A generic peptide screening method using 0.1% TFA for binding conditions in both hydrophilic and hydrophobic purification steps

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Reversed phase chromatography and its limitations

Reversed phase chromatography (RPC) is often the first-choice screening method for peptide purifications and results in high target purity at high yields in a single step. However, its drawbacks cannot be ignored (Fig. 1).

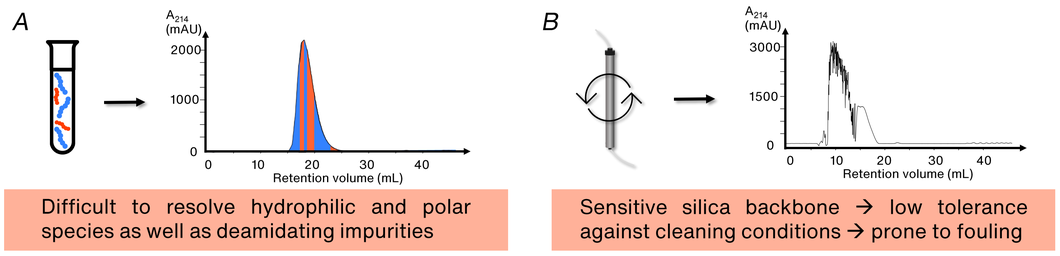

Figure 1. Illustration of the limitations around RPC. Difficult to resolve hydrophilic and polar species as well as deamidating impurities **(A)** and RPC being prone to fouling due to its low tolerance to harsh cleaning conditions **(B).**

Implementation of ion exchange chromatography (IEX)

An orthogonal hydrophilic IEX resin that tolerates harsh chemicals can be introduced upstream of the RPC column. For ease of performance, we used a traditional RPC binding buffer, 0.1% TFA, pH 2, including acetonitrile, when employing CIEX prior to RPC.

Method

Bivalirudin was purified using

- Stand-alone RPC *or*
- Stand-alone CIEX *or*
- CIEX + RPC

Analytical RPC was used for evaluation of the comparison and purification progress. The chromatographic conditions are laid out in Table 1.

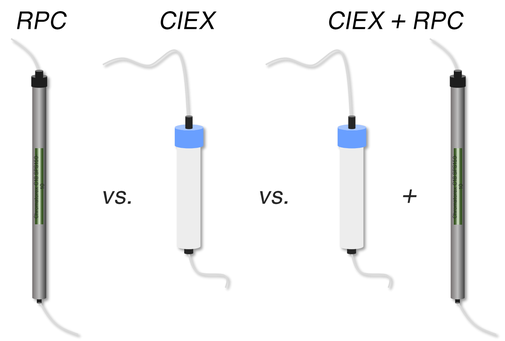

Figure 2. Stand-alone RPC *vs.* stand-alone CIEX *vs.* CIEX + RPC purification.

When operating at a low pH, unknown sequences are usually bound to the CIEX resin (sulphonate ligand) due to amino acids becoming protonated.

Table 1. Chromatographic running conditions.

Semi-preparative RPC	Chromatorex C18 SPS150-10, 10 µm, 4.6x250 mm
Analytical RPC	Ascentis® ES-C18, 2.7 µm, 2.1x150 mm
CIEX	WorkBeads™ 40S, 45 µm, 6.6x100 mm
RPX & CIEX binding buffer	0.1% TFA, 5% ACN, pH 2
RPC elution buffer	0.1% TFA, 80% ACN, pH 2
CIEX elution buffer	0.1% TFA, 5% ACN, 1M NaCl, pH 2
Preparative RPC flow rate	360 cm/h (1 mL/min)
CIEX flow rate	150 cm/h (0.9 mL/min)

Bivalirudin

Bivalirudin is a 20 amino acid (2.2 kDa) thrombin inhibitor, used as a blood thinner (Fig. 3). At pH 2 the peptide will bind to the CIEX ligand since its isoelectric point is around 3.7.

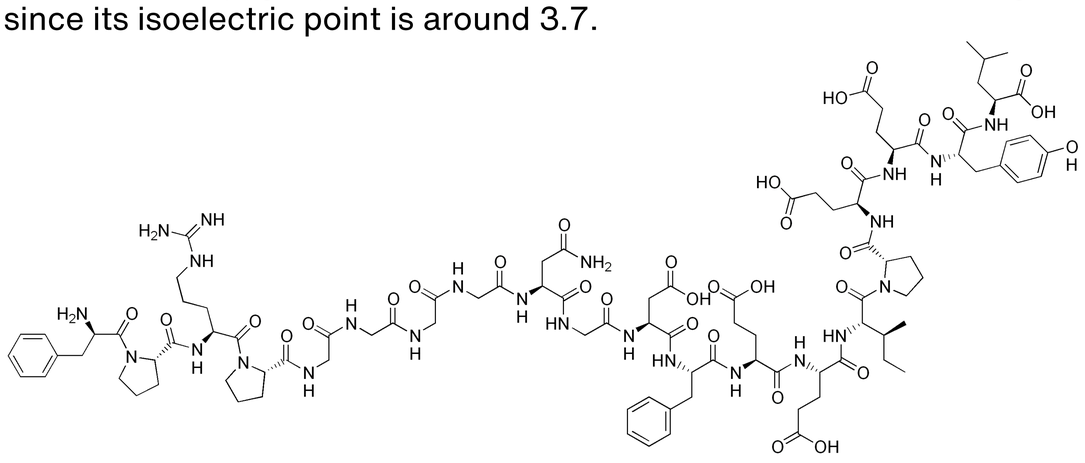

Figure 3. 20 aa bivalirudin peptide structure, 2.2 kD, isoelectric point 3.7. Source: Wikimedia Commons.

Result

- Stand-alone CIEX shows major improved purity of the crude sample (Fig. 4 and 5A and B).
- A purity of 94.7% at 97% yield was obtained using stand-alone RPC (Fig. 4 and 5B).
- CIEX-RPC gives 98.9% purity at 90% yield (Fig. 5B).
- Early eluting impurities, not properly resolved on RPC, were significantly reduced when CIEX was implemented (Fig. 5A).

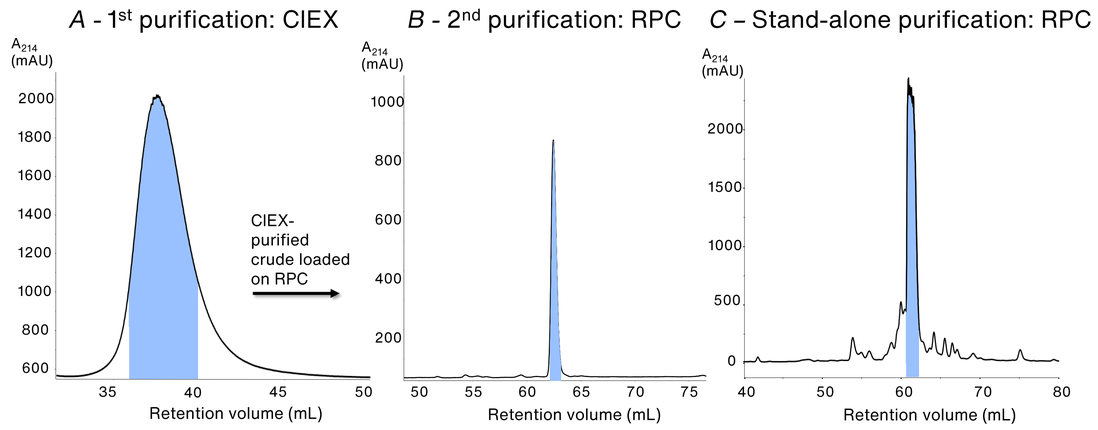

Figure 4. Chromatographic purification profiles for bivalirudin crude using only CIEX, CIEX-RPC or only RPC. Sample was loaded onto WorkBeads 40S **(A)** where the marked fractionation pool was collected and further purified on semi-preparative RPC **(B)** or the sample was only loaded onto semi-preparative RPC as a stand-alone purification step **(C)**. UV traces at 214 nm are shown as solid black lines and collected pool volumes as blue areas.

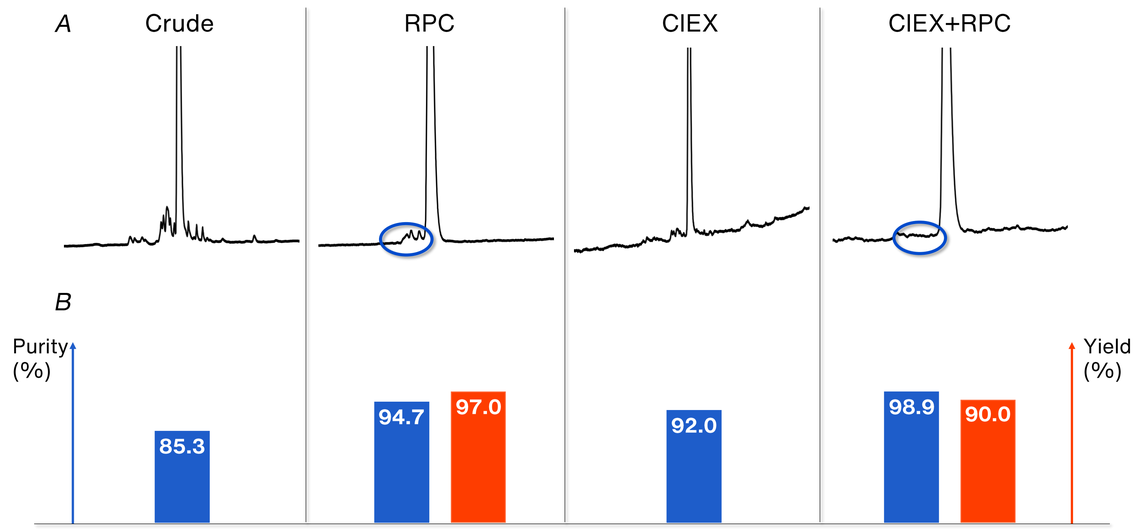

Figure 5. RPC analyses of the starting crude sample and purified pools from the one-step (IEX or RPC) vs. two-step purification (CIEX–RPC) **(A)**. Circles mark early peak impurities present (RPC) and removed (CIEX+RPC). Percentage of purity (blue bars) and yield (orange bars) for each purification step **(B)** .

CIEX + RPC resulted in a purity of close to 99% at a yield of 90%, which was notably higher purity than using RPC alone and with only a small yield loss.

Conclusions

The early eluting impurities *i.e.,* the less hydrophobic impurities, which were not properly resolved on RPC were significantly reduced when CIEX was added, demonstrating the orthogonality of the two methods. This complementarity will facilitate and improve more difficult purifications, especially for more polar peptides.

Initial IEX runs are easily set up using RPC-compatible buffers for screening purposes. Purification set-up can be optimized using more IEX-specific buffers if retention and separation are not good enough. Finally, this screening method also facilitates purification of peptides with unknown isoelectric points.

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