Purification of monoclonal antibodies by phenyl boronate chromatography

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This thesis focus on the feasibility of using aminophenyl boronate (APB) chromatography as the first step in the downstream processing of antibodies in order to reduce manufacturing costs, either by replacing Protein A chromatography or by reducing the loading of impurities before the Protein A step.The first step was to study the interaction of human immunoglobulin G (IgG) with the different components of the ligand. For this the binding of IgG and typical protein impurities (albumin and insulin) to the following resins was studied and compared: controlled pore glass (CPG); aminopropyl-CPG; aniline-CPG and APB-CPG. The chromatograms obtained showed that when the adsorption buffer was supplemented with 150 mM NaCl, electrostatic interactions were minimized, and most of the impurity proteins were collected in the flow-through while IgG was selectively recovered after elution with a competitive compound. These studies identified 20 mM HEPES, 150mM NaCl at pH 8.5 as the best binding buffer. In the next step, different elution buffers supplemented with competitive diols (sorbitol and mannitol) were evaluated in order to find an alternative to the standard elution buffer (1.5 M Tris-HCl, pH 8.5) and 50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl at pH 8.5 was found a viable alternative. The efficiency of the APB ligand was then compared with protein A, and a purification process combining two consecutive chromatographic steps on APB-CPG and protein A-CPG, respectively, was evaluated. The recovery of IgG was considerably lower than what was expected thus suggesting that more optimizations are required**.**

Keywords: monoclonal antibodies, aminophenyl boronate, affinity chromatography, purification, protein A

INTRODUCTION

The immune system comprises all the mechanisms by which a multicellular organism defends itself from foreign invaders such as bacteria, viruses, protozoa and fungi. This is the first line of defence against these invaders (1) (2).

There are two types of defence mechanisms: the innate or nonspecific system, and the adaptive immune system, which includes the selective action of lymphocytes and the production of specific antibodies (1) (2).

Antibodies are host proteins produced in response to the presence of foreign molecules in the body. They are synthesized primarily by plasma cells, a terminally differentiated cell of the B-lymphocyte lineage, and circulate throughout the blood and lymph where they bind to foreign antigens. Antibodies, also known as immunoglobulin's (Ig) are globular proteins (glycoprotein's) formed by four interconnected polypeptide chains with Y shaped (two heavy and two light chains) (1) (2).

Its primary structure (amino acid sequence) reveals a variable region and constant region. The constant region is located in the stem of the "Y", which interacts with other elements of the immune system and is identical in all types of antibodies, within the same class. The variable region is formed by the two upper arms of the "Y". It has a variable chemical response, unique to each antibody, and is complementary to a specific antigen, conferring specificity to the antibody (1) (2) (5).

The production of polyclonal antibodies is relatively simple compared with the production of mAbs. This production is done through the binding of antigen to carrier protein, which is introduced into the animal, generating antibodies. Then it is collected and purified it from serum antibodies using a chromatographic affinity column with the antigen immobilized on its surface. The selection of the animal is based on the need of the amount of antibodies (7).

Monoclonal antibodies (mAb) arise from a single B-lymphocyte, which is always producing the same antibodies in response to a pathogen (7).

In 1975, Kohler and Milstein published a revolutionary technique for producing monoclonal antibodies (mAbs) by hybridoma technology. With this new technology, mAbs have had a profound impact on medicine, giving an almost unlimited range of therapeutic or diagnostic reagents. Therapies using mAbs have become a major part of treatments in various diseases including transplantation, oncology, and autoimmune, cardiovascular and infectious diseases. The limitation of murine mAbs due to immunogenicity was overcome by replacement of the murine sequences with their human counterpart leading to the development of chimeric a humanized therapeutic antibodies. Incredible progresses were made in the development of display technologies, enabling not only the production of fully human antibodies but also the engineering of antibodies with altered properties such as molecular size, affinity and specificity. Furthermore, antibody engineering technologies are constantly advancing, allowing further adjustment of effectors function and serum half life. Optimal delivery of antibody to the target tissue remains to be addressed to avoid unwanted collateral damage as a result of systemic treatment, reaching a significant therapeutic effect (8).

Despite the wide range of preparative chromatography modes that can be used for the purification of monoclonal antibodies (mAbs), most purification schemes use protein A affinity chromatography as the capture step, which is due to its specificity for the region (Fc) of antibodies, their physical and chemical stability, and the ease and simplicity for the development process. The degree of purification achieved in this step is high, helping to simplify the rest of the purification process. Usually small amounts of contaminants, mainly high molecular weight aggregates, remaining host cell proteins and leached protein A, are still present after this step, and will be further removed in one or two subsequent polishing step. The protein A chromatography has been used for purification of antibodies, even though its high cost, and there are concerns about leaching of the ligand. Over time, this technique has been optimized for high capacity, high yields, cleaning and re-use, and is the basis of the "platform manufacturing" process in the purification of antibodies in the final stages of development (10) (5) (13).

In 1874, L. Vignon and G. Bouchardat attempted to titrate the boric acid (H_3BO_3), finding it impossible unless there was a second compound in solution, such as glycerol in equal concentrations. Other studies by these scientists and others over the following fifty years have shown that titration of borate depends on the interaction of covalent bonds with the 1,2-cis-diol group (15).

MATERIAL AND METHODS

Materials and biologics

Tris(hydroxylmetrhyl)aminomethane (Tris base) was obtained from Eurobio (Les Ulis, France). Sodium chloride was obtained from Panreac Quimica Sau (Barcelona, Spain). Sodium phosphate monobasic anhydrous (NaH_2PO_4), D-sorbitol, D-mannitol, phosphate buffered saline, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Citric acid

monohydrate and Hydrochloride acid were obtained from Merck (Darmstadt, Gemany). All other chemicals were of analytical or HPLC grade. Water used in all experiments was obtained from Milli-Q purification system (Millipore, Bedford, MA, USA).

Human immunoglobulin G (IgG) for therapeutic administration (product name: Gammanorm) was obtained from Octapharma (Lachen, Switzerland). Human serum albumin (HSA) and insulin were purchased from Sigma. A CHO clarified cell culture supernatants (cCCS) (37mg IgG/l; 1.1% protein purity, 2.2% HPLC purity) was used to evaluate the feasibility of the PB resin. A serum-free medium was used for production and was supplemented with HAS and insulin.

Chromatographic Study of interactions between IgG and the various constituents of the column

All chromatography experiments were carried out in AKTA Purifier system from Amersham Biosciences (Uppsala, Sweden) equipped with a Unicorn 5.1 data acquisition and processing software. Conductivity, pH, and UV absorbance at 280nm of the outlet stream was continuously monitored. Two different binding buffers were screened, namely (i) 20mM HEPES, pH 8.5, and (ii) 20mM HEPES with 150mM NaCl, pH 8.5. The elution buffer was 1.5M Tris-HCl, pH 8.5.

Prior to feed injection, the columns were equilibrated with fourteen point three column volumes (CVs) of adsorption buffer ate a flow rate of 1.0 ml/min. Pure protein samples (500µl, 1 mg IgG/ml, 2 mg HSA/ml, and 1 mg insulin/ml all prepared in adsorption buffer) were injected at 0.5 ml/min. The sample loop was emptied with at least three-times its volume. After washing the unbound or weakly retained molecules with five column volumes of the adsorption buffer at 1 ml/min, bound material was eluted following a 10 min linear gradient with the elution buffer at 1 ml/min. Flow-through and eluted fractions were collected on a Frac-950 fraction collector, from Amersham Biosciences and analyzed for IgG content. The chromatograms obtained with different adsorption buffers were recorded and compared in terms of peak area and retention time.

The columns CPG, CPG-Aniline, and CPG-aminopropyl were provided by doctoral researcher Rimenys Carvalho Jr. The CPG-Phenyl Boronate (CPG-APB) (figure 10) and CPG-Protein A (CPG-PA) were obtained from Millipore (Billerica, MA).

Chromatography on phenyl boronate resin

All chromatography experiments were carried out in AKTA Purifier system as previously described. Conductivity, pH, and UV absorbance at 280nm of the outlet stream was continuously monitored. Two different binding buffers and eleven elution buffers were screened to determine the best conditions for IgG adsorption. The binding buffers tested were: (i) 20mM HEPES, pH 8.5, and (ii) 20mM HEPES with 150mM NaCl, pH 8.5. The tested elution buffers were: (i) 1.5M Tris-HCl, pH 8.5, (ii) 50mM Tris-HCl, 100mM Sorbitol pH 8.5, (iii) 50mM Tris-HCl, 200mM Sorbitol pH 8.5, (iv) 50mM Tris-HCl, 250mM Sorbitol pH 8.5, (v) 50mM Tris-HCl, 100mM Mannitol pH 8.5, (vi) 50mM Tris-HCl, 200mM Mannitol pH 8.5, (vii) 50mM Tris-HCl, 250mM Mannitol pH 8.5, (viii) 50mM Tris-HCl, 200mM Sorbitol, 200mM NaCl pH 8.5, (ix) 50mM Tris-HCl, 200mM Mannitol, 200mM NaCl pH 8.5, (x) 200mM Tris-HCl, 200mM Sorbitol pH 8.5, (xi) 200mM Tris-HCl, 200mM Mannitol pH 8.5.

Prior to feed injection, columns were equilibrated with fourteen point three column volumes (CVs) of adsorption buffer ate a flow rate of 1.0 ml/min. Pure protein samples (2 ml, 250 mg IgG/ml, prepared in adsorption buffer) and CHO cCCS (2 ml CHO cCCS, 37µg IgG/ml) were injected at 0.5 ml/min. The sample loop was emptied with at least three-times its volume. After

washing the unbound or weakly retained molecules with five column volumes of the adsorption buffer, bound material was eluted following a 5 min linear gradient with the elution buffer. Flow-through and eluted fractions were collected and analyzed for IgG and protein content. The chromatograms obtained with different adsorption buffers were recorded and compared in terms of peak area and retention time.

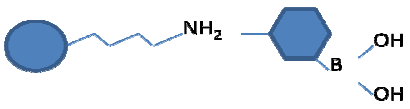

Figure 1- Schematic representation of the CPG-aminophenyl boronate resin

Chromatography on protein A resin

All chromatography experiments were carried out in AKTA Purifier system as previously described. Conductivity, pH, and UV absorbance at 280nm of the outlet stream was continuously monitored. Four different binding buffers and one elution buffer were screened to determine the best conditions for IgG adsorption. The binding buffers tested were: (i) 50mM NaH_2PO_4, 150mM NaCl, pH 7.4; (ii) 0.01M PBS, pH 7.4; (iii) 20mM HEPES, 150mM NaCl, pH 8.5; and (iv) 50mM Tris-HCl, 200mM Sorbitol, 200mM NaCl, pH 8.5. The elution buffer was 0.1 M citric acid, pH 3.0.

Prior to feed injection, columns were equilibrated with fourteen point three column volumes (CVs) of adsorption buffer ate a flow rate of 1.0 ml/min.

Pure protein samples (2 ml, 1 mg IgG/ml, prepared in adsorption buffer and CHO cCCS (2 ml CHO cCCS, 37µg IgG/ml) were injected at 0.5 ml/min. The sample loop was emptied with at least three-times its volume. After washing the unbound or weakly retained molecules with five column volumes of the adsorption buffer, bound material was eluted following a 10 min linear gradient with the elution buffer. Flow-through and eluted fractions were collected and analyzed for IgG and protein content. The chromatograms obtained with different adsorption buffers were recorded and compared in terms of peak area and retention time.

Purification of IgG in CHO cells

A 2-step purification process was evaluated for the purification of IgG from the CHO cCCS, using in first step the CPG-APB chromatography and in the second step CPG-PA (protein A) chromatography.

All chromatographic experiments were carried out in AKTA Purifier as described before. Conductivity, pH, and UV absorbance at 280nm of the outlet stream was continuously monitored. For the CPG-APB chromatography the binding buffer used was 20mM HEPES with 150mM NaCl pH 8.5, and the elution buffer was 50mM Tris-HCl, 200mM Sorbitol, 200mM NaCl pH 8.5. For the CPG-PA chromatography the binding buffer used was 0.01 M PBS, pH 7.4, and the elution buffer was 0.1 M citric acid pH 3.0.

Prior to feed injection, columns were equilibrated with fourteen point three column volumes (CVs) of adsorption buffer ate a flow rate of 1.0 ml/min.

CHO cCCS were injected at 0.5 ml/min first in column CPG-APB (2 ml CHO cCCS, 37µg IgG/ml). Flow-through and eluted fractions were collected and the pool of the eluted fractions containing IgG was injected in column CPG-PA (500 µl PB eluted pool, 28.96 mg IgG/ml). As always, the sample loop was emptied with at least three-times its volume. After washing the unbound or weakly retained molecules with five column volumes of the adsorption buffer, bound material was eluted following a 10 min linear gradient with the elution buffer.. The chromatograms obtained with different adsorption buffers were recorded and compared in terms of peak area and retention time. The fractions both columns

were analyzed for the IgG and protein content.

IgG quantification

The concentration of IgG was determined by analitycal protein A chromatograph using a PA ImmunoDetection sensor cartridge from Applied Biosystems (Foster City, CA, USA) as described in (18). In the initial experiments the binding buffer was composed by 10mM phosphate, 150mM NaCl pH 7.4, and the elution buffer was composed by 12mM HCl, 150mM NaCl pH 2-3. In further experiments the binding buffer was changed to 50mM NaH_2PO_4, 150mM NaCl pH 7,4. Samples were previously diluted four times with binding buffer.

Protein gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate the fractions purity collected from the purification experiments. The buffer sample composition was 62.5mM Tris-HCl, pH 6.2, 2% SDS, 0.01% bromophenol blue, 10% glycerol and 0.1 M DTT. All samples collected were diluted prior to denaturation at 100ºC for 10 min. Samples were applied in a 12% acrylamide gel, prepared from a 40% acrylamide/bis stock solution (29:1) from Bio-Rad (Hercules, CA, USA), and ran at 90mV using a running buffer that contained 192mM glycine, 25mM Tris, and 0.1% SDS, pH 8.3. Gels were firstly stained with Coomassie Brilliant Blue for 1h, and then destained in a solution of 30% of ethanol and 10% of acetic acid for one more hour. Repeat the last step one more time.

Protein Quantification

The quantification of total protein in CHO cells and in the samples collected in the AKTA Purifier was performed with the Bradford method using a Coomassie assay reagent provided by Pierce (Rockford, IL, USA). The protein standard used was bovine serum albumin (BSA). Absorbance was measured at 595 nm in a microplate reader from Molecular Devices (Sunnyvale, CA, USA). Protein purity was determined by dividing the concentration of IgG determined by Protein A HPLC by the concentration of total protein determined using the Bradford method.

RESULTS AND DISCUSSION

Chromatographic Study of interactions between IgG and the various constituents of the column

In order to use phenyl boronate as an alternative capture step to protein A affinity chromatography in the purification process of monoclonal antibodies, it is necessary to test the affinity of typical protein impurities that are typically present in cell culture supernatants. Towards the new ligand, aminophenyl boronic acid.

Initially tests were performed only with three typical protein components found in the cell culture supernatant, the insulin, antibody (IgG) and human serum albumin (HSA). A mock/control run will also be performed in order the see the changes input in the chromatogram baselines due to both adsorption and elution buffers. Four types of columns (CPG, CPG-aminopropyl, CPG-Aniline, and CPG phenyl boronate) were used to understand the binding of the proteins to the different groups of the resin. In each of these columns, the solutions are injected with two different adsorption buffers, 20 mM HEPES, pH 8.5 and 20 mM HEPES, 150 mM NaCl, pH 8.5 and only one elution buffer, 1.5 M Tris-HCl, pH 8.5. The following results were obtained by processing data through the program UNICORN 5.1. The chromatograms represented below were subtracted by the control run, canceling any interference of the buffer constituents.

The recovery of IgG is different comparing the different columns and the two binding buffers. When the binding buffer was

supplemented with NaCl (20 mM HEPES, 150mM NaCl, pH 8.5) the recovery of IgG in flow-through increased with a concomitant decrease in the elution step, for the three first columns. These results show that the NaCl eliminates the non-specific binding of IgG to the constitutes of the column CPG-APB. In this column the recovery is the same in both binding buffers, showing that the NaCl does not influence the affinity of IgG towards the APB lingand.

These results thus suggest that 20mM HEPES with 150mM NaCl at pH 8.5 is the best binding buffer, because it eliminates the unspecific binding of the IgG to the column.

Chromatography on phenyl boronate resin

The next step of this thesis was to evaluate different elution buffers for CPG-APB column, in order to find an alternative to the 1.5 M Tris-HCl buffer at pH 8.5, which is too concentrated and too expensive for the downstream process. The tested elution buffers were: (i) 1.5 M Tris-HCl, pH 8.5, (ii) 50 mM Tris-HCl, 100 mM Sorbitol, pH 8.5, (iii) 50 mM Tris-HCl, 200 mM Sorbitol, pH 8.5, (iv) 50 mM Tris-HCl, 250 mM Sorbitol pH 8.5, (v) 50 mM Tris-HCl, 100 mM Mannitol pH 8.5, (vi) 50 mM Tris-HCl, 200 mM Mannitol pH 8.5, (vii) 50 mM Tris-HCl, 250 mM Mannitol pH 8.5, (viii) 50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl pH 8.5, (ix) 50 mM Tris-HCl, 200 mM Mannitol, 200 mM NaCl pH 8.5, (x) 200 mM Tris-HCl, 200 mM Sorbitol pH 8.5, (xi) 200 mM Tris-HCl, 200mM Mannitol pH 8.5.

Table 1 - Table of protein purity of IgG, in Flow-through and elution steps after purification from a CHO cell supernatant using CPG-APB column. With 20mM HEPES at pH 8.5 binding buffer, and four elution buffer.

% protein purity in CHO cells			
Elution Buffer	**Flow-through**	**Elution**	
		Tested Buffer	1.5 M Tris-HCl pH 8.5
200 mM Tris-HCl, 200 mM Sorbitol pH 8.5	0.29	0.47	58.86
200 mM Tris-HCl, 200 mM Mannitol pH 8.5	0.90	0.49	74.35
50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl pH 8.5	0.30	11.36	2.46
50 mM Tris-HCl, 200 mM Mannitol, 200 mM NaCl pH 8.5	1.11	3.98	37.10

Table 2 - Table of protein purity of IgG, in Flow-through and elution steps after purification from a CHO cell supernatant using CPG-APB column. With 20mM HEPES with 150 mM at pH 8.5 binding buffer, and four elution buffer.

% protein purity in CHO cells			
Elution Buffer	**Flow-through**	**Elution**	
		Tested Buffer	1.5 M Tris-HCl pH 8.5
200 mM Tris-HCl, 200 mM Sorbitol pH 8.5	0.36	11.02	124.26
200 mM Tris-HCl, 200 mM Mannitol pH 8.5	1.46	0.48	95.59
50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl pH 8.5	0.93	15.51	3.21
50 mM Tris-HCl, 200 mM Mannitol, 200 mM NaCl pH 8.5	0.84	1.55	38.33

We can see that from all the elution buffers tested, only the 50 mM Tris-HCl, 200 mM sorbitol, 200 mM NaCl at pH 8.5 leads to a higher purity in the first elution, even though this is low, although the starting purity of the feed solution was even more lower (0.96%). The remaining samples had a higher purity in the second elution, being unfavourable for the purpose of this work, which is the replacement of the elution buffer,1.5 M Tris-HCl at pH 8.5. Apart from a few values, most of the results were low, and that can be due to the low concentrations of IgG.

Purification of IgG with PA CPG resin

To test the recovery of IgG from a CHO cell supernatant using the PA CPG resin, four bniding buffers: i) 50 mM phosphate buffer, 150 mM NaCl at pH 7.4 ii) 0.01 M PBS at pH 7.4; iii) 20mM HEPES, 150mM NaCl buffer at pH 8.5 and iv) 50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl at pH 8.5. The first two buffers are commonly used in the purification of antibodies by Protein A chromatography, while the later buffers were used in the purification of antibody in the CPG-APB resin, found in this study, to test if this buffers have any interference with the protein A resin. Using as the elution buffer the 0.1M citric acid buffer at pH 3.0.

According to the total protein quantification, it was observed that after purification with protein A, more than 90% of total protein had been eliminated (eluted in the flow-through).

Table 3 - Protein purity in the fractions collected form PA-CPG column: flow-through and elution steps, using four binding buffer, and 0.1 M citric acid at pH 3.0 as elution buffer.

Protein Purity (%)		
Binding Buffer	**Flow-through**	**Elution**
50 mM phosphate buffer, 150 mM NaCl pH 7.4	0.12	109
0.01 M PBS pH 7.4	0.24	74
20 mM HEPES, 150 mM NaCl pH 8.5	0.14	122
50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl pH 8.5	0.19	141

While calculating the purity of the antibody (table 13) in the final samples, it was observed that most of the samples have a purity greater than 100%, this is due to the fact that the quantification obtained with the protein A is greater than the value obtained with the Bradford method, this can due to experimental errors, since no method is 100% robust.

Purification of IgG in CHO cells

The last work of this thesis was to test a purification process using first APB-CPG resin and then the PA-CPG resin.

In the purification CPG-APB resin used the binding buffer was composed by 20 mM HEPES, 150 mM NaCl, pH 8.5 and the elution buffer by 50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl, 8.5. Three CHO cCCS samples (2 ml) were injected; these three injections were made to see if the process would be robust and reproducible. In each run fractions of 1 ml were collected during the flow-through and of 0.5 ml during the elution.

Table 4 - Table of % recovery in CPG-APB column, in Flow-through and elution steps. With 20 mM HEPES, 150 mM NaCl at pH 8.5 binding buffer, and 50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl at pH 8.5 elution buffer.

% Recovery of IgG from the CHO cell supernatant		
Run	**Flow-through**	**Elution**
A1	14.0	45.68
A2	14.0	75.8
A3	14.0	66.2

The percentage of recovery of IgG in this resin for the three samples was not reproducible, with the first run exhibiting a considerably lower recovery yield of IgG in the elution pool. This low percentage may have been due to an operation error, so for the remaining analysis we will withdraw the samples collected from the first run. Table 16 shows the protein purity and purification factor.

Table 5 - Table of % protein purity and protein factor in CPG-APB column, in Flow-through and elution steps. With 20 mM HEPES, 150 mM NaCl at pH 8.5 binding buffer, and 50 mM Tris-HCl, 200 mM Sorbitol, 200 mM NaCl at pH 8.5 elution buffer.

	Protein Purity (%)		**Purification Factor**	
Run	**Flow-through**	**Elution**	**Flow-through**	**Elution**
A2	0.36	10.34	0.11	3.27
A3	0.12	2.89	0.12	2.89

After analysis of chromatograms of purification of the antibody with the CPG-APB resin, it was found the pool of flow-through and elution fractions that have peaks, injecting this samples in CPG protein A column (500ul) using as binding buffer 0.01 M PBS pH 7.4, and as elution buffer, 0.1 M citric acid pH 3.0. The recovery of IgG, the protein purity and the purification factor are presented in tables 17-18.

Table 6 - Table of % recovery in PA CPG column, in Flow-through and elution steps. With 0.01 M PBS at pH 7.4 binding buffer, and 0.1 M citric acid at pH 3.0 as elution buffer.

% Recovery of IgG from the CHO cell supernatant		
Run	**Flow-through**	**Elution**
A2	7.8	17.21
A3	8.73	21.35

Table 7 - Table of % protein purity and protein factor in PA CPG column, in Flow-through and elution steps. With 0.01 M PBS at pH 7.4 binding buffer, and 0.1 M citric acid at pH 3.0 as elution buffer.

	Protein Purity (%)		**Purification Factor**	
Sample	**Flow-through**	**Elution**	**Flow-through**	**Elution**
A2	3.68	13.44	1.17	4.26
A3	3.75	14.10	1.19	4.47

The recovery of IgG was considerable lower than what was expected; one would expect to obtain recoveries above 80%. There may have been several problems for these results, problems with the resin, and problems in the low injection volume in the PA CPG column, among others. It is necessary optimize the process with additional tests.

CONCLUSION

The first step was test the binding of human immunoglobulin G (IgG) to the various constitutes of CPG-amino phenyl boronate The chromatographic runs shown in the section 3.1.1 were repeated but the adsorption buffer was supplemented with 150 mM NaCl in order to avoid the presence of electrostatic interactions. The chromatograms obtained for the CPG, CPG-aminopropyl and CPG-aniline for the adsorption buffer 20 mM HEPES, 150 mM NaCl ate pH 8.5 are different with most of the proteins coming up in the flow-through. This shows that electrostatic interactions

were in the base of the adsorptions. The only column that maintained its profile was the CPG-APB, which corroborates that there is a specific interaction between IgG and the ligand, phenyl boronate. These results suggest that 20mM HEPES with 150mM NaCl at pH 8.5 is the best binding buffer.

The next step was to evaluate different elution buffers for CPG-APB column, in order to find an alternative to the 1.5 M Tris-HCl buffer at pH 8.5, which is too concentrated and too expensive for the downstream process. Were tested eleven elution buffers, decreasing the concentration of Tris-HCl and supplemented with competitive diols (sorbitol and mannitol). With all results obtained, was conclude that for the purification of the antibodis with CPG-APB it should be used a binding buffer composed by 20 mM HEPES, 150 mM NaCl at pH 8.5 and an elution buffer composed by 50 mM Tris-HCl, 200 mM Sorbitol and 200 mM NaCl at pH 8.5.

After these, the next step was to test the purification of IgG on protein A CPG resin (CPG PA), then test the purification process using first the resin CPG-APB then the PA CPG resin. The recovery of IgG for all loading buffer were identical, and can see that there is no interference of the buffers used in purification with CPG-APB resin.

The last step was test the purification process using first the resin CPG-APB then the PA CPG resin. The recovery of IgG was considerable lower than what was expected; one would expect to obtain recoveries above 80%. There may have been several problems for these results.

With all this results there are future work that need to be done. First there is necessary repeat the elution studies, to confirm the results obtained in this thesis, and if the method is robust. And then it is necessary optimize the last step of this work to achieve high recoveries.

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