# Development of a novel combined IEX-RP chromatographic process for the purification of bivalirudin

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**A novel coupled ion exchange and reverse phase chromatographic process was investigated in the purification of bivalirudin from a semi-crude mixture using Purolite Chromalite® resins. The highly hydrophobic backbone of the ion exchange resin Chromalite® PCG1200FS contributes to the purification by means of a pseudo-mixed mode purification. The peptide bivalirudin was purified from an initial 80-85 % sample to >99 % purity. The process also shows excellent recovery of the peptide samples, with >99 % yield obtained.**

## Introduction

Peptides represent a highly valuable section of the pharmaceutical market, making up 13% of newly FDA-approved drugs in 2017 [1]. However, the highly regulated nature [2] of pharmaceuticals requires the careful and thorough purification of these therapeutic agents. Additionally, as these drugs are not orally bioavailable [3], they must be directly injected - again mandating a highly pure compound. The biological aspect of peptides is responsible both for a high level of specificity and activity in treating conditions, however these also result in strong off-target effects if insufficiently purified or conjugated in the case of peptide conjugates [3, 4].

Separation of therapeutic peptides often relies heavily on reversed-phased HPLC (RP-HPLC), which is the most commonly used method in both the development and production of these species [5]. This method can be inefficient, however, as similarities between peptides produced industrially and their synthetic by-products result in challenging purifications [6]. The small differences in affinity for the stationary phase between the target peptide and by-products, as well as long retention times [7], and a significant effect of small changes in the mobile phase composition on the retention, further complicate purification [8].

A common solution to increase the separating power of RP-HPLC is to make use of an ion-pairing agent, such as trifluoroacetic acid, and conduct the separation at low pH; this coupled basic amino acids with the ion pairing agent, results in a net zero charge - increasing the affinity of the peptides to the stationary phase [6]. Currently, ion exchange purification is generally avoided due to the difficulty in removing polar non-peptide impurities [9] as the elution of peptides will generally occur alongside that of some quantity of these impurities. Additionally, it can be challenging to ensure the removal of peptide by-products due to the close relation in charge between these molecules [10].

The use of a highly hydrophobic cation ion exchange stationary phase offers the possibility to carry out the ion-pairing process in reverse - the basic amino acids in the peptide pair with the acidic groups on the resin, while the hydrophobic backbone stabilises the interaction and exploits the 'reverse-phase'-like attraction between the non-polar areas of the peptides and the resin to increase separation. This approach also facilitates the removal of upstream impurities, including host cell proteins and DNA/RNA, as well as offering the possibility for virus clearance due to the low pH of the process [5].

## Experimental

### Resin Data

| Chromalite® | PCG1200FS | 15AD2 |
|---|---|---|
| Particle Size | 20 - 50 µm | 12 - 18 µm |
| Pore Diameter | 300 - 500 Å | 200 - 300 Å |
| Surface Area | >600 $m^2/g$ | >500 $m^2/g$ |
| Functional Group | Sulfonic Acid | None |
| Volume Capacity | 0.69 eq/L | N/A |
| Polymer Backbone Basis | Styrene/DVB | Styrene/ DVB |

### Cation Exchange Chromatographic Purification

Described here is the full purification procedure following all development work: A GE Tricorn 5/200 column was packed with Purolite Chromalite® PCG1200FS resin according to standard practice, with an asymmetry of 0.8-1.8 as acceptable. The column was equilibrated with 5 column volumes (CV) of 20 mM citrate, 150 mM NaCl, pH 2.5 then 4 mL of a 10 mg/mL solution of crude bivalirudin applied to the column. The column was then washed with 3 CV of 20 mM citrate, 150 mM NaCl, pH 2.5 then a step elution carried out with 10 CV of 20 mM citrate, 1 M NaCl, pH 5 followed by a further elution with 10 CV dH2O. The process was carried out at a linear velocity of 300 cm/hr throughout.

### Reverse Phase Chromatographic Purification

A GE Tricorn 5/200 column was packed with Purolite Chromalite® 15AD2 resin according to standard practice, with an asymmetry of 0.8-1.8 as acceptable. The column was equilibrated with 5 column volumes (CV) of 0.1% trifluoroacetic acid (TFA) then 4 mL of the purified solution of bivalirudin applied to the column. The column was then washed with 3 CV of 0.1% TFA followed by a gradient elution carried out over 10 CV to a final concentration of 0.1% TFA in 60% acetonitrile. A final hold was carried out with 3 CV 0.1% TFA in 60 % acetonitrile. The process was carried out at a linear velocity of 300 cm/hr throughout.

### HPLC Quantification of Bivalirudin

HPLC was performed using an Ascentis RP-Amide 5 µm, 250 x 4.6 mm column attached to a Perkin-Elmer Flexar HPLC system, with detection by UV absorption at 214 nm. The mobile phases used were:

Mobile Phase A: 150 mM triethylamine set to pH 3.0 with phosphoric acid

Mobile Phase B: Acetonitrile

The mobile phase was run at 1 mL / min at 25°C following the below gradient:

25 minutes 80% A

1 minute 70% A

2 minutes 50% A

3 minutes 80% A

A calibration curve was generated from an external standard for bivalirudin to allow the quantification of the recovery levels:

| Bivalirudin Concentration (mg/mL) | Peak Area |
|---|---|
| 1 | 8816916 |
| 0.75 | 6737482 |
| 0.5 | 4484665 |
| 0.25 | 2186559 |
| 0.1 | 905432 |
| 0 | 0 |

This gave the equation for determining bivalirudin concentration

Concentration (mg/mL) = (Peak Area * 0.0000001) - 0.0012

Recovery was defined as the proportion of bivalirudin present in the fractions as a percentage of the bivalirudin present in the load sample.

### Regeneration Method for Chromalite® PCG1200FS

The column was equilibrated with 5 CV of 1 M NaOH then continued until the UV absorbance at 280 nm was stable. The column was then washed with 5 CV of dH2O then continued until the UV absorbance at 280 nm was stable. The column was equilibrated with 5 CV of 1 M HCl then continued until the UV absorbance at 280 nm was stable. The column was then washed with 5 CV of dH2O then continued until the UV absorbance at 280 nm was stable.

### Results and Discussions

Initial purification efforts were focused on the use of a cation exchange resin to conduct an initial clean-up of a crude preparation of bivalirudin, a 2.2 kDa therapeutic peptide used as a thrombin inhibitor. These experiments were conducted using Chromalite® PCG1200FS, a styrene/DVB strong acid cation exchange resin of particle size 50 µm and a highly hydrophobic core that offers stabilisation of peptides after the initial ionic attraction.

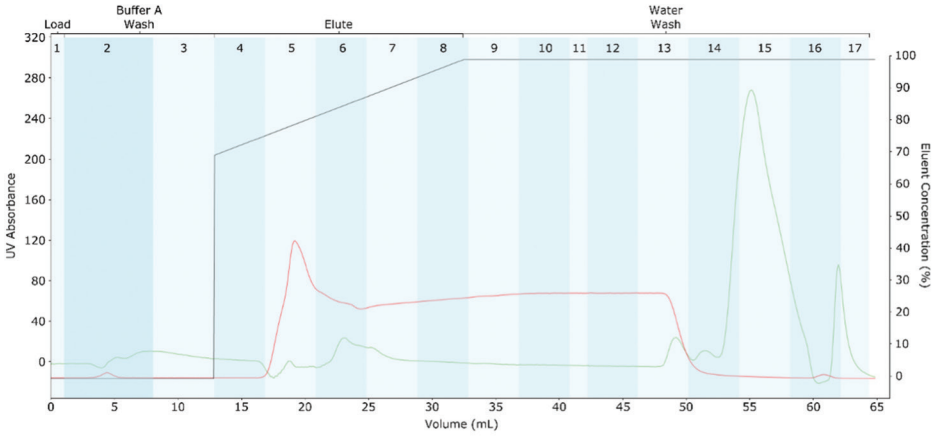


*Figure 1: Chromatogram of the purification of crude bivalirudin by Chromalite® PCG1200FS. UV absorbance at 280 nm shown in green, conductivity in red and % eluent in black. Fractions are identified by number at the top of the chromatogram.*

A preliminary gradient elution was conducted at loading of 2.5 mg bivalirudin per mL of resin and the UV trace is shown in Figure 1.

Interestingly, most of the elution occurs during the water wash. This indicates that the mode of binding is not only cation exchange, but that there is also some hydrophobic character to the binding that is not disrupted until the salt concentration in the eluent decreases sufficiently for elution. This is a common observation for Chromalite® resins, as they are designed to exploit the combination of ionic interactions for ion exchange purification, with a comparatively hydrophobic resin backbone to engage hydrophobic interactions simultaneously. The fractions 2, 6, 13, 14, 15, 16 and 17 were tested for bivalirudin content and purity by HPLC, giving the results reported in Table 1.

Purity is determined by comparing the proportion of bivalirudin peak area with that of all non-bivalirudin peaks, which is suitable as a first approximation of purity when standards cannot be generated for specific impurities. For example, fraction 15 has a total peak area (not shown in the table) of 4661753 and the peak area of bivalirudin (4482532) divided by the total peak area gives 0.96 or 96 % pure. This first test demonstrates the increase of the bivalirudin purity from 86 % in the crude sample to 96% in fraction 15. This shows both that the desired purity, >95% in the first 'capture' step, can be achieved by this method of purification and confirms that the bivalirudin is not eluted until the salt concentration is reduced, as very little bivalirudin is present in fractions other than 14-16.

Based on the observed elution from this initial test, the method was adjusted to a more straightforward stepwise elution process. In this experiment, 5 mg bivalirudin was loaded per mL resin and the resulting chromatogram is shown in Figure 2.

*Table 1: HPLC Data for fractions of crude bivalirudin purification by cation exchange.*

| Fraction | Bivalirudin Peak Area | Total Impurity Peak Area | Purity |
|---|---|---|---|
| Load | 1318733 | 206085 | 86% |
| 2 | 37682 | 88509 | 30% |
| 6 | 0 | 127158 | 0% |
| 13 | 38378 | 99853 | 28% |
| 14 | 708467 | 170344 | 81% |
| 15 | 4482532 | 179221 | 96% |
| 16 | 390895 | 210561 | 65% |
| 17 | 33811 | 263022 | 11% |

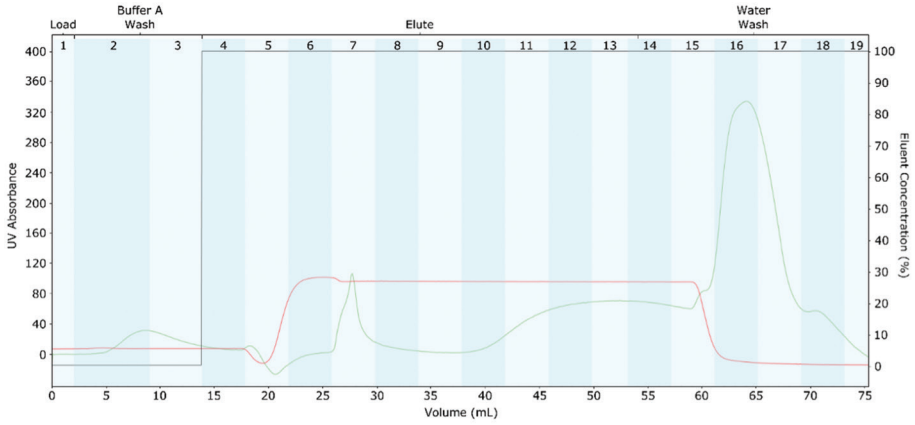


*Figure 2: Chromatogram of the purification of crude bivalirudin by Chromalite® PCG1200FS. UV absorbance at 280 nm shown in green, conductivity in red and % eluent in black. Fractions are identified by number at the top of the chromatogram.*

*Table 2: HPLC Data for fractions of crude bivalirudin purification by cation exchange*

| Fraction | Bivalirudin Peak Area | Total Impurity Peak Area | Purity | Bivalirudin (mg) | Recovery of Bivalirudin |
|---|---|---|---|---|---|
| Load | 58641336 | 11578990 | 84% | 5.86 | N/A |
| 2 | 5899 | 168763 | 3% | 0.00 | 0% |
| 7 | 0 | 250355 | 0% | 0.00 | 0% |
| 15 | 2312977 | 72130 | 97% | 0.92 | 16% |
| 16 | 11002671 | 464518 | 96% | 4.40 | 75% |
| 17 | 5077866 | 272681 | 95% | 2.03 | 35% |
| 18 | 245512 | 820174 | 23% | 0.10 | 2% |
| Total | | | | 7.45 | 128% |

*Table 3: HPLC Data for fractions of crude bivalirudin purification by cation exchange*

| Fraction | Bivalirudin Peak Area | Total Impurity Peak Area | Purity | Bivalirudin (mg) | Recovery of Bivalirudin |
|---|---|---|---|---|---|
| Load | 3198211* | 445213* | 88% | 12.75 | N/A |
| 2 | 220087 | 87182 | 72% | 0.15 | 1% |
| 7 | 0 | 159244 | 0% | 0.00 | 0% |
| 16 | 2852528* | 217750* | 93% | 11.36 | 89% |
| 17 | 2529208 | 1202963 | 68% | 1.01 | 8% |
| Total | | | | 12.52 | 98% |

** Diluted 1/10 for accurate quantification.*

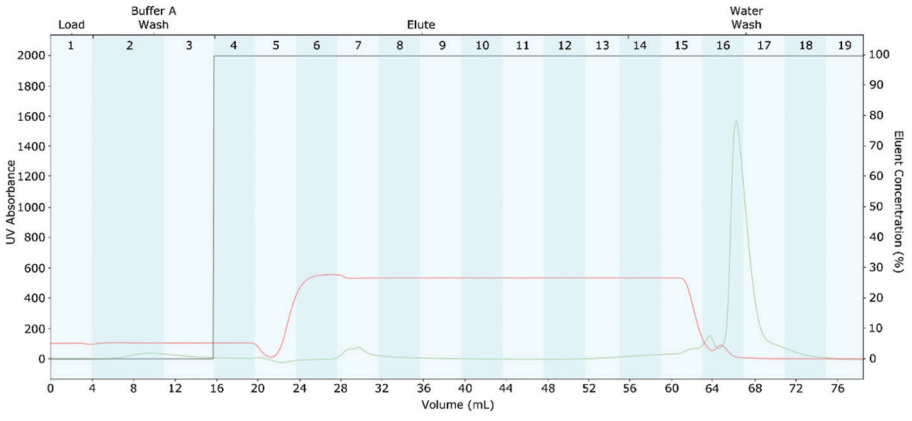


*Figure 3: Chromatogram of the purification of crude bivalirudin by Chromalite® PCG1200FS. UV absorbance at 280 nm shown in green, conductivity in red and % eluent in black. Fractions are identified by number at the top of the chromatogram.*

The elution is again observed in the water wash, in this case the increased load has resulted in a broadening of the elution peak as could be expected. The fractions 2, 7, 15, 16, 17 and 18 were tested for bivalirudin content and purity by HPLC, giving the results shown in Table 2 - a calibration was also carried out to quantify the amount of bivalirudin present and determine the % recovery of bivalirudin.

The three fractions present in the large peak, 15; 16 and 17, show a very high level of purity for bivalirudin and also recover a significant majority of the initial bivalirudin mass. The recovery above 100% is likely due to insufficient dilution of the load and fraction 16, which resulted in values outside the calibration range, where more error is likely to result.

Pooling of the fractions 15, 16 and 17 results in a fraction with recovery >90% and purity of 96%, shows a very good result, allowing for the erroneously high recovery observed due to insufficient dilution.

Following this good result, the method was carried out at the target loading of 10 mg bivalirudin per mL of resin. The chromatogram is shown in Figure 3.

In this case the increased load has resulted in the bivalirudin elution significantly eclipsing other peaks present in the crude mixture. The fractions 2, 7, 16 and 17 were tested for bivalirudin content and purity by HPLC, giving the results shown in Table 3.

The results show good recovery of bivalirudin from the crude mixture at a high level of purity. To maximise recovery, pooling of fractions 16 and 17 could be carried out, which would give a total recovery of 97% but would reduce the purity to 90% - which remains an excellent level of purity.

Based on these results, Chromalite® PCG1200FS represents a potentially well-suited resin to the initial purification of bivalirudin when used in a cation exchange purification as described above. The elution in the water wash is particularly useful, as this facilitates the direct application of the eluted bivalirudin fraction to a reverse phase process, without the need for a desalting step to remove interfering salts.

Following this cation exchange first step, a reverse phase polishing step was carried out using Chromalite® 15AD2, in order to gain further increases of purity with a target of >99% purity. A standard reverse phase gradient elution was carried out using 60% acetonitrile in 0.1% TFA in water and Figure 4 shows the chromatogram.

Significant fractionation of the remaining compounds can be observed, highlighting the need for the polishing step; fractions 7-13 were analysed for bivalirudin and impurity content (Table 4).

The results show a marked increase in the purity of bivalirudin from the IEX-purified sample, with purity > 99 % and recovery > 95%. A combined IEX-RP process can therefore be exploited for significant gains in the efficiency and cost of a peptide purification process.

To demonstrate that the purification could not be achieved without the initial ion exchange step, a comparison reverse phase-only purification of the crude bivalirudin sample was carried out using the Chromalite® 15AD2 column, Figure 5 shows the chromatogram.

In this purification, we can see that some separation is occurring, however there appear to be somewhat more species present in the solution than was observed in Figure 4. The fractions 8-15 were analysed by HPLC, shown in Table 5.

In this purification, only one fraction was purified to a greater standard than the loaded crude sample, and this represented <30 % of the recovered bivalirudin, indicating that the initial IEX step significantly contributes to the final purity of the bivalirudin.

## Conclusions

A novel combination of a low pressure IEX step followed by standard reverse phase polishing step in peptide purification was demonstrated using Chromalite® PCG1200FS and 15AD2. This simplified approach is both cost-effective and highly efficient when compared with a traditional RP-RP purification process, as the larger particle size of the IEX resin facilitates a higher flow rate and lower backpressure, which reduces both the cost of the resin and of the process equipment.

The flexibility of the Chromalite® range was demonstrated by the purification of bivalirudin from a semi-crude feed by a sulfonic acid-functionalised resin, with purity enhanced from ca. 80% to >90% with removal of organic scavengers and other aqueous impurities and complete recovery of the peptide loaded, followed by a reverse phase polishing step that increased the purity from >90% to >99%. A single reverse phase step following the same procedure as the polishing step could only improve the purity from 86% to 92% with a recovery of <30%.

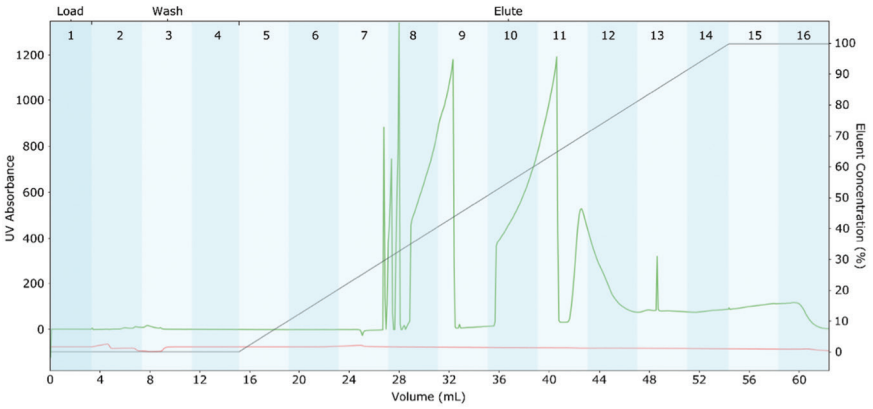


*Figure 4: Chromatogram of the purification of IEX-purified bivalirudin by Chromalite® 15AD2. UV absorbance at 280 nm shown in green, conductivity in red and % eluent in black. Fractions are identified by number at the top of the chromatogram.*

*Table 4: HPLC data for fractions of IEX-purified bivalirudin purification by reverse phase using Chromalite® 15AD2.*

| Fraction | Bivalirudin PA | Impurity PA | Purity | Bivalirudin (mg) | Recovery |
|---|---|---|---|---|---|
| Load | 1705140 | 106913 | 94.1% | 0.677 | |
| RP 7 | 0 | 5757 | 0% | 0 | 0% |
| RP 8 | 0 | 6045 | 0% | 0 | 0% |
| RP 9 | 0 | 5961 | 0% | 0 | 0% |
| RP 10 | 0 | 4577 | 0% | 0 | 0% |
| RP 11 | 1725051 | 12107 | 99.3% | 0.685 | 101% |
| RP 12 | 207443 | 43663 | 82.6% | 0.078 | 12% |
| RP 13 | 31799 | 6148 | 83.8% | 0.008 | 1% |
| Total | | | | 0.771 | 114% |

*Table 5: HPLC Data for fractions of crude bivalirudin purification by reverse phase*

| Fraction | Bivalirudin PA | Impurity PA | Purity | Bivalirudin (mg) | Recovery |
|---|---|---|---|---|---|
| Load | 5817532 | 946965 | 86% | 4.064 | |
| RP 8 | 0 | 4694 | 0% | 0 | 0% |
| RP 9 | 0 | 11973 | 0% | 0 | 0% |
| RP 10 | 0 | 61698 | 0% | 0 | 0% |
| RP 11 | 2717931 | 223177 | 92% | 1.082 | 27% |
| RP 12 | 8565461 | 1439039 | 86% | 3.421 | 84% |
| RP 13 | 524012 | 449183 | 54% | 0.205 | 5% |
| RP 14 | 56043 | 11342 | 83% | 0.018 | <1% |
| RP 15 | 1423 | 6707 | 18% | 0 | 0% |
| Total | | | | 4.726 | 116% |

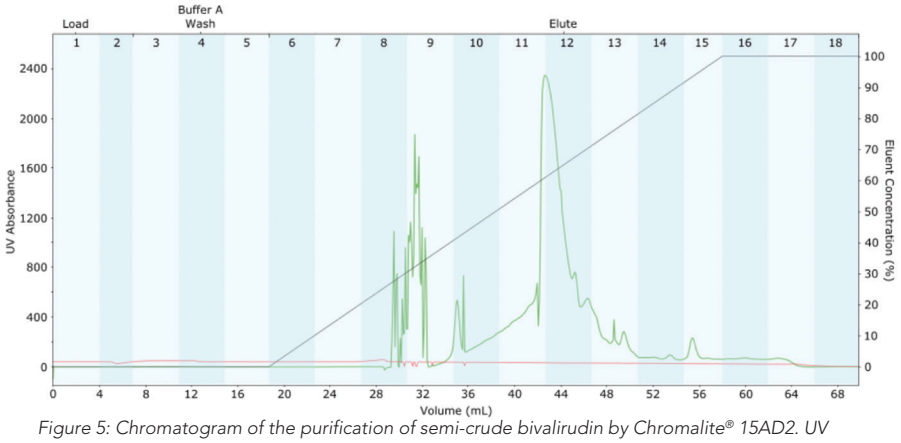


*Figure 5: Chromatogram of the purification of semi-crude bivalirudin by Chromalite® 15AD2. UV absorbance at 280 nm shown in green, conductivity in red and % eluent in black. Fractions are identified by number at the top of the chromatogram.*

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