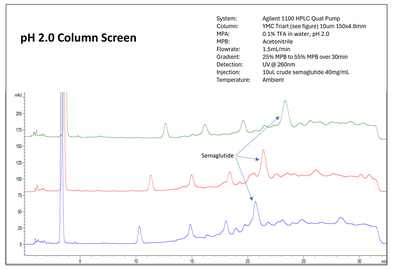
Since the isoelectric point (pI) of semaglutide is 5.4, pH 2.0 and pH 8.4 were chosen as the low and high pH eluents for screening column stationary phases.

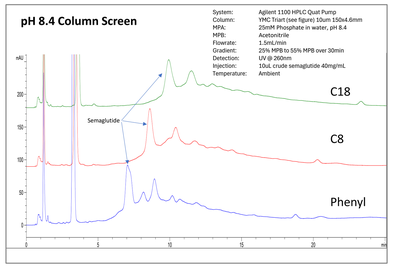
The pH 2.0 eluent used a 0.1% Trifluoroacetic acid (TFA) solution in water. The pH 8.4 eluent used a 25mM solution of monobasic sodium phosphate in water, that was pH adjusted with NaOH.

The gradient for these screening experiments was 25% MPB to 55% MPB over 30 min. The percentages were chosen based on experience with other GLP-1 peptides and a gradient that changes 1%/min is usually a good place to start with peptide RP chromatography.

The chromatographic profiles are quite different between the low pH and high pH runs for all three stationary phases. Semaglutide elutes later (at a higher amount of MeCN) in the low pH runs than it does in the high pH runs for all three columns.

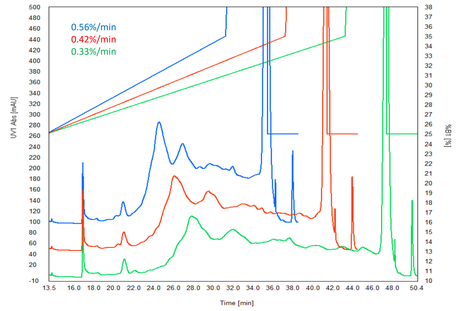
The phenyl column had the least retention of the three phases and has a slightly different selectivity than the C8 or C18 columns. The C8 and C18 have similar selectivity with the C18 column being more retentive. The Triart Prep C8-S was chosen as for the rest of this work





Gradient Screen

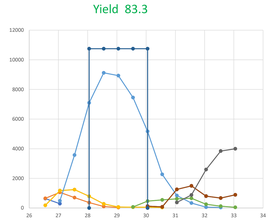
The Triart Prep C8-S column with Phosphate pH 8.4 as MPA and MeCN as MPB. A suitable gradient of was determined to run between 25% MPB and 35% MPB. See the "Determine Partitioning Range" section of this poster. The data in this section shows the chromatograms for different gradient times, which changes the slope of the gradient. See the figure to the right. A lower slope provides longer retention and usually better separation but a takes more time, uses more eluent and yields less concentrated fractions. A higher gradient slope reduces the retention and usually reduces the separation but takes less time, uses less eluent and yields more concentrated fractions.



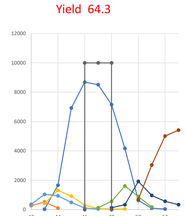
Evaluation of the Three Gradient Slopes

The separations from the three different gradient slope experiments were characterized by collecting fractions across the entire main peak and these fractions were assayed by analytical HPLC. See the "MCSGP Step 1" section of this Poster". When the peak area vs fraction number is plotted for all of the peaks found in these analytical results, the true profile of components in this region of the chromatogram can be visualized and quantitated. Below are the visualized profiles and fractions that would be pooled to generate material with 94% - 95% purity for the three different gradients.

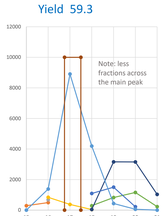
30 min Gradient
Purity 94.3
Yield 83.3



24 min Gradient
Purity 94.6
Yield 64.3



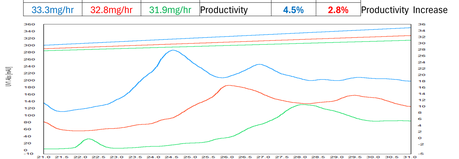
18 min Gradient
Purity 95.1
Yield 59.3



Calculated Results

The results for the different pooled materials can be calculated. See the table to the right. The steeper gradients provide more concentrated product solutions with higher productivity but will need more cycles and could use more solvent. Below is an overlay of the chromatograms from the three different gradients. The color shaded regions indicate the area pooled in this example.

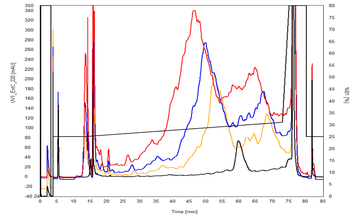
18 min Gradient	24 min Gradient	Optimized 30 min Gradient	
95.10%	94.60%	94.30%	Purity
59.30%	64.30%	83.30%	Yield
0.59mL	1.32mL	1.98mL	Product Volume
12.9mg/mL	6.46mg/mL	4.94mg/mL	Product Conc
13.7min	15.8min	18.4 min	Switch Time -25% -15% Switch Time Decrease
33.3mg/hr	32.8mg/hr	31.9mg/hr	Productivity 4.5% 2.8% Productivity Increase



Loading Study

A loading study is necessary to determine the maximum loading where the required purity is obtained, and the yield is optimized for a batch method. MCSGP processes can often use higher loads than single column batch methods because MCSGP processes recycle the eluting material where the product overlaps the impurities and this product is collected in the next run.

During development, any increase in loading, will require another experiment that profiles the prep chromatogram (fractions collected, HPLC data generated, and peak profile plotted) and the MCSGP wizard to generate a new MCSGP method.

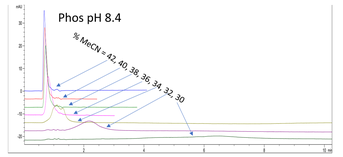


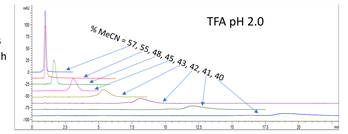
Determine Partitioning Range

With RP chromatography, most peptides have a narrow % MeCN range of eluent where the peptide partitions between the mobile phase and the stationary phase. When the % MeCN is below this range, the peptide is stuck to the stationary phase and doesn't move. When the % MeCN is above this range, the peptide remains in the eluent and is unretained. Determining this range is very useful for peptide chromatography, especially with MCSGP.

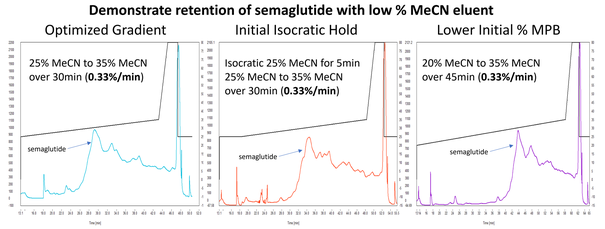
One method to determine the partitioning range, is to run a series of isocratic runs where the % MeCN is changed in small steps. The figures to the right show semaglutide runs with isocratic % MeCN steps for both pH 8.4 phosphate and pH 2.0 TFA.

With phosphate at pH 8.4, semaglutide is effectively unretained above 38% MeCN and is significantly retained below 30% MeCN. With TFA at pH 2.0, Semaglutide is effectively unretained above 55% MeCN and is significantly retained below 40% MeCN.

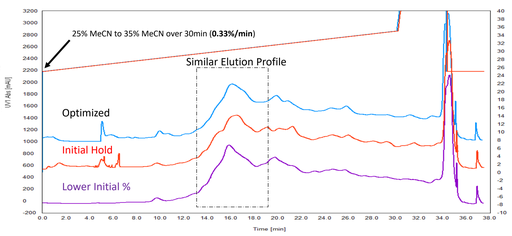




Demonstrate retention of semaglutide with low % MeCN eluent

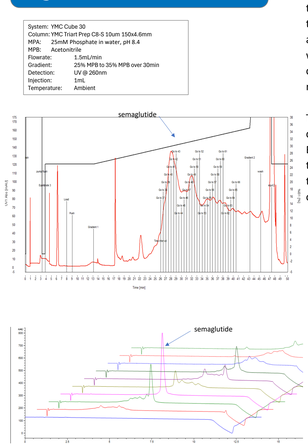


Above are 3 chromatograms that show gradients with MeCN slopes of 0.33%/min. The 1st is a linear gradient from 25% to 35%. The 2nd has a 5 min isocratic hold before the 25% to 35% gradient. The 3rd has a gradient that goes from 20% to 35% but has the same 0.33% slope as the others. To the right is an overlay of the same 3 chromatograms but the time axis has been shifted to align the gradient. The chromatographic profile for the elution of semaglutide is very similar for all 3 chromatograms.

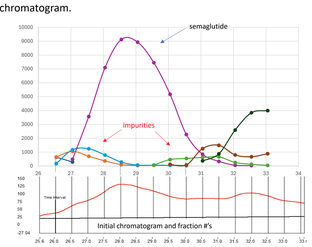


MCSGP Step 1 Single Column Batch Run

The first step when making an MCSGP process from a single column linear gradient batch method, is to run the single column method on the Cube. The slope of the gradient in this single column method will be the slope of the gradient in the MCSGP methodology. However, the linear gradient in the MCSGP method can start at a higher percentage of MPB and it can end at a lower percentage of MPB. The MCSGP Wizard software has tools that will predict things like yield, purity and throughput if the purity and concentration of the target peak is well characterized in the single column method.



The chromatogram to the left, shows a semaglutide single column chromatogram and all of the fractions collected to characterize this method. Below the chromatogram to the left, is the analytical runs that generated the data to characterize the method. Below is a plot of the PA vs Fraction # to recreate the semaglutide peak and the impurities that eluted in this part of the chromatogram.

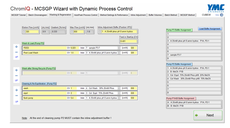


MCSGP Step 2 MCSGP Wizard

The 2nd step is to use the Cube software has an MCSGP Wizard that takes single column batch data that was run on a Cube and with minimal user input, converts this methodology into an MCSGP method. A graphical interface allows the user to position lines on the batch chromatogram to define where the MCSGP method will recycle material and where it will collect the product.

The wizard is designed to simply setup how the methodology loads the sample and how the columns are washed and re-equilibrated between each run.

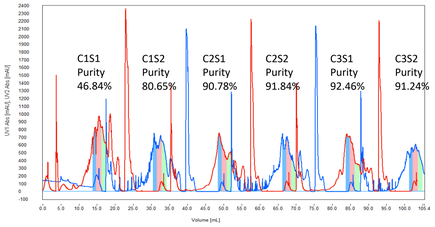
The MCSGP Wizard also setup up Autopeak, which uses the UV detector signal to trigger the start of the different MCSGP segments. This is key to a rugged and reproducible MCSGP methods.



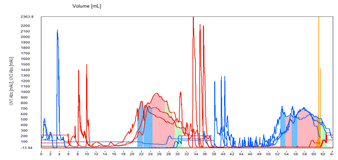
MCSGP Step 3 Run MCSGP

The 3rd step is to run the MCSGP process. The first criteria to evaluate for an MCSGP process is that equilibrium is reached. When the process is in equilibrium, the amount of material removed is equal to the amount of material added for each run and the resulting chromatograms are consistent. The process in this work was close to reaching equilibrium but ran out of feed stock before equilibrium was obvious and material wasn't available to repeat the experiment.

The chromatograms produced by an MCSGP process can be displayed linear with time as in the figure immediately below or it can be displayed as an overlay of each cycle. In the overlaid version, there is a series of chromatograms that display both column 1 and column 2.



The performance characteristics for these MCSGP and batch processes can be compared. Throughput was considerably higher for MCSGP. The purity was slightly lower for the MCSGP process. This could have been corrected by adjusting where the product is collected in the process, but material was not available. The yield was higher for the MCSGP process as expected because of the overlap recycling. MCSGP used a little more solvent. Most of the solvent consumption was due to the line dilution and column wash / re-equilibrium steps. These steps were not fully optimized, and the amounts used could be significantly reduced.



	MCSGP	Batch	Comparison
Runtime	35 min per cycle (17.5 min per switch)	50 min	
Injection Volume	1.1mL per cycle (0. 55mL per switch)	1mL per run	
Throughput (feed stock)	1.9mL / hour	1.2mL / hour	MCSGP is 58% higher
Throughput (crude mass)	47.5mg/hour	30mg/hour	MCSGP is 58% higher
Purity	91.6%** (avg of 4 switches)	94.3%	MCSGP is 2.7% lower
Yield	91.1% (avg of 4 switches)	83.3%	MCSGP is 7.8% higher
Solvent used	78.5mL per cycle (135mL/hour)	45mL per run (54mL/hour)	MCSGP is 74% higher
g Crude per L	350g crude per L solvent	550g crude per L solvent	MCSGP is 36% lower

Conclusions

In this poster, we presented a Peptide Separation Plan and described details about the different components. We presented column screening at both low pH and higher pH. The Triart C8 column was selected to for this work. The gradient screening section precented three different gradients that were screened. The gradient with the lowest slope was found best for a single column batch method but a higher sloped gradient would be better for MCSGP. The final part of the plan presented here discusses the role that loading studies play in prep process development.

The key to twin-column MCSGP methodology is the ability to stack the recycled material on the head of the next column with inline dilution. In this poster we present an approach for the determination of the partitioning range for a compound and how to demonstrate that ability to stack the material on the head of a column.

The final topic presented in the poster is a 3-step process to convert a single column batch method to an MCSGP process. The 1st step is to run the method on a Cube and characterize the desired peak and impurities within the region the desired peak elutes. The 2nd step is to use the MCSGP wizard to convert the single column batch data to an MCSGP method. The 3rd and final step is to run the process, demonstrate equilibrium and to evaluate the performance characteristics for the MCSGP process.

YMC America
8 Charlestown St.
Devens, MA 01434

JPreston@YMCAmerica.com