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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 243–249
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Anion-exchange chromatography using short monolithic columns as a complementary technique for human serum albumin depletion prior to human plasma proteome analysis

Tatjana Čerk Petrič [a], Peter Brne [b], Boštjan Gabor [b], Lidija Govednik [b], Miloš Barut [b,*], Aleš Štrancar [b], Lucija Zupančič Kralj [c]

[a] *Lek Pharmaceuticals d.d., Verovškova 57, SI-1526 Ljubljana, Slovenia*
[b] *BIA Separations d.o.o., Teslova 30, SI-1000 Ljubljana, Slovenia*
[c] *Faculty of Chemistry and Chemical Technology, Aškerčeva 5, SI-1000 Ljubljana, Slovenia*

Received 13 December 2005; received in revised form 12 June 2006; accepted 12 June 2006
Available online 27 July 2006

Abstract

In order to enable the detection of low abundance proteins from human plasma, it is necessary to remove high abundance proteins. Among them, human serum albumin and immunoglobulin G represent more than 75% of all such proteins. In this paper, the characterization of short monolithic columns was performed followed by the optimization of a multidimensional approach, known as conjoint liquid chromatography, to deplete human serum albumin and immunoglobulin G from a human plasma sample. Two different chromatographic modes were used: ion-exchange chromatography and affinity chromatography. A monolithic stationary phase (convective interaction media disk) bearing strong anion-exchange groups and another immobilized with protein G were placed in series into one housing. The optimal binding conditions were found that removed a majority of human serum albumin and immunoglobulin G from the human plasma sample. This method was compared to the depletion using a combination of pseudo-affinity and affinity columns. The results of the human serum albumin and immunoglobulin G depletion were confirmed by 2D electrophoresis. It has been shown that anion-exchange and affinity chromatography using convective interaction media monolithic columns can represent an efficient complementary technique for human serum albumin and immunoglobulin G removal from human plasma.
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Keywords: Monolithic column; Immunoglobulin G depletion; Human serum albumin depletion; Conjoint liquid chromatography; 2D electrophoresis; Proteomics; Human plasma proteome

1. Introduction

The human plasma proteome is the largest representative of the human proteome present in any sample. It consists of a large variety of proteins that have different chemical and physical properties and are present in plasma in different concentrations (the dynamic range of protein concentration is greater than 10 orders of magnitude) [1]. Human serum albumin (HSA) and immunoglobulin G (IgG) represent over 75% of all proteins present in plasma and their high abundance masks the detection and determination of the low abundance proteins which are potential biomarkers for various diseases, e.g., cancer and are therefore of great biological importance [2]. These low abundance proteins can only be detected if the high abundance proteins are efficiently and almost completely removed. Hence, strategies for the depletion of high abundance proteins are becoming of great interest.

The most common technique for removing IgG and HSA from a sample is chromatography. Chromatographic methods represent an essential tool for the isolation of biological compounds on the laboratory scale. These methods can offer high specificity and selectivity, permitting the recovery of biological compounds with adequate purity and under conditions preserving their biological features [3]. Besides ion-exchange chromatography, bioaffinity chromatography is most frequently used as it is supposed to be the most selective [4].

Bioaffinity chromatography is a form of adsorption chromatography, which is based upon the ability of biologically

* Corresponding author. Tel.: +386 1 426 56 49; fax: +386 1 426 56 50.
E-mail address: milos.barut@monoliths.com (M. Barut).

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doi:10.1016/j.jpba.2006.06.019

active substances to specifically and reversibly bind complementary substances which are generally called ligands. Given the varied nature of biologically active materials, affinity ligands represent a wide range of chemical compounds [5].

Proteins A and G are two bioaffinity ligands which, by being immobilized on different matrices like, e.g., porous particles, monolithic stationary phases [6–8] or membranes [9] are able to selectively bind IgG. Protein G has the ability to bind all four subclasses of IgG, whereas Protein A does not bind IgG3 [10].

As an alternative to naturally occurring ligands, pseudo-affinity ligands have been introduced for HSA removal. These dye-ligands are commercially available, inexpensive, can be immobilized easily and are more stable than natural ligands [11]. There are several dyes available, e.g., Procion Red HE-3B and Procion Blue MX-R [12]. However, Cibacron Blue F3GA is by far the most commonly used dye-ligand [11]. The main drawback of dyes is their moderate selectivity for proteins. To solve this problem, a new generation of dyes has been designed in silico, called biomimetic dyes, which are supposed to mimic natural ligands. They are expected to exhibit increased affinity and therefore better purifiy the targeted protein [13].

Immunoaffinity chromatography is by far the most selective; however, it may also be the most expensive chromatographic technique. Ion-exchange chromatography offers an adequate alternative to affinity chromatography and it is the most widely used technique for protein separation [4].

Because of their diversity, relative flexibility of use and relatively low cost, ion-exchangers have been introduced to isolate proteins from a relatively crude plasma fraction or as a polishing step to eliminate unwanted proteins, DNA and even virus particles. As many plasma proteins carry a negative charge at almost neutral pH, anion-exchangers are often used [3]. Anion-exchangers have minor selectivity compared to affinity ligands, however by altering the pH or ionic strength of the binding or elution buffer selectivity can be improved.

In this paper, the use of an anion-exchanger in combination with an affinity column was used for the depletion of human serum albumin and immunoglobulin G from human plasma. The aim was to demonstrate the possibility of using a less selective "ligand" for the depletion of high abundant serum albumin as an alternative to using an affinity or pseudo-affinity ligands.

2. Experimental

2.1. Chromatographic equipment

A gradient chromatography workstation, consisting of two pumps, an injection valve with a 20 μl sample loop and UV detector (280 nm) from Knauer (Berlin, Germany) was used. Chromatography data were processed using Eurochrom 2000 software (Knauer).

2.2. Columns

Convective interaction media (CIM) monolithic disks bearing strong (quaternary amine—CIM® QA) anion-exchange groups or Protein G ligand (CIM® Protein G) both from BIA Separations (Ljubljana, Slovenia) were used throughout the experimental work. As the affinity column for HSA depletion, a prototype unit CIM HSA depletion monolithic disk prepared by BIA Separations was used. The conjoint liquid chromatography column was constructed by stacking one CIM QA and one CIM Protein G disks, or one CIM HSA depletion and one CIM Protein G disks in one CIM housing, all from BIA Separations [14].

Chromatographic conditions used in the experimental work are described in the captions to the figures.

2.3. Model substances and chemicals

HSA was purchased from Sigma (St. Louis, MO, USA) and human IgG was obtained from Octapharma (Vienna, Austria).

All chemicals used, i.e., NaCl, TRIS buffer, glycine, HCl, K and Na phosphate buffers were p.a. quality from Merck (Darmstadt, Germany).

2.4. Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis

SDS-PAGE under non-reducing conditions, using a gradient 4–20% gel (Cambrex, Rocland, USA), was carried out according to the Laemmli protocol [15] using a Mini Protean II system (Bio-Rad, Hercules, USA). The SDS-PAGE gels were run at 200 V for 55 min. A discontinuous TRIS–Glycine buffering system was used. Protein bands were visualized using silver staining according to the protocol of GE Healthcare (Uppsala, Sweden).

2.5. 2D Electrophoresis

2D electrophoresis was used to separate proteins in the first dimension by isoelectric focusing and in the second dimension by molecular weight using SDS-PAGE electrophoresis. Isoelectric focusing (IEF) was undertaken using pre cast Immobiline™ Dry Strip pH 3–10 NL gradient 18 cm gels using an IPG phor unit (GE Healthcare). Prior to the analysis, the samples were concentrated by precipitation using a 2D Clean-up kit (GE Healthcare). The protein pellet was dissolved in 380 μl of a rehydration solution prior to loading. On each gel, 90 μg of protein were loaded. The IPG strip and sample were covered with Dry Strip Cover Fluid (GE Healthcare) and ran at a constant voltage of 500 V for 2 h, 1000 V for 2 h and 8000 V for 4 h, followed by a gradient voltage from 8000 to 1000 V within 6 h according to the manufacturer instructions (GE Healthcare). After that, SDS-PAGE electrophoresis was performed using 12.5% resolving gel, discontinuous TRIS–Glycine buffering system and Hoefer DALT instrument (GE Healthcare). Gels were electrophoresed at constant voltage 200 V for 6.5 h. Protein bands were visualized using the silver staining procedure according to the protocol of GE Healthcare. The pictures of 2D gels were taken by an Olympus E500 Camera and the spots on the gels were determined and analyzed with Dymension 2.02 software (Syngene, Fredericks, USA).

3. Results and discussion

In order to enable the detection of biomarkers (which are commonly low abundance proteins) in complex body fluids, it is necessary to efficiently remove high abundance proteins. For example, human serum albumin (HSA) and immunoglobulin G (IgG) represent more than 75% of all proteins in human serum/plasma. Their high concentration masks the detection of proteins that are present in much lower concentrations. Affinity chromatography with its high specificity may be the method of choice for achieving their detection. For instance, the use of an affinity column with immobilized protein G is one of the most common and best-defined methods for binding IgG. The picture with HSA is somehow different. For years, the specific removal of HSA has been carried out using immobilized textile dyes, like, e.g., Cibacron Blue. Because of their lower specificity, biomimetic ligands have been introduced. However, since HSA is a carrier of many different substances, it is not prudent to completely remove HSA because some of the biomarkers of interest may be bound to this carrier. Another drawback of the mimetic ligands lies in the fact that their selectivity is limited to a fairly narrow range of sample concentrations and the capacity is flow dependent. If the sample amount loaded onto a column with an immobilized mimetic ligand is too low, there is a high probability that some other proteins, rather than the HSA alone, will bind to the matrix. On the other hand, the dynamic binding capacity can be exceeded, especially at increased linear velocities [16]. Because of this, complementary and more robust methods are needed to replace these affinity methods. The use of a novel stationary phases that exhibit flow unaffected properties may improve method robustness giving more degrees of freedom to the end user. One simple, yet important, possibility for removing HSA is the use of ion-exchange materials. Ion-exchangers are not selective enough, however, they offer one additional feature, i.e., the possibility of selective fractionation of the sample by applying different binding and elution conditions [17]. By selecting the appropriate chromatographic conditions, it is possible to obtain from a complex sample a low concentration of HSA and enough low abundance proteins that can be visualized by 1D or 2D electrophoresis. Combined with the results obtained by other methods, e.g., affinity, one can look for differences in the electropherograms and attempt to identify biomarkers by comparing a healthy and diseased person. The use of a monolithic column, which offers a flow unaffected dynamic binding capacity can shorten the time for sample preparation and increase screening throughput.

Therefore, in this work, the removal of HSA from human plasma was carried out on a strong anion-exchange CIM QA short monolithic column. The loading capacity of a CIM QA monolithic column is from 5 to 10 mg of protein depending on the chromatographic conditions and sample used. In all experiments, the maximum amount of proteins loaded on the QA column was in the range of 2 mg, i.e., approximately 50% of the lowest dynamic binding capacity value so as not to exceed the column's binding capacity. The binding of a protein to an anion-exchanger is obtained by selecting the conditions (buffer, pH, ionic strength) that cause the negatively charged protein groups to bind to the positively charged matrix. All proteins with a negative charge will bind, however, the binding strength will vary due to the different isoelectric points and surface characteristics. By increasing the concentration of sodium chloride to the binding mobile phase, some of the less strongly bound proteins will be eluted from the matrix and found in the flow-through fraction. By carefully adjusting the elution conditions, most of the HSA will not bind to the matrix, while the majority of other proteins will still bind and afterwards can be eluted by even higher sodium chloride concentration. The elution and the flow-through fractions can then be combined and applied to the SDS-PAGE to check for the presence of other proteins.

In the first step, the minimum NaCl concentration in the binding buffer that is needed to suppress HSA binding to the matrix was established. This was done using different mobile phases made of 20 mM phosphate buffer, pH 7.2 and different amounts of NaCl in the range from 0.05 to 0.6 M. Human plasma was mixed with the above-mentioned mobile phases in the range of 1:3 (v/v) and 100 μl of the diluted plasma samples were applied to the CIM QA short monolithic column. Flow-through and elution fractions were collected and tested by SDS-PAGE for the presence of HSA. The results obtained revealed that the most pronounced effect is obtained in the NaCl range between 0.1 and 0.15 M NaCl. When using 0.1 M NaCl there was still a lot of HSA in the elution fraction, while with 0.15 M NaCl, most of the HSA was found in the flow-through fraction and only a small fraction of HSA was still present in the elution fraction. With higher NaCl concentrations (more than 0.15 M NaCl) the quantity of other proteins in the elution fraction was decreased as more and more proteins were not binding to the matrix and were instead coeluted with the HSA. Therefore, it was concluded that the conditions using a 20 mM phosphate buffer containing 0.15 M NaCl, pH 7.2, were the most suitable for further work. This result was the first step towards developing an efficient fractionation scheme. However, one should bear in mind that by using these conditions, many other proteins are present in the fraction containing HSA and therefore are lost for further analyses, unless additional steps are introduced.

After establishing the conditions for HSA removal using a CIM QA disk, the next step was to construct a monolithic column, which would be used for the simultaneous removal of both, HSA and IgG. In doing this, the main characteristic of the monolithic columns were exploited and a CLC column was made by simply placing one QA and one protein G CIM disk in the same housing. The Protein G monolithic column is capable of completely removing IgG when the amount of IgG loaded onto the column is less than 3 mg. The CLC column was first washed with the binding mobile phase. After that, 100 μl of the diluted human plasma (1:3, v/v) was applied. Part of the proteins did not bind to the CLC column and ran directly through the column (Fig. 1, Part I). After that, a stepwise gradient was applied to the mobile phase with 0.15 M NaCl, where part of the proteins were eluted (Fig. 1, Part II), followed by an increasing NaCl concentration up to 1 M to remove proteins that were more strongly bound to the anion-exchanger (Fig. 1, Part III). Following this, the mobile phase was changed to 0.1 M glycine–HCl, pH 2.0 which caused the elution of IgG from the protein G column (Fig. 1, Part IV).

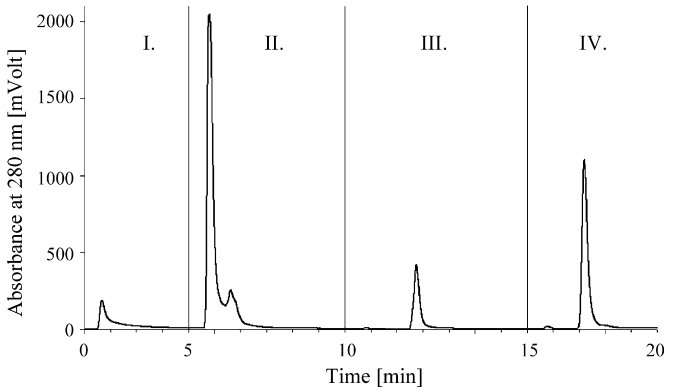

Fig. 1. Albumin and IgG depletion using CIM QA and CIM Protein G monolithic disks placed in a sequence in CIM disk housing. Binding buffer: 20 mM Na-phosphate buffer, pH 7.2 (Part I); elution buffer: 20 mM Na-phosphate buffer, pH 7.2 containing 0.15 M NaCl (Part II); 20 mM Na-phosphate buffer, pH 7.2 containing 1 M NaCl (Part III) and 0,1 M glycin–HCl (Part IV); flow rate 1 ml/min; human plasma sample (1:3, v/v) diluted with 20 mM phosphate buffer, pH 7.2.

All of these fractions were collected and further characterized by 1D electrophoresis as shown in Fig. 2.

From Fig. 2, one can see that most of the HSA and IgG had been removed using the CLC monolithic column at the applied chromatographic conditions. The majority of the HSA can be found in lane 3, while many other proteins and only a small amount of HSA can still be found in lane 4 (fraction eluted with 1 M NaCl). On the other hand, IgG is found in lane 5 only (fraction eluted with 0.1 M glycine–HCl, pH 2.0). The flow-through fraction and the fraction eluted with 1 M NaCl were mixed together and this combined HSA and IgG depleted fraction was

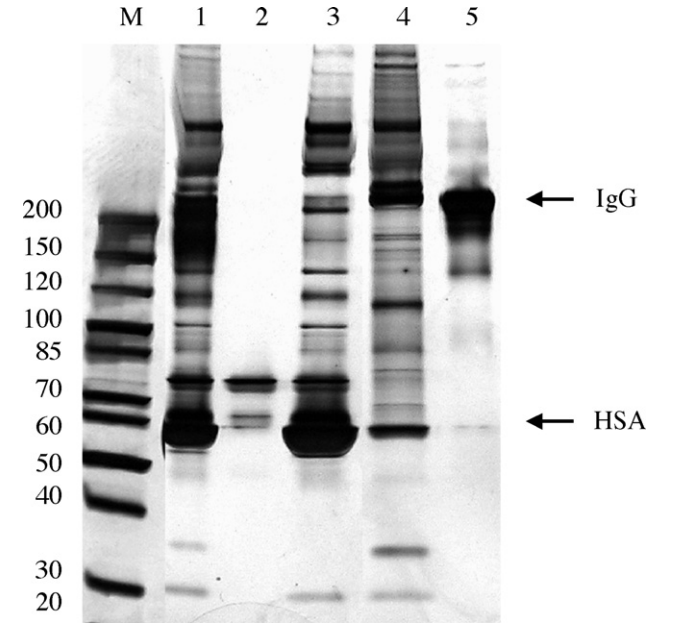

Fig. 2. SDS-PAGE separation under non-reducing conditions of four fractions of sample using different mobile phases. M- marker; (1) Sample (human plasma sample in 20 mM phosphate buffer pH 7.2); (2) flow-through fraction; (3) fraction eluted with 20 mM phosphate buffer pH 7.2 containing 0.15 M NaCl; (4) fraction eluted with 20 mM phosphate buffer pH 7.2 containing 1 M NaCl; 5-fraction eluted with 0,1 M glycin–HCl.

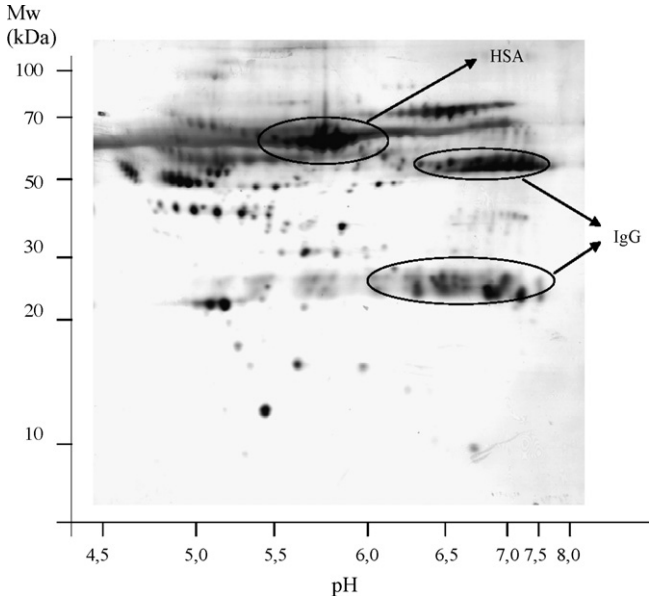

Fig. 3. 2D electrophoresis of whole plasma that was not depleted for high abundance proteins. First dimension: pH 3–10 IEF (18 cm); second dimension: 12.5% SDS-PAGE (20 cm). Protein load: 90 μg.

first concentrated using a precipitation method and afterwards applied to 2D electrophoresis. The 2D gel of the depleted combined fraction was compared to the 2D electrophoresis of the human plasma (see Figs. 3 and 4). By comparing both pictures, one can see that albumin and IgG were efficiently removed thus enhancing the visualization of some of the previously unseen spots. This is especially important for proteins in the lower molecular mass range as these low molecular proteins may serve as diagnostic markers. However, in addition to the appearance of these spots that had been invisible before, some were lost due

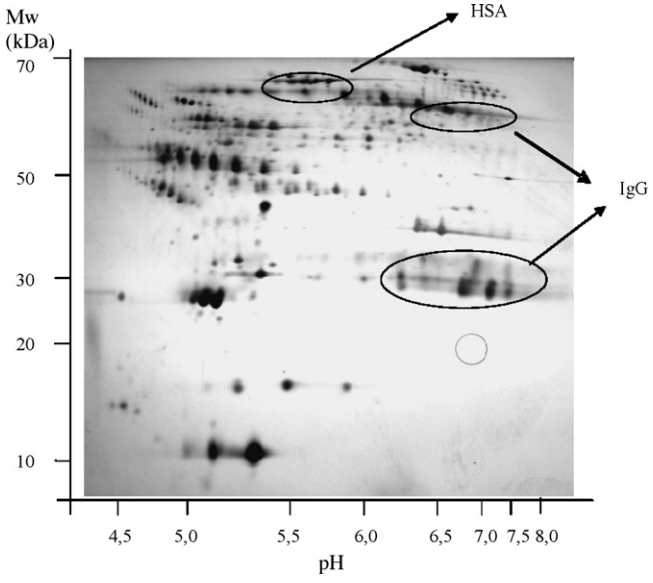

Fig. 4. 2D electrophoresis of whole plasma after the removal of albumin and IgG (depletion) with the CLC monolithic column made of QA and Protein G disks. First dimension: pH 3–10 IEF (18 cm); second dimension: 12.5% SDS-PAGE (20 cm). Protein load: 90 μg.

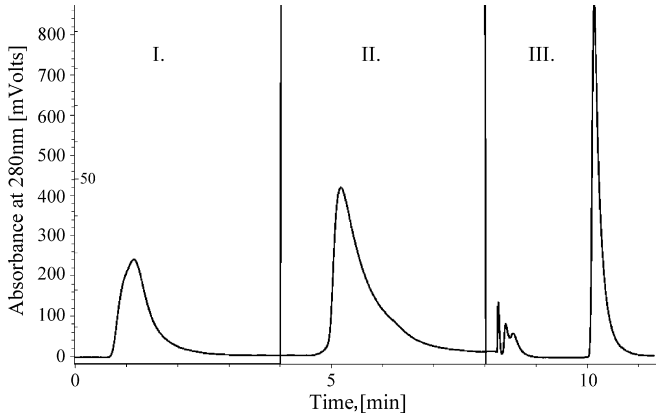

Fig. 5. Albumin and IgG depletion using CIM HSA depletion disk and CIM Protein G monolithic disk placed in a sequence in CIM disk housing. Binding buffer: 50 mM K-phosphate buffer pH 7.5 (Part I); elution buffer: 50 mM K-phosphate buffer, pH 7.5 containing 1.5 M KCl (Part II) and 0.1 M glycin–HCl (Part III); flow rate 1 ml/min; human plasma sample (1:3, v/v) diluted with 50 mM K-phosphate buffer, pH 7.5.

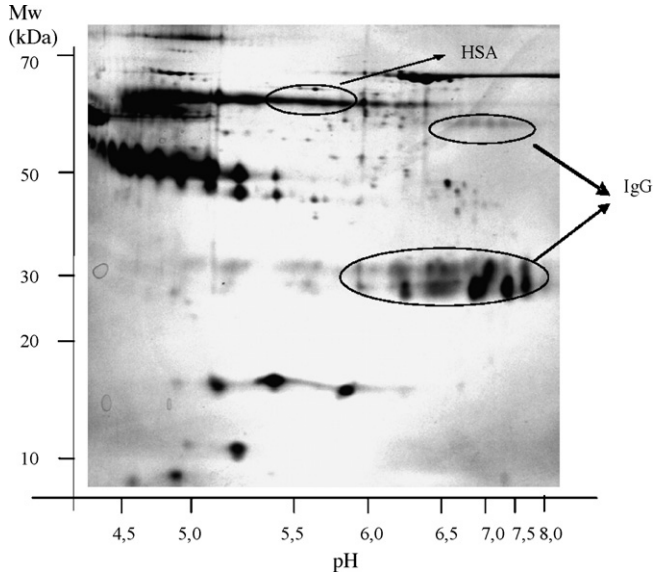

Fig. 6. 2D electrophoresis of the whole plasma sample after removal of HSA and IgG using HSA depletion and Protein G disks (flow-through fraction, Part I from Fig. 5). First dimension: pH 3–10 IEF (18 cm); second dimension: 12.5% SDS-PAGE (20 cm). Protein load: 90 μg.

to being bound to albumin or due to "non-specific" binding to the chromatographic matrix. From inspecting lane 3 in Fig. 2 (the fraction with the majority of albumin), we see that many other proteins are also present in this fraction. There are two questions that should be addressed: How does this compare to a more specific albumin depletion method using pseudo-affinity ligands? Is it possible to add an additional step in the depletion scheme in order to separate these proteins from albumin and than pool them into the already depleted sample, thus broadening the analysis window?

To answer the first question, the depletion of IgG and HSA was carried out by using a CLC column made from a combination of Protein G and HSA depletion monolithic disks [18]. The CLC column was connected to the HPLC and first equilibrated with the binding buffer. Human plasma was mixed with the binding buffer in the range of 1:3 (v/v) and 100 μl of the diluted plasma samples were applied to the CLC column. Part of the sample did not bind to the column and was found in the flow-through fraction (Fig. 5, Part I). Albumin bound to the HSA depletion column was eluted by a step change to the mobile phase with 1.5 M KCl (Fig. 5, Part II) and IgG was eluted by a stepwise change to a lower pH with the use of 0.1 M glycine–HCl, pH 2 (Fig. 5, Part III). The depleted sample (from Part I in Fig. 5) was loaded onto a 2D gel (see Fig. 6). This was then compared to the 2D electropherogram obtained by a CLC column consisting of a QA and Protein G monolithic disks, see Fig. 4. When comparing Fig. 6 to Fig. 4, one can see that additional spots appeared and many disappeared, however, in most cases, the spots were different than the ones seen when an anion-exchanger was used. In both cases, (HSA depletion with the use of an anion-exchange and with the use of a pseudo-affinity columns), around 1250–1300 spots were identified on the 2D gels. From this point of view, both techniques performed similarly. When both 2D gels where further compared, it was found that approximately 500–600 spots were located at the same position on both gels, while the remaining 600–700 spots were different. Based on this, it can be concluded that the two techniques have a similar selectivity, but due to the different fingerprint of spots may be considered complementary.

Lastly, an additional depletion step was tested since the pseudo-affinity albumin depletion step lacks selectivity. This is expected as the ligand is small and therefore can bind proteins other than albumin as well. The initial sample of human plasma or serum that is usually applied to the affinity column contains a plethora of different proteins that compete with albumin for the binding site. After the ion-exchange step, there is a fraction containing albumin and a certain amount of other proteins that might be important and should be retained (lane 3 in Fig. 2). Therefore, it is possible to apply this fraction onto a pseudo-affinity column and verify its selectivity for albumin removal. To do this, the fraction eluted with 0.15 M NaCl from the CLC column consisting of a QA and Protein G monolithic disks (Fig. 1 Part II) was loaded onto an HSA depletion disk. The 2D gel of the flow-through fraction (Fig. 7) was compared to the 2D electrophoresis gel of the loaded fraction (Fig. 8). From Fig. 8, one can see that HSA is still present in the fraction eluted from the CLC column. After the additional cleaning using the HSA depletion disk. (Fig. 7), it can be seen that the presence of HSA is significantly less. There is a big increase in the spot number (from 928 to 1400), while some spots are missing. The missing spots might represent fragments of albumin. By taking this final albumin depleted fraction and mixing it with the previous two fractions, it is possible to get a representative sample from which albumin and IgG were efficiently removed, while many other proteins remained and could be visualized by 2D electrophoresis and later on used for additional screening.

The question of method selection is based upon whether the spot (potential biomarker) that is being searched for becomes visible. It is clear that anion-exchange chromatography using

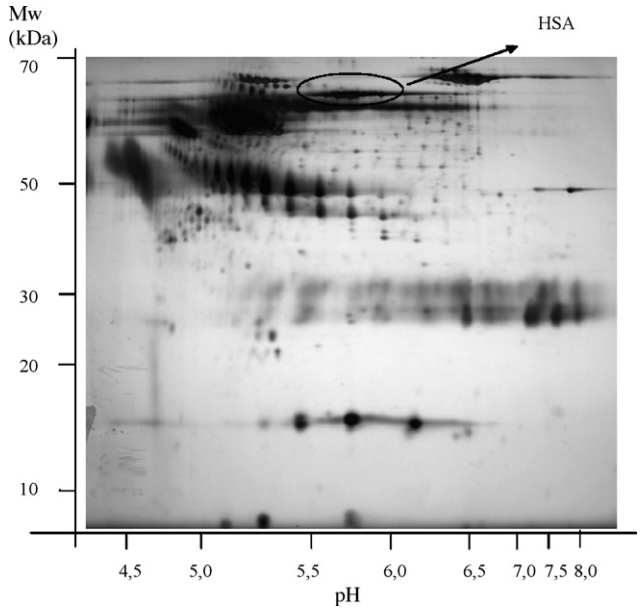

Fig. 7. 2D electrophoresis of the flow-through fraction eluted with 0,15 M NaCl from CLC column consisting of a QA and protein G disks (Part II, Fig. 1) and loaded on the column consisting of a HSA depletion disk. First dimension: pH 3–10 IEF (18 cm); second dimension: 12.5% SDS-PAGE (20 cm). Protein load: 90 μg.

CIM monolithic columns can represent a complementary technique for HSA removal from human plasma samples. This approach gives the end-user the possibility of developing different fractionation schemes – also with the use of other ion-exchange, e.g., cation-exchange, columns – with the aim of selectively depleting complex samples and enabling the study of different and smaller portions of the human serum or plasma proteome.

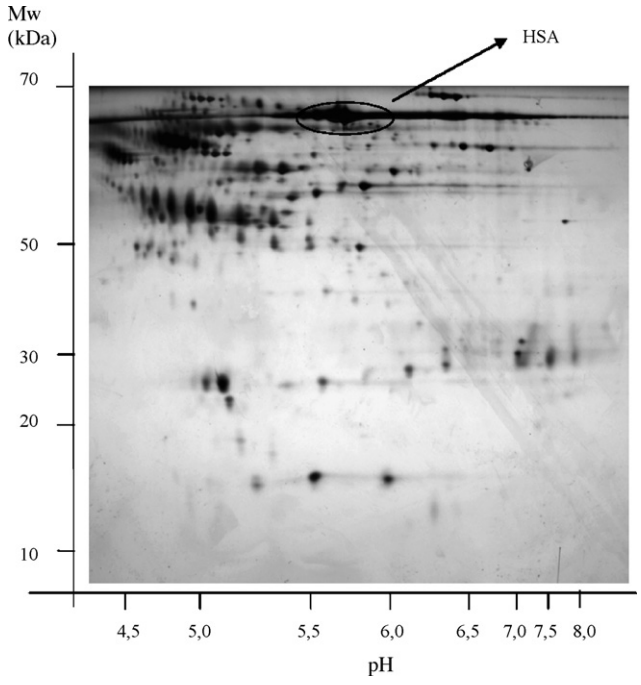

Fig. 8. 2D electrophoresis of the fraction eluted with 0.15 M NaCl from the CLC column consisting of a QA and Protein G disk (Part II, Fig. 1). First dimension: pH 3–10 IEF (18 cm); second dimension: 12.5% SDS-PAGE (20 cm). Protein load: 90 μg.

4. Conclusion

A conjoint liquid chromatography method obtained by placing an anion-exchange (CIM QA) and an affinity (Protein G) monolithic disk in the same housing was used for an efficient removal of high abundance proteins, HSA and IgG, from human plasma. This method was compared to a chromatographic method using a combination of Protein G and an HSA depletion monolithic disk. The analysis of the obtained gels demonstrated that the two methods have similar selectivity and similar performance, but approximately 50% of the protein spots revealed were different. From this result, the method using an ion-exchanger could represent a complementary technique to affinity chromatography for HSA removal from human plasma samples. Finally, the albumin fraction from the QA disk monolithic column still contained potential biomarkers which could be further fractionated with the use of a pseudo-affinity column that completely removed albumin. The remaining proteins could then be pooled to the depleted fractions. The overall results demonstrate the possibility of tailoring a fractionation scheme according to the portion of the human plasma proteome that needs to be processed.

Acknowledgements

The authors wish to thank Dr. Polona Jamnik and Prof. Peter Raspor from the Biotechnical Faculty, University of Ljubljana for their assistance in evaluating 2D gels with the Dymension software. The authors are also very grateful to Darryl Glover for his careful reading of the manuscript and language polishing.

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