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Development of an intermediate chromatography step in an insulin purification process. The use of a High Throughput Process Development approach based on selectivity parameters

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A B S T R A C T

Recent innovations in designing purification processes for biopharmaceutical production have enabled initial screening (optimization) of chromatographic conditions for binding to be performed in miniaturized batch format. The present report demonstrates the possibility of using this format to screen for selectivity and illustrates the need for careful adjustment of protocols when highly abundant, tightly-binding impurities are present in the sample. This batch format approach was used to choose a chromatography medium (resin) from a selection of available resins for the purification of recombinant insulin expressed in *E. coli* and to screen binding and elution conditions.

Subsequent optimization was performed in small packed columns using a Design of Experiments (DoE) approach with statistical modeling before scaling up to a small pilot scale experiment. In this study insulin was effectively purified from the more tightly-binding C-peptide, and a reduction in insulin variants was also noted using the optimized conditions.

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1. Introduction

Designing purification processes for biopharmaceutical proteins at industrial scale is challenging, as there are numerous possible combinations of chromatography media and conditions. Fast process development has lately come in focus. In order to facilitate a "short time to market" the development of these processes should be performed as early as possible even though only small amounts of pure API (Active Pharmaceutical Ingredient) may be available.

Protein purification is comprised of sequential unit operations including several chromatographic steps, where each chromatography step is dedicated to a specific purpose [1], i.e. Capture, Intermediate (purification) and Polishing. To speed up the optimization of conditions for chromatographic purification, parallel formats (both miniaturized columns and batch format) have been introduced, often called a High Throughput Process Development (HTPD) approach [2–5]. Determination of protein binding capacities in batch format is an old and well established method often used for single component systems but to our knowledge not used for complex samples with highly abundant impurities. The batch format is an excellent tool for screening of chromatography media, covering a large experimental space by using for example 96-well plates filled with chromatography resins, which were used in this study.

Capto™ MMC, a multimodal cation exchanger medium, was used for the initial capture step, where pro-insulin was captured from a cell culture harvest subjected to appropriate pre-treatment. The capture step was developed, as is the intermediate step described herein, following the HTPD approach, but concentrating more on binding capacity than selectivity. While binding capacity in the separation is still important in the intermediate chromatography step selectivity in binding and elution is even more in focus. Thus, screening experiments should not only assess capacities and yield but should also indicate where highest product purity may be achieved. As shown in this work, the batch format methodology can be used for developing this step, for optimization of the selectivity using crude material from a regular process.

Through good media choice and careful optimization of chromatographic conditions, it may be possible to effectively select and purify a protein even when other proteins that may bind the media as tightly as the target are present in high concentrations. In the present report, efficient cleavage of proinsulin leads to high

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concentrations of a strongly binding C-peptide, creating a potential challenge to efficient purification of the insulin molecule [6–9]. This report focuses on the development of a process that overcomes this challenge, ensuring high purity and yield of insulin despite the abundance of the C-peptide.

2. Materials and methods

2.1. Sample preparation

The collected proinsulin material from the capture step was first crystallized, resolved in 100 mM glycine buffer pH 8 and then cleaved enzymatically, at pH 8, using Carboxypeptidase B (C9584) and Trypsin (T0303), from Sigma-Aldrich (St. Louis, MO, USA). Cleavage was monitored by the reversed-phase chromatography (RPC) method described below. Cleavage was interrupted by reducing pH to 4 by adding glacial acetic acid when the insulin peak reached its maximum size, i.e. after approximately 15.5 h at 12 °C. This material was used as starting material for the intermediate step.

2.2. Analytical reversed phase chromatography

A Kromasil™ 100-3.5 C4 (150 × 4.6 mm) column (AkzoNobel Pulp and Performance Chemicals, Bohus, Sweden) was used. Buffer A was prepared from 50 mM monobasic sodium phosphate and 100 mM sodium perchlorate solutions, adjusted to pH 2.5 with perchloric acid, this solution was mixed with 30% in acetonitrile (v/v). Buffer B was 50% acetonitrile and 50% of the 50 mM monobasic sodium phosphate and 100 mM sodium perchlorate solutions (v/v), pH 2.5.The column was eluted by a linear gradient from 26% B to 38.7% B for 13 min; followed by a linear gradient from 38.7% B to 100% B for 1 min and maintained at 100% B for 4 min after which the column was re-equilibrated at 26% B. An ÄKTAmicro™ chromatography system (GE Healthcare Life Sciences, Uppsala, Sweden) was used. Detection was made at 280 nm.

2.3. High-throughput screening study of chromatography media and conditions

Separate studies were performed to optimize binding and elution conditions. In both cases the 96-well format was used. Before applying sample, PreDictor™ plates, (GE Healthcare, Uppsala, Sweden) were conditioned with 200 μl binding buffer (50 mM acetate, pH 4 in 10% ethanol) for 3 min at 1100 rpm on a MTS 2/4 digital microtiter shaker (IKA, Stanfun, Germany), followed by removal of the liquid using a Whatman UniVac 3 Vacuum manifold (GE Healthcare, Uppsala, Sweden) or a 5810 R centrifuge (Eppendorf, Hamburg, Germany). Sample was added to the wells and incubated for 60 min on the microtiter shaker at 1100 rpm.

3. Binding and elution studies

The binding study was performed using PreDictor plates with either low (2 μl or 6 μl) or high (20 μl) chromatography media volume. The chromatographic media studied were Capto MMC, Capto S, Capto SP ImpRes and SP Sepharose™ Fast Flow (all GE Healthcare, Uppsala, Sweden), supplied in prepacked PreDictor plates either as single media plates or as media screening plates. Media screening plates contained Capto S, Capto MMC and SP Sepharose Fast Flow. For Capto SP ImpRes plates containing only this medium were used.

For the binding study with cleaved proinsulin, 300 μl sample (cleaved material, total protein concentration 8–12 mg/ml, diluted with binding buffer to a final insulin concentration of approximate 1.3 mg/ml) was added once to the low media volume PreDictor plates. For the study with high volume media consecutive loadings of insulin were used and total protein concentration in the loading sample was also decreased (to approximately 0.8 mg/ml in respect to insulin) to ensure the initial loading was below saturation of the media. The liquid fraction from the flow through was taken for analysis. Insulin binding was determined at four different ethanol concentrations (0–50%) and four different NaCl concentrations (0–300 mM). Even though the experiments were performed as replicates in the 96-well plate only one well per condition was subjected to HPLC-analysis for determination of concentration and purity. Some of the replicates where used for conformity analysis.

High-volume (20 μl) single-medium plates filled with Capto SP ImpRes and SP Sepharose Fast Flow were used for the elution studies and 200 μl of a solution of cleaved material (diluted 1:1 with binding buffer) was loaded into each well. After the loading step 3 wash steps were performed (3 × 200 μl binding buffer) followed by 3 elution steps (3 × 200 μl). Elution conditions were screened at four different ethanol concentrations (30–50%) and eight different NaCl concentrations (100–450 mM).

Calculation of static binding capacity was performed using the Assist software (GE Healthcare, Uppsala) where the start sample and content in flow through fraction was used.

3.1. Chromatography optimization and robustness studies

The Design of Experiments (DoE) module of an ÄKTA™ avant 25 system was used to design the elution optimization study. A Central Circumscribed (CCC) design was used and was based on two variables: ethanol (37–58%) and NaCl concentration (128–272 mM). After the first elution step the salt concentration was raised to 1 M and ethanol to 50% in order to elute the C-peptide. Optimization of this step was not considered necessary. Insulin was dissolved in equilibration buffer (50 mM sodium acetate buffer, pH 4.0 containing 10% ethanol) and loaded onto 1 ml Tricorn™ 5/50 columns (GE Healthcare, Uppsala, Sweden) packed with SP Sepharose FF and Capto SP ImpRes media at 20 mg insulin/ml chromatography medium. Amount of insulin loaded was well below the dynamic binding capacity, in order to stay under the limit where fibril formation of insulin could be a risk [10–12]. Flow rate was maintained at 0.4 ml/min, i.e. corresponding to a residence time of 2.5 min.

Eleven experiments were performed on each of the two chromatography columns. Different elution profiles were obtained depending on the ethanol and NaCl concentrations. Elution fractions were collected, pooled according to the appearance of the chromatogram and analyzed for purity. Cleaning in Place (CIP) of columns was performed between experiments using 1 M NaOH for 3 column volumes (CV) at a flow rate of 0.4 ml/min.

To check the robustness of the optimized elution conditions a study was made on the Capto SP ImpRes, packed in a Tricorn 5/50 column, A Full Factorial 2-Levels DoE based on variation in sample load (20 mg/ml chromatography medium ±10%), ethanol concentration (47.5% ± 1%) and NaCl concentration (128 mM ± 8 mM) was used. Eleven experiments were performed and variation of Insulin purity was investigated and the response was insulin purity. MODDE™ software (Umetrics, Umeå, Sweden) was used to analyze the DoE experiments.

3.2. Scale-up

The developed method was scaled-up 40 and 400 times using HiScale™ 16/40 and AxiChrom™ 50/300 columns, respectively (both GE Healthcare, Uppsala, Sweden), packed with Capto SP ImpRes to bed heights of 20 cm. Columns were equilibrated with 5 CV Buffer A (50 mM sodium acetate, pH 4.0 containing 47.5% ethanol). Protein sample was loaded on the column to 20 mg protein/ml medium. After loading, the columns were washed with 3

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