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Monoclonal Antibody Purification with Reduced Turbidity by Using High-Salt Elution Buffer During Protein A Chromatography.

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Abstract:
During purification of monoclonal antibodies, viral inactivation/removal step is a mandatory step to comply with the regulatory guidelines which commonly performs at low pH about 3.5 and further neutralizes it to a pH about 7.0. Neutralization of monoclonal antibodies results formation of high turbidity which is troublesome for further processing such as filtration and other chromatography processes and it eventually leads to filter clogging and increases column back pressure. In this work, we propose a new optimized process of affinity chromatography (Protein A) by using a high salt elution buffer which reduces turbidity more than 80%. A systematic study of different concentration of salts were analysed and it reveals that salt concentration greater than 100mM in Protein A elution buffer reduces turbidity during neutralization of monoclonal antibodies when compared to low salt concentration of elution buffer. We also found that elution of monoclonal antibodies during Protein A chromatography at pH 3.5 results higher reduction of turbidity compared to pH 3.0. These findings provide both cost and time effective for large scale production of monoclonal antibodies as it eliminates the use of multiple filters throughout the purification process.

Keywords: Affinity Chromatography, Protein A Chromatography, Column back pressure, Turbidity, Monoclonal antibody (mAb), Neutralization, Viral inactivation.

Introduction:

Monoclonal antibodies are being used widely to treat diseases such as cancer, autoimmune diseases etc. One such monoclonal antibody named Omalizumab was developed to treat Asthma, allergic asthma and chronic idiopathic urticaria which are almost invariably accompanied by elevated levels of IgE [1]. Omalizumab is an anti-IgE drug that binds with the IgE and thereby inhibits the binding of Immunoglobulin E (IgE) to its high affinity receptors present on the surface of the mast cells [2]. With rapid increase in the demand of the biologics and drugs has led to significant growth in the upstream

processes for the production leading to increase the production of drugs from milligram per litre to grams per litre [3,4]. The substantial increase in the upstream productivity, creates pressure to deliver drug product, resulting in the improvement of the downstream processes to enhance the efficiency and the quality of the drug [5].

It was realised in 1970 that monoclonal antibodies have high therapeutic potential and can be used to treat diseases as unlike polyclonal antibodies they are homogeneous and mono-specific, due to which are they commonly used in the development of therapeutics and drugs [6]. In past decade, the need to produce purified monoclonal antibodies has advanced exponentially, to meet the need for the drugs and to meet the increased commercial demand the upstream processes are improved such that in large scale these upstream processes can yield up to multi-kilogram of monoclonal antibodies [3,4]. The challenge is therefore to develop the purification processes which efficiently purifies these monoclonal antibodies at low price [4]. There are many purification processes that separate protein of interest from the impurities. Some of the well-known downstream purifications techniques, ion exchange chromatography, hydrophobic interaction chromatography (HIC), Ceramic hydroxyapatite chromatography (CHT), affinity chromatography, Size- exclusion chromatography, gel filtration, etc. that removes the impurities and reduces the turbidity during viral inactivation, to attain efficiency [7].

However, various chromatography and other purification methods are being used for purifications of monoclonal antibodies, formation of high turbid protein solution at neutralization step has become troublesome. During protein purification through Protein A chromatography, elution at low pH and neutralization of low pH treated protein mixture results high turbidity formation and it leads to filter clogging that is used for further purifications of proteins during the manufacturing of mammalian cell-based biotherapeutics which makes the downstream operations very expensive. Furthermore, a turbid protein solution causes high back pressure during chromatography process complicating manufacturing operations and multiple use of filters that directly impacts the cost and operational time of the process [8]. This study addresses the reduction of turbidity in protein solution eluted from affinity chromatography e.g., Protein A and Protein G Affinity Chromatography which further reduces the burden of using multiple column and filtration steps during downstream operations. Protein A and G is derived from *Staphylococcus* and *Escherichia* can be used to purify antibodies from serum, ascitic fluid, the supernatants of hybridoma cells, etc. since these proteins are highly specific for purifying polyclonal IgG [9]. Thus, it is well known that protein A chromatography is commonly used for capturing monoclonal antibodies. Since it has high binding capacity, selectivity while effectively separating impurities protein of interest from impurities such as high molecular weight species (HMW) and low molecular weight species (LMW), host cell proteins (HCP) [10].

Turbid elution pools and high column back pressure is common during elution of monoclonal antibodies (mAbs) by acidic pH in Protein A chromatography when antibody composition is subjected to viral inactivation treatment [11]. These turbid elution pools are irreversible and hence are filtered. To filter these pH-induced precipitates of HCPs multiple filters (such as depth filter and aseptic filtration) are required in line with Protein A column [6].

Filtration is a critical unit operation that is used for primary and secondary clarification during the manufacturing of mammalian cell-based biotherapeutics. However, continuous manufacturing processes require consistent use of filtration over a long period, with potential unpredictable variations in feed stream attributes, which is a challenge currently facing the industry. Also, this turbid elution can clog chromatography column as well as the filters and can hinder the continuous manufacturing process [11].

Moreover, an increase in turbidity will ultimately lead to the use of multiple filters or the use of a filter having a large filtration area, which will directly impact the cost and the operational time of the process. Furthermore, depth filtration is used to remove precipitates and turbidity after neutralization of low pH virus inactivated product.

This study interestingly found that elution of protein with high salt concentration buffer i.e., more than 100mM during affinity chromatography reduces the turbidity of the protein mixture during or post neutralization.

Materials and Methods:

All chromatographic processes were carried out using an AKTA Pure 150 system from Cytiva. The concentration of protein samples was determined by measuring absorbance at 280nm using Shimadzu Spectrophotometer. Mabselect Sure LX resin media obtained from Cytiva. XK50/40 column was obtained from Cytiva or Vantage columns were obtained from Millipore Corporation. Turbidity measurements were measured using TN100 Portable Turbidimeter from Thermo scientific. All Chemicals were obtained from JTB or Merck Millipore and were of GMP grade.

A monoclonal antibody molecule capable to bind to IgE molecule expressed in the Chinese Hamster Ovary (CHO) cell line is captured using Protein A (Mab Select Sure LX, Cytiva) packed in the XK50/40 column or in Vantage columns VL11* 30.

Methods:

Protein A Chromatography elution with 25 mM acetate at pH 3.5±0.1:

A monoclonal antibody molecule capable to bind to IgE molecule expressed in Chinese Hamster Ovary (CHO) cell line is captured using Protein A (Mab Select Sure LX, Cytiva) packed in Vantage columns VL11 X 30 Millipore column. The residence time is 4 min for all the phases. After equilibration with Tris Acetate + 150 mM NaCl, pH 6.8-7.2 clarified harvest is loaded at ≤40 mg/mL of the resin. After loading column washes with equilibration buffer followed by wash 2 and wash 3 buffers followed by elution with 25 mM Acetate, pH 3.5±0.1 buffer as mentioned in Table 1. The affinity chromatography step is operated in bind and eluate mode and collection is done from 500 mAU (2.5AU/cm) ascending to 500 mAU (2.5AU/cm) descending of the peak. Elute is further subjected to viral inactivation and neutralization. Turbidity was measured at protein A elute stage.

The experimental design for the Protein A is summarized in Table 1. The effect of salt concentration of elution buffer and pH during the protein A chromatography on turbidity of protein mixture is shown in Table 5.

Table 1: Experimental design for Protein A chromatography

Step	Buffer	Residence time (min)	Column volume (CV)
WFI	WFI	4	2-3
Sanitization	0.1 M NaOH	4	2-3
Equilibration	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	3-5
Load	Clarified Harvest	4	Till end of load volume

Wash 1	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	3-5
Wash 2	20 mM Tris-acetate + 1 M NaCl + 0.1% Polysorbate 20, pH 7.0 ± 0.2	4	3-5
Wash 3	30 mM Tris-acetate, pH 5.5 ± 0.2	4	5-7
Elution	25 mM Acetate, pH 3.5 ± 0.1	4	3
Neutralization wash	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	2-3
Sanitization	0.1 M NaOH	4	2-3
Neutralization wash	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	3-5
Storage	2% Benzyl alcohol in Wash 3 buffer	4	2-3

Virus Inactivation & Neutralization of Protein A Eluate:

For virus inactivation pH of protein, A Elute is adjusted to pH 3.5 with 1N HCl and incubates at room temperature for 50 minutes. After 50 minutes of incubation, a sample is neutralized to pH 6.2 with a 1M tris base for 20 min.

Turbidity Measurements:

NTU of neutralized protein A elute is measured in nephelos turbidity units (NTU) using a calibrated portable nephelometer (Orbeco Hellige, Farmingdale, N.Y., U.S.A.). Turbidity was measured in the 0 to 200 NTU range. Neutralized protein A elute is further filtered with a 0.2µm filter.

Protein A Chromatography elution with 125 mM Acetate, pH 3.5 ±0.1:

In another experiment, a monoclonal antibody molecule capable to bind to IgE molecule expressed in the Chinese Hamster Ovary (CHO) cell line is captured using Protein A (Mab Select Sure LX, Cytiva) packed in the XK50/40 column. The residence time is 4 min for all the phases. After equilibration with Tris Acetate+ 150 mM NaCl, pH 6.8 – 7.2 clarified harvest is loaded at ≤40 mg/mL of the resin. After loading column washes with equilibration buffer followed by wash 2 and wash 3 buffers followed by elution with 125 mM Acetate, pH 3.5±0.1 buffer as mentioned in Table 2. The affinity chromatography step is operated in bind and eluate mode and collection is done from 500 mAU (2.5AU/cm) ascending to 500 mAU (2.5AU/cm) descending of the peak. Elute is further subjected to viral inactivation and neutralization. Turbidity was measured at protein A elute stage.

The experimental design for the Protein A is summarized in Table 2. The effect of salt concentration of elution buffer and pH during the protein A chromatography on turbidity (NTU data) of protein mixture is shown in Table 5 respectively.

Table 2: Experimental design for Protein A chromatography

Step	**Buffer**	**Residence time (min)**	**Column volume (CV)**
WFI	WFI	4	2-3
Sanitization	0.1 M NaOH	4	2-3

Equilibration	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	3-5
Load	Clarified Harvest	4	Till end of load volume
Wash 1	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	3-5
Wash 2	20 mM Tris-acetate + 1 M NaCl + 0.1% Polysorbate 20, pH 7.0 ± 0.2	4	3-5
Wash 3	30 mM Tris-acetate, pH 5.5 ± 0.2	4	5-7
Elution	125 mM Acetate, pH 3.5 ± 0.1	4	3
Neutralization wash	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	2-3
Sanitization	0.1 M NaOH	4	2-3
Neutralization wash	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	3-5
Storage	2% Benzyl alcohol in Wash 3 buffer	4	2-3

Virus Inactivation & Neutralization of Protein A Eluate:

For virus inactivation pH of Protein A Elute is adjusted to pH 3.5 with 1N HCl and incubates at room temperature for 50 minutes. After 50 minutes of incubation, a sample is neutralized to pH 6.2 with a 1M tris base for 20 min.

Turbidity Measurements:

NTU of neutralized protein A elute is measured in nephelos turbidity units (NTU) using a calibrated portable nephelometer (Orbeco Hellige, Farmingdale, N.Y., U.S.A.). Turbidity was measured in the 0 to 200 NTU range. Neutralized protein A elute is further filtered with a 0.2µm filter.

Protein A Chromatography elution with 200 mM Acetate, pH 3.5 ± 0.1:

In another experiment, the monoclonal antibody is captured using Protein A (Mab Select Sure LX, Cytiva) packed in Vantage columns VL11 X 30 Millipore column. The residence time is 4 min for all the phases. After equilibration with Tris Acetate + 150 mM NaCl, pH 6.8 – 7.2 clarified harvest is loaded at ≤40 mg/mL of the resin. After loading column washes with equilibration buffer followed by wash 2 and wash 3 buffers followed by elution with 200 mM Acetate, pH 3.5±0.1 buffer as mentioned in Table 1. The affinity chromatography step is operated in bind and eluate mode and collection is done from 500 mAU (2.5AU/cm) ascending to 500 mAU (2.5AU/cm) descending of the peak. Elute is further subjected to viral inactivation and neutralization. The experimental design for Protein A is summarized in Table 3 and the effect of salt concentration of elution buffer and pH during the protein A chromatography on turbidity (NTU data) of protein mixture is shown in Table 5.

Table 3: Experimental design for Protein A chromatography

Step	Buffer	Residence Time (min)	Column Volume (CV)
WFI	WFI	4	2-3
Sanitization	0.1 M NaOH	4	2-3
Equilibration	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	5
Load	Clarified Harvest	4	Till loading volume
Elution	200 mM Acetate, pH 3.5 ± 0.1	4	3pl
Neutralization wash	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	2-3
Sanitization	0.1 M NaOH	4	2-3
Neutralization wash	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	3-5
Storage	2% Benzyl alcohol in Wash 3 buffer	4	2-3

Virus Inactivation & Neutralization of Protein A Eluate:

For virus inactivation pH of protein, A Elute is adjusted to pH 3.5 with 1N HCl and incubates at room temperature for 50 minutes. After 50 minutes of incubation, a sample is neutralized to pH 6.2 with a 1M tris base for 20 min.

Turbidity Measurements:

NTU of neutralized protein A elute is measured in nephelos turbidity units (NTU) using a calibrated portable nephelometer (Orbeco Hellige, Farmingdale, N.Y., U.S.A.). Turbidity was measured in the 0 to 200 NTU range. Neutralized protein A elute is further filtered with a 0.2µm filter.

Effect of pH during Protein A elution at pH 3.0± 0.1:

Further to study the effect of pH another experiment is carried out. A monoclonal antibody molecule capable to bind to IgE molecule expressed in Chinese Hamster Ovary (CHO) cell line is captured using Protein A (Mab Select Sure LX, Cytiva) packed in Vantage columns VL11 X 30 column. The residence time is 4 min for all the phases. After equilibration with Tris Acetate+ 100 mM NaCl, pH 6.8–7.2 clarified harvest is loaded at ≤40 mg/mL of the resin. After loading column washes with equilibration buffer followed by wash 2 and wash 3 buffers followed by elution with 200 mM Acetate, pH 3.0±0.1 buffer as mentioned in Table 3. The affinity chromatography step is operated in bind and eluate mode and collection is done from 50 mAU (0.25AU/cm) ascending to 50 mAU (0.25AU/cm) descending of the peak. Elute is further subjected to viral inactivation a neutralization. Turbidity was measured at protein A elute stage.

The experimental design for the Protein A step is summarized in Table 4 and table 5 shows the NTU data for effect of salt concentration of elution buffer and pH during the protein A chromatography on protein mixture.

Table 4: Experimental design for Protein A

Step	Buffer	Residence Time (min)	Column Volume (CV)
WFI	WFI	4	2-3
Sanitization	0.1 M NaOH	4	2-3
Equilibration	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	5
Load	Clarified Harvest	4	Till loading volume
Elution	200 mM Acetate, pH 3.5 ± 0.1	4	3pl
Neutralization wash	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	2-3
Sanitization	0.1 M NaOH	4	2-3
Neutralization wash	20 mM Tris-acetate + 150 mM NaCl, pH 7.0 ± 0.2	4	3-5
Storage	2% Benzyl alcohol in Wash 3 buffer	4	2-3

Virus Inactivation & Neutralization of Protein A Eluate:

For virus inactivation pH of protein, A Elute is adjusted to pH 3.5 with 1N Acetic acid and incubates at room temperature for 50 minutes. After 50 minutes of incubation, a sample is neutralized to pH 6.2 with 1M Tris base.

Turbidity Measurements:

NTU of neutralized protein A elute is measured in nephelos turbidity units (NTU) using a calibrated portable nephelometer (Orbeco Hellige, Farmingdale, N.Y., U.S.A.). Turbidity was measured in the 0 to 200 NTU range. Neutralized protein A elute is further filtered with a 0.2µm filter.

Results and Discussion:

In protein purification turbidity plays an important role, protein solutions with higher turbidity have higher insoluble particles which may be aggregates or other impurities and have a negative effect on the purity and yield of the protein mixture. High turbidity also damages the cassettes and columns by clogging them. Thus, higher turbidity is an indication of lesser purity and higher impurity concentration and vice versa.

To observe the impact on the turbidity due to change in the salt concentration during elution, a comparative study is performed between elution of antibodies at low salt concentration compared to elution of antibodies at high salt concentration.

Table 5: Turbidity data of Protein A Chromatography step

Example	Elution Buffer	Protein A Elute (NTU)	Neutralized Protein A Elute (NTU)
Example 1	25 mM Acetate, pH 3.5±0.1	3.3	227.0
Example 2	125 mM Acetate, pH 3.5±0.1	10.2	103.0
Example 3	200 mM Acetate, pH 3.5±0.1	6.7	36.1
Example 4	200 mM Acetate, pH 3.0±0.1	5.4	42.5

From the results shown in Table 5, it is observed, in Example 1 when protein gets eluted with 25 mM acetate buffer at pH 3.5, shows the turbidity 227 NTU which is significantly higher than protein eluted with 125 mM acetate buffer, at pH 3.5±0.1 (Example 2) showing turbidity of 103 NTU and protein eluted with 200 mM acetate buffer, at pH 3.5±0.1 (Example 3) showing turbidity of 36.1 NTU. Further when example 3 is compared with example 4 it is observed, reduction in pH of elution buffer increased the turbidity from 36.1 NTU to 42.5 NTU. Lower NTU value directly improves the subsequent depth filtration performance as well as decreases the area requires for the unit operation.

Conclusion:

Conventional Protein A based chromatography elution usually generates high turbidity after neutralization steps which affects the filtration process and further it requires use of multiple filters and columns during downstream operations. In this work, we identified an optimized Protein A chromatography process and our results revealed that performing the elution of protein mixture through affinity chromatography with elution buffer having high salt concentration which is greater than 100mM at pH 3.0-3.5 significantly reduced turbidity of protein mixture consisting of omalizumab during neutralization.

In our study, suggests that the role of low salt concentration (<100mM) in elution buffer which is commonly used in affinity chromatography affects the neutralization step by increasing the turbidity. Our study sheds new light and provides cost and time effective purification process for large scale production of monoclonal antibodies by eliminating multiple use of filters throughout the purification process.

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