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Industrial Purification of Pharmaceutical Antibodies: Development, Operation, and Validation of Chromatography Processes

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Introduction

Recombinant monoclonal antibodies are becoming a great success for the biotechnology industry. They are currently being studied in many clinical trials for treating a variety of diseases, and recently several have been approved for treating cancer (Carter *et al.*, 1992; Anderson *et al.*, 1996; Baselga *et al.*, 1996; Bodey *et al.*, 1996; Longo, 1996). Although there are several types of antibodies produced in different types of cell lines, the most clinically significant antibodies are full-length humanized IgG_1 produced in CHO cells. This review describes the methods used to purify these antibodies at industrial scale, focusing on chromatography processes, and with particular reference to recent work at Genentech.

Routine laboratory purification of antibodies has been well described (for example see Scott *et al.*, 1987), but the considerations for large-scale production of pharmaceutical-grade antibodies are much different than those for laboratory scale. There are extreme purity requirements for pharmaceutical antibodies, and routine large-scale production requires high yield and process reliability. To gain regulatory approval, the process must be completely validated to run consistently within specified limits, so the process should be designed to facilitate validation.

Large-scale production of antibodies as pharmaceutical products is a complex

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Abbreviations: CV, column volume; HCCF, harvested cell culture fluid; CHOP, Chinese hamster ovary proteins; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; CE, capillary electrophoresis; HPLC, high-performance liquid chromatography; ppm, parts per million (ng/mg); LOQ, limit of quantitation; SEC, size exclusion chromatography; pI, isoelectric point; GMP, good manufacturing practice; g/l, when describing column loads this is grams of antibody per litre of column volume.

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endeavour, including a manufacturing process with multiple steps and significant analytical support. Antibody manufacturing includes cell banking and cell culture, recovery, filling (possibly including lyophilization), finishing, and packaging. Product recovery includes harvest, which is removal of cells and cell debris by tangential flow filtration or centrifugation (van Reis *et al.*, 1991), chromatography for antibody purification, and formulation by tangential flow filtration. Here we focus on process chromatography, which must reliably produce highly purified antibody.

To satisfy the stringent purity requirements for pharmaceutical antibodies, an extensive analytical control system is integrated with the manufacturing process at all steps, particularly on release of the final product. The analytical control system includes assays for product-related variants (including charge and glycosylation variants), often using ion exchange HPLC or CE (Hunt *et al.*, 1996; Hunt and Nashabeh, 1999), but these variants are typically controlled during cell culture and are not removed during chromatography. To ensure that no variants are formed during purification, antibody stability is controlled during chromatography by limiting extremes of pH, temperature, and other process variables to reduce the amount of oxidation, deamidation, aggregation, and other variant-formation routes.

Many pharmaceutical proteins require a significant clearance of product-related variants. An example of this is insulin-like growth factor, where several product-related variants (such as a single amino acid oxidation and clipped forms) are removed to <1% during purification (Fahrner *et al.*, 1998, 1999b). The acceptable level of product-related variants is an issue which dates to the first proteins produced by recombinant DNA technology. The resolution and sensitivity of current analytical technology permits the definition of very minor differences among the product protein population. The fact that variants can be discovered does not automatically indicate that they need to be removed or even controlled. For example, the DNA sequence for IgG_1 antibodies codes for a lysine at the C-terminus of each heavy chain. During cell culture, one or both of these lysines are usually removed, leading to three charged populations (zero, one, or two lysines). This variability has no impact on the ability of the antibody to bind its target antigen or effect any biological activity. Therefore, the product definition would allow for all three species. The same approach can be extended to other product variants. It is necessary to characterize the molecular source of the variation and demonstrate that the variation has no effect on potency or safety.

From a recovery standpoint, one of the most significant advantages to using antibodies produced in CHO cells is that the level of product-related variants can be effectively controlled during cell culture so that little or no variants must be removed during recovery. This level of control during cell culture allows the use of a streamlined, three-step recovery process. Instead of focusing on the removal of product-related variants, the process is concerned with the clearance of pharmaceutical impurities such as virus, DNA, host cell proteins, endotoxin, and small molecules. This recovery process consists of protein A affinity chromatography, cation exchange chromatography, and anion exchange chromatography.

Antibody recovery

Since no single chromatography step can achieve the necessary antibody purity, the

three process steps must be integrated to meet the requirements of purity, yield, and throughput. In addition, the process must be robust, reliable, and amenable to validation.

The primary consideration is purity. While yield and throughput may be necessary for an economically viable product, without meeting the purity requirements for biological pharmaceuticals there will be no product at all. Throughput and yield are becoming more important as many clinical indications for antibodies require very high doses. At our manufacturing plant, we typically use processes that purify a 5–10 kg antibody batch in less than three days with greater than 65% overall process yield.

PURITY CONSIDERATIONS

Although pharmaceutical antibodies do not require the removal of product-related variants that complicate the purification of some proteins, other purity requirements are extreme. There are six main purity considerations for the recovery of pharmaceutical antibodies.

1. Host cell proteins

Host cell proteins are present in high amounts (sometimes >1,000,000 ng/mg) in the harvested cell culture fluid. They are typically removed during purification <5 ppm, a total reduction of at least 10^5. In our studies, the level of host cell proteins was measured quantitatively by ELISA (Chen, 1996) and qualitatively by SDS-PAGE.

For the ELISA, affinity purified goat anti-CHOP antibodies were immobilized on microtitre plate wells. Dilutions of the pool samples were incubated in the wells, followed by an incubation with peroxidase-conjugated goat anti-CHOP. The horseradish peroxidase enzymatic activity was quantified with *o*-phenylenediamine. Samples were serially diluted 2-fold in assay diluent so that the absorbance reading fell within the range of the standard curve (1.5 ng/ml to 400 ng/ml).

To analyse the antibody by SDS-PAGE, the pool samples were run under reducing and non-reducing conditions on one-dimensional Novex 8–16% Tris–glycine gels. Samples were loaded at 2.5 μg/lane for non-reducing conditions and 5.0 μg/lane for reducing conditions. The gels were silver-stained using the Novex silver express kit. The samples were compared to a reference standard for identification of product related bands.

2. DNA

The World Health Organization set a requirement for DNA in biopharmaceutical formulations of <10 ng/dose. DNA is present at high levels in the harvested cell culture fluid (>1,000,000 pg/mg) and must be removed to <10 ng/dose levels. During validation studies DNA may be spiked into the load to demonstrate clearance.

In our studies, the level of DNA was measured using the Molecular Devices Threshold DNA assay kit. The typical range of detection of the Threshold Total DNA assay was between 6.3 and 400 pg/ml. Samples were assayed at a minimum of 3 dilutions with and without a 100 pg spike of DNA. This procedure was used to evaluate DNA recovery because some buffers, impurities and proteins are known to

inhibit the detection of DNA and inhibit spike recovery. The mean value for all sample dilutions falling within the range of the standard curve and meeting spike recovery acceptance criteria was used.

3. Aggregate

The main product-related variant that must be reduced is aggregated forms of the antibody (mostly dimer) because of the possible immunogenicity of the aggregate. The aggregate content in the HCCF is about 5–15% for many antibodies, and it is typically reduced to below 0.5% in the final bulk. The primary step used to remove aggregate is cation exchange chromatography.

In our studies, aggregate was measured by size-exclusion chromatography. A Bio Sil SEC-250 7.5 x 300 mm column from BioRad was run at 1 ml/min using a mobile phase containing 50 mM NaH_2PO_4/50 mM Na_2HPO_4/0.15 M NaCl, pH 6.8. The column was equilibrated with the mobile phase buffer and 20 µl volumes of blank, standard, control and study samples were sequentially injected and run on the SEC for analysis.

4. Small molecules

The harvested cell culture fluid contains many small molecules, originating from the media components and created during cell culture by the CHO cells. Rather than determining the level of all small molecules, a few representative marker molecules are measured. Here we present the results from measurements of insulin and Pluronic F-68.

The level of Pluronic F-68 was measured using a 500 MHz NMR. NMR detects hydrogen-containing molecules based on magnetic moments. Pluronic has a characteristic peak in the spectrum with a chemical shift of 1.1 ppm, which was used for quantification. Peak areas in samples were compared with the standards. The Pluronic F-68 standard curve was run in process buffers, and covered the range of 25 µg/ml to 1024 µg/ml. As controls, the conditioned protein A pool was analysed unspiked and spiked with 25 µg/ml Pluronic F-68.

The level of insulin in the pool samples was determined by a competition ELISA. The monoclonal antibody to insulin was immobilized on microtitre plate wells. Diluted samples and biotinylated insulin were placed in the antibody immobilized wells. The insulin and biotinylated-insulin compete for binding to the antibody. The amount of bound biotinylated-insulin was detected with alkaline phosphatase–streptavidin and *p*-nitrophenyl phosphate substrate. All samples were assayed in wells coated with non-immune mouse antibody in place of the specific monoclonal antibody. This control showed that binding to the plate is mediated by the specific monoclonal antibody and not by a non-specific interaction.

5. Leached protein A

During protein A affinity chromatography, some protein A leaches from the column and ends up in the antibody pool. Because protein A can be immunogenic and cause other physiological reactions (Gagnon, 1996), leached protein A must be cleared during downstream chromatography.

The level of protein A in our samples was determined by a sandwich ELISA (Lucas *et al.*, 1988). Chicken anti-protein A antibody was immobilized on microtitre wells; Protein A binds to the coat antibody. The amount of bound protein A was detected with chicken anti-protein A labelled with biotin, followed by streptavidin–HRP and then the substrate *o*-phenylenediamine dihydrochloride and hydrogen peroxide. The reaction was stopped by adding sulphuric acid. The product was quantified by reading an absorbance at 490 nm. All samples were initially diluted to 0.2 mg/ml antibody in assay diluent. Samples were then serially diluted 2-fold with sample/standard diluent which contained 0.2 μg/ml antibody. Samples were assayed as a dilution series to ensure that antibody excess was reached. Values were calculated as the average of all results within the reporting range (0.78–25 ng/ml).

6. Virus

Harvested cell culture fluid may have 10^4 or more retrovirus-like particles per ml, and biological pharmaceuticals are allowed to have 1 theoretical virus particle per 10^6 doses, so the recovery process must provide significant virus clearance. The validation and test procedures for viral clearance are complicated and are beyond the scope of this chapter. However, the process is capable of clearing virus to acceptable levels. In general, the protein A affinity chromatography step provides 10^7 ('7 logs') of virus clearance (10^4 by removal and 10^3 by low-pH inactivation in the elution pool), and the anion exchange chromatography step provides 10^4 (4 logs) of viral clearance by removal. If this level of viral clearance is not sufficient, additional process steps such as viral filtration may be required.

Purity calculations

For all quantitative assays, the level of impurity in the sample is calculated by multiplying the measured value by the sample dilution. Since samples may be diluted to differing extents to avoid matrix interference, the absolute sensitivity (LOQ) of the assay will be influenced by the required sample dilution. Because values are often reported in ppm or ng of impurity per mg of product (not ng/ml), the reported sensitivity will also depend on the product concentration in the sample.

THREE-STEP RECOVERY PROCESS

The purity, yield, and throughput requirements can be achieved using three chromatography steps: protein A affinity chromatography, followed by cation exchange chromatography, followed by anion exchange chromatography. Protein A and cation exchange chromatography are run in bind-and-elute modes, while the anion exchange chromatography is run in flow-through mode (for antibodies with pI greater than about 8). Running in these modes in this order produces a high-yield process capable of meeting the purity requirements (*Table 12.1*).

We present methods that may be applied to many antibodies, but it is important to note that some antibodies may have specific considerations, such as susceptibility to aggregation, oxidation, deamidation, or other stability problems. In these cases, adjustments to the process may have to be made. For example, an antibody that is

Table 12.1. Typical yield and purity values for the three-step antibody recovery process

	Yield (%)	Host cell proteins (ng/mg)	DNA (pg/mg)	Endotoxin (EU/mg)	Protein A (ng/mg)	Aggregate (%)
HCCF	–	250,000–1,000,000	100,000–1,500,000	5–100	–	5–15
Protein A	>95	200–3000	100–1000	<0.005	3–35	5–15
Cation	75–90	25–150	<10	<0.005	<2	<0.5
Anion	>95	<5	<10	<0.005	<2	<0.5

highly prone to deamidation may require a limit on its exposure to high pH (>8) during recovery. An important part of process development is determining the stability of the antibody, since product stability will strongly influence the specific parameters used during recovery.

The first step in the process is protein A affinity chromatography (*Figure 12.1*). The majority of the purification occurs during protein A affinity chromatography (*Table 12.1*), which clears host cell proteins, DNA, and endotoxin. In addition, it removes insulin and Pluronic F-68 to less than detectable levels. However, it does not clear aggregate, and it adds protein A into the pool.

Protein A is a bacterial cell wall protein that binds specifically to antibodies, and it binds particularly well to human IgG_1. When immobilized onto chromatography

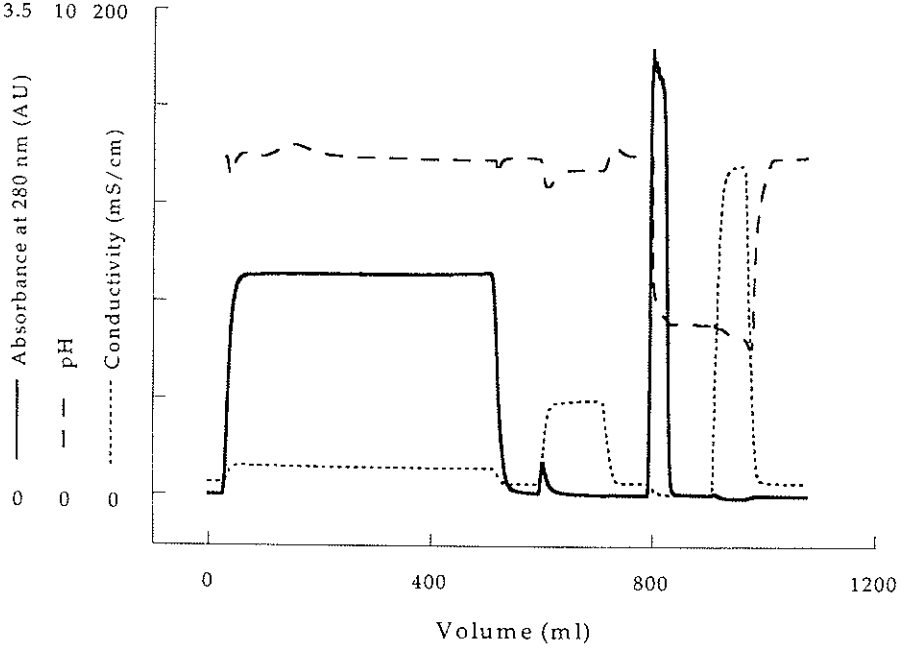

Figure 12.1. Chromatogram from a typical protein A affinity chromatography run. A 1.0 cm inner diameter × 20 cm length column was packed with Prosep A chromatography media. Four buffers were used. Buffer A was 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1; buffer B was 25 mM Tris, 25 mM NaCl, 5 mM EDTA, 0.5 M tetramethylammonium chloride pH 7.0; buffer C was 0.1 M acetic acid, pH 3.5; and buffer D was 2 M guanidine HCl, 10 mM Tris, pH 7.5. The column was equilibrated with 5 column volumes of buffer A, loaded to 20 g/l, washed with 3 column volumes of buffer A, washed with 3 column volumes buffer B, washed with 3 column volumes of buffer A, eluted with 5 column volumes of buffer C, and regenerated with 3 column volumes of buffer D. The column was run at 550 cm/h.

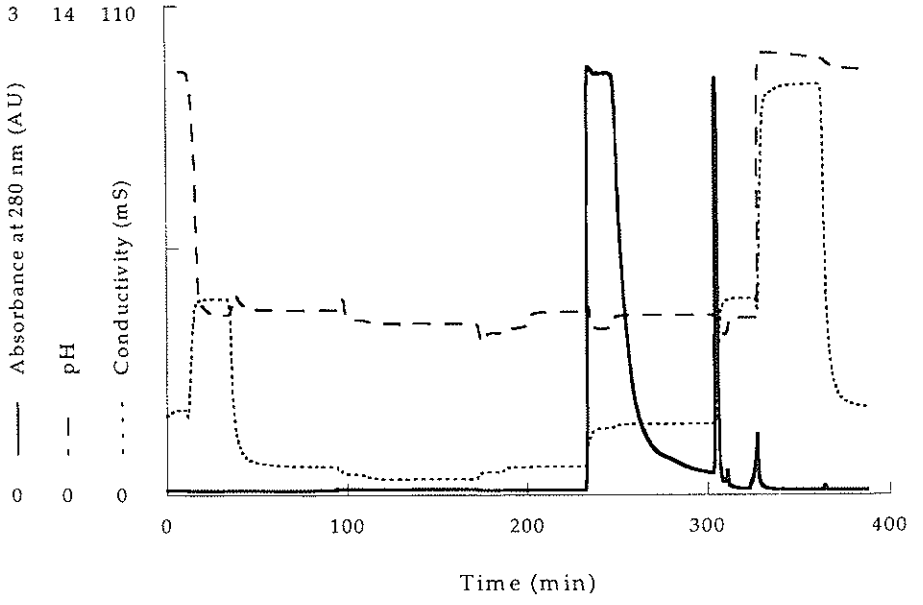

Figure 12.2. Chromatogram from a typical cation exchange chromatography run. The column was 0.66 cm inner diameter × 20 cm length, packed with Poros 50 HS. The column was washed with 2 CV of 0.016 M MES/0.004 M NaMES/0.500 M NaCl, pH 5.5, then equilibrated with 5 CV of 0.016 M MES/0.004 M NaMES/0.060 M NaCl, pH 5.5, loaded to 40 g/l, washed with 5 CV of 0.016 M MES/0.004 M NaMES/0.060 M NaCl, pH 5.5, eluted with 5 CV of 0.016 M MES/0.004 M NaMES/0.160 M NaCl, pH 5.5, regenerated with 2 CV of 0.016 M MES/0.004 M NaMES/0.500 M NaCl, pH 5.5, sanitized with 2 CV of 0.5 N NaOH, and stored in 3 CV of 0.1 N NaOH.

media, protein A provides a technique for purifying recombinant antibodies because it can selectively bind antibodies in complex solutions, allowing impurities to flow through (Ey *et al.*, 1978; Surolia *et al.*, 1982; Lindmark *et al.*, 1983; Reis *et al.*, 1984). Protein A affinity chromatography is by far the most effective type of chromatography for removal of host cell proteins and small molecules, and this is the main reason that it is used for antibody purification.

In the past, the harvested cell culture fluid was often concentrated before the first chromatography step to decrease the loading time. With the development of high-titre cell culture (typically >0.5 g/l) and protein A affinity chromatography media capable of high capacity at high flow rate (typically 20 g/l at 40 CV/h), the need to concentrate the harvested cell culture fluid has been eliminated. In our three-step process, the harvested cell culture fluid is loaded directly onto the protein A column. Because protein A affinity chromatography media is expensive, a smaller column is cycled several times to purify a single batch. This is possible because of the high flow rates that can be achieved for protein A columns.

Cation exchange chromatography (*Figure 12.2*) is the second step. It uses a negatively charged group (typically sulphopropyl) immobilized to the chromatography media. Cation exchange chromatography clears host cell proteins, aggregate, and leached protein A (*Table 12.1*). The antibody binds to the negatively charged sites on the column, and it is eluted with a step gradient to high salt. Host cell proteins, aggregate, and leached protein A elute in the regeneration phase, after the antibody

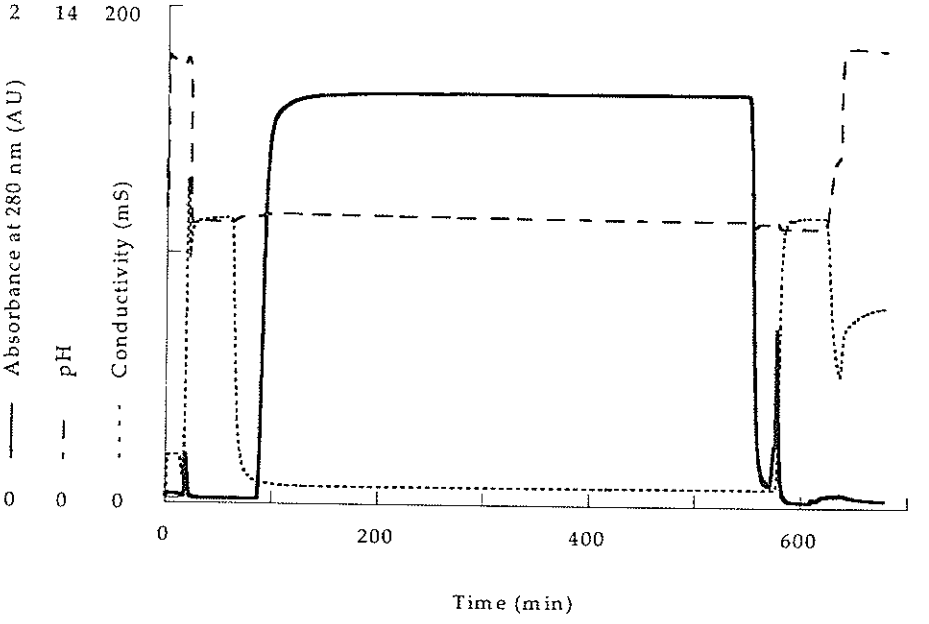

Figure 12.3. Chromatogram from a typical anion exchange chromatography run. The column was 0.66 cm inner diameter x 20 cm length, packed with Q Sepharose Fast Flow. The column was washed with 3 CV of 0.180 M Tris HCl/0.07 M Tris Base/2.0 M NaCl, pH 8.0, equilibrated with 4 CV of 0.018 M Tris HCl/0.007 M Tris Base/0.05 M NaCl, pH 8.0, loaded to 100 g/l, washed with 7 CV of 0.180 M Tris HCl/0.07 M Tris Base/2.0 M NaCl, pH 8.0, regenerated with 3 CV of 0.25 M Tris/2.0 M NaCl, pH 8.0, sanitized with 2 CV of 0.5 NaOH, and stored in 3 CV of 0.1 NaOH, 3 CV.

has eluted. Cation exchange columns can be loaded to >40 g/l, which allows the batch of antibody to be purified in a single cycle on a reasonably sized column.

Anion exchange chromatography (*Figure 12.3*) is the last chromatography step. It uses a positively charged group (typically quaternary amine) immobilized on the chromatography media. Anion exchange chromatography can be run in flow-through mode, which means that the antibody product flows through the column while the impurities bind. It removes DNA and residual host cell proteins. These impurities are removed from the column with a regeneration step, typically 0.5–1 M NaOH.

These three steps together comprise a process that, while meeting stringent purity and throughput restrictions, still produces a high yield of antibody (*Table 12.1*). The protein A affinity step has >95% yield, the cation exchange step has >75% yield, and the anion step has >95% yield, for an overall >65% process yield, which is exceptional for an industrial process with these extreme purity requirements. By choosing and sizing columns correctly and running them under conditions for high capacity, the throughput requirements can be met.

PROCESS VALIDATION

Validation is a regulatory requirement to demonstrate that a process, when operated within set parameters, can consistently produce a specified product. The complete validation plan is extensive and includes validation of process equipment, software, utilities, equipment cleaning, and analytical methods.

According to the U.S. FDA, 'Process validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes' (FDA's Guidelines on General Principles of Process Validation, May 1, 1987). For chromatography processes, this means in part that processes must be validated at extremes of operating parameters such as load, conductivity, pH, and column lifetime.

An important part of the validation effort is developing and writing validation protocols. A validation protocol is 'a written plan stating how validation will be conducted, including test parameters, product characteristics, production equipment, and decision points on what constitutes acceptable test results' (ibid.). The chromatography validation studies should be carefully designed in advance, and data generated during process development is often used to determine validation ranges and critical process variables.

Some validation studies must be performed at manufacturing scale. This includes the validation of process purity, where the levels of impurities are measured at each process step over several (usually three) runs. Validating the removal of impurities can eliminate the necessity to measure these impurities in each batch prior to release. The specific impurities to be measured are determined in advance, and a table similar to *Table 12.1* is constructed showing the measured levels across the process. Consistent results can then be demonstrated for consecutive runs.

Several studies that are not practical to do at manufacturing scale may be performed at laboratory scale (Sofer, 1996). These include viral clearance, hold times for product pools and buffers used in production, and column parameter and re-use. In the following sections, we present data from studies that validated the column operating ranges (parameter validation) and the column lifetime (re-use validation). This data also serves to illustrate the constraints under which the processes must operate, which may in turn affect the development effort.

Parameter validation determines the effects of the variation of process conditions on the product and the process, because processes must be robust within the licensed operating parameters (Kelley *et al.*, 1997). Typical variables that are studied during characterization are load, buffer conductivity, and buffer pH. The effect on the product and process is measured by yield and purity. Column lifetime should be prospectively determined, and re-use validation determines a limit on the number of times a chromatography column may be re-used or cycled (Seely *et al.*, 1994).

Both parameter and re-use validation were performed at laboratory scale. When using laboratory scale studies as part of the overall chromatography validation plan, every parameter except column diameter must be the same as manufacturing scale. To ensure comparability to the manufacturing process, all process parameters, including buffers, volumes (measured in CV), and column heights were the same as the manufacturing process. Only the column diameter was changed. The buffers were prepared according to the manufacturing batch records using GMP raw materials.

Protein A affinity chromatography

DEVELOPMENT AND OPERATION

The basic protocol of a protein A affinity column is straightforward: bind at neutral pH and elute at acid pH. This simple bind/elute chemistry does not leave much room

for purification optimization, but since protein A affinity chromatography provides extreme purification in a single step, even an unoptimized process can produce a highly purified antibody. The optimization effort typically focuses not on purity but on throughput.

Protein A affinity media is expensive compared to ion exchange media – more than 30 times the cost. While the ion exchange process columns are sized so that a batch of antibody can be purified on a single cycle on the column, protein A affinity columns are sized to run several cycles to purify a single batch in order to minimize the cost of the column (as well as minimizing the cost of replacing the column if it is damaged). This cycling requires throughput optimization in order to purify the antibody in a reasonable amount of time. One important factor in optimizing throughput is the column capacity.

Capacity is affected by many variables, including the type of protein A affinity chromatography media, ligand density, the antibody concentration in the load, the column temperature and column length, the buffer, conductivity, and pH of the load, and the flow rate (Katoh *et al.*, 1978; Tu *et al.*, 1988; Fuglistaller, 1989; Kamiya *et al.*, 1990; Kang and Ryu, 1991; Schuler and Reinacher, 1991; Van Sommeren *et al.*, 1992). Of these variables, the simplest to control for production and the ones that will have the most significant impact on capacity are the column length, the flow rate, and the chromatography media. Bed height and flow rate both affect the breakthrough capacity; together bed height and flow rate determine the residence time (Fahrner *et al.*, 1999a).

Several types of chromatography media are available for process applications. They include Sepharose Fast Flow (crosslinked agarose), Poros 50 (polystyrene-divinylbenzene), and Prosep (controlled-pore glass). In a study comparing these sorbents (Fahrner *et al.*, 1999c), we found that the sorbent type and flow rate had a strong effect on breakthrough capacity (*Figure 12.4*). Flow rate had the strongest effect on Sepharose; while both Poros and Prosep were less strongly affected by flow rate, Poros had a higher capacity at all flow rates. The type of media had a strong effect on breakthrough capacity, but it did not strongly affect the purity of the antibody (*Table 12.2*). For example, the amount of host cell proteins in the purified antibody pools ranged from 2.5 mg/g to 4.9 mg/g. The amount of host cell proteins in the load was approximately 950 mg/g (950,000 ppm), so these numbers represent a range from 380-fold clearance to 190-fold clearance. The Poros sorbent may have the least non-

Table 12.2. Comparison of protein A affinity chromatography sorbents

	Poros 50	Prosep	Sepharose
Pressure drop (psi h $cm^{-2} \times 10^{-3}$)	3.2	0.3	1.1
Purified antibody			
Yield (%)	104 ± 1	103 ± 2	100 ± 2
DNA (ng/mg)	41 ± 3	40 ± 4	29 ± 2
Host cell proteins (mg/g)	2.5 ± 0.2	3.7 ± 0.2	4.9 ± 1.2
Protein A (ng/mg)	4.6 ± 0.5	3.1 ± 0.5	5.7 ± 1.7

(Data from Fahrner *et al.*, 1999c.) Values for yield (percent loaded antibody in the purified pool), host cell proteins (mg host cell proteins per g antibody), DNA (ng DNA per mg antibody), and protein A (ng protein A per mg antibody) were for runs using a 10 cm column length and 500 cm/h flow rate (50 CV/h), loaded to their capacity determined at 1% breakthrough. Values are the average of three runs, plus or minus one standard deviation. Load material was clarified Chinese hamster ovary cell culture fluid.

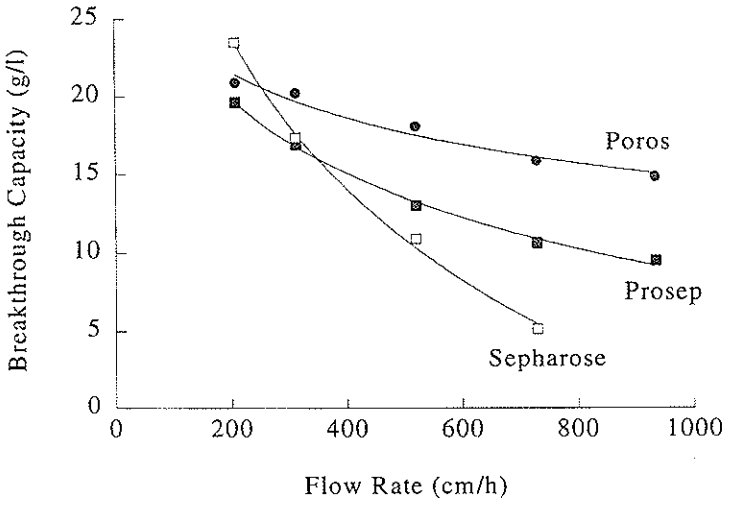

Figure 12.4. Effect of sorbent and flow rate on the breakthrough capacity for protein A affinity chromatography. Data from Fahrner *et al.* (1999c).

specific binding since Poros 50 has the lowest value for host cell proteins. However, even the lowest clearance (for Sepharose) represents a 99.48% removal of host cell proteins, leaving a small amount of host cell proteins that can be cleared downstream.

In our work, we use Prosep A (controlled-pore glass) because it provides good purity, low protein A leakage, and good throughput. It is also easy to pack due to a fast settling rate and high permeability. We have found that it withstands re-use reasonably well, and for several antibodies we have validated its lifetime to 300–400 cycles.

Although the development of protein A affinity chromatography does not focus on purity, purity is still a concern. One way to decrease the amount of host cell proteins in the elution pool when using Prosep media is by using an intermediate wash with tetramethylammonium chloride (TMAC). Since the base material for Prosep is controlled-pore glass that is made from silica, any exposed silica surface may bind proteins. TMAC is known to elute proteins from silica (Chandha and Sulkowski, 1981; Reifsnyder *et al.*, 1996), and in the chromatogram in *Figure 12.1*, a peak is apparent when TMAC is introduced into the column. This peak is not nearly as large when washing with other salts such as sodium chloride or sodium sulphate, which supports the theory that TMAC is eluting host cell proteins that are bound to the exposed silica.

Development of the elution stage is concerned with the elution buffer (Narhi *et al.*, 1997). For elution at pH <3, either citrate or acetate may be used. Some antibodies may partially or completely precipitate in one of these buffers, but generally either citrate or acetate will produce high recovery yield. The elution buffer should be used in an amount that will produce a pool of pH <3.8, so that no adjustment is required for viral inactivation, which requires pH <3.8 for 15–30 min.

The protein A column may typically be run in 4–10 cycles to purify a single batch. Since each cycle is only about 1 hour long, this cycling allows rapid throughput while reducing the cost of the column. The load material must be assayed for antibody concentration prior to loading, usually by an analytical protein A affinity assay. After

Table 12.3. Results from the protein A affinity chromatography re-use study

Cycle	Yield (%)	CHOP (ng/mg)	DNA (ng/mg)	Insulin (ng/mg)	Protein A (ng/mg)	Pluronic F-68 (ng/mg)
5	101	516	1.3	<1.5	6.0	<1246
150	103	525	1.5	<2.0	17.9	<1743
300	96	1455	0.5	<1.7	18.9	<1475
340	90	1604	0.1	<1.8	34.3	<1989

determining the antibody concentration, the load is split up into the minimum amount of cycles that can purify the batch. This may underload the column for each cycle. Because the measurement of the load volume may be inaccurate, it is important to have an air sensor on the load line to end loading of the last cycle if the load runs out early. During elution, the pool begins when the absorbance reaches a predetermined value. The pooling may end when either absorbance or volume reaches a predetermined value. Typically, the pH of the elution pool is adjusted to >5 before holding for an extended amount of time because the antibody may be unstable in the elution pool due to the low pH.

RE-USE VALIDATION

Because protein A affinity chromatography media is expensive, column re-use is a significant concern. For one of our antibodies, we validated the life of the column to 340 cycles. A laboratory scale study was performed to demonstrate that the quality of the product purified by the protein A affinity chromatography step was not affected by multiple re-use of the Prosep A resin. Representative cycles (5, 150, 300, and 340) over the course of the study were selected and the pools were analysed for yield and purity (host cell proteins, DNA, protein A, insulin, and Pluronic F-68). These pools were also analysed by SDS-PAGE under reducing and non-reducing conditions. Chromatograms for these selected cycles were compared to a reference chromatogram.

The yield and purity (host cell proteins, DNA, insulin, protein A and Pluronic F-68) for the four representative cycles are shown in *Table 12.3*. The yields ranged from 90% to 103%. Although there is an apparent decrease in yield with increasing cycle number, a yield of 90% is acceptable. The levels of DNA ranged from 0.1 to 1.5 ng/mg. The levels of host cell proteins ranged from 516 to 1604 ng/mg. An approximate three-fold variation in host cell protein levels in the protein A pool does not significantly affect the final product purity, given that the process has been validated to consistently clear host cell proteins by greater than one thousand-fold in subsequent steps. Insulin levels were consistently below the limit of quantitation (LOQ <2 ng/mg). Protein A levels ranged from 6.0 to 34.3 ng/mg. These levels were less than the manufacturing action limit of 50 ng/mg. The levels of Pluronic F-68 were consistently below the LOQ (<2000 ng/mg). Although the change in host cell proteins and leached protein A suggested ageing of the column, the extent of this was not sufficient to warrant reducing the column lifetime to less than 340 cycles.

Pool samples run on SDS-PAGE, under reducing and non-reducing conditions, were consistent over the course of the 340 cycles (*Figures 12.5A* and *12.5B*). A small band appears in cycle 300 (lane 10, non-reduced) but does not appear in cycle 340

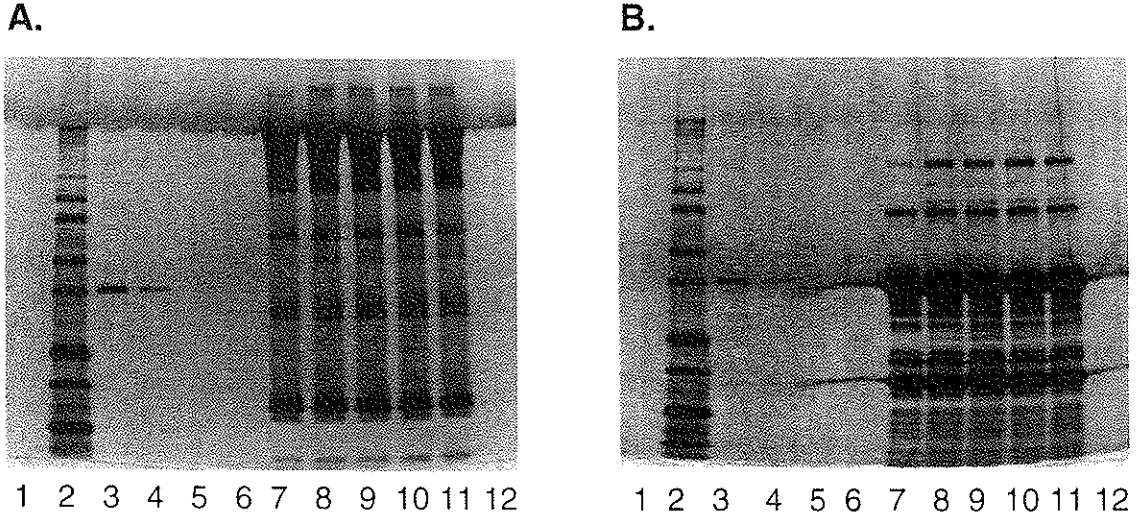

Figure 12.5. (A) Non-reduced and (B) Reduced SDS-PAGE of elution pools from the protein A affinity chromatography re-use validation study. For both gels, lane 1: buffer blank, 2: Molecular weight standards (200, 116, 97, 66, 55, 36, 31, 21, 14 kD), 3: 20 ng BSA, 4: 2 ng BSA, 5: buffer blank, 6: buffer blank, 7: antibody standard, 8: pool from cycle 5, 9: cycle 150, 10: cycle 300, 11: cycle 340.

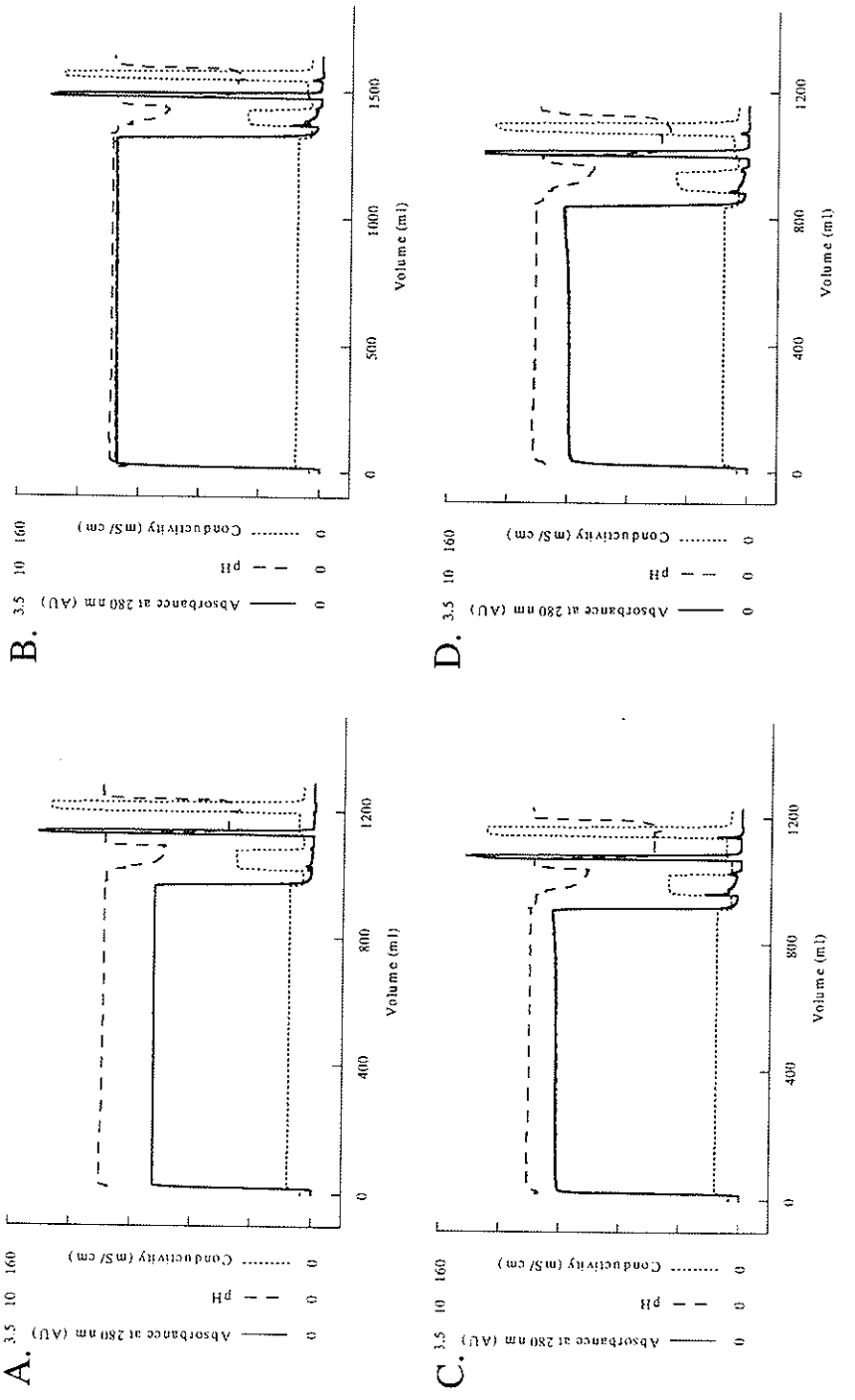

Figure 12.6. Chromatograms from the protein A affinity chromatography re-use validation. (A) Cycle 5. (B) Cycle 150. (C) Cycle 300. (D) Cycle 340.

(lane 11, non-reduced), indicating that this is not a trend. The chromatograms from the representative cycles were compared with the chromatogram from cycle 5, the reference chromatogram (*Figure 12.6*). The peak shape and the absorbance, pH, and conductivity profiles of the reference chromatogram were consistent throughout the study.

Cation exchange chromatography

DEVELOPMENT AND OPERATION

Cation exchange chromatography is used as the intermediate purification step for recombinant antibodies. Although the protein A affinity step greatly reduces the amount of host cell proteins, DNA, and endotoxin, these impurities must be further removed. In addition, the protein A affinity step does not reduce the level of aggregate, and it introduces protein A molecules into the purified antibody. Cation exchange chromatography reduces the level of host cell proteins, DNA, endotoxin, aggregate, and leached protein A. It will also reduce the level of any misformed antibody (for example, antibody with two heavy chains and one light chain). Some host cell proteins flow through the column during load, some elute with the antibody, but the majority elute during the regeneration phase. During chromatography, the leached protein A and aggregate also elute during the regeneration phase.

Development of the cation exchange step focuses on several aspects: the chromatography media, the wash and elution conditions, and the load onto the column. For large-scale use, several vendors can supply chromatography media, including Pharmacia of Uppsala, Sweden (which makes Sepharose) and PerSeptive Biosystems of Framingham, MA, U.S.A. (which makes Poros). We have found that the best media depends on the antibody. For the antibody shown in *Figure 12.2*, Poros 50 HS separated aggregate better than SP Sepharose Fast Flow, providing higher yield at equivalent purity.

There are two critical variables to investigate when developing the wash and elution conditions: the buffer pH and the amount of salt in each buffer. The pH of the separation will be determined in part by the stability of the antibody, and before finalizing the separation pH the stability of the antibody at that pH should be evaluated. We have found that a pH of 5.5 is often optimal for bind-and-elute cation exchange chromatography of antibodies. This pH is high enough that antibody stability is not a problem, and it is low enough to provide sufficient capacity. The typical column capacity at pH 5.5 and a load conductivity <8 mS/cm is about 40 g/l. The conductivity of the protein A affinity chromatography pool is low (<5 mS/cm), so capacity is not greatly affected by pH until the pH is >7 (about 2 pH units below the antibody pI), when capacity may decrease.

The elution conditions are optimized in series of experiments where the column is loaded to capacity and eluted using varying concentrations of sodium chloride or another salt. The collected pool is analysed for yield, purity (aggregate, host cell proteins, DNA, and protein A), and peak width. Generally, at lower salt concentrations the antibody may not completely elute and yield will be low, and at higher salt concentrations the yield will be high but aggregate and host cell proteins will begin eluting with the antibody. This balance between purity and yield will then be the focus

of the development effort. The width of the eluted peak is important for process applications, since a large peak will need a large tank for collection, and the peak volume will affect the throughput of the next step since a larger peak will take a longer time to load. At higher salt concentrations the width will be narrow and at lower salt concentrations the peak will be broader.

If pH is also a factor during development of the elution conditions, the varying salt conditions are applied at varying pH values. This will produce a complex interaction between pH, salt concentration, yield, and purity.

Impurities are removed from the column by increasing the conductivity with a regeneration buffer wash. For development purposes, this wash may be 1 M NaCl or some other appropriate salt so that the regeneration fraction may be assayed for host cell proteins, DNA, and antibody to ensure mass balance. Later, in production, this high-salt wash is generally replaced with sanitization buffer such as 0.5 or 1 M NaOH. In addition to eluting proteins due to the high ionic strength, NaOH also degrades proteins and other molecules and strips them from the column.

Operation at large scale is straightforward except for two factors: column size and peak collection. Since the run time for a typical cation exchange column is about 8 hours, column cycling is not desirable. Therefore the column is usually sized to purify a batch of antibody in a single cycle. The maximum column diameter may be determined by plant size or other considerations, so often the bed height (or column length) will need to be adjusted to have a column size sufficient to purify the batch of antibody. The bed height may affect the separation, so this parameter should be defined during development or the effect of the bed height on the separation should be evaluated during development. The bed height is typically 20–30 cm.

Peak collection begins when the absorbance reaches a pre-set value. It ends when either a pre-set absorbance or volume is reached. When developing elution conditions, the balance between purity, yield and peak width may result in elution conditions where the aggregate is not baseline resolved from the antibody. In this case, rather than eluting in the regeneration, some aggregate may elute in the tail of the main antibody peak. When this occurs, special attention needs to be paid to the pooling conditions so that an antibody peak low in aggregate can be collected. Often, by ending the pool at a relatively high absorbance, a low-aggregate peak can be collected without greatly affecting yield.

PARAMETER VALIDATION

Because the salt concentration and pH of the elution buffer has a strong effect on the antibody separation, the effect of these variables was carefully validated. A laboratory scale cation exchange column was packed (6.84 ml volume, 0.66 cm diameter and 20 cm bed height). The column was packed at the maximum flow rate of the study (200 cm/h). The packed column was sanitized with 0.5 N NaOH (2 CV) and stored with 0.1 N NaOH (3 CV). The control chromatography run was performed with a load of 40 g/l and a flow rate of 100 cm/h. Values measured during the parameter validation are compiled in *Table 12.4*.

Variation in antibody loaded onto the cation exchange resin (10 to 50 g antibody per litre of resin) had no significant effects on the chromatography, recovery, or the quality of the product in the pool. Several load-dependent effects were evident in the

Table 12.4. Results from the cation exchange parameter chromatography validation study

Load (g/l)	Flow rate (cm/h)	Buffer pH	Buffer addition	Yield (%)	Pool concentration (g/l)	Aggregate (%)	DNA (log clearance)
10	–	–	–	82	1.63	0.00	>1.85
20	–	–	–	88	3.48	0.00	>2.18
30	–	–	–	91	5.44	0.57	>2.37
40*	100	5.5	–	86	6.74	0.51	>2.47
50	–	–	–	89	8.58	0.66	2.27
–	200	–	–	87	7.00	0.71	2.3
–	50	–	–	86	6.92	0.59	>2.48
–	–	5.6	–	91	7.26	1.55	>2.5
–	–	5.4	–	82	6.43	0.00	2.04
–	–	–	– 5 mM NaCl	89	6.84	0.49	>2.47
–	–	–	+ 5 mM NaCl	90	6.87	0.95	>2.48

* indicates the control run
– indicates that the baseline condition was used

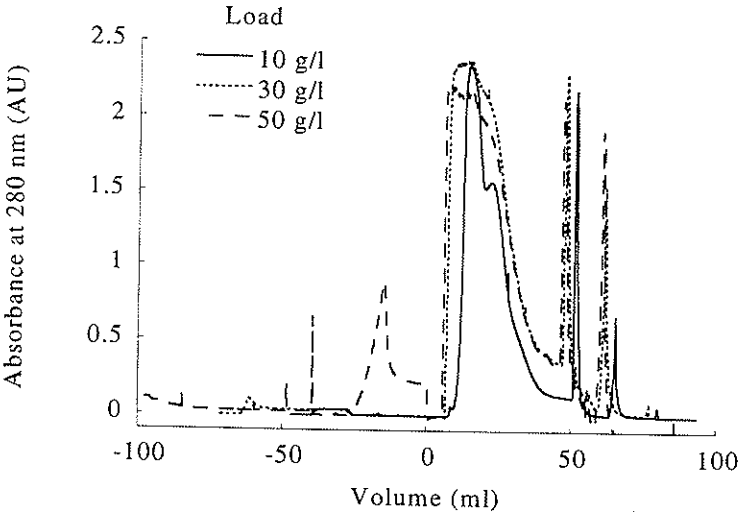

Figure 12.7. Overlaid chromatograms from the cation exchange load validation study. The x-axis is volume from the start of elution. Except for the amount of antibody loaded, conditions are the same as in *Figure 12.2*.

chromatograms (*Figure 12.7*). An increase in absorbance during loading showed some breakthrough at 50 g/l loads, but the amount of protein lost due to breakthrough was minimal (1.8% of the product loaded on the column) and had no significant effect on the recovery of product in the elution pool. Although the elution peak showed some broadening with increasing loads (initially on the tailing edge, then on the leading edge), the broadening of the elution peak does not effect the performance of the chromatography step since the defined pooling criteria (five column volumes) accommodates the largest load. As the overall mass of the load increased, the components which elute in the regeneration and sanitization peaks increased as well.

The antibody yield in the elution pool was independent of the mass of antibody loaded on the column and was approximately 85% of the protein loaded. This consistent recovery of product in the pre-set pool volume results in a concentration of antibody in the pool proportional to the antibody loaded.

The antibody quality in the pool was not affected by the amount of antibody loaded, as determined by size exclusion chromatography and DNA analysis. In addition, the relative SDS-PAGE purity of these pool samples was equivalent to the antibody produced at manufacturing scale, and a significant reduction of the highest molecular weight band seen in the non-reduced load sample was observed in each pool sample. The later finding is consistent with the SEC data of the load and pool samples. Monomeric antibody was measured at 95.4% in the load and 99.3% in the pool samples. This improvement in product quality is a result of aggregate protein being retained on the cation exchange resin during elution and is unaffected by load amount. Furthermore, the measured reduction of DNA from the load to the pool samples was a minimum of 2 logs regardless of the antibody applied to the cation exchange resin.

At the flow rate range of 50 to 200 cm/h, there was no difference in the cation exchange chromatography of antibody. There was no peak broadening either on the leading or tailing edge of the elution profile due to change in flow rate. The yield,

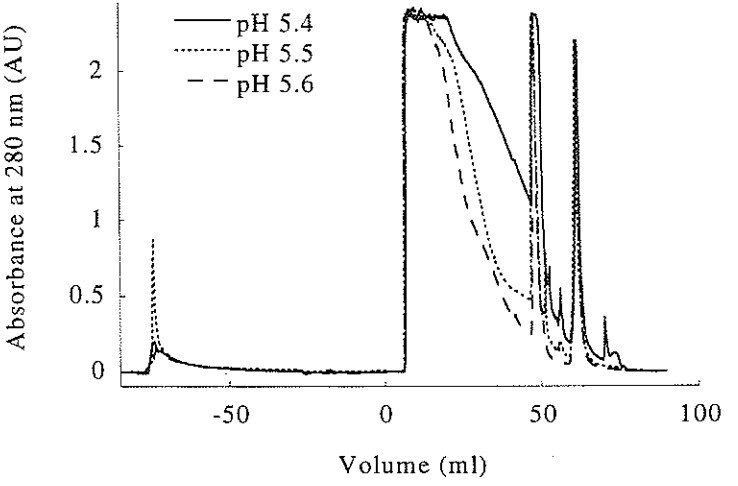

Figure 12.8. Overlaid chromatograms from the cation exchange pH validation study. The x-axis is volume from the start of elution. Except for the pH of the buffers, conditions are the same as in *Figure 12.2*.

SDS-PAGE, SEC, and DNA were consistent for all flow rates. The yield of antibody in the pools ranged from 87% to 89%. The reduced and non-reduced SDS-PAGE gel for pool samples demonstrated equivalence to the cation exchange pools produced at manufacturing scale. Finally, the DNA reduction from the load to the pool of the cation exchange chromatography was a minimum of 2.3 logs regardless of the flow rate applied to the system.

At the pH range of 5.4 to 5.6, the cation exchange chromatography and the antibody quality was not significantly affected. While buffer pH change within this range does have an effect on the elution profile (*Figure 12.8*), the recovery of product and the antibody quality in the eluate pool was acceptable. Although increasing buffer pH leads to an elution profile which approaches baseline at a faster rate, the product recovery yield of antibody in the cation exchange pool is at least 82.6%. In addition, the amount of monomeric antibody as determined by SEC is at least 98.5%. Finally, the DNA is reduced by a minimum log factor of 2.04 regardless of the buffer pH used to perform cation exchange chromatography of antibody.

With conductivity changes of ± 5 mM NaCl in each buffer used for cation exchange chromatography, the chromatography and the antibody quality was not significantly affected. With these changes in buffer conductivity, a minimal shift in the elution profile was noted. However, the resulting pool is acceptable as measured by product recovery yield, SDS-PAGE, SEC, and DNA analysis. The yield was consistent among runs, ranging from 87.1% to 89.9%. The SDS-PAGE and the SEC indicate product equivalent to the reference product. The amount of monomer is at least 99.0% in the pools produced by change of conductivity. Finally, the amount of DNA in these pools is more than 2.5 log less than the amount of DNA present in conditioned protein A pool and independent of the buffer conductivity.

The findings of this study demonstrated that this chromatography can withstand the extremes of each parameter tested to produce pools of acceptable antibody yield and purity.

Table 12.5. Results from the cation exchange chromatography re-use study

Cycle	Yield (%)	CHOP (ppm)	DNA (ppm)	Insulin (ppm)	Protein A (ppm)	Aggregate (%)
1	88.7	75	<0.006	<4.61	<3.9	0.67
11	90.4	90	0.060	<3.9	0.68	
21	90.0	85	0.012	<4.56	<3.9	0.44
31	90.6	63	0.058	<3.9	0.44	
41	95.9	35	0.015	<4.40	<3.9	0.33
50	88.3	34	0.011	<4.74	<3.9	0.30
Average	90.6	64	0.48			
Standard deviation	2.4	22	0.15			

RE-USE VALIDATION

To demonstrate that the quality of the product purified by the cation exchange chromatography step is not affected by multiple re-use of the column, a laboratory scale study was performed. A 1.6 cm inner diameter × 20 cm length column (column volume = 38 ml) was packed with cation exchange media. 50 cycles were performed on the laboratory scale column using the same operational parameters and setpoints/ranges as used in manufacturing. Because the column used at manufacturing scale is often repacked between manufacturing campaigns, the laboratory scale column was periodically repacked. The column was placed in storage buffer after each cycle. During re-use, to simulate varying process conditions encountered in production, the load of the column was changed each cycle, varying between 10–50 g/l.

Six representative pools over the course of the study were analysed for total protein and purity (SDS-PAGE, aggregate, protein A, DNA, and host cell proteins), and four representative pools were analysed for insulin (*Table 12.5*).

Yield varied from 88.7% to 95.9%, with no significant change from cycle 1 (88.7%) to cycle 50 (88.3%). DNA varied from <0.006 ppm to 0.06 ppm, with no significant change from cycle 1 (<0.006 ppm) to cycle 50 (0.011 ppm). Host cell proteins varied from 34 ppm to 90 ppm, with no significant change from cycle 1 (75 ppm) to cycle 50 (34 ppm). The level of protein A in the pools was less than detectable (<3.9 ppm). The levels of insulin were less than detectable. Aggregate varied from 0.30% to 0.67%, with no significant change from cycle 1 (0.67%) to cycle 50 (0.30%). None of the observed fluctuations are significant enough to impact the product quality or the downstream process.

The SDS-PAGE gels, both reduced and non-reduced, are shown in *Figures 12.9A* and *12.9B*. No significant new bands or change in the profile of protein distribution appeared over the course of 50 cycles, and bands on the pool samples were of comparable density to the reference material. In addition, the chromatograms were consistent with respect to absorbance, pH, and conductivity.

Yield, host cell proteins, aggregate, and DNA did not change significantly through the cycles. Insulin and protein A were reduced to non-detectable levels in all cycles assayed. The results of this study demonstrated that the cation exchange column performs consistently throughout 50 cycles. Product quality, as measured by yield, host cell proteins, DNA, insulin, and protein A, remained consistent throughout 50 cycles. Column performance, as measured by comparing chromatograms and by column integrity, remained consistent throughout 50 cycles.

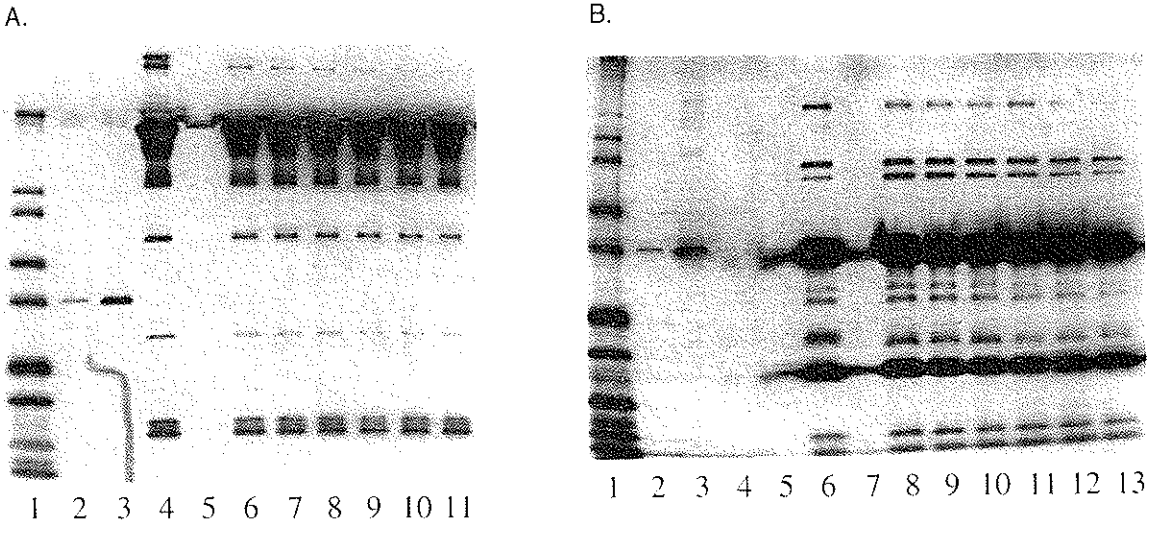

Figure 12.9. (A) Non-reduced and (B) Reduced SDS-PAGE of elution pools from the cation exchange chromatography re-use validation study. (A). Lane 1: Molecular weight standards (200, 116, 97, 66, 55, 36, 31, 21, 14 kD), 2: 2 ng BSA, 3: 50 ng BSA, 4: antibody standard, 5: buffer blank, 6: pool from cycle 1, 7: cycle 11, 8: cycle 21, 9: cycle 31, 10: cycle 41, 11: cycle 50. (B). Lane 1: Molecular weight standards, 2: 2 ng BSA, 3: 50 ng BSA, 4: buffer blank, 5: buffer blank, 6: antibody standard, 7: buffer blank, 8: pool from cycle 1, 9: cycle 11, 10: cycle 21, 11: cycle 31, 12: cycle 41, 13: cycle 50.

Anion exchange chromatography

DEVELOPMENT AND OPERATION

The isoelectric point of many antibodies is high (often >8 and sometimes >9), so anion exchange chromatography run in flow-through mode offers a high-yield method for final purification of antibodies with a high pI. For antibodies with a pI lower than about 8, the anion exchange chromatography step may be run in bind-and-elute mode, which may offer advantages over the flow-through mode, such as additional clearance of leached protein A.

For anion exchange chromatography run in flow-through mode, the pH of the load material is raised to about 0.5–1 pH unit below the pI of the antibody, the conductivity of the load material is adjusted to <7.5 mS/cm, and the antibody is then pumped through the column. Because the pH of the load is less than the pI of the antibody, the antibody will flow through. Since the pI of most host cell proteins is less than the pH of the load, most host cell proteins will bind to the column. Under these conditions, DNA and endotoxin will also bind strongly to the column. The purified antibody is collected after it flows through the column, and the impurities are removed from the column during the regeneration phase.

Development of the flow-through step is straightforward. The antibody is run through the column at various levels of pH and conductivity. In general, at any value of pH, decreasing the conductivity will increase the clearance of host cell proteins. Since the conductivity of the cation exchange pool may be relatively high (>12 mS/cm), the conductivity of the load onto the anion exchange column is controlled by dilution of the cation exchange pool with water. After dilution, the pH is adjusted to the appropriate value.

Since the throughput of the anion exchange column will be determined by the volume of the load, it is best to have the dilution as small as possible. Running the column pH just below antibody binding will allow the least dilution (highest conductivity), so the column is typically run at a pH that is 0.5–1 pH units below the antibody pI. When the pH is only about 0.2 units below the pI of the antibody, decreasing the conductivity below a critical value will allow the antibody to bind, resulting in yield loss. For our antibodies, we typically need a 2× dilution to reduce the conductivity to <7.5 mS/cm and the step is run at pH 8.

Like the cation exchange column, contaminants are removed from the column by increasing the conductivity with a regeneration buffer wash. For development purposes, this wash may be 1 M NaCl (or some other appropriate salt) so that the regeneration fraction may be assayed for host cell proteins, DNA, and antibody to ensure mass balance. Later, in production, this high-salt wash is generally replaced with sanitization buffer such as 1 M NaOH. In addition to eluting proteins due to the high ionic strength, it also degrades proteins and other molecules and strips them from the column.

In production, the column is sized for throughput. Because only a small amount of host cell proteins, DNA, and endotoxin bind to the column, the column dimensions could in principle be very small. However, with most commonly used anion exchange resins, the flow rate is limited. For example, when packed into a process-scale column the flow rate limit on Q Sepharose Fast Flow is about 200 cm/h. With this low flow

rate, using a column sized for binding capacity of the impurities would dramatically increase the run time. For this reason, anion exchange columns are typically about the same size as the cation exchange (about 100–200 l), and are loaded with about 100 g/l antibody.

PARAMETER VALIDATION

This study examined the effects in purification performance induced by changes in the process parameters used in the antibody anion exchange chromatography step. We examined the effect of total amount of antibody to be loaded, flow rate, buffer pH, and buffer conductivity.

A laboratory scale anion exchange column (0.66 cm diameter, 19 cm bed height and a column volume of 6.5 ml) was prepared. The column was sanitized with 0.5 N NaOH (2 CV) and stored in 0.1 N NaOH (3 CV).

Separate samples were conditioned (as performed in manufacturing) and loaded to study each parameter change. The conductivity of the load was adjusted during the conditioning by increasing or decreasing the dilution of the load with purified water. The conductivity of the load was matched to the conductivity of the elution/wash buffer and the conductivity of the elution/wash buffer was altered by changing the salt concentration in the buffer. The pH of the load and buffers was adjusted by titration with 1.5 M Tris.

A control chromatography was performed with an antibody load of 100 g/l and at a flow rate of 76 cm/h as defined for manufacturing scale. Each of the remaining chromatography experiments was performed with one variable changed from the control conditions. The results from this study are compiled in *Table 12.6*.

Analysis of the recovery yields for the different antibody loads showed no significant differences. The recovery yields for the anion exchange step were comparable and range between 95 and 100%. SDS-PAGE analysis of the anion exchange pools from the different loads showed no additional bands when the load of antibody increased from 50 g/l to 125 g/l. These results show that the capacity of the anion exchange column to remove contaminants is unchanged even at the highest load of 125 g/l. The shape of the chromatograms during the different load experiments show no unexpected discrepancies, and the level of host cell proteins was <4 ppm.

Table 12.6. Results from the anion exchange chromatography parameter validation study

Load (g/l)	Flow rate (cm/h)	Buffer pH	Buffer adjustment	Yield (%)	Host cell proteins (ng/mg)	DNA (pg/mg)
125	–	–	–	96.6	<4	<9
100*	76	8.0	(50 mM)	97.6	<4	<9
75	–	–	–	101.1	<4	<9
50	–	–	–	101.4	<4	<9
–	–	8.5	–	101.5	<4	<9
–	–	7.5	–	101.2	<4	<9
–	–	–	+ 50 mM	98.9	<4	<9
–	–	–	– 25 mM	99.2	<4	<9
–	140	–	–	100.0	<4	<9
–	40	–	–	99.1	<4	<9

* indicates the control run

The performance of the anion exchange step was evaluated at two flow rates (40 cm/h and 140 cm/h). The product recovery yields at the low and high flow rate showed no significant changes when compared to the standard anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different flow rate experiments showed no additional bands when compared to the standard pool, and the level of host cell proteins was consistently <4 ppm. These results showed that the capacity of the anion exchange column to remove protein contaminants is not affected over the flow rate range tested. The shape of the chromatograms from the different flow rate experiments is equivalent with only variations in load volume. No discrepancies were found in the chromatograms.

The performance of the anion exchange step was evaluated at two conductivity values. The load conductivity and the equilibration/elution buffer conductivity were changed to 4.4 mS and 13.32 mS in separate experiments. The product recovery yields at the low and high conductivities showed no significant changes when compared to the control anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different conductivity experiments showed no additional bands when compared to the standard anion exchange pool, and host cell proteins were <4 ppm. These results demonstrated that the capacity of the anion exchange column to remove protein contaminants is not affected over the conductivity range tested. The performance of the anion exchange step was evaluated at two pH ranges. The product recovery yields at the low and high pH showed no significant changes when compared to the control anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different pH experiments showed no additional bands when the pH was changed from 8.5 to 7.5, and the level of host cell proteins was consistently <4 ppm. The results demonstrated that the anion exchange step performs adequately over the range of all the parameters tested.

RE-USE VALIDATION

A 1.6 cm inner diameter × 19 cm length column (column volume = 38 ml) was packed with Q Sepharose chromatography media. After the column was cycled 50 times, the column was unpacked and an aliquot of the used resin was packed into a 0.66 cm inner diameter × 19 cm length column (column volume = 6.5 ml), and a DNA spike challenge was performed on the column.

Several measured values for the re-use study are shown in *Table 12.7*. Yield varied from 101.0% to 109.7%, with no significant change from cycle 1 (107.6%) to cycle

Table 12.7. Results from the anion exchange chromatography re-use study

Cycle	Yield (%)	CHOP (ng/mg)	DNA (ng/mg)
1	107.6	<7	<3
11	106.4	<7	<3
21	108.5	<7	<3
31	109.7	<3	<3
41	101.0	<3	<3
50	106.6	<3	<3
Average	106.6		
Standard deviation	2.8		

50 (106.6%). The level of host cell proteins was <7 ppm for all cycles. DNA in the Q-Sepharose pool was less than detectable for all pools.

In the SDS-PAGE gels, both reduced and non-reduced, no significant new bands appeared over the course of 50 cycles, and bands on the pool samples were of comparable intensity to the reference material. The chromatograms were consistent with respect to absorbance, pH, and conductivity.

The results of this study demonstrate that the anion exchange column performs consistently throughout 50 cycles. Product quality, as measured by yield and host cell proteins, remained consistent throughout 50 cycles.

Conclusions

With the increasing use of monoclonal antibodies as pharmaceuticals, there is a need for robust, reliable, cost-effective processes. As a central part of an overall manufacturing system, the three-step recovery process we described can meet the requirements for purity, throughput, and yield. Integrating the protein A affinity, cation exchange, and anion exchange chromatography steps can provide sufficient clearance of host cell proteins, DNA, endotoxin, virus, small molecules, and aggregate. With correctly sized columns and process equipment, each batch of antibody can be recovered in less than three days. By developing and running each step correctly, an overall process yield of 70% can be achieved. The process is robust, reliable, and can be validated to operate within a range of operating parameters.

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