APPLICATIONS

Change Selectivity, Not the Stationary Phase, for Synthetic Peptide Multi-Step Purifications

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In this technical note, we present peptide purifications that use multi-step chromatographic processes accomplished with a single stationary phase for increased efficiency with time and cost savings.

Introduction

Natural peptides are short polymers made by linking amino acids and there is a growing interest in using peptides as therapeutic agents. The techniques necessary for large scale manufacturing of these peptides are well developed and require purification to obtain the desired target component(s). Crude synthetic peptide mixtures contain many closely related components and it is difficult, if not impossible, for one purification process to separate the desired component from the mix of the other components. Often these purifications are multiple-step, but chromatography is always used for at least one or more of these steps. Peptides are also well suitable for UV detection, which is convenient for chromatographic purifications.

Selectivity is the ability of a chromatographic method to separate a mixture into its different components. When multiple chromatographic steps are employed, the conventional idea is to use two or more complementary forms of chromatography such as ion-exchange, gel permeation, affinity, or reversed phase separations to take advantage of their different selectivities. Changing the stationary phase is an effective way to obtain different selectivities, but this can be both expensive and time consuming in a large scale purification process. Fortunately the very nature of peptides allows for changes in selectivity based on changes in pH or the choice of organic solvent used in the separation. These changes are relatively inexpensive and easily implemented utilizing a single column approach.

Amino acids are categorized by their side chains as non-polar, hydrophobic, polar uncharged, 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 with ionization being the most significant property. Some amino acids have both acidic and basic ionizable side chains so 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 silica is neutral or negatively charged. How the peptides interact with the stationary phase can change based on the type(s) of organic solvents used in the eluent. This is related to the polarity of the peptide and is mainly due to the non-ionizable side chains.

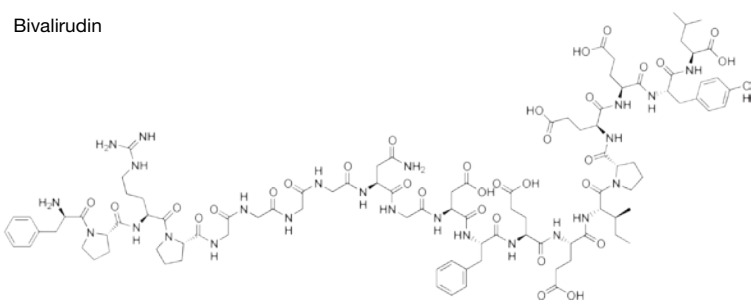
In this technical note, we present peptide purifications that use multi-step chromatographic processes accomplished with a single stationary phase for increased efficiency with time and cost savings. The peptides used for this work are Bivalirudin and Terlipressin as depicted in **Figure 1**.

Table 1. Natural Amino Acids Classification

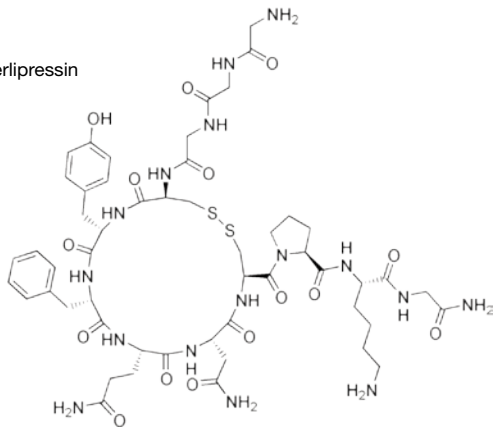
Non-polar hydrophobic residues	Polar uncharged residues	Positively charged residues (basic)
Alanine	Asparagine	Arginine
Isoleucine	Cysteine	Histidine
Leucine	Glutamine	Lysine
Methionine	Glycine	**Negatively charged residues (acidic)**
Phenylalanine	Serine	
Proline	Threonine	Aspartic acid
Tryptophan	Tyrosine	Glutamic acid
Valine		

Figure 1. Chemical Structures for Bivalirudin and Terlipressin

Bivalirudin



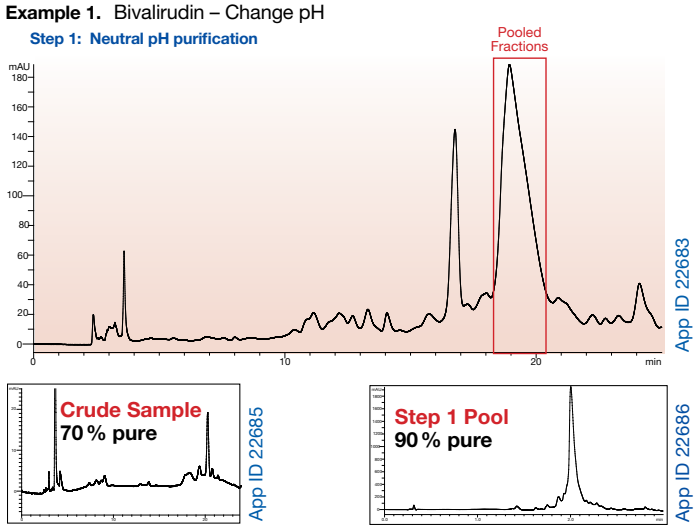
Terlipressin

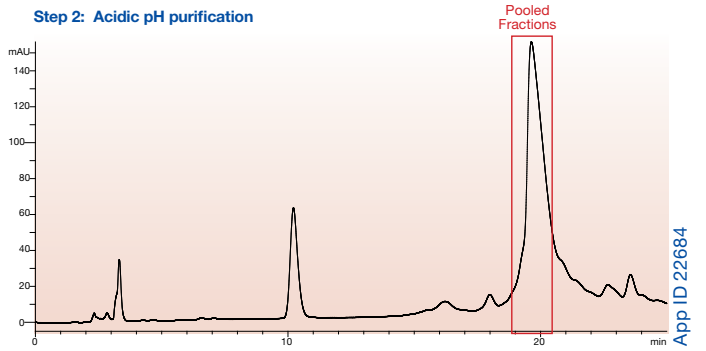


Results and Discussion

Example 1 using crude Bivalirudin demonstrates how effective a multi-step purification can be performed when the pH is changed from one step to another, even when the eluent uses the same organic component. Bivalirudin has an isoelectric point of 3.41 and has a significant percentage of amino acids with acidic side chains (See **Table 2**). When the eluent has neutral pH, both the acidic side chains and the silica are charged. Under these conditions, the stationary phase interactions are significantly influenced by these charged species. When the eluent has low pH, both the acidic side chains and the silica are uncharged. The hydrophobic and polar uncharged side chains of Bivalirudin have significant interactions with the stationary phase.

Example 1. Bivalirudin – Change pH





Step 1 Conditions

Column: Luna® 10 µm-*PREP* C18(3)
Dimensions: 250 x 4.6 mm
Part No.: 00G-4616-E0
Mobile Phase: A: 20 mM Potassium phosphate pH 7.0
B: Acetonitrile

Gradient:	Time (min)	B (%)
	0	10
	2.5	10
	22.5	20
	30.0	20

Flow Rate: 1.0 mL/min
Temperature: Ambient
Detection: UV @ 254 nm
Sample: Bivalirudin Initial Material

Step 2 Conditions

Mobile Phase: A: 0.1 % TFA in Water
B: Acetonitrile

Gradient:	Time (min)	B (%)
	0	10
	5	10
	20	20
	25	20

Sample: Bivalirudin Step 1 Product

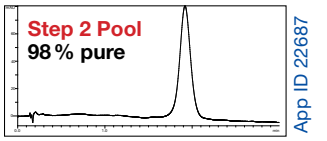


Table 2. Amino Acids Distribution in Bivalirudin and Terlipressin

	Isoelectric point	Acidic side chains	Basic side chains	Non-polar side chains	Polar uncharged side chains
Example 1 Bivalirudin	3.41	5	1	7	7
Example 2 Terlipressin	9.75	0	1	4	7

Analytical Methods

HPLC System: Agilent 1100
Analytical QC Conditions:

Example 1: Bivalirudin – Change pH

Column: Kinetex® 2.6 µm XB-C18 (App ID: 22685)
Dimensions: 50 x 4.6 mm
Part No.: 00B-4496-E0
Mobile Phase: A: 0.1 % TFA in Water
B: Acetonitrile

Gradient:	Time(min)	B (%)
	0.0	10
	2.75	10
	22.5	20
	25	20

Flow Rate: 1.0 mL/min
Temperature: Ambient
Detection: UV @ 220 nm
Sample: Bivalirudin

App ID: 22686 and 22687 Conditions same as above, except as noted:

Gradient:	Time(min)	B (%)
	0.0	10
	0.25	10
	1.0	30
	1.75	30
	2.0	40

Flow Rate: 2.0 mL/min

Example 2: Terlipressin – Change Organic

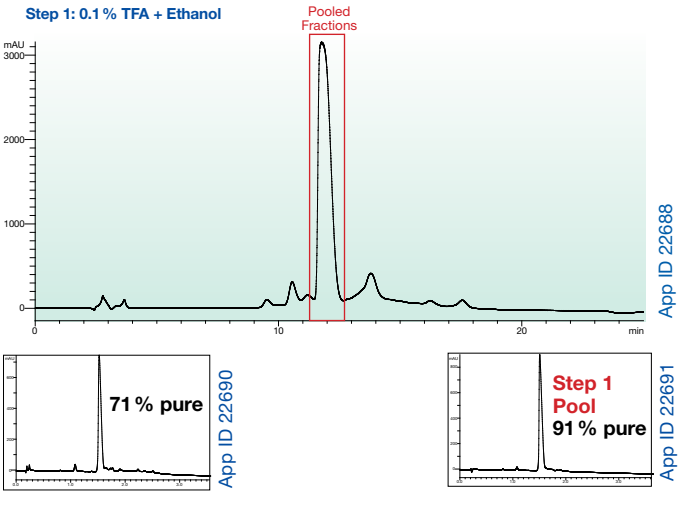
Column: Kinetex 5 µm C18 (App ID: 22690) (App ID: 22691) (App ID: 22692)
Dimensions: 50 x 4.6 mm
Part No.: 00B-4601-E0
Mobile Phase: A: 0.1 % TFA in Water
B: Acetonitrile

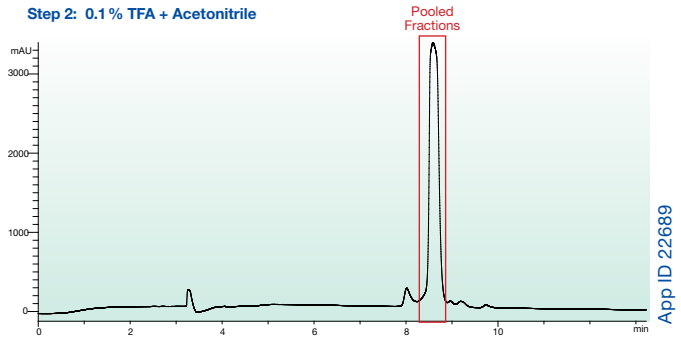
Gradient:	Time(min)	B (%)
	0	10
	3	40
	3.1	10
	5	10

Flow Rate: 2.0 mL/min
Temperature: Ambient
Detection: UV @ 210 nm
Sample: Terlipressin

Example 2 using crude Terlipressin demonstrates how effective a multi-step purification can be when the type of organic solvent used in the eluent is changed from one step to another, even when the pH remained constant. Terlipressin has an isoelectric point of 9.75 and is mostly comprised of non-polar and polar uncharged side chains (See **Table 2**). When the eluent has low pH, the peptide interacts well with the uncharged silica and stationary phase. The chromatographic behavior of the peptide differs when the strong solvent in the eluent is capable of hydrogen bonding like Ethanol and not with Acetonitrile. This is most likely due to the significant amount of uncharged polar side chains.

Example 2: Terlipressin – Change Organic



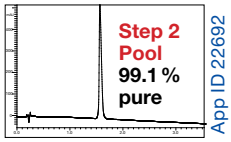


Step 1 Conditions

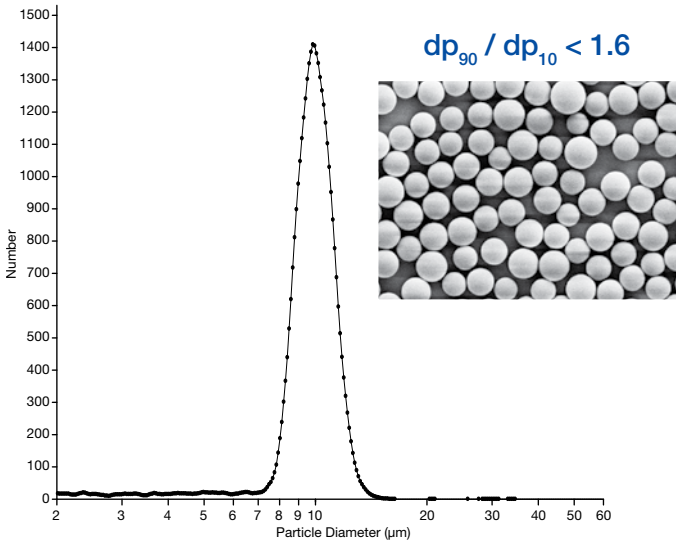
Column:	Luna® 10 µm-*PREP* C18(3)
Dimensions:	250 x 4.6 mm
Part No.:	00G-4616-E0
Mobile Phase:	A: 0.1 % TFA in Water B: Ethanol
Gradient:	**Time (min) B (%)** 0 10 20 40
Flow Rate:	1.0 mL/min
Temperature:	Ambient
Detection:	UV @ 210 nm
Sample:	Terlipressin Initial Material

Step 2 Conditions

Mobile Phase:	A: 0.1 % TFA in Water B: Acetonitrile
Gradient:	**Time (min) B (%)** 0 5 20 20 21 5 25 5
Sample:	Terlipressin Step 2 Product



Graph 1. Luna(3) Particle Size Distribution Curve



Conclusion

The column used for large scale purifications can be a significant part of the total cost of the purification process. This cost is not solely based on the stationary phase inside the column but also needs to include the cost of the hardware and the time needed to pack and unpack these columns. As shown, a considerable amount of time and expense can be saved by using the same column for each step in a multi-step purification process while only modifying the mobile phase. The two examples presented in this technical note utilize the Luna 10 µm-*PREP* C18(3) stationary phase. The Luna(3) media has a narrower particle size distribution (See **Graph 1**) providing superior performance with lower backpressure over the previous Luna and Luna(2) media. This new Luna(3) media offers a more uniform, stable, and reproducible chromatography bed that results in longer lifetime with increased productivity. These examples demonstrate the versatility achievable with a single stationary phase and column in a multi-step purification process for better throughput and overall total economy.

APPLICATIONS

Ordering Information

Luna 10 µm-*PREP*	Scout Columns		HPLC Bulk Media		
Phases	**250 x 4.6 mm**	**250 x 10 mm**	**1 kg**	**5 kg**	**10 kg**
C18(3)	00G-4616-E0	00G-4616-N0	04K-4616	04L-4616	04M-4616
C8(3)	00G-4623-E0	00G-4623-N0	04K-4623	04L-4623	04M-4623

Contact your Phenomenex technical consultant or local distributor for additional bulk packings and quantities not listed.

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