

Peptide Purification Method Development: Application for the Purification of Bivalirudin

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In this technical application, we present the purification method development and scale up experiments performed on a crude sample of Bivalirudin prepared by solid phase synthesis.

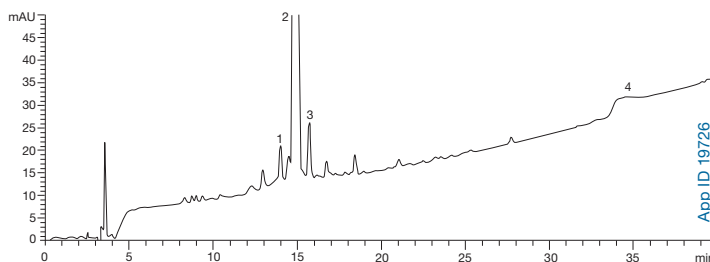
Introduction

The Active Pharmaceutical Ingredient (API), Bivalirudin shown in **Figure 1** is a 20 amino acid peptide containing one basic and 5 amino acid residues. With a chemical formula of $C_{98}H_{138}N_{24}O_{33}$ and a molecular weight of 2180.3 g/mol, Bivalirudin is also known as Angiomax. It is a direct thrombin inhibitor indicated for use as an anticoagulant.

We screened the following silica-based stationary phases: Luna[®] C5, Luna C8(2), Luna C18(2), Luna Phenyl-Hexyl, Gemini[®] C18, and Synergi[™] Polar-RP, as well as, the following buffers: trifluoroacetic acid (TFA), triethyl ammonium phosphate (TEAP), ammonium acetate, and potassium phosphate. The result of the phase screening, buffer used, and the scale up experiment for the best tested condition are presented.

Several reversed phase (RP) sorbents were tested in combination with various chromatographic conditions for the purification of Bivalirudin, a fairly hydrophilic peptide that elutes at 27 % of Acetonitrile on a C18 bonded sorbent. The crude peptide was synthesized by solid phase peptide synthesis (SPPS) and after TFA cleavage the crude peptide was obtained with a crude purity of 71.3 % as shown in **Figure 2**. The sample was produced by CS Bio, Co. and the synthesis was not optimized.

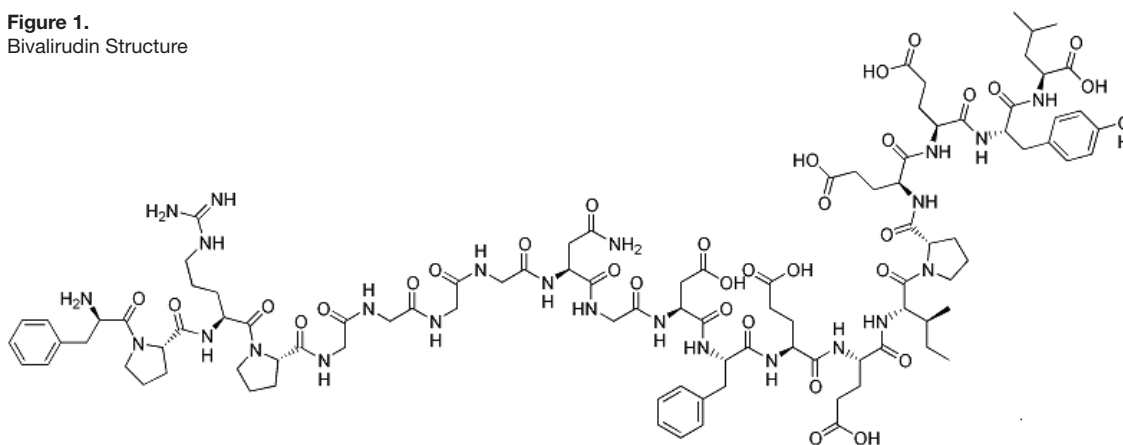
Figure 2.
Bivalirudin – Crude Purity



Peak No.	Time (min)	Area	Area %
1	14.01	547	9.7
2	14.87	3611	71.3
3	15.53	652	13.1
4	35.90	258	5.9

Column: Luna 5 μ m C8(2)
Dimensions: 250 x 4.6 mm
Part No.: 00G-4249-E0
Mobile Phase: A: 0.1 % TFA in Water
 B: 0.1 % TFA in Acetonitrile
Gradient: 20-60 % B in 40 min, hold 80 % B for 5 min
Flow Rate: 1 mL/min
Temperature: 25 °C
Detection: UV @ 220 nm
Injection Volume: 5 μ L
Sample Concentration: 1.5 mg/mL in water

Figure 1.
Bivalirudin Structure



H-D-Phe-Pro-Arg-Pro-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH

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Material and Methods

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were purchased from EMD (San Diego, CA, USA). The crude bivalirudin sample was kindly provided by CS Bio, Co. (Menlo Park, CA, USA). All HPLC columns were obtained from Phenomenex® (Torrance, CA, USA). All chromatography were performed on an Agilent® 1100 HPLC system from Agilent Technologies (Santa Clara, CA, USA) equipped with a quaternary pump, degasser, variable UV wavelength detector and autosampler.

Results and Discussion

Screening Experiments

Based on the peptide molecular weight and hydrophilicity, the following sorbents were tested for selectivity: Luna C5, Luna C8(2), Luna C18(2), Luna Phenyl-Hexyl, Gemini C18, and Synergi Polar-RP.

For screening purposes, sorbents with particle size of 5 µm and 4 µm were chosen. Using a column selector, we were able to evaluate 6 sorbents with a variety of mobile phases in less than 24 hours. Peptide purification is a complex process where a number of chemical additives can be added to the mobile phases in order to improve the separation of impurities from the targeted product. In **Table 1** are listed some of the most common mobile phase additives used in peptide purification as well as the recommended operational pH.

Table 1.
Mobile Phase Additive and pH for Peptide Purification

Compound	Formula	pK _a	Recommended pH
TFA	CF ₃ CO ₂ H	0.3	1.5-2.3
MSA*	CH ₃ SO ₃ H	-1.9	1.5-2.2
Formic acid	HCO ₂ H	3.8	2.8-4.8
Acetic acid	CH ₃ CO ₂ H	4.8	3.8-5.8
TEAP (Phosphate 1)	TEAH ₂ PO ₄	2.2	1.5-3.2
TEAP (Phosphate 2)	(TEA) ₂ HPO ₄	7.2	6.2-8.2
Potassium phosphate	KH ₂ PO ₄	7.2	6.2-8.2
Ammonia	NH ₄ OH	9.2	8.2-9.0
Ammonium acetate	NH ₄ CH ₃ CO ₂	9.2	6.0-9.0
Ammonium bicarbonate	NH ₄ HCO ₃	9.2	7.8-9.0
Ammonium formate	NH ₄ HCO ₃	9.2	7.8-9.0

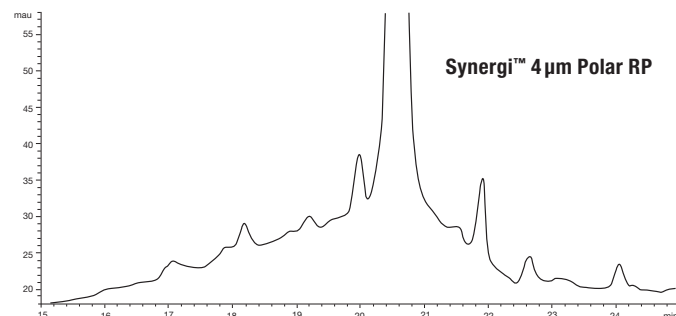
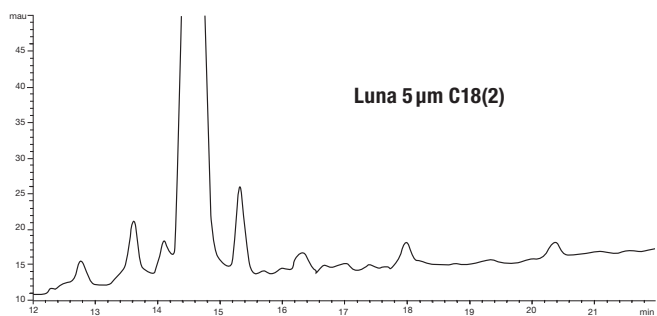
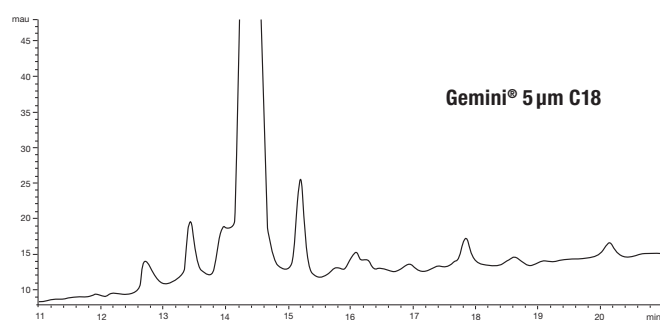
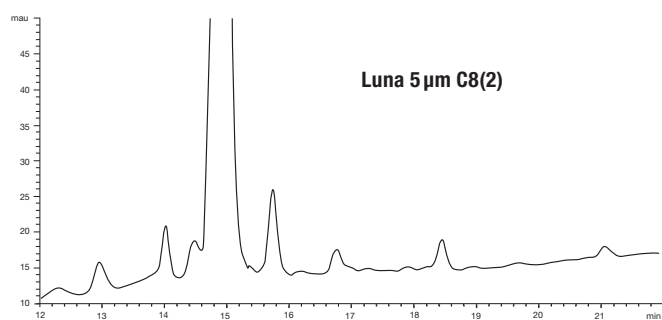
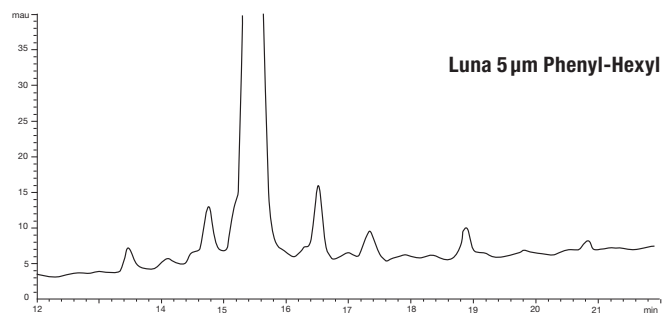
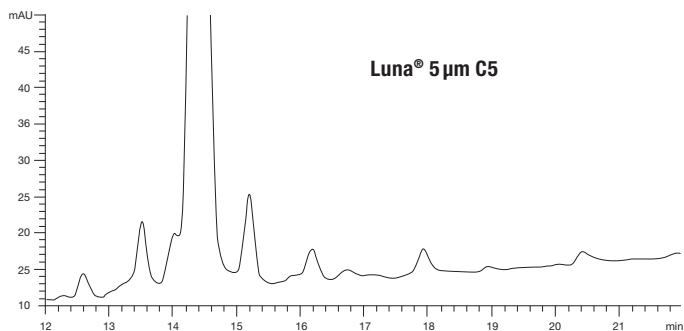
*MSA = methanesulfonic acid

The mobile phase additives tested for in this purification development study were TFA, TEAP, potassium phosphate, and ammonium acetate respectively presented in **Figures 3, 4, 5, and 6**. Each chromatogram represented is an enlargement of the region of interest. The results suggest that the best separation is achieved on Luna C8(2) and Luna C18(2) using ammonium acetate pH 4.7 or potassium phosphate pH 7 as mobile phase additives as depicted respectively in **Figures 5 and 6**.

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Figure 3.
0.1% TFA Screening Results

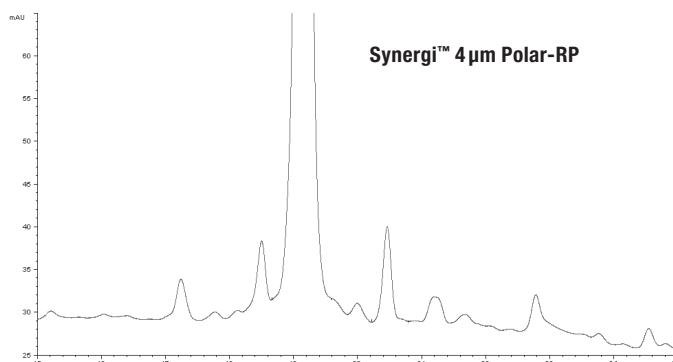
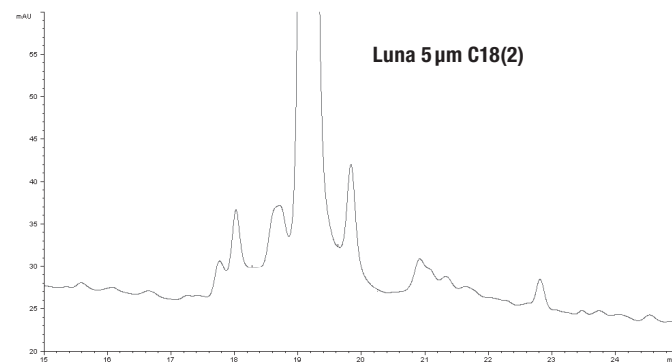
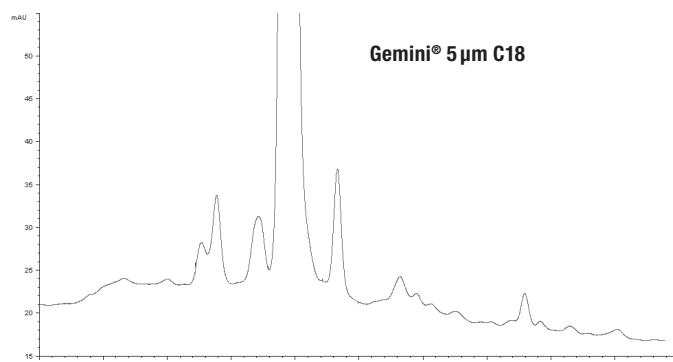
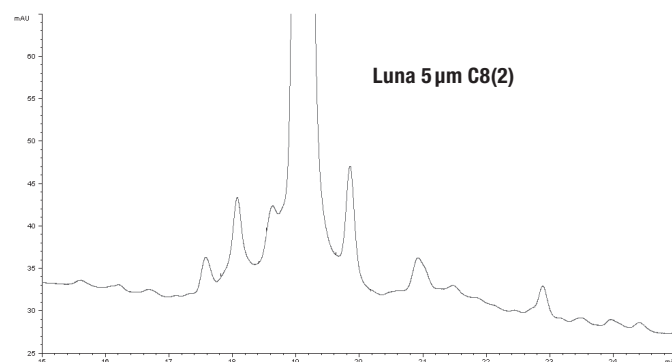
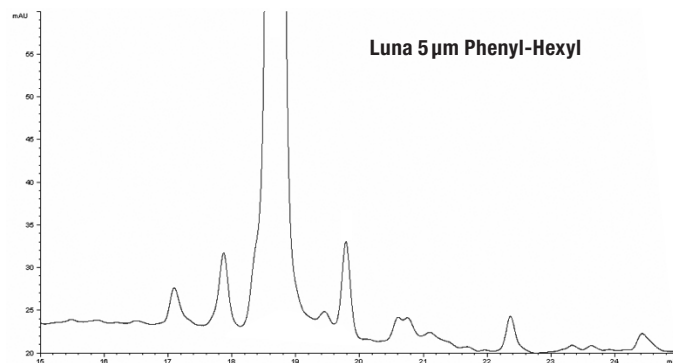
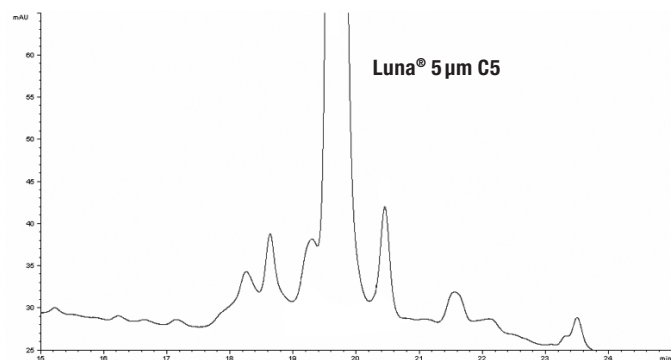


Column: As noted
Dimensions: 250 x 4.6 mm
Mobile Phase: A: 0.1% TFA in Water
B: 0.1% TFA in Acetonitrile
Gradient: 20-60 % B in 40 min (except for Synergi Polar-RP with 15-55 % B in 40 min) to 80 % in 5 min
Flow Rate: 1 mL/min
Temperature: 25 °C
Detection: UV @ 220 nm
Injection Volume: 5 μL
Sample Concentration: 1.5 mg/mL in water

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Figure 4.
20 mM TEAP pH 2.3 Screening Results

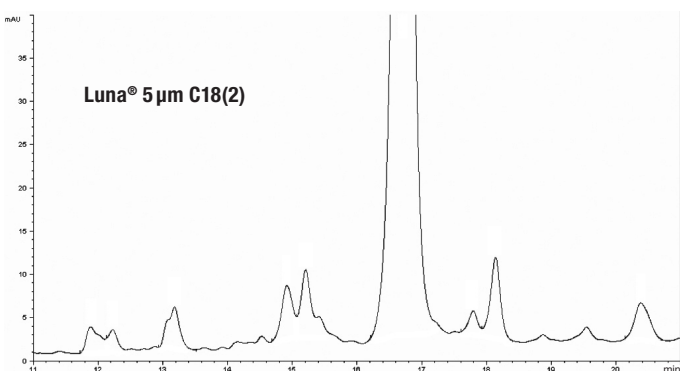
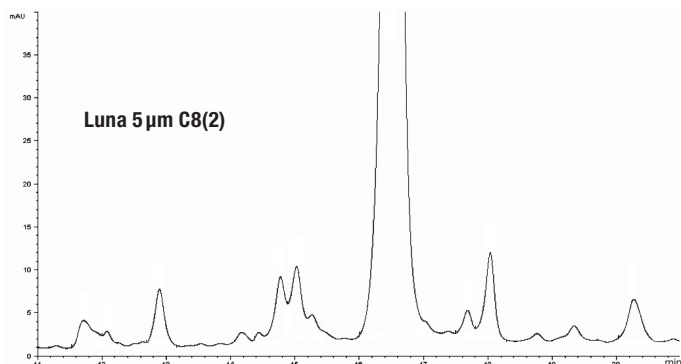


Column: As noted
Dimensions: 250 x 4.6 mm
Mobile Phase: A: 150 mL of Acetonitrile in 1350 mL of 20 mM (TEAP) pH 2.3
B: 900 mL of Acetonitrile in 600 mL of 20 mM (TEAP) pH 2.3
Gradient: 10-90 % B in 40 min
Flow Rate: 1 mL/min
Temperature: 25 °C
Detection: UV @ 220 nm
Injection Volume: 5 μL
Sample Concentration: 1.5 mg/mL in water

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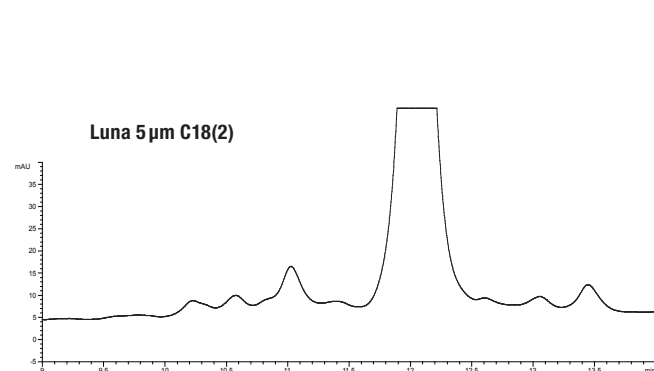
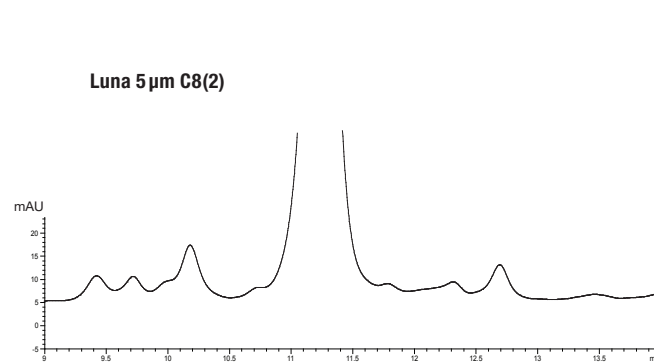
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Figure 5.
20 mM Potassium Phosphate pH 7 Results



Column: As noted
Dimensions: 250 x 4.6 mm
Mobile Phase: A: 20 mM Potassium phosphate pH 7 in Water
 B: 0.1 % TFA in Acetonitrile
Gradient: 5-45 % B in 40 min
Flow Rate: 1 mL/min
Temperature: 25 °C
Detection: UV @ 220 nm
Injection Volume: 5 µL
Sample Concentration: 1.5 mg/mL in water

Figure 6.
20 mM Ammonium Acetate pH 4.7 Results



Column: As noted
Dimensions: 250 x 4.6 mm
Mobile Phase: A: 20 mM Ammonium acetate pH 4.7 in Water/Acetonitrile (1:9)
 B: 20 mM Ammonium acetate in Water/Acetonitrile (1:1)
Gradient: 0 to 100 % B in 40 min
Flow Rate: 1 mL/min
Temperature: 25 °C
Detection: UV @ 220 nm
Injection Volume: 5 µL
Sample Concentration: 1.5 mg/mL in water

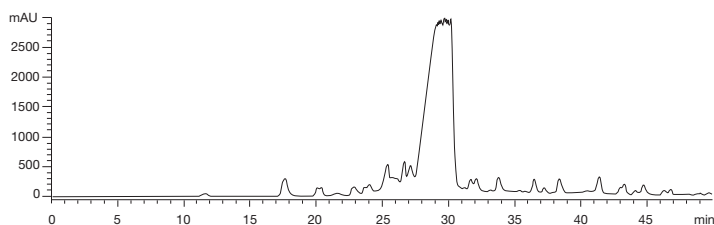
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Overload Experiments

The targeted purity for the final product was an overall purity greater than 98.0 % with no single impurity greater than 1.0 %. Based on the preliminary screening experiments, overload experiments were performed on Luna® C8(2) 100 Å 10µm-*PREP* 250 x 4.6 mm column. For purification development, it is best to use the same column length and particle size than the ones intended for scale up to a large column. The elution profile for the overload experiment using ammonium acetate at pH 4.7 is shown in **Figure 7**. The purification fractions collected were analyzed on a Luna C8(2) 5µm 250 x 4.6 mm column and the results are shown in **Table 2**.

Figure 7.
Purification Elution Profile at 1.5 % Specific Load



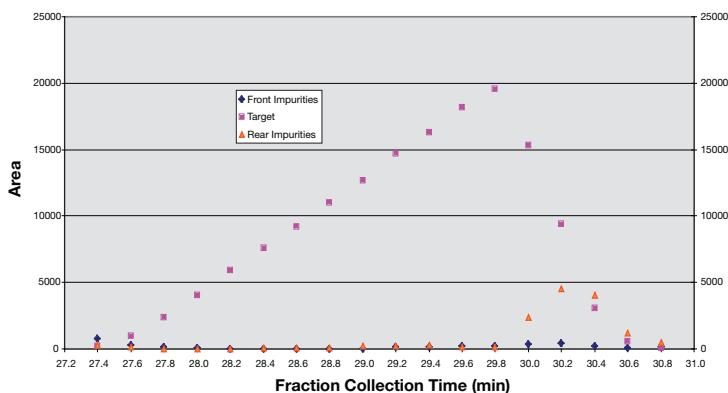
Column: Luna 10µm-*PREP* C8(2)
Dimensions: 250 x 4.6 mm
Part No.: 00G-4324-E0
Mobile Phase: A: 100 mM Ammonium acetate pH 4.7 in Water
 B: Acetonitrile
Gradient: 10 to 50 % B in 40 min; hold at 80 % B and 20 % C for 5 min; re-equilibration at 90 % A and 10 % for 10 min
Flow Rate: 1 mL/min
Temperature: 25 °C
Detection: UV @ 280 nm
Injection Volume: 5 mL
Sample Concentration: 7 mg/mL in water

Table 2.
Purification - Fraction Analysis

RT (min)	Front Impurities (Area)	Target Compound (Area)	Rear Impurities (Area)
27.4	781	200	296
27.6	247	985	122
27.8	107	2397	0
28.0	59	4012	0
28.2	0	5890	0
28.4	0	7619	39
28.6	0	9170	39
28.8	0	11003	74
29.0	0	12657	224
29.2	125	14659	238
29.4	151	16303	245
29.6	183	18191	158
29.8	190	19599	114
30.0	370	15293	2339
30.2	451	9379	4548

Analytical HPLC Conditions:

Same conditions as **Figure 1** using Luna 5µm C8(2) 250 x 4.6 mm column



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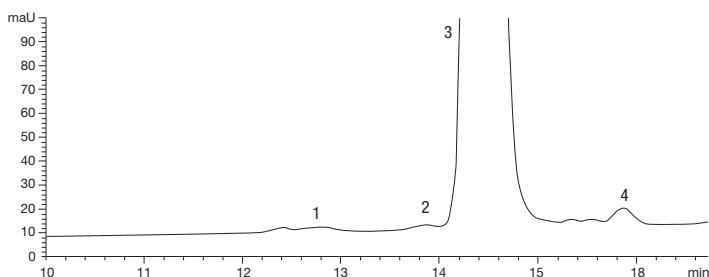
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Fractions with purity > 96 % were combined and pooled to recover 80 % of the peptide loaded with an overall purity of 98.53 % with the greatest single impurity at 0.93 % as demonstrated in **Figure 8**. Similar overloading experiments were performed using potassium phosphate producing lower recovery for the same purity criteria (data not shown).

Figure 8.
Purification Recovery and Final Product Purity



11 Combined fractions 27.8 – 29.8 min;
Recovery 80.5 % with purity ≥ 98.5 %

Peak No.	Time (min)	Area	Area %
1	12.74	73.7	0.35
2	13.83	40.6	0.19
3	14.37	21118.7	98.53
4	15.858	200.5	0.93

Conclusions

Crude Bivalirudin obtained by SPPS with a crude purity of 71.3 % was purified by reversed phase chromatography using Luna C8(2) 100 Å 10 µm-PREP as sorbent. The best recovery and throughput was achieved by using ammonium acetate at pH 4.7 as a mobile phase which allowed us to prepare a Bivalirudin sample with purity greater than 98.0 % and a recovery of 80 % in a single stage purification. Additionally, it is important to point out that the final product was prepared as an acetate salt form which most of the time is the required salt form used as a peptide pharmaceutical ingredient.

Ordering Information

Scout Columns (mm)

Luna (100 Å)		
Phases	250 x 4.6	250 x 10
10 µm-PREP		
C18(2)	00G-4324-E0	00G-4324-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
Silica(2)	00G-4322-E0	00G-4322-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	00G-4271-N0

Synergi (80 Å)		
Phases	250 x 4.6	250 x 10
10 µm		
Polar-RP	00G-4351-E0	00G-4351-N0

Gemini (110 Å)		
Phases	250 x 4.6	250 x 10
10 µm		
C18	00G-4436-E0	00G-4436-N0

Bulk Media

Luna (100 Å)							
Phases	100 g	1 kg	5 kg	10 kg	50 kg	100 kg	
10 µm-PREP							
C18(2)	04G-4324	04K-4324	04L-4324	04M-4324	04N-4324	04P-4324	
C8(2)	04G-4323	04K-4323	04L-4323	04M-4323	04N-4323	04P-4323	
C4(2)	04G-4460	04K-4460	04L-4460	04M-4460	04N-4460	04P-4460	
Phenyl-Hexyl	04G-4325	04K-4325	04L-4325	04M-4325	04N-4325	04P-4325	
Silica(2)	04G-4322	04K-4322	04L-4322	04M-4322	04N-4322	04P-4322	
15 µm							
C18(2)	04G-4273	04K-4273	04L-4273	04M-4273	04N-4273	04P-4273	
C8(2)	04G-4272	04K-4272	04L-4272	04M-4272	04N-4272	04P-4272	
Phenyl-Hexyl	04G-4286	04K-4286	04L-4286	04M-4286	04N-4286	04P-4286	
Silica(2)	04G-4271	04K-4271	04L-4271	04M-4271	04N-4271	04P-4271	

Synergi (80 Å)							
Phases	100 g	1 kg	5 kg	10 kg	50 kg	100 kg	
10 µm							
Polar-RP	04G-4351	04K-4351	04L-4351	04M-4351	04N-4351	04P-4351	

Gemini (110 Å)				
Phases	100 g	1 kg	5 kg	10 kg
10 µm				
C18	04G-4436	04K-4436	04L-4436	04M-4436

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Ordering Information

Axia™ Packed Preparative HPLC Columns (mm)

Luna (100 Å)				
Brand	Phase	250 x 21.2	250 x 30	250 x 50
Luna	10 µm C5	00G-4092-P0-AX	00G-4092-U0-AX	00G-4092-V0-AX
Luna	10 µm PREP C18(2)	00G-4324-P0-AX	00G-4324-U0-AX	00G-4324-V0-AX
Luna	10 µm PREP C8(2)	00G-4323-P0-AX	Inquire	00G-4323-V0-AX
Luna	10 µm PREP Phenyl-Hexyl	00G-4325-P0-AX	Inquire	Inquire
Synergi	10 µm Polar-RP	00G-4351-P0-AX	00G-4351-U0-AX	00G-4351-V0-AX
Gemini	10 µm C18	00G-4436-P0-AX	00G-4436-U0-AX	00G-4436-V0-AX

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