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APPLICATION NOTE

Optimizing the buffer conditions for a glycopeptide conjugate API purification

Glycopeptides represent one of the most important classes of antibiotics relating to the emerging global challenge in treating resistant bacterial infections. They can either be chemically synthesized or biologically produced, both methods generate crude peptides contaminated with extensive amounts of process-related impurities (failure sequences, chemical modifications, bioburden, *etc.*), that need to be removed. All therapeutic peptides have stringent purity requirements which may present a challenge in the downstream purification procedure. Reversed phase chromatography (RPC) is the gold standard for peptide purification but is not always sufficient to remove all impurities. Introducing orthogonality into the purification scheme in the form of adding a cation exchange chromatography (CIEX) step can facilitate this problem. Here we demonstrate a workflow for screening and optimizing the buffer conditions for a CIEX-RPC purification of a glycopeptide conjugate API, which outperformed the more traditional RPC-RPC setup.

Glycopeptides as antibiotics

The covid-19 pandemic has spurred the entire pharmaceutical industry, with antibiotic development and production being no exception. In 2021 the global antibiotic market was valued at USD 47 million and is expected to reach USD 65 million by 2027. Bringing forth novel antibiotics as well as engineering existing antibiotics to improve treatment efficacy for the increasing prevalence of bacterial infections is trending worldwide. As reported by the WHO (2018) glycopeptides represent one of the most important classes of antibiotics relating to the emerging global challenge in treating antibiotic resistant bacterial infections including those caused by methicillin-resistant *Staphylococcus aureus* (MRSA). Glycopeptide drugs are generally administrated as a last resort when treating life threatening infections caused by Gram-positive bacteria. They act by disrupting cell wall synthesis, hence inhibiting bacterial growth.

Peptide Purifications

Nowadays, the regulatory authorities recommend the use of combinations of orthogonal separation methods for purification of biomolecules. Peptides can be recombinantly produced in a cell-based expression system, insulin being an example, or synthesized using *e.g.* solid-phase peptide synthesis (SPPS). The different peptide production methods both pose various challenges regarding process-related impurities.

Crude peptides from, e.g., *Escherichia coli*, contain many host cell related impurities, such as lipids, nucleic acids, endotoxins etc. Crude synthetic peptide feeds (solid phase synthesis or liquid phase synthesis) mainly contain impurities such as truncations, deletions and potentially chemically modified sequences. Conjugation of peptides to another molecule further complicates the purification. Regardless of whether conjugation is part of the method or not, there are very strict purity requirements for therapeutic peptides. Thus the recommendation is to use orthogonal purification methods to reach a final purity of the targets.

Orthogonal methods - RPC *vs*. IEX

The fact that all individual impurities must be kept low (the reporting threshold is of 0.1% and identification threshold is of 0.5% for synthetic peptides) puts high demands on the downstream purification processes. Reverse phase chromatography (RPC) is the most commonly and frequently used peptide separation technique. As for all purification strategies, this technique has its own challenges and disadvantages. In addition to the resins being expensive, sensitive and prone to fouling, some peptide species cannot be separated on RPC to a sufficiently high purity. To circumvent these drawbacks and to improve the peptide separation, we recommend including ion exchange chromatography (IEX) as a capture step upstream of the RPC step. IEX is dependent on the ionic interactions between the immobilized ion groups on the resin and charged groups in the peptide.

Both cation and anion exchangers have been used with success for peptide purifications. CIEX is more common than AIEX, but which one to use ultimately depends on the peptide sequence.

Case study – Glycopeptide conjugate API

The target molecule was a small (<2 kDa) glycopeptide conjugate API, with an isoelectric point >8. The conjugate is a small entity that affects the net charge of the glycopeptide to enhance the cell penetration abilities and thereby improve the effectiveness against more cell types. This peptide conjugate feed had proven difficult to purify with RPC due to one main impurity eluting close to the target. This impurity caused long term issues in the purification process, leading to variation in retention times and lower yields over time. Since this impurity needs to be removed to get a robust purification process, IEX was evaluated as an orthogonal technique. First, a test purification was performed using generic mobile phases and later, different buffer conditions were explored and optimized to obtain a robust scale-up process with preparative loadings. Purities and yields were evaluated, and CIEX-RPC *vs.* the more common RPC-RPC approach was compared (Figure 1).

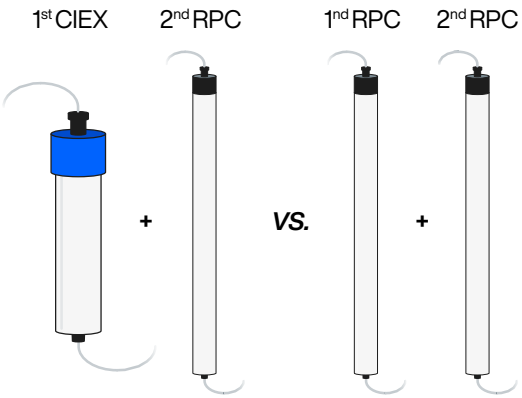

Figure 1. CIEX + RPC *vs.* RPC + RPC purifications.

Pre-study

The purpose of the pre-study was to evaluate if an improved separation between the target peak and main impurity peak could be achieved with IEX compared to using RPC as the capture step. As peptides can differ extensively in molecular size and chemical properties there is no optimized recipe that can be used for all peptide purifications, meaning it can be challenging knowing which combination of stationary phases and mobile phase compositions to apply. However, we have observed that various peptides can be purified using traditional RPC buffers in the CIEX purification step. Here, traditional RPC buffers refers to using 0.1% trifluoroacetic acid (TFA), pH 2, and 5-20% acetonitrile (ACN) in the mobile phase. 0.1% TFA in the mobile phase will not constitute a proper buffer system, but it will be good enough to separate most peptide species if they are stable at that pH. At such acidic conditions, the amino acids will be protonated at both the amine and carboxyl groups, ensuring adsorption of the peptide to a CIEX resin.

In this study, the same generic RPC buffers were used during the pre-study in both the CIEX and the RPC steps with the difference that the elution mobile phase contained 1 M NaCl for the CIEX elution step and 80% acetonitrile for the RPC elution step (Table 1).

Table 1. Screening mobile phases compositions.

	Binding mobile phase	Elution mobile phase
CIEX	0.1% TFA, 5% ACN, pH 2.0	0.1% TFA, 5% ACN, 1 M NaCl, pH 2.0
RPC	0.1% TFA, 5% ACN, pH 2.0	0.1% TFA, 80% ACN, pH 2.0

Since the glycopeptide conjugate purified in this study is a small peptide that contains few hydrophobic side chains, a C18 resin with relatively small pore sizes is chosen which usually offers the best potential separation for such targets. The same RPC resin was used in both the pre-study and the optimized scale up studies. For the ion exchange step WorkBeads™ 40S was used, which is an agarose-based CIEX resin optimal for peptide purification due to beads with small pores and a narrow pore size distribution. WorkBeads 40S is thus an excellent choice as an upstream purification step if a cation exchange chromatography resin is preferred prior to the RPC step(s). See Table 2 for the columns used.

TFA and acetonitrile as additives

- TFA is an ion-pairing agent used in RPC to enhance the hydrophobic interactions between the sample and the ligands (e.g. C18) and to minimize mixed-mode interactions, such as ionic interactions.
- ACN changes the polarity of the mobile phase and prevents non-specific hydrophobic interactions, also avoiding mixed-mode interactions.

Table 2. Columns used in this study.

Semi-preparative RPC column	Chromatorex™ C18 SPS150-10, 10 µm, 4.6x250 mm (Fuji Silysia)
Analytical RPC column	SVEA™ Gold C18, 5 µm, 4.6x250 mm (Nanologica)
CIEX column	GoBio™ Mini S 1 mL (WorkBeads 40S, 45 µm) (Bio-Works)

CIEX separates the crude peptide

Figure 2B shows the impurity trace for the studied peptide on the analytical RPC column. The main impurity eluted very close to the target peak at the retention time (RT) of 1.02 and was difficult to remove, thus the need for an orthogonal technique such as IEX is obvious. For the first step, CIEX was performed by loading a small amount of the sample onto a GoBio Mini S 1 mL column. A 0–100% linear gradient over 20 columns volumes (CV) was applied at a flow rate of 150 cm/h (1 mL/min). The purification showed that the main impurity eluted with baseline separation prior to the target peptide peak (Figure 2A) facilitating easy collection of the target. Analysis of the fractions was carried out using analytical RPC where a comparison with the crude peptide was done. The results shows that CIEX removed the majority of the difficult impurity (Figure 2C) and that it is beneficial to add this step.

Optimization of buffer conditions

CIEX

For the CIEX separation we wanted to elute our target using a salt gradient, since the peptide lacked acidic amino acids and thus a pH gradient was not applicable. There are many aspects to consider when deciding on which mobile phase composition to use.

We decided to use 20 mM ammonium acetate, where the following parameters were evaluated; gradient, pH, elution salts and ACN concentration (Table 3).

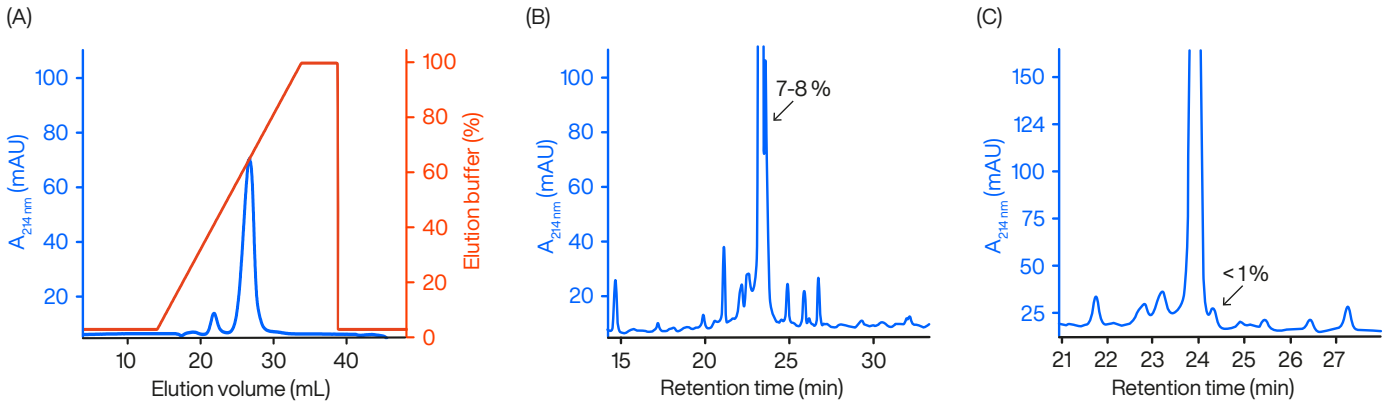

Figure 2. (A) CIEX purification using WorkBeads 40S. The blue trace represents the absorbance at 214 nm and the red trace represents the concentration of elution mobile phase. (B) Zoom in on the impurity profile of the crude sample using Nanologica SVEA Gold C18 (C) Zoom in of the impurity profile post CIEX purification using Nanologica SVEA Gold C18. The percentage represents the amount of the main impurity.

Table 3. Summary of purification outcome when varying the CIEX purification conditions.

Binding mobile phase	Elution mobile phase	pH	Linear gradient	Baseline separation	Peak width$_{½\ height}$ (mL)		Peak area of evaluated area (%)		Comment
					Pre-peak	Main peak	Pre-peak	Main peak	
20 mM $NH_4CH_3CO_2$	1 M $CaCl_2$	5.2	10-40%	No	0.78	1.18	13	87	Overall, very poor baseline separation when using $CaCl_2$ as eluent
	1 M $CaCl_2$		0-30%	No	0.68	1.55	14	86	
20 mM $NH_4CH_3CO_2$, 10% ACN	1 M $CaCl_2$, 10% ACN	5.2	10-40%	No	1.2	1.2	100	100	
	1 M $CaCl_2$, 10% ACN		0-30%	No	0.36	1.25	11	89	
20 mM $NH_4CH_3CO_2$	1 M NaCl	5.2	20-85%	Yes	2.63	2.06	21	75	Broadest peak width
20 mM $NH_4CH_3CO_2$, 10% ACN	**1 M NaCl, 10% ACN**	**5.2**	**20-85%**	**Yes**	**1.18**	**1.6**	**16**	**83**	**Most narrow peak width**
20 mM $NH_4CH_3CO_2$	1 M NaCl	4	20-85%	Yes	1.42	2.04	5	93	Drifting baseline
20 mM $NH_4CH_3CO_2$	1 M NaCl	7	20-85%	Yes	1.75	1.93	10	89	Broad peak width

Choosing your peptide IEX buffer system - quick guide

- Choose a buffer composition that preferably does not have the same UV absorption as your target. Peptides generally have UV absorption between 190-220 nm.
- Not all buffering salts can be combined with all elution salts due to the precipitation risk e.g., citrate precipitates in calcium chloride.
- Addition of an organic solvent, such as ACN is common in peptide purification.
- Peptides may often require low ionic concentration for maximal binding.

Two different elution salts were evaluated; $CaCl_2$ and NaCl. NaCl was shown to give superior separation between the two peaks. Moreover, pH 5.2 with the addition of 10% ACN gave the best separation. As a result, using 20 mM ammonium acetate, 10% ACN, pH 5.2, for the CIEX mobile phases was considered the best option (as highlighted in table 3). A follow up study investigated the impurity profile when adding 0, 10, 20 or 30% ACN in the mobile phases (Figure 3).

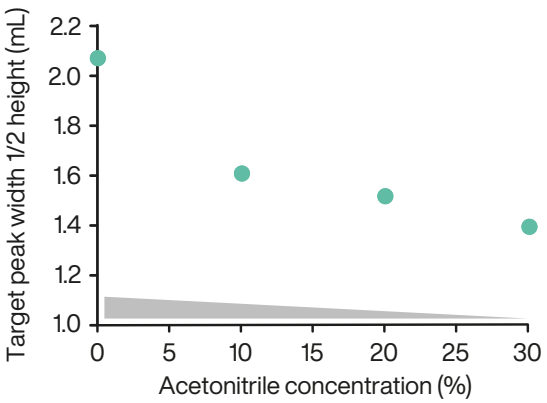

Figure 3. The peak width at half height is plotted against the acetonictrile concentration. The selectivity factor is subsequently decreasing with increasing acetonitrile concentration as depicted in plot.

As expected, increasing the ACN-concentration gives a sharper peak since the hydrophobic interactions are suppressed as the separation is mainly depending on electrostatic interactions, but meanwhile the selectivity between the pre-peak and the main target declines as the target is eluting earlier in the salt gradient. This resulted in the decision that 10% ACN offered the best combination of separation and peak shape.

CIEX mobile phases

Binding mobile phase	20 mM $NH_4CH_3CO_2$, 10% ACN, pH 5.2
Elution mobile phase	20 mM $NH_4CH_3CO_2$, 10% ACN, 1 M NaCl, pH 5.2

RPC

After optimization of preparative CIEX conditions, the RPC conditions were studied. To facilitate orthogonal selectivity in the two RPC steps, mobile phases using different pH are preferred. This is because the pH of the mobile phase influences the retention of the peptide due to the peptide interactions with the resin. In the same manner, using different buffer types generally has a strong effect on the retention of the target peptide.

Ion pairing agents such as TFA can facilitate improved separation but is preferably avoided due to its low tolerance in the final product and the need for it to later be removed.

Three different mobile phase compositions were evaluated (Table 4). When working with sodium phosphate buffers, as in this application, it is important to not exceed 60% ACN in elution and washing steps to avoid precipitation. The same applies for ammonium acetate, where around 90% is the limit. The phosphate buffers gave the most promising results and were selected for the preparative purification setups.

The order of the two RPC steps does not matter as long as they provide orthogonal separation. In this study, we decided to use a pH of 6.5 in the first capture RPC step and a pH of 2.2 in the second polishing RPC step. Moreover, the buffers with pH 2.2 were also used for the RPC step in the CIEX–RPC setup.

RPC mobile phases

Binding mobile phase 1st RPC	20 mM NaH_2PO_4, pH 6.5
Elution mobile phase 1st RPC	20 mM NaH_2PO_4, 50% ACN, pH 6.5
Binding mobile phase 2nd RPC	20 mM NaH_2PO_4, pH 2.2
Elution mobile phase 2nd RPC	20 mM NaH_2PO_4, 55% ACN, pH 2.2

When the mobile phases were set, the elution conditions were evaluated, i.e., length in CV and slope of the linear gradient (data not shown). For peptides, it is most common to use gradient elution since step elution often causes peak broadening.

Table 4. Summary of the purification outcome when varying parameters for the purification conditions for the RPC step.

Binding mobile phase	Elution mobile phase	pH	Linear gradient	Baseline separation	Comment
20 mM NaH_2PO_4	20 mM NaH_2PO_4, 55% ACN	2.2	0–100%	No	Main impurity appears as a shoulder on the main peak
20 mM $NH_4CH_3CO_2$	20 mM $NH_4CH_3CO_2$, 80% ACN	4.5	0–100%	No	No visual separation due to drifting baseline
20 mM NaH_2PO_4	20 mM NaH_2PO_4, 50% ACN	6.5	0–100%	No	Main impurity appears as a shoulder on the main peak

Main study: preparative purification using IEX + RPC *vs.* RPC + RPC

In the main study, scaled up conditions to verify the robustness of the method were applied with a load of 25.0 mg crude peptide/mL resin for the CIEX-RPC purification and 10.8 mg crude peptide/mL resin for the RPC-RPC purification.

The sample load differs considerably depending on the complexity of achieving a good separation

- 20–30 mg peptide/mL IEX resin is a good rule of thumb
- 7–24 mg peptide/mL RPC resin is a good starting point

The goal was to remove the main impurity from the target peptide. Depending on the required purity of the target molecule, the yield will be affected (see the black trace in figure 4A and 4C). Here, the target peak was collected from the fractions with low abundance of the main impurity to get a total purity of 92% and pooled. These pooled fractions were further loaded onto the second RPC polishing step (Figure 4B and 4D). All collected pools (as visualized by the purity profile with green bars in the elution profiles) were analyzed for purity, yield, and abundance of the main impurity.

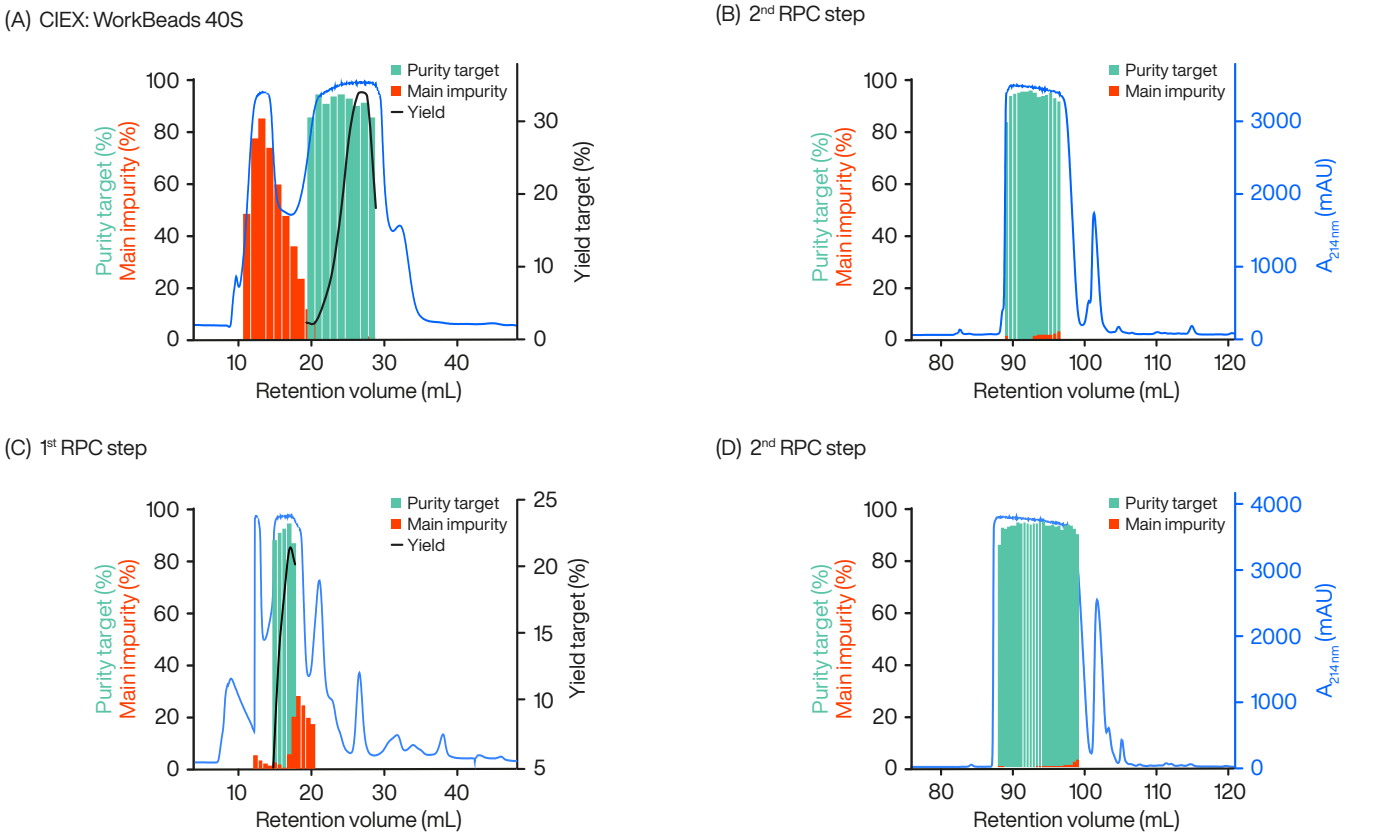

Figure 4. Purity profiles for the chromatographic steps. (A) CIEX elution profile. (B) Polishing RPC elution profile (post CIEX). (C) Prep RPC elution profile. (D) Polishing RPC elution profile (post prep RPC). Blue trace corresponds to absorbance at 214 nm, green bars are the purity of the target, red bars are the abdundance of the main impurity, and the black trace corresponds to the target yield in the collected pools.. Flow rate: 150 cm/h (0.9 mL/min) for the IEX step and 360 cm/h (1 mL/min) for the RPC steps.

Figure 4A shows that the main impurity (red bars) is resolved on the CIEX column, where the target peak easily can be collected. However, the main impurity did not resolve as well on the RPC column (Figure 4C), but the overall purity was still improved compared to the feed. Figures 4B and 4D are the respective RPC polishing steps for the two setups, showing the target purity in each eluted fraction compared to the main impurity. By combining the orthogonal techniques CIEX and RPC, a purity of 96% at a yield of 89% was reached compared to a purity of 95% at a significantly lower yield of 78% for RPC-RPC (Table 5). Importantly, the main impurity was significantly reduced, <1%, using both setups.

Table 5. Purity, yield and main impurity from the CIEX, CIEX + RPC, RPC and RPC + RPC purifications.

	Purity	Yield	Main impurity
Crude peptide	78%	100%	7.5%
CIEX	92%	90%	<1%
CIEX + RPC	96%	89%	0.6%
1st RPC	92%	79%	2.5%
RPC + RPC	95%	78%	0.9%

Conclusions

For challenging peptide purifications, the use of RPC is not always enough to remove problematic impurities. Here we have shown the advantage of employing orthogonality into a difficult purification by adding CIEX prior to RPC. For the glycopeptide conjugate API studied here the difficult main impurity could be decreased to below 1% at a yield of 89%, and a total purity of 96% using this combination of chromatography techniques.

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