

High-throughput process development and scale-up of an intermediate purification step for recombinant insulin

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This Application note describes high-throughput process development with PreDictor[™] plates and Assist software to develop the intermediate purification step of insulin derived from *E. coli*. This revealed the ion exchanger Capto[™] SP ImpRes to be the most promising medium (resin) and identified binding and elution conditions that gave good separation of insulin from truncated forms of insulin. Further column studies using 1 mL Tricorn[™] columns and ÄKTA[™] avant 25 chromatography system confirmed that the developed conditions were robust. Finally, the method was scaled up, first 40-fold and then 400-fold, with both purity and recovery exceeding 90%. This figure is a good basis for achieving the required purity (98% or more) in a following polishing step not described here.

Introduction

The ever-increasing need for higher throughput in process development has led to the introduction of new high-throughput process development (HTPD) tools. In purification development workflows, such HTPD methods have led to significant efficiency gains seen as a reduction in both the time and amount of sample required for selecting chromatographic media and establishing running conditions.

PreDictor 96-well filter plates (prefilled with BioProcess™ chromatographic media from GE Healthcare) simplify rapid, high-throughput screening during process development. Conditions defined using PreDictor plates can be verified and optimized in column format with HiScreen™ or HiTrap™ prepacked columns. An automated chromatography system such as ÄKTA avant 25 will provide both buffer preparation and Design of Experiments (DoE) functionality to simplify this task. The same system can also be used for initial scale-up to pilot scale in, for example, HiScale™ 16/40 columns. Further scale-up can be performed on ÄKTA avant 150 system and columns from the AxiChrom™ range.

This Application note describes a complete workflow from PreDictor plate screening to a 400 mL column volume process for the intermediate purification of insulin on the Capto SP ImpRes ion exchanger. This intermediate purification is the second chromatography step that follows the initial capture of pro-insulin using Capto MMC (a multimodal cation exchanger) described in an earlier Application note (1). Note that several unit operations take place between these two steps, including enzymatic cleavage (Fig 1) of the pro-insulin to form insulin. These operations are not described here.



Fig 1. Structure of insulin (composed of A- and B-chain) after enzymatic cleavage.



Materials and methods PreDictor screening experiments for binding/elution

Whenever possible, the PreDictor plate screening experiments were performed with fully automated protocols using a Tecan™ Freedom EVO™-2 200 Robotic System. Otherwise, manual protocols were used. Liquid removal was by vacuum or centrifugation throughout the study. The sample used in all experiments was generated by enzymatic cleavage of lyophilized pro-insulin obtained from BIOMM S.A., Belo Horizonte, Brazil. Detailed procedures of how to perform the screening experiments can be found in the high-throughput process development handbook (2).

PreDictor plates used were media screening plates (2 μ L/6 μ L media volumes) plus low-volume (6 μ L) and high-volume (20 μ L) single-medium plates. All plates were filled with cation exchange chromatography media.

Assist software was used to support the PreDictor workflow from setting-up the experimental design to evaluating the plate data. MODDE™ software was also used for some optimization work.

Analysis of experiments

All insulin samples of interest were analyzed on an ÄKTAmicro™ system using an analytical RPC method based on a phosphate/ perchlorate (pH 2.5) system with acetonitrile as organic modifier. The column used was a Kromasil™ 100-3.5 C4; 150 × 4.6 mm. Figure 2 shows a typical chromatogram of a start sample where insulin and its main impurities are monitored.

Column:	Kromasil 100–35 C4; 150 × 4.6 mm
Sample:	Enzyme-cleaved pro-insulin
Buffer A:	50 mM phosphate, 100 mM perchlorate buffer, pH 2.5:acetonitrile (70:30)
Buffer B:	50 mM phosphate, 100 mM perchlorate buffer, pH 2.5:acetonitrile (50:50)
Flow rate:	1 mL/min
System:	ÄKTAmicro



Fig 2. Separation of a cleaved start sample of insulin on the C4 RPC column.

The resulting insulin concentration in the flowthrough fraction (i.e. the fraction collected after binding) or the first elution fraction for each condition was used as input data for Assist software, which generated extrapolated response surfaces for the experimental evaluation.

Chromatograms were evaluated as follows:

 $Concentration_{sample} = Concentration_{cleaved sample} \times Peak area_{sample} / Peak area_{cleaved sample}$

Column experiments

Tricorn 5/50 columns (1 mL) were used for most small-scale studies such as the optimization and robustness studies. The columns were packed according to the instructions for the individual medium. HiScale 16/40 column was packed to bed height of 20 cm. AxiChrom 50/300 was packed to a bed height of 20.5 cm using predefined Intelligent Packing methods in UNICORN™ 6.1 software (3, 4). Column efficiencies were tested with 1% acetone in 20% ethanol.

All column experiments, comprising those used in the screening, optimization, robustness studies, and scale up, were performed on chromatography systems suitable for the column dimensions. The systems used for the development work were ÄKTA avant 25 and ÄKTA avant 150, while ÄKTAmicro was used for analyses.

Results

Binding experiments in PreDictor plates

Insulin is a small, relatively hydrophobic peptide with a limited solubility close to its pl. An earlier solubility investigation had revealed the possibility of using $pH \le 4$ for binding. As pH 4 is the pH used to terminate the enzymatic cleavage of pro-insulin to insulin in the preceding step, it was decided not to adjust pH further. The two factors investigated in the binding experiments were NaCl and ethanol concentrations. An organic solvent is needed to improve chromatographic performance of insulin. Initially, three candidate media were screened: Capto S, SP SepharoseTM Fast Flow, and Capto MMC. Figure 3 shows that all three bound insulin in ethanol. Capto S and SP Sepharose Fast Flow both bound at low NaCl concentration, while Capto MMC appeared to have higher binding at elevated levels.

At this stage in the process development work, a new ion exchange medium (Capto SP ImpRes) was commercialized. Capto SP ImpRes is a high-flow agarose medium with a smaller particle size than SP Sepharose Fast Flow (approx. 40 vs 90 μ m) but has almost identical pressure-flow properties. Its binding properties were investigated and the result was very similar to that of SP Sepharose Fast Flow.

Based on the binding experiments, all media except Capto MMC were considered for further studies, although based on particle size, the Capto SP ImpRes was assumed to have higher peak resolving capability. Further results from Capto S are not shown here as the initial column studies showed less promising results.

The binding conditions chosen for all the following experiments were 10% (v/v) ethanol without addition of NaCl and no further adjustment of pH.



Fig 3. Binding at different NaCl and ethanol concentrations in PreDictor CIEX screening plates. SP Sepharose Fast Flow and Capto S both bound at lower NaCl concentrations than Capto MMC. Assist software was used in obtaining this data.

Elution experiments in PreDictor plates

The pH used in the binding experiments (pH 4) was maintained in the elution study. Its purpose was to find promising elution conditions for insulin and to study selectivity during elution, that is, identify conditions that separated insulin from truncated insulins as well as from pro-insulin. Ethanol and NaCl concentration in the elution step were the two factors varied.

Insulin was loaded to about 80% of the binding capacity found in the binding experiment after adding 10% (v/v) ethanol to the sample. Ethanol in the elution was varied between 30% and 50% and the NaCl concentration between 100 and 450 mM NaCl. Yield in the two first fractions and purity in the first elution fraction were determined and results are visualized in Figure 4.

The blue area of each media indicates more than 80% yield after two elution fractions. After the first elution, the

maximum purity that was achieved with SP Sepharose Fast Flow was just above 70% (shown as red shaded area). This purity was also found in the fractions from Capto SP ImpRes, but at lower NaCl concentrations.

Closer examination of the Capto SP ImpRes results (Fig 5) revealed that even higher purity could be obtained at relatively low NaCl concentrations while still maintaining a yield of 80% in the two first elution fractions. Analyzing all fractions also revealed that the C-peptide (the most abundant impurity) needs high NaCl concentration to elute, while the truncated insulins eluted at the same conditions as for insulin (or conditions close to these).

The above results show that the column chromatography study should be performed at an ethanol concentration in the range of 40 and 50% and at salt concentrations between 150 and 250 mM NaCl.







Fig 5. Overlay view of yield and purity results for Capto SP ImpRes with a higher purity setting than in Fig 3. Blue – yield over 80%, red – purity over 77%. Overlapping areas correspond to conditions fulfilling both criteria. The higher purity seen with Capto SP ImpRes offers a window of opportunity to explore in further column experiments. Assist software was used in visualizing these data.

Column optimization studies using the DoE module in ÄKTA avant 25

The study to optimize elution from Capto SP ImpRes was performed in small columns (Tricorn 5/50) using ÄKTA avant 25 system with a built-in DoE module in UNICORN software. Loading was performed at pH 4 in 10% (v/v) ethanol. Insulin was loaded at 20 mg/mL medium, well below the dynamic binding capacity in order to stay under the limit where fibril formation of insulin could be a risk (5).

Elution conditions were varied using a Central Composite Circumscribed (CCC, reference 6) as indicated in Figure 6. Buffers were prepared in-line using the ÄKTA avant 25 Buffer Prep module from stock solutions of 200 mM sodium acetate, pH 4 buffer, 4 M NaCl, ethanol, and ultrapure water.



Fig 6. CCC design used in the Capto SP ImpRes optimization study in a Tricorn 5/50 column using ÄKTA avant 25 system with its built-in DoE module.

The conditions shown were used for the first elution step, which was followed by a second step at 1 M NaCl and 50% ethanol in 50 mM sodium acetate buffer pH 4.0. This second step was used to elute the C-peptide. The more difficult separation of insulin from its truncated forms was achieved isocratically in the first elution. Good statistical models for the results were obtained using retention volume as response (see Fig 7 for the Capto SP ImpRes models).

To visualize the conditions giving good separation, a sweetspot analysis was performed on the optimization study results (see green area in Fig 8). An operating space, that is, the largest difference in elution volume between the insulin peak and the truncated insulin peak, was identified. Within this space, the condition with the lowest addition of NaCl and a moderate ethanol concentration was selected for further evaluation.

A) Retention volume insulin peak (mL)



B) Retention volume truncated insulins (mL)







Fig 8. Sweet spot analysis of the retention behavior of insulin and truncated forms at different salt and ethanol concentrations. Blue area shows conditions where insulin has a retention volume of 22 to 26 mL. The green area indicates conditions where, at the same time, the second peak (truncated insulins) has a retention volume of 28.5 to 31 mL, that is, the so-called sweet spot. MODDE software was used in obtaining these data.

Figure 9 compares the best conditions for Capto SP ImpRes and SP Sepharose Fast Flow. Capto SP ImpRes gives a highresolution separation of insulin and the truncated insulin forms, while SP Sepharose Fast Flow cannot resolve these components (the truncated forms are concentrated in the tail-end of the insulin peak). Table 1 shows the purity achieved in the column experiments for Capto SP ImpRes and SP Sepharose Fast Flow.

Table 1. Purification of insulin on Capto SP ImpRes and SP Sepharose Fast Flow under chosen elution conditions performed in Tricorn 1 mL columns

Sample	Insulin purity (%)	Insulin yield (%)	Truncated insulins (%)
Starting material	64	-	11.5
Capto SP ImpRes	91	95	2.8
SP Sepharose Fast Flow	80	92	11.5

Column robustness studies using the DoE module in ÄKTA avant 25

The Capto ImpRes robustness study was performed in Tricorn 5/50 by varying sample volume, NaCl, and ethanol concentration, which is visualized by the three-dimensional space as shown in Figure 10. Table 2 shows conditions for the center-point and ranges in the chromatographic runs. All conditions investigated showed similar results, that is the variation in purity, insulin, and truncated insulins were of the same magnitude as the replicates in the center point (Fig 10). Insulin purity varied between 90 and 92% and content of truncated insulins between 2.4 and 2.8%. In all cases, the achieved yield was close to 95%. A separate study using three different lots of Capto SP ImpRes, run at the centerpoint, also gave a robust result.

Columns:	Tricorn (5/50) packed with A) Capto SP ImpRes and B) SP Sepharose Fast Flow.
Sample load:	20 mg insulin at pH 4 in 10% (v/v) ethanol
Equilibration buffer:	50 mM sodium acetate, pH 4 in 10% ethanol
Elution:	A) First step, 50 mM sodium acetate, pH 4 in 47.5% (v/v) ethanol and 128 mM NaCl (10 CV); second step at 1 M NaCl and 50% ethanol (v/v) in 50 mM sodium acetate buffer pH 4.0 (10 CV) B) first step, 50 mM sodium acetate, pH 4 in 47.5% (v/v) ethanol and 200 mM NaCl (10 CV); second step at 1 M NaCl and 50% ethanol (v/v) in 50 mM sodium acetate buffer pH 4.0 (10 CVs).
Flow rate:	0.4 ml/min
System:	ÄKTA avant 25

A) Capto SP ImpRes





Fig 9. Comparison of optimal resolution between insulin (I) and its truncated forms (T) on Capto SP ImpRes A) and SP Sepharose Fast Flow B). The improved resolution observed with Capto SP ImpRes reduced the amount of truncated insulins. The C-peptide (C) eluted after increasing the salt concentration to 1 M NaCl, that is, 100% B corresponds to 4 M NaCl.





Fig 10. Experimental design and results for the robustness study shown as responses for purity and truncated insulin in the insulin fraction. Experiment I, J, and K are the center points and the results indicate that the developed method is robust within the tested limits.

Table 2. Summary of conditions, center-point and ranges (in bold) in the robustness study performed on Capto SP ImpRes

Column:	Tricorn 5/50			
Sample:	2 column volumes (CV) insulin			
	(cleaved), pH 4, 10% (v/v) ethanol,			
	range ± 0.2 CV			
Elution step 1 (insulin):	10 CV			
	50 mM sodium acetate, pH 4 47.5% (v/v) ethanol, range ± 1% 128 mM NaCl, range ± 8 mM (center point)			
Step elution 2 (C-peptide):	4 CV			
	50 mM sodium acetate, pH 4			
	1 M NaCl			
Residence time:	2.5 min (0.4 mL/min, 120 cm/h)			

Scaling up

Column bed height was increased to 20 cm and was kept during further scale-up to larger column diameters. The columns were HiScale 16/40 (16 mm diameter) and AxiChrom 50/300 (50 mm diameter) packed with Capto SP ImpRes, which gave respective media volumes of 40 mL and 400 mL.

The columns were equilibrated with 50 mM sodium acetate, pH 4 containing 47.5% ethanol, but the feed sample contained only 10% (v/v) ethanol. Elution was performed with 50 mM sodium acetate, pH 4 containing 47.5% ethanol and 130 mM NaCl. The sample load was kept constant during scale-up but in order to keep linear flow rate at 120 cm/h, residence time was, increased from 2.5 min to 10 min. Fractions from the flowthrough and eluted peaks were analyzed on the RPC column.

Figure 11 and Table 3 show the scale-up results and purity data for each column diameter. Both demonstrate that the intermediate step for insulin purification was scaled up successfully from a 1 mL Tricorn 5/50 Capto SP ImpRes column via 40 mL HiScale 16/40 up to a 400 mL AxiChrom 50 column run on ÄKTA avant 150 system. Using this scale-up approach, purity of the target protein exceeded 92% and yield was greater than 95%.

Table 3. Purity and recovery data from the scale-up experiments

Column	Scale-up factor	Feed loaded (mL)	Insulin eluted (mL)	Insulin purity (%)	Yield (%)
Tricorn 5/50	1	2	2	92	95
HiScale 16/40	40	80	60	93	95
AxiChrom 50/300	400	800	600	92	102





Conclusions

High-throughput screening with PreDictor plates and Assist software enabled quick selection of two potential chromatography media and identified promising binding and elution conditions for the intermediate purification of recombinant insulin expressed in *E. coli*. This gave a fast and confident start to process development for this chromatographic step.

Additional experiments on the two selected media (SP Sepharose Fast Flow and Capto SP ImpRes) were run on 1 mL Tricorn columns to further optimize purification conditions. This smallcolumn work clearly showed that the purity of collected insulin was higher using the recently introduced Capto SP ImpRes. This medium gave improved separation of insulin from its truncated forms compared with SP Sepharose Fast Flow.

When the optimized protocol was also confirmed to be robust, the process was successfully scaled up from a 1 mL Tricorn 5/50 column to a 400 mL AxiChrom 50 column. The resulting purity for this production-scale intermediate purification step was in excess of 90% and the yield was 98% or more. This purity was increased further in a following polishing step (not described here) and the desired final purity of 98% was therefore easily achieved. The lower content of truncated forms of insulin achieved in the intermediate purification of this study would benefit the final polishing step by reducing the purification burden placed on it.

The overall outcome of this study demonstrates the value of introducing high-throughput methods into process development workflows.

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