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LARGE-SCALE CHROMATOGRAPHY OF RECOMBINANT PROTEINS

ERICH HOCHULI
Central Research Units, F. Hoffmann-La Roche & Co. Ltd., CH-4002 Basle (Switzerland)

SUMMARY

When recombinant proteins are expressed in bacterial cells and subsequently grown in fermentation tanks, there remains the problem of recovering the product in pure form. The empirical knowledge gained upon recovery of recombinant proteins indicates that a one-step purification process is very unlikely to succeed. However, combinations of modern techniques, such as immunoaffinity chromatography or immobilized-metal affinity chromatography, with classical techniques, such as ion-exchange chromatography, seem to be suitable for large-scale recovery of recombinant proteins.

INTRODUCTION

Modern molecular biology has provided methods for transferring human genes into host cells, *e.g.*, bacteria resulting in the expression of the coded proteins. After the microorganisms have grown in large fermentation tanks, the desired product must be recovered in pure form. Large-scale purification of proteins by traditional multi-step chromatographic separations is very tedious and affords low overall yields. Development of efficient large-scale purification is lagging behind progress in the expression and fermentation technology of recombinant proteins. There is a need for a more rational approach to separation strategies. Adsorption-based affinity chromatography may be an attractive method. The importance of affinity chromatography with immobilized monoclonal antibodies for the large-scale purification of pharmaceutical proteins has been reviewed[1,2].

Immobilized metal affinity chromatography is a well known technique for protein purification. Porath *et al.*[3,4] and Sukowski[5] have shown that this technique is well suited for selective fractionation of proteins according to their content of exposed histidine side chain. The chelating ligands iminodiacetic acid and tris(carboxymethyl)ethylenediamine were bound covalently to oxirane-activated agarose, and the resulting gels were charged with metal ions such as Cu^{2+}, Zn^{2+} or Ni^{2+}. These resins have since been used successfully for the purification of many peptides and proteins[6] and may also have potential for large-scale chromatography.

In recent years, very attractive genetic approaches to large-scale purification of recombinant proteins have been developed. Hybrid proteins were prepared by fusing the coding sequence of the protein of interest with the coding sequence of a

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peptide with high affinity for an affinity resin together with the sequence of a specific cleavage site. Such fusion proteins could efficiently be purified, taking advangage of the specific binding of the affinity peptide (affinity tail) to the affinity resin. After purification of the fusion protein, the affinity tail could be split off at the designed cleavage site. One approach to protein purification was described with staphylococcal Protein A, which was used as an affinity tail owing to its strong affinity to immunoglobulin G (IgG)[7]. Other gene fusion systems have been developed, based on fusion to β-galactosidase[8] and polyarginine[9]. Recently, the large-scale purification of human insulin-like growth factor I by means of the protein A gene fusion system has been published[10].

In this report, some results obtained with large-scale immunoaffinity and immobilized-metal affinity chromatography of recombinant proteins are presented.

Expression in *Escherichia coli* and subsequent purification by immunoaffinity chromatography of an interferon with very promising activity as a therapeutic agent, interferon α-2a, has been demonstrated by Staehelin *et al.*[11]. However, while scaling up this process, it was discovered that the purified interferon α-2a was not homogeneous. Besides oligomers, formed by intermolecular disulphide bonds[12], two monomeric forms, which have been termed slow and fast monomers[13], were observed. In addition, an interferon fragment, encompassing amino acids 23–165 of interferon α-2a[14], was detected. It could be shown that the slow monomer represents a form with only one intact disulphide bridge, whereas the amino acid sequence and the correct disulphide bridges of interferon α-2a fast monomer could be confirmed (Fig. 1).

An improved purification process to separate the monomers from the oligomers of interferon α-2a has been described by Tarnowski *et al.*[15]. However, large-scale production of interferon α-2a fast monomer, the molecule with the correct di-

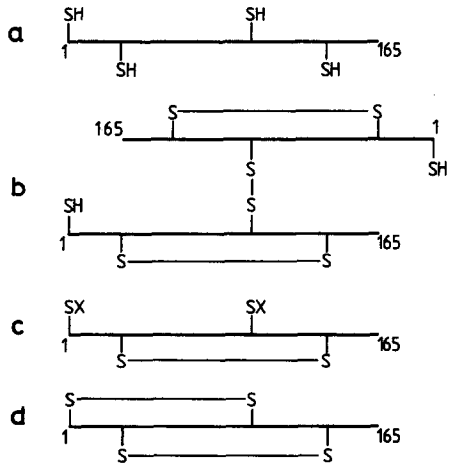

Fig. 1. Schematic structure of different interferon α-2a species. The amino acid sequence deduced from the nucleotide sequence of the gene indicates that interferon α-2a is a protein with 165 amino acids of which four cysteines are in positions 1, 29, 98 and 138. (a) Reduced interferon α-2a molecule; (b) dimer formed via intermolecular disulphide cross-linkage; (c) slow monomer, the molecule with only one intact disulphide bond; (d) fast monomer, the molecule with the correct disulphide bonds.

sulphide bridges, prompted the inclusion of copper chelate chromatography in the purification process[16].

A new nickel chelate adsorbent which has a remarkable selectivity for peptides and proteins containing neighbouring histidine residues was recently introduced[17]. This finding brought about immobilized-nickel affinity chromatography of fusion proteins containing an histidine affinity peptide.

EXPERIMENTAL

Large-scale chromatography of interferon α-2a

In the first step interferon α-2a was extracted from the cell paste of the recombinant organism[18]. After removal of the cell debris by centrifugation, the clear crude extract was concentrated by ultrafiltration. The next step was affinity chromatography with monoclonal antibodies immobilized on a solid support. The immunoadsorbent was prepared by coupling monoclonal antibody, raised against interferon α-2a, to *N*-hydroxysuccinimide ester-activated Sepharose fast flow (Pharmacia), as described previously[16]. A column (26 cm × 10 cm) was packed with 2000 ml of immunoadsorbent and automated for recycling with a computer system. The microprocessor was programmed to process one batch of 300 l in ten cycles on the affinity column. In one cycle, 30 l of concentrated crude extract were pumped with a flow-rate of 4 l/h from an holding tank through a pressure controller and a filter to the column. The filter system protected the column by trapping particles. The effluent from the column was monitored by an UV monitor (Uvicord S, LKB) at 280 nm. When loading of crude extract was complete, the selector valve was switched to the next step and the column was washed sequentially with 1.5 column volumes, each of wash buffer 1 (0.3 *M* guanidine–HCl, 0.3% Triton X-100, 0.1 *M* Tris–HCl, pH 7.5), wash buffer 2 (0.5 *M* sodium chloride, 0.2% Triton X-100, 0.025 *M* Tris–HCl, pH 7.5), wash buffer 3 (0.1 *M* sodium thiocyanate, 0.1% Triton X-100, 0.025 *M* Tris–HCl, pH 7.5) and rinse buffer (0.15 *M* sodium chloride; 0.1% Triton X-100). The column effluents were discarded. After switching to elution with diluted acetic acid (0.2 *M* acetic acid, 0.1% Triton X-100, 0.15 *M* sodium chloride), the column eluate was collected, yielding 3.8 l of interferon α-2a solution. Finally, after equilibration with three column volumes of wash buffer 1, the column was ready for the next cycle. The eluates from the ten cycles were pooled. Thus, from one batch of 300 l concentrated crude extract with a specific activity* of $1.5 \cdot 10^6$ Units/mg, 38 l of product with a specific activity of $1.7 \cdot 10^8$ Units/mg were obtained.

The different forms of interferon α-2a (Fig. 2) were separated by immobilized-copper affinity chromatography. A 38-l volume of the immunoadsorbent eluate was loaded at a flow-rate of 2.5 l/h on a column(26 cm × 14 cm) packed with 4000 ml of copper chelate adsorbent. The adsorbent was prepared by coupling iminodiacetic acid to epibromohydrin-activated Sepharose CL 6B (Pharmacia) as described previously (Fig. 3)[16]. The interferon α-2a forms were eluted with diluted acetic acid. With elution buffer 1 (0.025 *M* acetic acid, 0.1 *M* sodium chloride, 0.1% Tween-20) first the fragment (1.4 column volumes), then the slow monomer (2.4 column vol-

* Interferon activity was determined by a cytopathic effect inhibition assay with vesicular stomatitis virus and a bovine kidney cell line[19]. Protein was determined by the method of Lowry *et al.*[20].

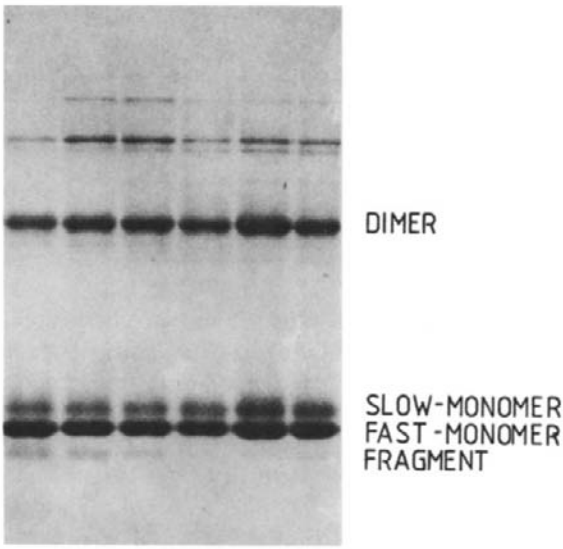

Fig. 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis under non-reducing conditions (without 2-mercaptoethanol present) of six different interferon α-2a preparations. Dimer: interferon formed by an intermolecular disulphide bond. Slow monomer: interferon with only one intact disulphide bridge. Fast monomer: interferon with the correct disulphide bridges. Fragment: interferon fragment encompassing amino acids 23–165.

umes), followed by the fast monomer (3.2 column volumes) were eluted. With elution buffer 2 (0.2 *M* acetic acid, 0.2 *M* sodium chloride, 0.1% Tween-20) the oligomers were recovered. The fast monomer was pooled for processing in the next steps.

To remove the non-ionic detergent Tween-20, used in the previous step, a cation-exchange column (CM-52 cellulose from Whatman, U.K.) was included in the process. In this chromatographic procedure, the interferon was first adsorbed in elu-

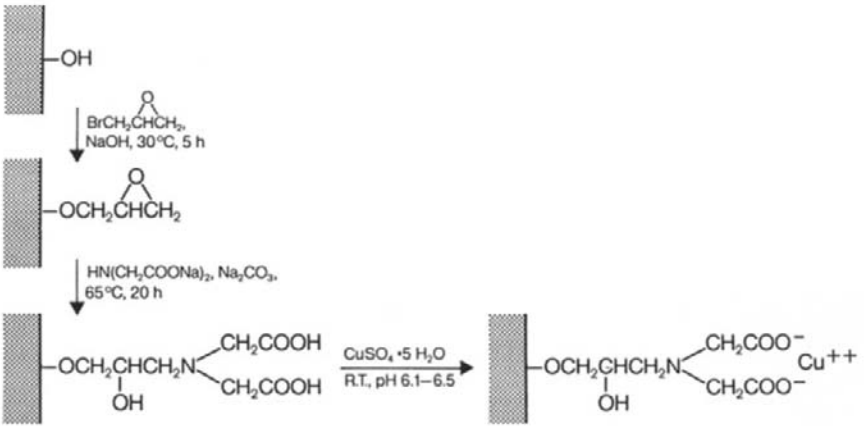

Fig. 3. Synthesis of the copper chelate adsorbent.

tion buffer 1, the detergent was washed out and then the product was eluted with elution buffer 3 (0.025 *M* acetic acid, pH 5, 0.12 *M* sodium chloride).

The last step of the interferon α-2a purification was gel chromatography using Sephadex G-50 (Pharmacia) as described previously[15]. The final product was stored frozen at −20°C.

Chromatography of fusion proteins

DNA elements have been prepared which, after integration into expression vectors, directed the production of fusion proteins, consisting of a protein of interest and an affinity peptide with neighbouring histidine residues[21]. The recombinant fusion proteins synthesized in *E. coli* were extracted by sonification in 0.05 *M* sodium phosphate buffer, pH 8 (10 g cells in 60 ml extraction buffer). After centrifugation the supernatant was directly pumped on the nickel nitrilotriacetate (NTA)-chelate column (Fig. 4), prepared as described[17]. When loading of the crude extract was complete, the column was first washed with extraction buffer and then the product was eluted with a linear gradient from pH 8 (extraction buffer) to 5 (0.05 *M* sodium phosphate, pH 5).

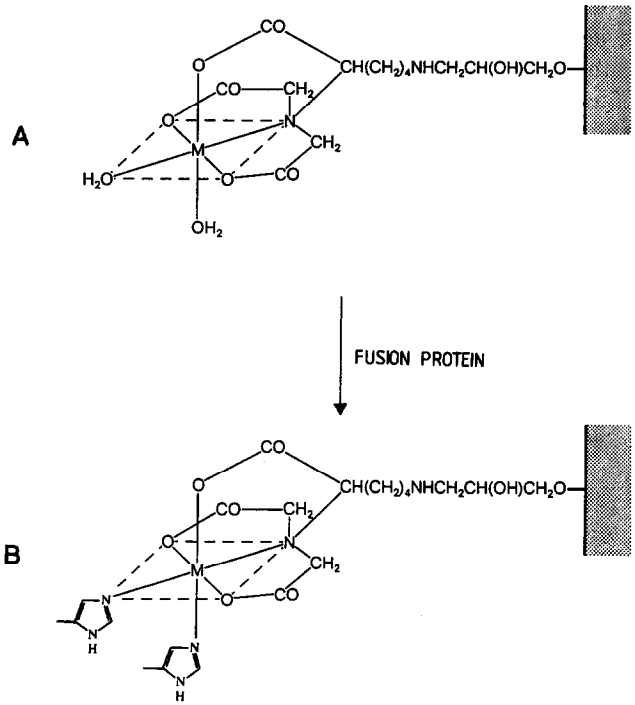

Fig. 4. Schematic structure of the NTA adsorbent. (A) Two valencies are occupied by H_2O and are disposable for selective protein interactions; (B) two valencies are occupied by imidazole from the protein containing neighbouring histidine residues.

TABLE I

ANTIBODY SHEDDING FROM THE IMMUNOADSORBENT COLUMN

Number of cycles	*Interferon (mg/ml)*	*Mouse IgG*	
		ng/ml	*ppm*
1	0.26	11.4	44
2	0.44	9.3	21
3	0.36	5.2	14
4	0.51	3.4	7
5	0.29	2.7	9
6	0.27	1.6	6
7	0.35	4.6	13
8	0.56	1.9	3

RESULTS

Mouse immunoglobulin contamination

Immunoaffinity chromatography of interferon α-2a was complicated by the problem of immunoglobulin contamination. Although the capacity of the immunoadsorbent column for interferon was not diminished after several hundreds of cycles, detectable levels of mouse IgG were removed from the adsorbent in the eluates. However, it could be demonstrated that this contamination was removed below detectable levels in the subsequent purification steps (data not shown). For the detection and quantification of mouse IgG, a specific enzyme immunoassay was used. In a second experiment, a freshly prepared immunoadsorbent column was tested for antibody shedding during the first eight cycles of operation. As seen in Table I, the shedding rate of 44 ppm in the first cycle dropped to a more or less constant level after three cycles.

TABLE II

PURIFICATION OF AN INTERFERON α-2a PREPARATION SPIKED WITH ANTIBODY

ND = Not detected.

	Interferon (mg/ml)	*Mouse IgG*	
		ng/ml	*ppm*
Immunoadsorbent column			
Eluate	0.26	2.5	9.6
Eluate + mouse IgG	0.26	60	230
Copper chelate column			
Flow-through		ND	
Eluate monomer		ND	
Eluate oligomers	0.15	10	67
CM cellulose column			
Flow-through		ND	
Eluate	0.37	0.5	1.4

TABLE III

BACTERIAL ENDOTOXIN CONCENTRATIONS

Purification step	*EU/ml*
Crude extract	20000
Immunoaffinity column	30
Copper chelate column	10
Ion-exchange column	< 1
Gel chromatography column	< 1

In a third experiment, an immunoadsorbent eluate was spiked with the monoclonal antibody used for the adsorbent synthesis. This solution was then followed through the purification process of interferon α-2a. As seen in Table II, most of the mouse IgG is removed in the subsequent purification steps.

The results of the experiments described indicate that the purification process of interferon α-2a is satisfactory with regard to contamination from mouse IgG.

Bacterial endotoxin contamination

Interferon α-2a, synthesized by *E. coli*, is initially contaminated with endotoxins, the presence of which prohibits its therapeutic use. To demonstrate that the endotoxins are removed from interferon α-2a in the purification procedure developed, process validation experiments were carried out. Table III shows the endotoxin concentrations measured after each purification step. The contamination was determined by the Limulus Amoebocyte Lysate (LAL) test[22]. The test has a sensitivity of < 1 EU/ml where 1 EU endotoxin unit is defined as 0.1 ng *E. coli* endotoxin.

These results indicate that the purification process of interferon α-2a removes the endotoxins efficiently.

Copper contamination

In order to determine whether copper was leached from the copper chelate adsorbent, a procedure was developed for measuring copper in the purified interferon α-2a by atomic absorption spectroscopy. Each lot of the drug substance was tested, and all lots were found to have consistently low levels of copper, as is demonstrated for three different lots in Table IV.

From these results, it can be calculated that for the interferon α-2a dosage form of $3 \cdot 10^6$ units the copper content is less than 1 ng.

TABLE IV

COPPER CONCENTRATION IN THREE LOTS OF INTERFERON α-2a

Lot No.	*ng Cu/mg interferon*
84-09	30
84-10	50
84-12	40

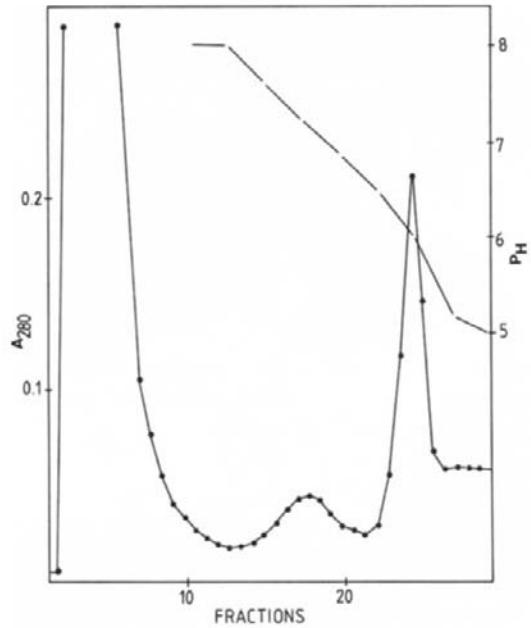

Fig. 5. Chromatography of a fusion protein on the Ni^{2+}–NTA adsorbent. A 140-ml volume of a crude extract from *E. coli* was loaded (200 ml/h) on a column (14 cm × 5 cm) packed with 280 ml of the nickel adsorbent. The histidine affinity peptide-containing product was elute with a 1000-ml gradient from pH 8 to 5.

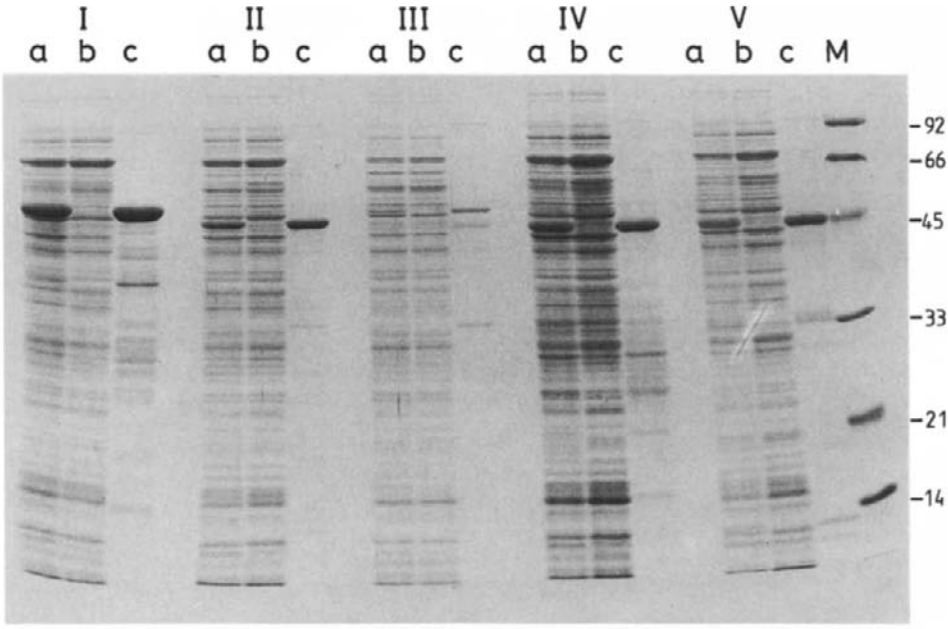

Fig. 6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of five different fusion proteins (I–V). (a) Crude extract loaded onto the column; (b) contaminants passed through the column; (c) eluted product; M = molecular-weight standard.

Purification of fusion proteins with the Ni^{2+}–NTA adsorbent

Fig. 5 shows the chromatogram of a fusion protein containing an affinity peptide with neighbouring histidine residues. Without further purification, a crude extract from *E. coli* was loaded on the Ni^{2+}–NTA column and eluted with a linear gradient from pH 8 to 5. Fig. 6 shows the results of the purification of five different fusion proteins on the Ni^{2+}–NTA adsorbent. All proteins, even the rather weakly expressed protein III, were purified to more than 85% purity in a single chromatographic step.

DISCUSSION

An ideal adsorbent for immobilized-metal affinity chromatography must, on the one hand, strongly complex the metal ions and, on the other hand, permit reversible interactions between immobilized metal ions and proteins. Immobilized iminodiacetic acid (IDA resin) largely fulfils these requirements for Cu^{2+} but only to a limited extent for Ni^{2+} or Zn^{2+}, since the latter are only weakly bonded. Nickel(II) ions are washed out from IDA resin upon loading with strong chelating proteins or peptides, *e.g.*, His-His-Leu-Gly-Gly-Ala-Lys-Glu-Ala-Gly-Asp-Val. However, chelate adsorbents of Ni^{2+} or Zn^{2+} are of particular interest, as Ni^{2+} and Zn^{2+} have the ability to complex six ligands, whereas Cu^{2+} preferably binds four in its inner coordination sphere.

A new nitrilotriacetic acid adsorbent has recently been introduced[17]. This quadridentate chelating adsorbent (NTA resin) occupies four positions in the coordination sphere of metal ions with coordination number six (Fig. 4). The remaining two ligand positions in the octahedral coordination sphere are disposable for selective protein interactions.

The new metal NTA adsorbent, which is more stable to metal leaching than the metal IDA adsorbent, was tested for its specificity to bind peptides and proteins containing histidine residues[17]. The results revealed that the adsorbent charged with Ni^{2+} is specific for proteins and peptides having neighbouring histidine residues (Fig. 4).

Affinity chromatography as outlined was designed according to genetic principles by using a novel metal chelate adsorbent. It may be a generally applicable method for the recovery of any recombinant protein synthesized in bacteria, yeast or mammalian cells, but there are still several problems to be solved. One difficulty is the specific and complete cleavage of the affinity tail after recovery of the fusion protein. Some promising results have been obtained, but the cleavage method still needs improvement. However, for many applications, removal of the affinity peptide is not necessary, because it comprises only a few amino acids.

The process described for the purification of interferon α-2a, synthesized by *E. coli*, was designed to accomplish two purposes: to produce a product with maximum purity and to be highly reproducible. Although the specificity of an immunoadsorbent for one protein is very high, an one-step purification process seems to be out of the question. For the large-scale purification of recombinant proteins to homogeneity, immunoaffinity chromatography in combination with other chromatographic techniques is necessary. Despite initial scepticism, immobilized-metal affinity chromatography has proven to be a good method for large-scale purification of recom-

binant proteins for pharmaceutical applications. For the purification of interferon α-2a, this technique, in combination with immunoaffinity chromatography, seems to be an effective method for the recovery of this new drug substance on a produciton scale.

The recovery of recombinant proteins for therapeutic use sets different purity standards. Since these proteins are produced in host cells such as bacteria, there is concern that trace amounts of proteins, DNA or endotoxins from the host organism might be present in the drug substance. Also, purification of proteins requires the addition of various chemicals in order to perform specific separation functions and to protect against destruction of the protein during purification. These chemicals should not contaminate the final product because of their toxicological and pharmacological properties. Therefore, process validation studies are necessary to determine whether these possible contaminants are removed by the various chromatographic steps. Mouse IgG removed from the immunoadsorbent, endotoxins extracted from the host organism and the leakage of copper ions from the copper chelate adsorbent serve as illustrations of these important considerations.

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