# Practical Issues in the Industrial use of Hydroxyapatite for Purification of Monoclonal Antibodies

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## Abstract

Hydroxyapatite has been reported to offer unique abilities for removal of leached protein A and aggregates from monoclonal antibodies for more than 15 years, but how it works and how to build it into a robust overall manufacturing procedures are just beginning to be appreciated. This presentation will begin with discussion of the primary retention mechanisms and how to manipulate them to remove aggregates, leached protein A, DNA, endotoxin, and virus. These capabilities easily support a broadly applicable purification platform consisting of protein A affinity, hydroxyapatite, and anion exchange chromatography. They further suggest feasibility of a 2-step platform employing only protein A and hydroxyapatite. A simple approach to method development will be described. Suggestions will be made for selection and preparation of buffers to support maximum performance, reproducibility, and media life.

## Introduction

Hydroxyapatite is a crystalline mineral of calcium and phosphorus with a structural formula of $Ca_{10}(PO_4)_6(OH)_2$. Sets of 5 calcium doublets (C-sites) and pairs of phosphate triplets (P-sites) are arranged in a repeating geometric pattern. Unlike most other chromatography adsorbents, CHT is both the ligand and the support matrix. Hexagonal cross section nanocrystals are agglomerated and fused into porous ceramic spheres at high temperature to form a stable chromatography adsorbent. CHT is available as Type I and Type II. Type I supports higher capacity for IgG. 20 micron media in 1–2 mL columns is ideal for scouting. 40 micron media is better suited for large scale industrial applications.

Recent publications have revealed CHT to be a powerful method for polishing protein A purified monoclonal antibodies (Table 1). It achieves this performance by cooperation between two distinct retention mechanisms: metal affinity and cation exchange. Metal affinity occurs between carboxyl clusters on proteins and CHT calcium in a manner similar to EDTA. Phosphorylated solutes such as DNA, endotoxin, and lipid enveloped virus also interact strongly with CHT calcium through a related mechanism. Calcium affinity interactions are very strong and cannot be eluted with even saturated sodium chloride. Phosphate is required for elution. Cation exchange occurs between protein amino residues and negatively charged CHT phosphates. Simple phosphate gradients have been the traditional choice for eluting hydroxyapatite. The high affinity of phosphate for calcium allows it to behave as competitor for CHT calcium–protein interactions, and its conductivity as a salt allows it to dissociate cation exchange interactions between proteins and CHT.

## Controlling selectivity for IgG purification

IgG antibodies generally exhibit neutral to mildly alkaline isoelectric points (pI). This is consistent with a preponderance of amino residues and a smaller complement of carboxyl residues. This in turn suggests that calcium affinity between IgG and CHT should be fairly weak, while cation exchange between IgG and CHT phosphate should be fairly strong. Simple phosphate gradients can dissociate both but do not permit independent control of the two mechanisms, however it is possible to elute IgG with a gradient of neutral salt at a fixed low concentration of phosphate. The low phosphate weakens IgG affinity for calcium without substantially reducing the cation exchange interaction, which can then be eluted with the neutral salt. This strategy gives better purification performance than traditional phosphate gradients (Table 2). 10 mM phosphate is sufficient for most IgG monoclonals. A small percentage require only 5 mM phosphate and a similar proportion may require up to 15 mM. This approach has proven to give consistent results for a diverse array of monoclonals, including murine, chimeric, and human IgG of several subclasses.

IgG aggregates elute after monomeric IgG, usually in a well resolved peak, or series of peaks in which larger aggregates elute later (Figure 1). Figure 2 shows the quality of aggregate removal that can be achieved, even with antibody preparations burdened by more than 40% aggregates. Recent work indicates that substituting potassium phosphate and chloride for sodium phosphate and chloride, supports better separation between monomer and aggregate populations. Substitution of acetate for chloride has the opposite effect (Figure 1A-D).

The primary contaminants of protein A purified IgG behave very differently from IgG. Protein A is acidic, with a pI of about 4.8. This is consistent with the large proportion of glutamic and aspartic acid residues in its structure, which give it a strong affinity for CHT calcium. Complexes of leached protein A and IgG bind to CHT by a combination of the strong calcium interaction of the protein A and the strong cation exchange interaction of the IgG. Elevated phosphate concentrations are required for elution. Leached protein levels in the IgG monomer fraction are commonly reduced to concentrations beneath the sensitivity limit of current assays (Table 1). The quality of the separation appears to be best for most antibodies at pH 6.5

DNA, endotoxin, and lipid enveloped viruses are heavily phosphorylated. Phosphorylated molecules have a strong affinity for CHT calcium and require even higher phosphate concentrations for elution than protein A-IgG complexes. Experimental data indicate that the lower the phosphate concentration during the chloride gradient, the more effective the removal of DNA, and especially endotoxin (Table 3). A minimum of 5mM phosphate is necessary to maintain the stability of CHT at pH 6.5.

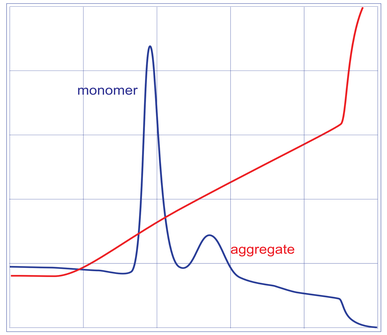


**Figure 1A. KCl**

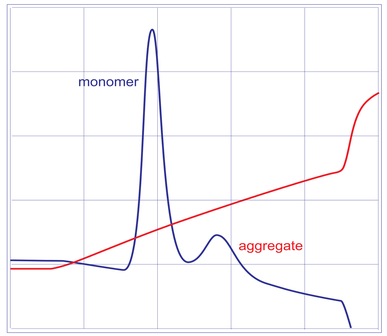


**Figure 1B. NaCl**

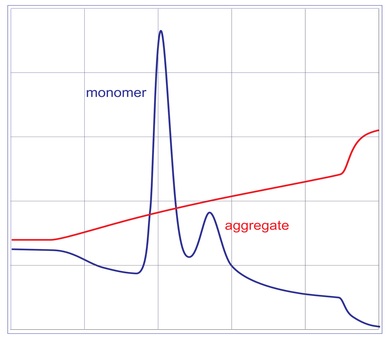


**Figure 1C. KAc**

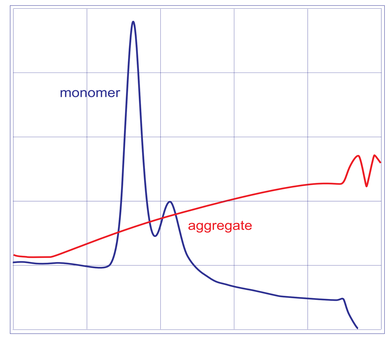


**Figure 1D. NaAc**

**Figure 1A-D.** The influence of different Hofmeister ions on resolution between aggregates and monomeric IgG. Profile A was generated at constant 5mM KPO4 pH 6.7, with a 25CV linear gradient to 1M KCl. Profile B was generated in 5mM NaPO4 pH 6.7 with a 25CV linear gradient to 1M NaCl. Profile C was generated at constant 5mM KPO4 pH 6.7 with a 25CV linear gradient to 1M K acetate. Profile D was generated in 5mM NaPO4 pH 6.7 with a 25CV linear gradient to 1M Na acetate. All runs were conducted at a linear flow rate of 600 cm/hr on a 1mL Atoll MediaScout 5/50 column (5mm x 5cm) prepacked with CHT Type I, 20 micron. The potassium profiles provide better separation than the sodium profiles, and the chloride profiles provide better separation than acetate.

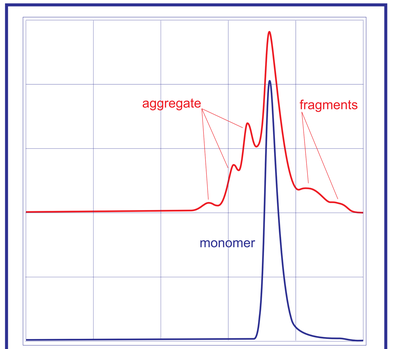


**Figure 2.** Analytical size exclusion profiles of highly aggregated chimeric IgG1 after protein A purification (red profile) and after polishing on CHT Type I 40 micron (blue profile).

**Table 1. Contaminant reduction**

| Contaminant | Method | Reduction |
|---|---|---|
| **Aggregates** | HPSEC | 1-2 logs |
| **Protein A** | Cygnus | 1-2 logs |
| **CHOP** | ELISA | 2 logs |
| **DNA** | Picogreen | > 3 logs |
| **Endotoxin** | LAL | > 4 logs |
| **aMULV** | Infectivity | > 4 logs |
| **xMULV** | Infectivity | > 3 logs |
| **MVM** | Infectivity | 2 logs |
| **PPV** | Infectivity | > 1 log |

**Table 2. Phosphate v chloride gradients**

| Parameter | Phosphate | Chloride |
|---|---|---|
| **Aggregate** | < 1% | < 1% |
| **Protein A** | < 1ppm | < 1ppm |
| **CHOP** | < 72ppm | < 12ppm |
| **DNA** | < 7ppm | < 1ppm |
| **Endotoxin** | < 5EU/mL | < 0.1EU/mL |

Mouse/human chimeric IgG1

**Table 3. Phosphate concentration and contaminant removal**

| Phosphate | 5mM | 10mM | 15mM |
|---|---|---|---|
| **Protein A** ng/mL | BDT | BDT | BDT |
| **DNA** ng/mL | <1.0 | <1.0 | 3.9 |
| **Endotoxin** EU/mL | <0.05 | 1.0 | 1.6 |

BDT: below detection limit

**For more information**

Pdf files of recent publications describing IgG purification with CHT can be downloaded at www.validated.com.

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