



**HAL**  
open science

## Capture of human monoclonal antibodies from a cell culture supernatant by phenyl boronate chromatography

Ana Margarida Azevedo, Ana Gabriela Gomes, Luis Borlido, Ines F.S. Santos, Duarte Miguel F. Prazeres, Maria Raquel Aires-Barros

### ► To cite this version:

Ana Margarida Azevedo, Ana Gabriela Gomes, Luis Borlido, Ines F.S. Santos, Duarte Miguel F. Prazeres, et al.. Capture of human monoclonal antibodies from a cell culture supernatant by phenyl boronate chromatography. *Journal of Molecular Recognition*, 2010, 23 (6), pp.569. 10.1002/jmr.1068 . hal-00589484

**HAL Id: hal-00589484**

**<https://hal.science/hal-00589484v1>**

Submitted on 29 Apr 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Capture of human monoclonal antibodies from a clarified cell culture supernatant by phenyl boronate chromatography

Ana M. Azevedo, A.G. Gomes, L. Borlido, I.F.S. Santos,  
D.M.F. Prazeres, M.R. Aires-Barros

IBB – Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

## ABSTRACT

In this work, we investigated the feasibility of using phenyl boronate (PB) chromatography for the direct capture of monoclonal antibodies from a CHO cell supernatant. Preliminary results, using pure protein solutions have shown that PB media can bind to human antibodies, not only at strong alkaline conditions but also at acidic pH values. In fact, antibodies have been found to bind in the pH range 5.5-8.5. On the other hand, insulin and human serum albumin (HSA) did not bind at alkaline pH but at lower pH, which reflects the importance of non-specific interactions with the matrix. Different binding and eluting buffers were evaluated for the capture of IgG from a CHO cell supernatant and the most promising results were obtained using 20 mM HEPES at pH 8.5 as binding buffer and 1.5 M Tris-HCl as eluting buffer. Using a step elution, all IgG was recovered in the elution pool with a maximum purification factor of 56. A gradient elution allowed a further increase of the final purity, yet achieving a slightly lower yield. IgG recovery was around 85% and the purification factor was 76. The highest purity was obtained when the pH of the cell supernatant feed was previously adjusted to 8.5. Starting from an initial protein purity of 1.1% and HPLC purity of 2.2%, after PB adsorption, a final protein purity of 85% and a HPLC purity of 88% was achieved.

**Key-Words:** Monoclonal antibodies; phenyl boronate; affinity chromatography; downstream processing; capture; purification

## INTRODUCTION

Therapies based on monoclonal antibodies (mAbs) are one of the fastest growing and most lucrative segments of the pharmaceutical industry. Since the first approval of a monoclonal antibody for the treatment of acute kidney transplant rejection, in 1986, more than 24 mAbs have been approved for the treatment of a vast number of diseases, including different types of cancers, autoimmune diseases and transplant rejection, while hundreds are in clinical trials or under development. Given their low potency and increasing market potential, large amounts of pure mAbs are required for the treatment of chronic diseases. This increasing product demand has challenged biotechnologists to increase production capacity and improve the conventional purification technology.

Currently, almost all marketed mAbs are produced by mammalian cell culture using either Chinese hamster ovary (CHO) or mouse myeloma (NS0) cells (1, 2). Advances in molecular biology and protein engineering have led to remarkable improvements in cell culture productivities, with antibody titers exceeding 10 g/l for the CHO expression system (3). With capacity bottlenecks moving towards downstream purification areas, the need for a broader strategic approach for the purification of monoclonal antibodies is being increasingly recognized as the key to improve the overall process performance (4).

The downstream processing of mAbs at manufacturing scales normally includes several steps in order to ensure a high clearance of different types of impurities, including host cell proteins and DNA, viruses, metabolites and product-related impurities (e.g. high molecular weight aggregates and low molecular weight clipped species) (5). The purification of mAbs in a platform-based approach is typically composed by three chromatographic steps, including an affinity capture step on a protein A based matrix, and two orthogonal steps for the removal of viruses (5). The affinity capture step relies on the specific interaction of the antibody Fc part with the immobilized protein A, a cell wall protein of *Staphylococcus aureus* (6). This affinity step not only originates purities higher than 95% with high yields (5), but is also very flexible in terms of conductivity and pH of the feed stream, allowing the direct capture of antibodies from the clarified cell culture. This is impracticable with other capture alternatives, especially ion exchange and hydrophobic interaction chromatography that require a pre-conditioning step upstream. Nevertheless, protein A is the most expensive step in the downstream processing of mAbs accounting for 50% of the total costs (7). Indeed, the costs associated with this unit operation are almost one order of magnitude higher than the costs associated with other traditional chromatographic operations (8).

Novel affinity-based separations have emerged from the development of synthetic ligands including peptides obtained by combinatorial libraries and artificial ligands generated by *de novo* process designs (9, 10), although so far with limited applicability by big pharma companies. Non-chromatographic alternatives including membrane chromatography, tangential flow filtration, high gradient magnetic fishing, aqueous two-phase extraction, precipitation and crystallization have also been described (4, 11, 12).

Although several alternatives to the established platform have been proposed, newer and more economic methods are still being pursued to facilitate the manufacturing of large amounts of mAbs that comply with the stringent impurity clearance requirements stipulated by regulatory agencies (FDA, EMEA). With this work, the feasibility of using phenyl boronate (PB) as an affinity ligand for the purification of mAbs has been investigated. The PB ligand is a useful tool for the specific capture and isolation of *cis*-diol-containing molecules, such as carbohydrates, glycoproteins, glycopeptides, enzymes, RNA, nucleotides, nucleic acids, and nucleosides (13-15). The ligand interacts predominantly with *cis*-diol groups by forming reversible covalent ester bonds. Antibodies are glycoproteins, bearing N-linked oligosaccharide at the asparagine residue of the CH2 domain of the Fc portion. Although the carbohydrate moiety exhibits some heterogeneity with respect to the terminal sugars attached, fucose, galactose and mannose, all containing 1,2-*cis*-diol groups, can be typically found in mAbs. This opens up wide perspectives for the use of PB as a ligand for the purification of mAbs. Preliminary work developed by Brena and co-workers have already shown the ability of PB to bind antibodies and other glycosylated proteins present in blood serum (16). More recently, a two-dimensional high-performance liquid chromatography setup combining a PB and a reversed phase column, demonstrated that boronate chromatography was best described as mixed-mode interaction (17), confirming the initial studies by Brena *et al.* that indicated that non-specific interactions could play a role in the binding of proteins to the PB ligand.

## **MATERIALS AND METHODS**

### ***Materials and Biologicals***

Tris(hydroxymethyl)aminomethane (Tris base) and glycine were obtained from Eurobio (Les Ulis, France). Sodium chloride was obtained from Panreac Quimica Saur (Barcelona, Spain). Sodium phosphate monobasic anhydrous (Na<sub>2</sub>HPO<sub>4</sub>), D-sorbitol, ethylenediaminetetraacetic acid (EDTA), phosphate buffered saline (PBS), and 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Magnesium chloride ( $MgCl_2$ ) was obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical or HPLC grade. Water used in all experiments was obtained from a 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 was purchased from Sigma and insulin (product name: ActRapid) from Novo Nordisk (Bagsværd, Denmark). A CHO clarified cell culture supernatants (cCCS) (37 mg 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 human serum albumin and insulin

### ***Chromatography on phenyl boronate resin***

All chromatographic experiments were carried out in an Äkta Purifier system from Amersham Biosciences (Uppsala, Sweden) equipped with a Unicorn 5.1 data acquisition and processing software. Conductivity, pH and UV absorbance at 280 nm of the outlet stream was continuously monitored. Different binding and elution buffers were screened to determine the best conditions for IgG adsorption and elution using at least two independent replicated assays. The tested binding buffers at pH 8.5 were: i) 20 mM HEPES; ii) 20 mM HEPES, 150 mM NaCl; iii) 20 mM HEPES, 100 mM  $MgCl_2$ ; iv) 20 mM HEPES, 15 mM Tris; v) 50 mM glycine, 100 mM  $MgCl_2$ ; vi) 10 mM phosphate, 150 mM NaCl; vii) 50 mM phosphate, 300 mM NaCl; viii) 100 mM borate buffer; ix) 100 mM borate buffer, 150 mM NaCl and x) 100 mM borate buffer, 300 mM NaCl. The tested elution buffers were: i) 20 mM sodium acetate pH 5; ii) 150 mM NaCl pH 2-3; iii) H<sub>2</sub>O; iv) 0.15mMTris-HCl, pH 8.; v) 0.15 M Tris-HCl, 0.1 M sorbitol, pH 8.5; vi) 0.15 M Tris-HCl, 0.1 M EDTA, pH 8.5; vii) 0.5 M Tris-HCl, pH 8.5; viii) 1 M Tris-HCl, pH 8.5; ix) 1.5 M Tris-HCl, pH 8.5 and x) 0.6 M citrate, pH 7.5.

Prior to feed injection, columns were equilibrated with 10 column volumes (CV) of adsorption buffer (20 mM HEPES at pH 8.5, unless otherwise stated). Pure protein samples (500  $\mu$ l, 1 mg IgG/ml, 2 mg HSA/ml and 1 mg insulin/ml all prepared in adsorption buffer) and CHO cCCS (2 ml CHO cCCS, 37  $\mu$ g 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 5 CVs of the absorption buffer, bound material was eluted following a step gradient or a 10 min linear gradient with the elution buffer (1.5 M TrisHCl at pH 8.5, unless otherwise stated). Flow-through and eluted

fractions were collected on a Frac-950 fraction collector, from Amersham Biosciences and analyzed for IgG and protein content. The chromatograms obtained with the different adsorption and elution buffers were recorded and compared in terms of peak areas and antibody recovery.

### ***IgG quantification***

The concentration of IgG was determined by analytical protein A chromatograph using a PA ImmunoDetection sensor cartridge from Applied Biosystems (Foster City, CA, USA) as described elsewhere (18). The binding buffer was composed by 10 mM phosphate, 150 mM NaCl, pH 8.5 and the elution buffer was composed by 12 mM HCl, 150 mM NaCl, pH 2-3. Samples were previously diluted (at least 4 times) with a dilution buffer composed by 0.05% Tween 80 in binding buffer.

### ***Protein quantification***

The quantification of proteins 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 micro plate 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.

### ***Protein gel electrophoresis***

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate the fractions' purity collected from the PB column. The buffer sample composition was 62.5 mM Tris-HCl, pH 6.2, 2% SDS, 0.01% bromophenol blue and 10% glycerol. All samples collected were diluted prior to denaturation in reducing conditions with dithiothreitol at 100°C for 5 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 90 mV using a running buffer that contained 192 mM glycine, 25mM Tris and 0,1% SDS, pH 8.3. Gels were firstly stained with Coomassie Brilliant Blue and, whenever the intensity of the bands was too low, the gels were subsequently stained with silver nitrate.

### ***Determination of purity by HPLC***

HPLC purity of the fractions eluted from the PB column was determined by size-exclusion chromatography (SEC). Samples were diluted 5-times in PBS (phosphate buffer saline) and run in isocratic mode in a TSK-Gel Super SW3000 column equipped

with a TSK-GEL Super SW guard column, both from Tosoh Bioscience (Stuttgart, Germany) at 0.35 ml/min for 25 min. HPLC purity was quantified by the ratio of the IgG peak area and the total area of the chromatogram subtracted by the total area of the corresponding buffer chromatogram (absorption buffer for flow-through fractions and the elution buffer for eluted fractions).

## **RESULTS AND DISCUSSION**

### ***Adsorption Studies***

In order to evaluate the feasibility of using PB chromatography to capture mAbs, different buffers were studied to check which conditions favoured the adsorption of IgG to a PB packed column. As the binding of 1,2-cis-diol groups to PB moieties occurs predominantly at alkaline pH values, a set of buffers at pH 8.5 were tested (see Materials and Methods section). All buffers allowed the binding of more than 90% of the loaded IgG (results not shown). However, 20 mM HEPES was selected as the standard adsorption buffer as it is reported in the literature to enhance the binding of cis-diol containing compounds to PB media (19, 20). Furthermore, and unlike the case of phosphate buffers, the binding of cis-diols has been reported to be independent of the concentration of HEPES (20).

### ***Elution Studies***

Since the elution of molecules adsorbed to PB is commonly achieved by a decrease in pH or by an increase in the concentration of a competing diol, a range of eluting agents were analyzed including among other sodium acetate at pH 5; NaCl pH 2-3; Tris-HCl pH 8.5. For this last buffer the molarity was varied from 0.15 to 1.5 M and components such as sorbitol and EDTA were added. Although a downward pH gradient may be useful for the elution of nucleotides from PB, this strategy was not efficient in the case of antibodies. At pH 5, no bound IgG was released whereas at pH 2-3 only 7% eluted. The most efficient elution agent was Tris: an increase in concentration from 0.15 to 1.5 M allowed an increase in the amount of eluted IgG from 73% to 100%. The addition of sorbitol to 0.15 M Tris improved the elution of IgG and allowed a recovery of 86% of the total IgG. Nonetheless, 1.5 M Tris was selected as the standard IgG elution, since it produced the narrowest and highest peaks.

### **Binding Capacity Studies**

Frontal chromatography experiments were carried out to determine the binding capacity of the PB resin by continuously feeding solutions of IgG in 20 mM HEPES, pH 8.5, with concentrations ranging from 1 to 10 g/l. The corresponding breakthrough curves were used to determine the dynamic binding capacity (DBC). A DBC of  $10.4 \pm 1.1$  mg IgG/ml resin was determined at 10% breakthrough. After resin saturation, bound IgG was eluted with 1.5M Tris, pH 8.5 and the eluted mass was subsequently quantified by off-line quantitative protein A HPLC, yielding  $15.2 \pm 1.8$  mg IgG/ml resin.

### **Selectivity Studies**

The binding of pure human IgG, human recombinant insulin and human serum albumin (HSA) to PB media was measured separately and compared under different adsorption conditions, namely buffer type, pH and ionic strength. These proteins were selected because they are typically added to serum-free cell culture media. Insulin is non-glycosylated and thus cannot participate in the esterification of boronic acid. In addition, serum albumin is characterized by a low content in carbohydrates, although highly glycosylated albumin can be found in the serum of patients (21)

The binding-and-eluting profiles of the three proteins under different adsorption conditions, namely pH and ionic strength, are shown in Fig. 1 and 2. IgG bound completely (>95%) in almost all conditions, except when a competitor (Tris) was added. In this case the binding of IgG decreased to 69%. The binding of insulin to the PB media was dependent on the pH but not on the ionic strength. At neutral pH, around 88% of insulin was adsorbed to the column, while at pH 8.5 binding was negligible except in the presence of  $MgCl_2$ , which is typically used as an enhancer (16). The binding of albumin however was found to be highly dependent on both pH and ionic strength (Fig. 1). At neutral pH, in both HEPES and PBS buffer, around 70% of albumin was adsorbed to the column, while at pH 8.5 and in HEPES buffer, only 12% of HSA was bound. The increase in ionic strength brought about by the addition of NaCl or  $MgCl_2$  increased the binding of HSA considerably to 65 and 91%, respectively.

These different binding profiles indicate that in addition to the esterification reaction between PB and *cis*-diol moieties, secondary interactions can play an important role, especially at neutral (and acid) conditions. In fact, binding of *cis*-diols to boronic acids is not as simple as it appears (22). Boronic acids, as a result of their deficient valence ( $sp^2$  hybridization state) contain an empty *p* orbital and adopt a trigonal, coplanar geometry. At neutral and acidic pH values, they are able to interact by charge transfer interactions with Lewis bases, such as the amino groups typically found in proteins (23). However, at alkaline pH values (namely 8.5), the trigonal boronic acid



group ionizes to form a tetrahedral hydroxyboronate anion. Although both phenyl boronic acid and phenyl boronate are able to bind to *cis*-diol groups, the equilibrium constant of the esterification reaction for tetrahedral complexes formation has been reported to be much higher (at least three orders of magnitude) than for the trigonal complexes (19). In addition, the tetrahedral boronate anion can no longer participate in charge transfer interactions, as the boron atom is now in a  $sp^3$  hybridization state. Secondary interactions are thus minimized at alkaline conditions. Nevertheless, the presence of a negative charge in the hydroxyboronate anion can induce electrostatic interactions, which may however be neglected by increasing the ionic strength. Although hydrophobic interactions can also play a role, they should be negligible taking into account the considerably low ionic strength of the adsorption buffers used and the fact that elution is promoted by 1.5 M Tris.

Taking in consideration the behavior of the three model proteins described above, one can conclude that at pH 8.5 and low ionic strength, secondary interactions are minimized and only proteins bearing carbohydrates, i.e., IgG and the glycosylated fraction of HSA, bind to the PB column. As the pH decreases, the boron atom adopts the trigonal conformation with one empty *p* orbital that can then interact with Lewis bases, including amino and carboxyl groups present in amino acid residues. Because both albumin and insulin are negatively charged at pH 8.5, the negative charge of the boronate anion can produce columbic repulsion providing additional selectivity. On the other hand, increasing the ionic strength masks this interaction and selectivity decreases.

### ***CHO Cell Supernatant***

The capture of IgG from a CHO cell supernatant containing serum albumin and insulin was evaluated. Fig. 3 compares the chromatographic runs obtained when 20 mM HEPES at pH 8.5 or PBS at pH 7 were used as adsorption buffers, and 1.5M Tris, pH 8.5 was used for step elution of bound IgG. Although both adsorption buffers allowed the subsequent recovery of all IgG upon elution, the final IgG purity in the collected fractions was different. At pH 7, the purification factor was as high as 15, whilst at pH 8.5 it was higher than 63. Size-exclusion chromatograms obtained for both the flow-through and elution pools are illustrated in Fig. 4 and compared with the feedstock. Residence times of 10 min, 10.7 min and 13.5 min were determined for IgG, HSA and insulin, respectively. The removal of HSA and insulin could then be determined by taking into account the initial and final area of the corresponding protein peaks. Both buffers allowed the removal of 98% of HSA. At pH 8.5, all insulin was removed whilst at pH 7 only 66% was removed.

In order to clarify whether the difference observed in the purification of IgG using PBS at pH 7 or 20 mM HEPES at pH 8.5 as adsorption buffers was only dependent on the pH, or if both buffer type and ionic strength could also play an important role, two other buffers were evaluated for the capture of IgG, namely 20 mM HEPES with 150 mM NaCl at pH 8.5, and 20 mM HEPES at pH 7. The chromatograms obtained for both runs were very similar to the ones represented in Fig. 3. SEC-HPLC analysis of the pools collected upon step elution, showed that the removal of insulin was not very effective at pH 7 for both, and that the removal of albumin was very similar for the different adsorption buffers. An extra run at pH 8.5 using 20 mM HEPES was also performed in which the pH of the CHO cell supernatant, initially at pH 7.2, was adjusted to pH 8.5 by the addition of NaOH. This has considerably enhanced the final purity from 1.1% to 63%, with a removal of 98% of HSA and 100% of insulin. Table 1 summarizes the performance parameters, including yield, protein purity, purification factor, HPLC purity and HPLC purification factor for the different adsorption buffers evaluated. The results show that both protein purity and HPLC purity are mostly dependent on the pH and not on the buffer type.

In order to check if the purity of the IgG-containing fractions could be further increased, the adsorption runs described above were repeated, but a gradient elution was performed instead. In the illustrative experiment shown in Fig. 5, the pH of the CHO supernatant feed was adjusted to pH 8.5 and 20 mM HEPES at pH 8.5 was used as the adsorption buffer. In all runs performed, two peaks eluted from the PB column and their relative height was dependent on the type of buffer as illustrated in Fig. 6, being the first peak considerably more intense at neutral and alkaline pH in the presence of NaCl. SEC-HPLC analysis showed that the first peak is rich in impurities (e.g. albumin and insulin) and the second peak in IgG. These results were corroborated by an SDS-PAGE analysis (Fig. 7 and 8). Comparing the density and intensity of the protein bands characteristic of the flow-through and elution pools, one can conclude that most proteins, including albumin, insulin and the majority of host cell proteins do not interact with the PB media. It is also interesting to notice that when HEPES is used as the adsorption buffer, the amount of proteins present in the first elution peak is considerably higher at pH 7 than at pH 8.5 (Fig. 7, lane 7 and 4, respectively). In addition, using HEPES at pH 8.5 as adsorption buffer and adjusting the pH of the supernatant to 8.5, the amount of proteins in the first elution peak is negligible (Fig. 8, lane 3) being only visible after silver staining. Also, in the gradient elution pool, albumin is still present although in very low concentration as its band is not visible in the SDS-PAGE after Coomassie staining, but only after silver staining.

Table 2 summarizes the performance parameters, including yield, protein purity, purification factor, HPLC purity and HPLC purification factor for the different adsorption buffers evaluated. With gradient elution, both protein purity (i.e. the IgG/protein ratio) and HPLC purity increased considerably with the corresponding purification factors reaching 76 and 39, respectively. Nonetheless, recovery in the second elution pool was slightly lower (around 85%) as a small part of IgG is lost in the first elution pool. In addition, IgG concentration in the second elution pool was lower (typically half) than the one obtained performing a step elution. For the IgG capture from the CHO cell supernatant adjusted to pH 8.5, using 20 mM HEPES at pH 8.5 as adsorption buffer, the concentration of IgG in the step elution pool was 33.6 mg/l and decreased to 16 mg/l in the gradient elution pool. In this case, protein purity topped 85%, starting from an initial purity of 1.1%.

## **CONCLUSION**

Phenyl boronate chromatography can be successfully used for the direct capture of antibodies from clarified cell supernatants, allowing not only a high IgG recovery but also a high clearance of protein impurities, including albumin, insulin and host cell proteins. The type of buffer, ionic strength and especially pH had a profound effect on the purity of the collected IgG fractions. Higher purification factors were obtained when adsorption was carried out at pH 8.5 and low ionic strength, and elution was performed with a 1.5 M Tris linear gradient. In addition, purity could still be increased by adjusting the pH of the cell supernatant feed to 8.5. Starting from a feedstock with 1.1% protein purity and 2.2% HPLC purity, IgG was recovered with a final protein purity of 85% and a final HPLC purity of 88%. The exceptional stability, selectivity, and low cost of PB ligands together with the operational advantages associated to porous glass media can lead to more efficient, less expensive, and safer chromatographic purification of antibodies at manufacturing scales. In conclusion, phenyl boronate chromatography can form the basis of a new platform for the downstream processing of monoclonal antibodies, either as an alternative to the traditional protein A capture step, or as a pre-purification step before protein A chromatography in order to increase the life time of this high-value resin. PB chromatography has been used before but mostly as a polishing step for the separation of glycoforms, as described by Zhang and co-workers (17). This work however shows that it can be used to capture antibodies directly from a clarified cell culture, which is highly advantageous in terms of process.

## Acknowledgments

A. M. Azevedo acknowledges the initiative “Ciência 2007” of the Portuguese ministry for science, technology and higher education (www.mctes.pt). A.G. Gomes and L.Borlido acknowledge Fundação para a Ciência e Tecnologia (FCT) for the PhD fellowship and BD/30786/2006 and BD/45077/2008. Authors wish also to acknowledge Fred Mann from Millipore for the kind gift of PROSEP-Phenyl Boronate resin.

## REFERENCES

1. Field, R. (2007) Recombinant human IgG production from myeloma and Chinese hamster ovary cells, in *Cell Culture and Upstream Processing* (Butler, M., Ed.), pp 57-78, Taylor & Francis, New York.
2. Curling, J. (2009) The Development of Antibody Purification Technologies, in *Process Scale Purification of Antibodies* (Gottschalk, U., Ed.), pp 25-52, Wiley, New Jersey.
3. Kelley, B., Blank, G., and Lee, A. (2009) Downstream processing of monoclonal antibodies: Current practices and future opportunities, in *Process Scale Purification of Antibodies* (Gottschalk, U., Ed.), pp 1-23, Wiley, New Jersey.
4. Azevedo, A. M., Rosa, P. A. J., Ferreira, I. F., and Aires-Barros, M. R. (2009) Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing, *Trends Biotechnol.* 27, 240-247.
5. Shukla, A. A., Hubbard, B., Tressel, T., Guhan, S., and Low, D. (2007) Downstream processing of monoclonal antibodies - Application of platform approaches, *J. Chromatogr. B* 848, 28-39.
6. Hober, S., Nord, K., and Linhult, M. (2007) Protein A chromatography for antibody purification, *J. Chromatogr. B* 848, 40-47.
7. Bergemann, K., Eckermann, C., Garidel, P., Grammatikos, S., Jacobi, A., Kaufmann, H., Kempken, R., and Pisch-Heberle, S. (2007) Production and downstream processing, in *Handbook of Therapeutic Antibodies* (Dübel, S., Ed.), pp 199-237, Wiley, Weinheim.
8. Ghose, S., McNerney, T., and Hubbard, B. (2007) Protein A affinity chromatography for capture and purification of monoclonal antibodies and Fc-fusion proteins: Practical considerations for process development, in *Process Scale Bioseparations for the Biopharmaceutical Industry* (Shukla, A. A., Etzel, M. R., and Gadam, S., Eds.), pp 463-489, Taylor & Francis, Boca Raton.
9. Roque, A. C. A., Lowe, C. R., and Taipa, M. A. (2004) Antibodies and genetically engineered related molecules: Production and purification, *Biotechnol. Prog.* 20, 639-654.
10. Roque, A. C. A., Silva, C. S. O., and Taipa, M. A. (2007) Affinity-based methodologies and ligands for antibody purification: Advances and perspectives, *J. Chromatogr. A* 1160, 44-55.
11. Przybycien, T. M., Pujar, N. S., and Steele, L. M. (2004) Alternative bioseparation operations: life beyond packed-bed chromatography, *Curr. Opin. Biotechnol.* 15, 469-478.
12. Low, D., O'Leary, R., and Pujar, N. S. (2007) Future of antibody purification, *J. Chromatogr. B* 848, 48-63.

13. Gomes, A. G., Azevedo, A. M., Aires-Barros, M. R., and Prazeres, D. M. F. (2010) Clearance of host cell impurities from plasmid-containing lysates by boronate adsorption, *J. Chromatogr. A* 1217, 2262-2266.
14. Liu, X.-C. (2006) Boronic acids as ligands for affinity chromatography, *Chin. J. Chromatogr.* 24, 73-80.
15. Ren, L., Liu, Z., Dong, M., Ye, M., and Zou, H. (2009) Synthesis and characterization of a new boronate affinity monolithic capillary for specific capture of cis-diol-containing compounds, *J. Chromatogr. A* 1216, 4768-4774.
16. Brena, B. M., Batista-Viera, F., Rydén, L., and Porath, J. (1992) Selective adsorption of immunoglobulins and glycosylated proteins on phenylboronate-agarose, *J. Chromatogr. A* 604, 109-115.
17. Zhang, B., Mathewson, S., and Chen, H. (2009) Two-dimensional liquid chromatographic methods to examine phenylboronate interactions with recombinant antibodies, *J. Chromatogr. A* 1216, 5676-5686.
18. Azevedo, A. M., Rosa, P. A. J., Ferreira, I. F., and Aires-Barros, M. R. (2008) Integrated process for the purification of antibodies combining aqueous two-phase extraction, hydrophobic interaction chromatography and size-exclusion chromatography, *J. Chromatogr. A* 1213, 154-161.
19. Bosch, L. I., Fyles, T. M., and James, T. D. (2004) Binary and ternary phenylboronic acid complexes with saccharides and Lewis bases, *Tetrahedron* 60, 11175-11190.
20. Springsteen, G., and Wang, B. (2002) A detailed examination of boronic acid-diol complexation, *Tetrahedron* 58, 5291-5300.
21. Dolhofer, R., and Wieland, O. H. (1980) Increased glycosylation of serum albumin in diabetes mellitus, *Diabetes* 29, 417-422.
22. Yan, J., Springsteen, G., Deeter, S., and Wang, B. (2004) The relationship among pKa, pH, and binding constants in the interactions between boronic acids and diols--it is not as simple as it appears, *Tetrahedron* 60, 11205-11209.
23. Hall, D. G. (2005) Structure, properties, and preparation of boronic acid derivatives. Overview of their reactions and applications, in *Boronic Acids*. (Hall, D. G., Ed.), pp 1-100, Wiley, Weinheim.

**Table 1.** Performance parameters obtained for the purification of IgG from a CHO cell supernatant using different adsorption buffers and step elution with 1.5 M Tris. CHO cCCS: 1.1% protein purity and 2.2% HPLC purity.

Adsorption buffer	Yield (%)	Protein Purity* (%)	PF†	HPLC Purity‡ (%)	HPLC PF
<b>PBS pH 7</b>	100 ± 1	26 ± 1	23	28 ± 1	13
<b>20mM HEPES, pH 7</b>	100 ± 1	29 ± 1	25	29 ± 2	13
<b>20mM HEPES, 150mM NaCl, pH 8.5</b>	100 ± 1	38 ± 1	33	38 ± 3	17
<b>20mM HEPES, pH 8.5</b>	100 ± 1	37 ± 2	32	38 ± 2	17
<b>20mM HEPES, pH 8.5 CHO @ pH 8.5</b>	97 ± 2	63 ± 3	56	74 ± 2	33

\*Ratio between IgG and protein concentration; † Ratio between final and initial protein purity; ‡ Area of IgG peak divided by the total area of chromatogram subtracted by the total area of the corresponding buffer; § Ratio between final and initial HPLC purity.

**Table 2.** Performance parameters obtained for the purification of IgG from a CHO cell supernatant using different adsorption buffers and a gradient elution with 1.5 M Tris. CHO cCCS: 1.1% protein purity and 2.2% HPLC purity.

Adsorption buffer	Yield (%)	Protein Purity* (%)	PF <sup>†</sup>	HPLC Purity <sup>‡</sup> (%)	HPLC PF <sup>§</sup>
<b>PBS pH 7</b>	73 ± 2	71 ± 2	63	65 ± 2	29
<b>20mM HEPES, pH 7</b>	100 ± 4	73 ± 2	64	67 ± 1	30
<b>20mM HEPES, 150mM NaCl, pH 8.5</b>	85 ± 4	63 ± 1	55	69 ± 1	31
<b>20mM HEPES, pH 8.5</b>	87 ± 3	73 ± 3	63	69 ± 2	31
<b>20mM HEPES, pH 8.5 CHO @ pH 8.5</b>	80 ± 2	85 ± 2	76	88 ± 1	39

\*Ratio between IgG and protein concentration; <sup>†</sup> Ratio between final and initial protein purity; <sup>‡</sup> Area of IgG peak divided by the total area of chromatogram subtracted by the total area of the corresponding buffer; <sup>§</sup> Ratio between final and initial HPLC purity.

## Caption of figures

**Figure 1.** Percentage of IgG (■), albumin (■) and insulin (■) present in the elution pool after loading 0.5 ml of pure protein solutions (1 g/l) in different adsorption buffers: i) 20 mM HEPES pH 8.5; ii) 20 mM HEPES, 150 mM NaCl pH 8.5; iii) 20 mM HEPES, 150 mM NaCl, 15 mM Tris pH 8.5; iv) 20 mM HEPES, 150 mM MgCl<sub>2</sub> pH 8.5; v) 20 mM HEPES pH 7; vi) 10 mM phosphate, 150 mM NaCl pH 7.

**Figure 2.** Binding of IgG (dark line), HSA (medium line) and insulin (light line) to PB column in different adsorption buffers: (a) 20 mM HEPES pH 8.5 and (b) PBS (10 mM phosphate, 150 mM NaCl, pH 7).

**Figure 3.** Capture of IgG from a CHO cell supernatant using PB chromatography with 20 mM HEPES at pH 8.5 (—) and PBS at pH 7 (—) as adsorption buffers. Bound proteins were eluted using a step gradient with 1.5 M Tris-HCl at pH 8.5. The IgG concentration determined by off-line HPLC analysis is also shown: (■) 20 mM HEPES at pH 8.5 and (◆) PBS at pH 7.

**Figure 4.** HPLC-SEC analysis of the purity of the flow-through (—) and elution (—) pools collected during the course of capture of IgG from a CHO cell supernatant using PB chromatography with PBS at pH 7 (a) and 20 mM HEPES at pH 8.5 (b) as adsorption buffers. The chromatogram of the feed (—) is also shown. Standard retention times: 10 min for IgG, 10.7 min for HSA and 14 min for insulin.

**Figure 5.** Purification of IgG from a CHO cell supernatant, adjusted to pH 8.5, using 20 mM HEPES pH 8.5 as adsorption buffer and a 10 min gradient elution (—). IgG concentration in collected fractions was determined by off-line HPLC analysis is also shown (■).

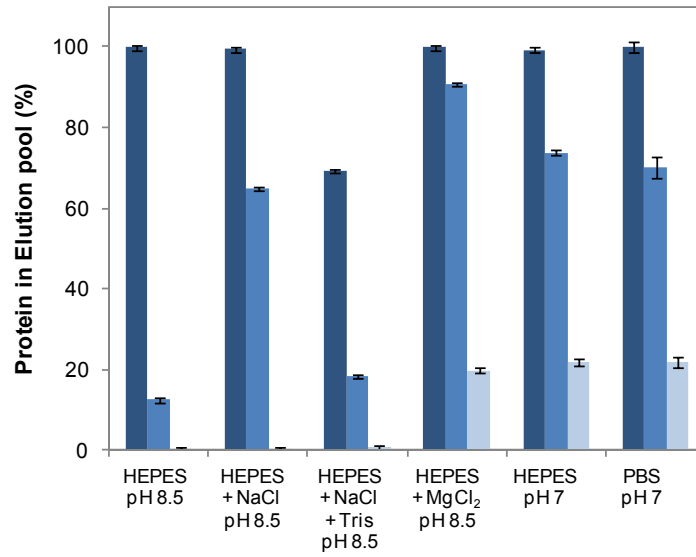
**Figure 6.** Effect of the adsorption buffer on the relative height of the elution peaks obtained for the purification of IgG from a CHO cell supernatant. From top to bottom (18 min peak): PBS at pH 7 (—); 20 mM HEPES pH 7 (—); 20 mM HEPES, 150 mM NaCl pH 8.5 (—); 20 mM HEPES pH 8.5 (—) and 20 mM HEPES pH 8.5 with CHO supernatant adjusted to pH 8.5 (—).

**Figure 7.** Silver stained SDS-PAGE of the different fractions collected during the purification of IgG from a CHO cell supernatant by PB chromatography using HEPES pH 8.5 (lanes 3-5) or HEPES pH 7 (lanes 6-8) as adsorption buffer and a gradient elution. Lanes ID: 1- CHO cell supernatant; 2- molecular weight standards (from bottom to top: 10, 15, 20, 25, 37, 50, 75, 100, 150, 200 kDa); 3- Flow-through fraction (HEPES pH 8.5); 4- Peak 1 (HEPES pH 8.5); 5- Peak 2 (HEPES pH 8.5); 6- Flow-through fraction (HEPES pH 7); 7- Peak 1 (HEPES pH 7); 8- Peak 2 (HEPES pH 7).

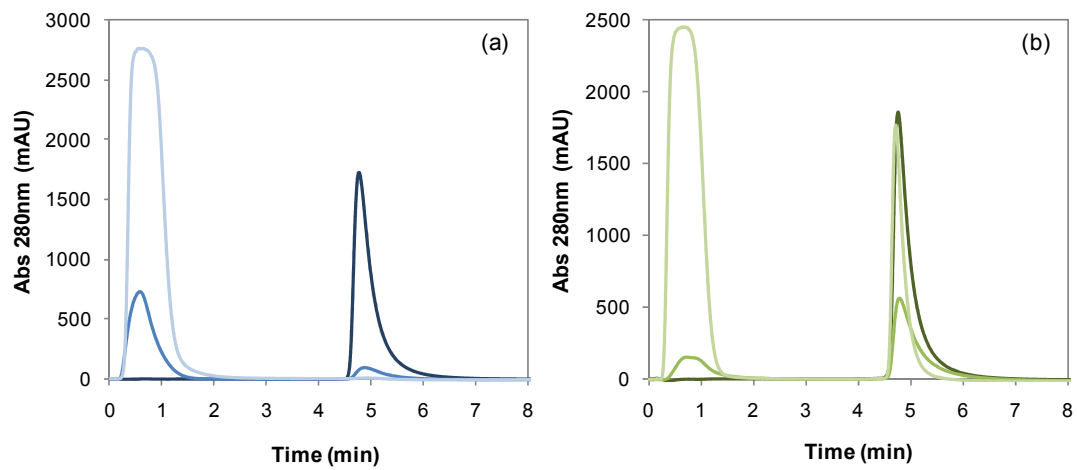
**Figure 8.** Silver stained SDS-PAGE of the different fractions collected during the purification of IgG from a CHO cell supernatant adjusted to pH 8.5 by PB chromatography using HEPES pH 8.5



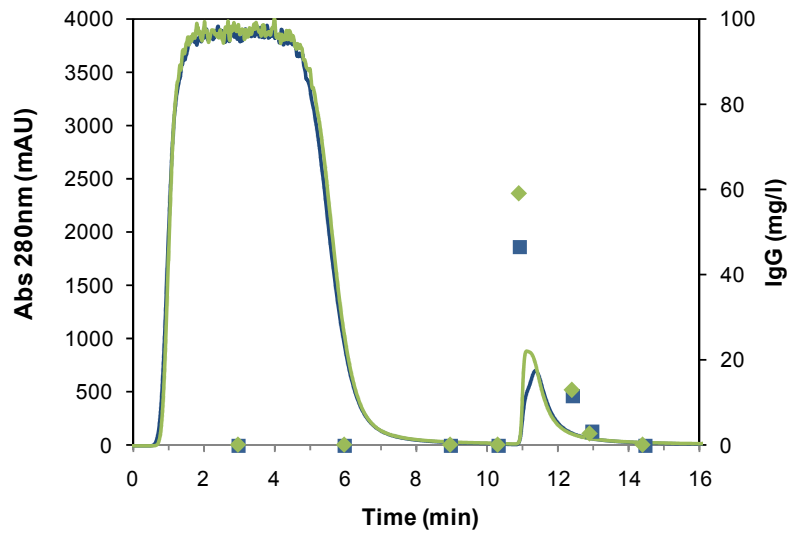
as adsorption buffer. Lanes ID: 1- molecular weight standards (from bottom to top: 10, 15, 20, 25, 37, 50, 75, 100, 150, 200 kDa); 2- Flow-through fraction of the gradient elution; 3- Peak 1 of the gradient elution; 4- Peak 2 of the gradient elution; 5- CHO cell supernatant; 6- Flow-through fraction of the step elution; 7- Elution fraction of the step elution; 8- Pure IgG from Gammanorm.



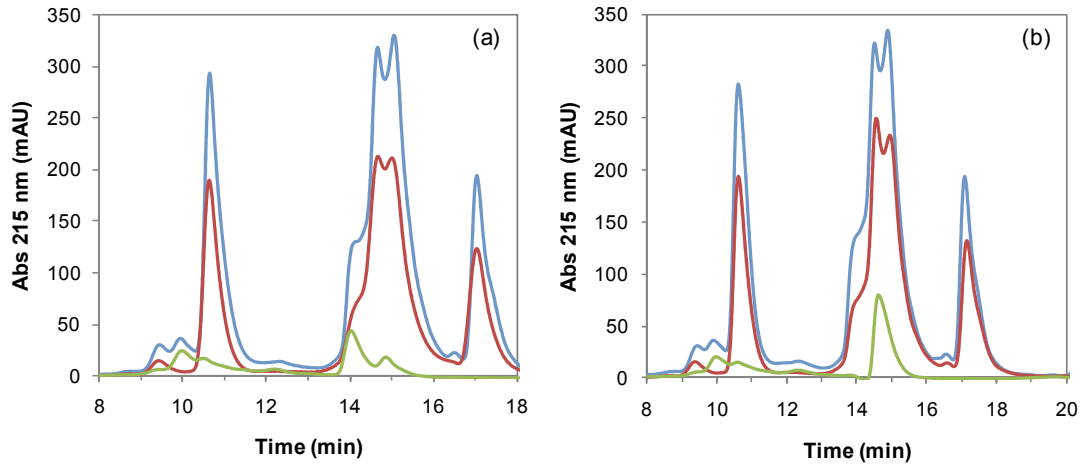
**Figure 1.** Percentage of IgG (■), albumin (■) and insulin (■) present in the elution pool after loading 0.5 ml of pure protein solutions (1 g/l) in different adsorption buffers: i) 20 mM HEPES pH 8.5; ii) 20 mM HEPES, 150 mM NaCl pH 8.5; iii) 20 mM HEPES, 150 mM NaCl, 15 mM Tris pH 8.5; iv) 20 mM HEPES, 150 mM MgCl<sub>2</sub> pH 8.5; v) 20 mM HEPES pH 7; vi) 10 mM phosphate, 150 mM NaCl pH 7.



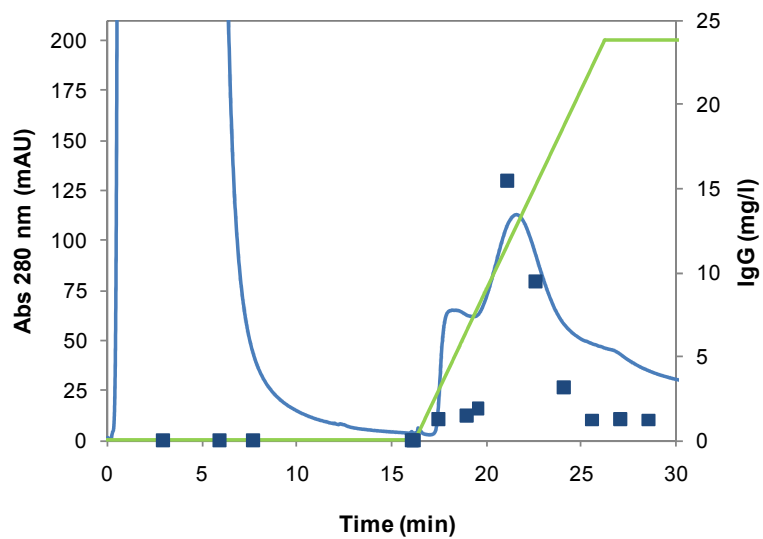
**Figure 2.** Binding of IgG (dark line), HSA (medium line) and insulin (light line) to PB column in different adsorption buffers: (a) 20 mM HEPES pH 8.5 and (b) PBS (10 mM phosphate, 150 mM NaCl, pH 7).



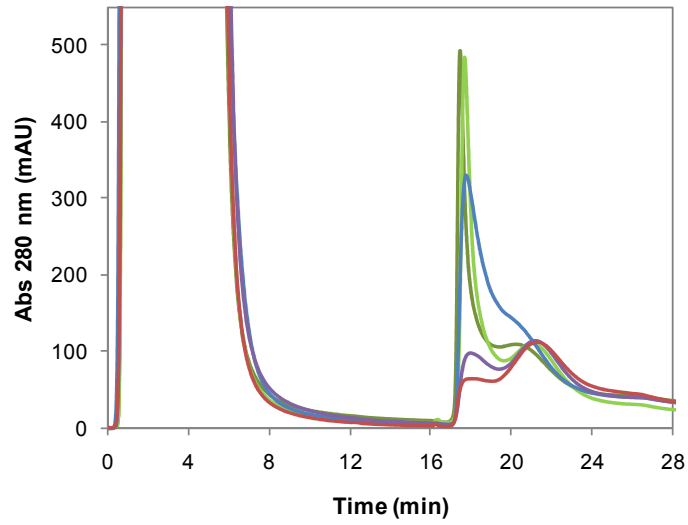
**Figure 3.** Capture of IgG from a CHO cell supernatant using PB chromatography with 20 mM HEPES at pH 8.5 (—) and PBS at pH 7 (—) as adsorption buffers. Bound proteins were eluted using a step gradient with 1.5 M Tris-HCl at pH 8.5. The IgG concentration determined by off-line HPLC analysis is also shown: (■) 20 mM HEPES at pH 8.5 and (◆) PBS at pH 7.



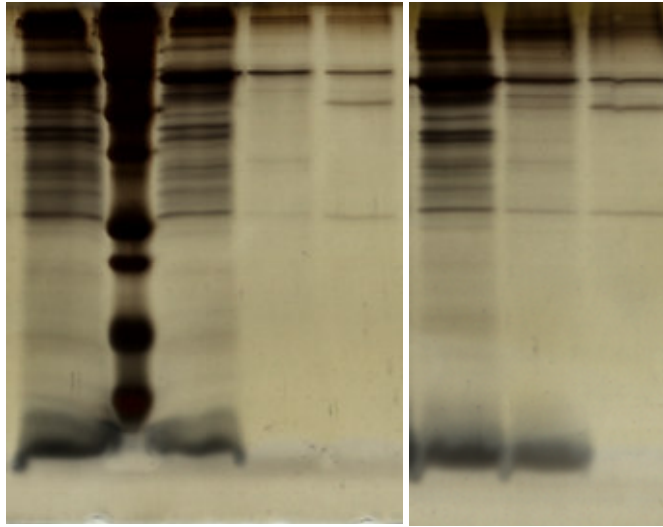
**Figure 4.** HPLC-SEC analysis of the purity of the flow-through (—) and elution (—) pools collected during the course of capture of IgG from a CHO cell supernatant using PB chromatography with PBS at pH 7 (a) and 20 mM HEPES at pH 8.5 (b) as adsorption buffers. The chromatogram of the feed (—) is also shown. Standard retention times: 10 min for IgG, 10.7 min for HSA and 14 min for insulin.



**Figure 5.** Purification of IgG from a CHO cell supernatant, adjusted to pH 8.5, using 20 mM HEPES pH 8.5 as adsorption buffer and a 10 min gradient elution (—). IgG concentration in collected fractions was determined by off-line HPLC analysis is also shown (■).

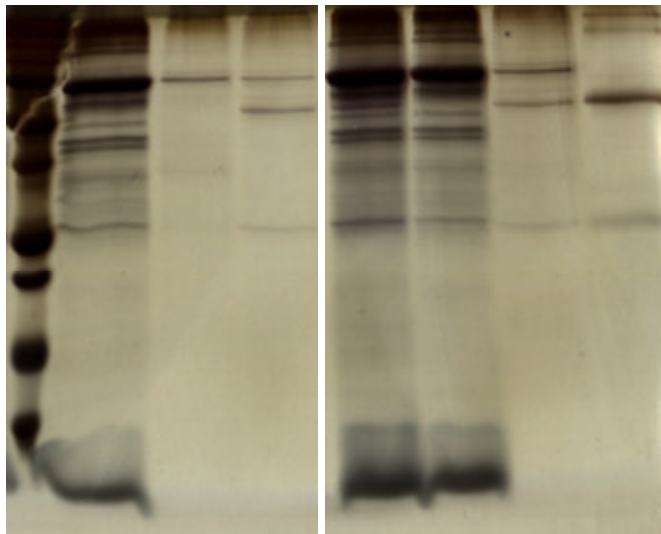


**Figure 6.** Effect of the adsorption buffer on the relative height of the elution peaks obtained for the purification of IgG from a CHO cell supernatant. From top to bottom (18 min peak): PBS at pH 7 (—); 20 mM HEPES pH 7 (—); 20 mM HEPES, 150 mM NaCl pH 8.5 (—); 20 mM HEPES pH 8.5 (—) and 20 mM HEPES pH 8.5 with CHO supernatant adjusted to pH 8.5 (—).



**Figure 7.** Silver stained SDS-PAGE of the different fractions collected during the purification of IgG from a CHO cell supernatant by PB chromatography using HEPES pH 8.5 (lanes 3-5) or HEPES pH 7 (lanes 6-8) as adsorption buffer and a gradient elution. Lanes ID: 1- CHO cell supernatant; 2- molecular weight standards (from bottom to top: 10, 15, 20, 25, 37, 50, 75, 100, 150, 200 kDa); 3- Flow-through fraction (HEPES pH 8.5); 4- Peak 1 (HEPES pH 8.5); 5- Peak 2 (HEPES pH 8.5); 6- Flow-through fraction (HEPES pH 7); 7- Peak 1 (HEPES pH 7); 8- Peak 2 (HEPES pH 7).





**Figure 8.** Silver stained SDS-PAGE of the different fractions collected during the purification of IgG from a CHO cell supernatant adjusted to pH 8.5 by PB chromatography using HEPES pH 8.5 as adsorption buffer. Lanes ID: 1- molecular weight standards (from bottom to top: 10, 15, 20, 25, 37, 50, 75, 100, 150, 200 kDa); 2- Flow-through fraction of the gradient elution; 3- Peak 1 of the gradient elution; 4- Peak 2 of the gradient elution; 5- CHO cell supernatant; 6- Flow-through fraction of the step elution; 7- Elution fraction of the step elution; 8- Pure IgG from Gammanorm.