Human Serum and Plasma Protein Depletion – Novel High-Capacity Affinity Column for the Removal of the "Top 14" Abundant Proteins

Application

Proteomics

Authors

Peter Mrozinski, Nina Zolotarjova, and Haiying Chen
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Abstract

The serum and plasma proteome are desirable biological samples due to their accessibility and representative complexity. This complexity is due, in part, to the wide dynamic range of protein concentrations. There is great hope that the investigation of these samples will lead to the discovery of new protein markers for disease diagnosis, therapeutic monitoring, and novel drug targets. The tremendous complexity of the plasma proteome presents an extreme analytical challenge with respect to sample preparation methods in proteome characterization. We present the results on a new device for the specific depletion of 14 high-abundant proteins from human biological samples.

Introduction

Depletion of high-abundant proteins in serum, plasma, and other physiological fluids has become routine and an accepted technique. These high-abundant protein components interfere with the identification and characterization of important low-abundant proteins by limiting the dynamic range for mass spectral and electrophoretic analyses. It is estimated that there are greater than 10^6 different proteins in human serum and plasma. In addition, the 30 most abundant proteins comprise approximately 99% of the total protein mass.

We are presenting the results on a new device for the specific depletion of 14 high-abundant proteins from serum and plasma. By depleting these 14 high-abundant proteins, we are removing approximately 94% of the total protein mass. This depletion column has the same specificity, reproducibility, and high efficiency as the industry standard Human 6 and Human 7 devices. The device depletes the 14 targeted proteins with robust performance for over 200 runs and excellent depletion efficiency as determined by enzyme-linked immunosorbent assay (ELISA). This depletion results in an improved dynamic range for proteomic analysis, which is limited in the detection of low-abundant proteins due to the masking effects of high-abundant proteins. Furthermore, the removal of the high-abundant proteins improves loading capacity on two-dimensional gel electrophoresis (2DGE) and liquid chromatography/mass spectrometry (LC/MS), resulting in the simplification of a complex system and enabling the detection of low-level proteins.

Experimental

Reagents and Equipment

Human plasma and serum were purchased from Rockland Immunologicals (D519-04) and Sigma Aldrich (S7023). Pre-cast SDS-PAGE gels were obtained from Invitrogen (Carlsbad, CA). The Multiple Affinity Removal System (MARS) Human 14 (4.6 mm id × 100 mm) and Buffers A and B were obtained from Agilent Technologies, Inc. (Wilmington, DE). An 1100 HPLC (Agilent Technologies, Inc.) consisting of a binary pump, a thermostatted autosampler with extended injection volume

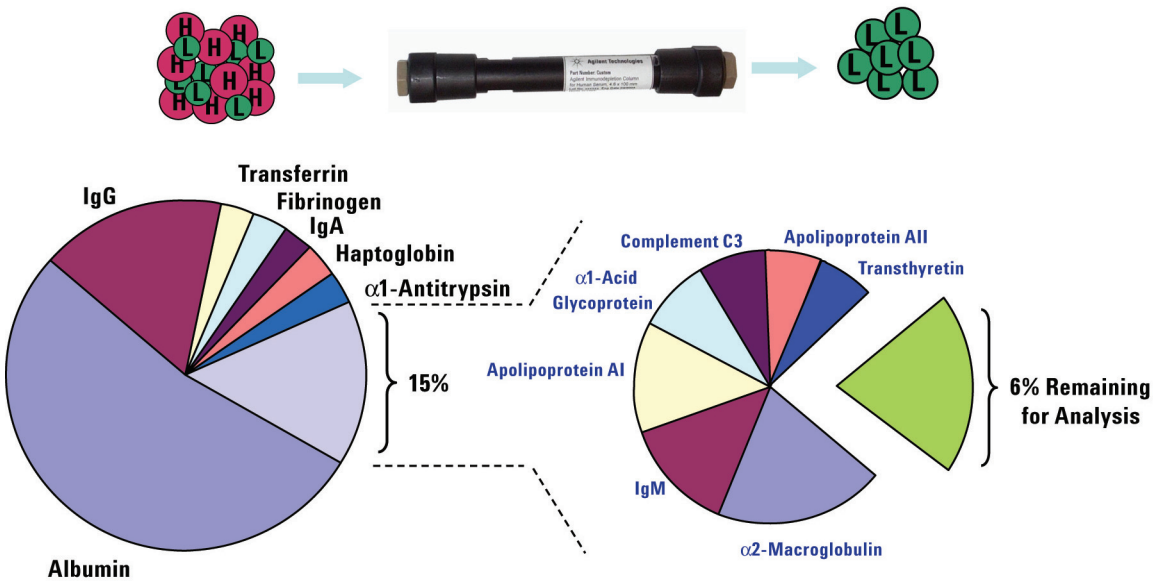


Figure 1. Multiple Affinity Removal System Human 14.

option, a thermostatted column compartment, a diode array detector, and a thermostatted analytical scale fraction collector were used for the affinity.

Affinity Column

The newly developed affinity column is an extension and improvement on the Agilent Multiple Affinity Removal System described and evaluated previously [1-3]. Based on affinity-purified polyclonal antibody binders, the Human 14 column also contains low molecular weight affibody ligands for the depletion of several proteins. The column requires a two-buffer system for operation. Buffers A and B are optimized to minimize coadsorption of nontargeted proteins and to ensure reproducibility of column performance and long column lifetime. Buffer A is a salt-containing neutral buffer, pH 7.4, used for loading, washing, and re-equilibrating the column. Buffer B is a low-pH urea buffer used for eluting the bound high-abundant proteins from the column.

Sample Preparation

Before injection onto a MARS column, the human plasma and serum was diluted 4X with Buffer A. The samples were transferred to a 0.22-µm spin filter and centrifuged for 1 min at 16,000 × g to remove particulates. Diluted plasma was prepared just prior to use and stored at 4 °C until injected.

ELISA Analysis of the Flow-Through Fraction

Standard sandwich ELISAs were used to determine the completeness of removal of targeted proteins from human plasma. Briefly, assay plates were coated with 100 µL of flow-through fraction proteins diluted 1:10 in Buffer A. After an overnight incubation at 4 °C, plates were washed with phosphate buffered saline (PBS), and the nonspecific binding sites were blocked with 200 µL of blocker solution (1xPBS/Casein Blocker, Bio-Rad) for 2 hours. After washing plates with PBS, 100 µL of antihuman antigen antibodies were added in blocker solution. Plates were incubated for 2 hours, then washed; a secondary horseradish peroxidase (HRP) conjugated antibody was added for 1 hour. After washing with PBS, liquid substrate (3,3′,5,5′—Tetramethylbenzidine [TMB] Liquid Substrate System, Sigma) was added and the absorbance was measured at 655 nm.

BCA Protein Assay and SDS-PAGE

Flow-through proteins and bound proteins were buffer exchanged into PBS and concentrated. Protein concentrations were analyzed using a bicinchoninic acid (BCA) protein assay kit (Pierce). Samples were stored at –80 °C until analysis. SDS-PAGE analysis was carried out using Invitrogen Tris-Glycine pre-cast gels (4% to 20% acrylamide, 10 wells, 1 mm). Proteins were visualized by Coomassie Blue staining with GelCode Blue (Pierce).

LC/MS/MS Analysis

To analyze the specificity of the immunodepletion, the bound fraction was resolved by SDS-PAGE. The entire bound fraction lane was cut into bands and processed with a Protein In-gel Typtic Digestion Kit (Agilent Technologies). Peptides were analyzed by LC/MS/MS on an Agilent HPLC-Chip/XCT Ultra Trap. Separation conditions were:

- RP Mobile Phase: [A]-H_2O w\0.1% FA, 3% ACN and [B]-ACN w\0.1% FA, 3% H_2O – 500 nL/min
- Enrichment Mobile Phase: H_2O w\0.1% FA, 3% ACN – 5 µL/min
- RP Gradient: 0% B at 4 min, 8% B at 4.25 min, 35% B at 39 min, 50% B at 44 min, 95% B at 44.1 min, 95% B at 46 min, 0% B at 47 min

The MS/MS data were analyzed with Spectrum Mill (Agilent Technologies, Inc.) version A.03.02 against the Swiss-Prot human database. The following filter was used after database searching: peptide score > 11, peptide % SPI > 70, and protein score > 20. Only fully tryptic peptides were considered, with one missed cleavage allowed.

Results and Discussion

The multiple affinity removal column for the depletion of 14 high-abundant proteins (Human 14) was developed and based on a Human 6 multiple affinity removal column. In addition to the affinity resins for the depletion of HSA, transferrin, haptoglobin, IgG, IgA, and α1-antitrypsin (Level I proteins), the column contains binders for the depletion of fibrinogen, α2-macroglobulin, α1-acid glycoprotein, complement C3, IgM, apolipoprotein AI, apolipoprotein AII, and transthyretin (prealbumin) – (Level II proteins). Based on affinity-purified polyclonal antibody binders, the Human 14 column also contains low molecular weight affibody binders for the depletion of several proteins. These binders were selected on the basis of specific and efficient target protein binding and their ability to function under specified loading and elution conditions. Affinity media was prepared in such way that various binders are covalently bound and cross-linked to the polymeric support in preferential orientation of the binding sites away from the solid-phase surface, providing maximum capacity for targeted proteins.

The protocol for using the column consists of loading the plasma/serum sample, collecting the flow-through (low-abundant) proteins, column washing, and eluting bound proteins (high-abundant proteins). Figure 2 shows overlays of representative chromatograms for the depletion process on a 4.6 mm id × 100 mm column. The average column capacity is about 20 µL of plasma or serum for the 4.6 mm id × 50 mm column, 40 µL for a 4.6 mm id × 100 mm column, 90 µl for a 7.5 mm id × 75 mm column, and 250 µL for a 10 mm id × 100 mm column. The immunoaffinity depletion process is easily automated using conventional HPLC or FPLC hardware.

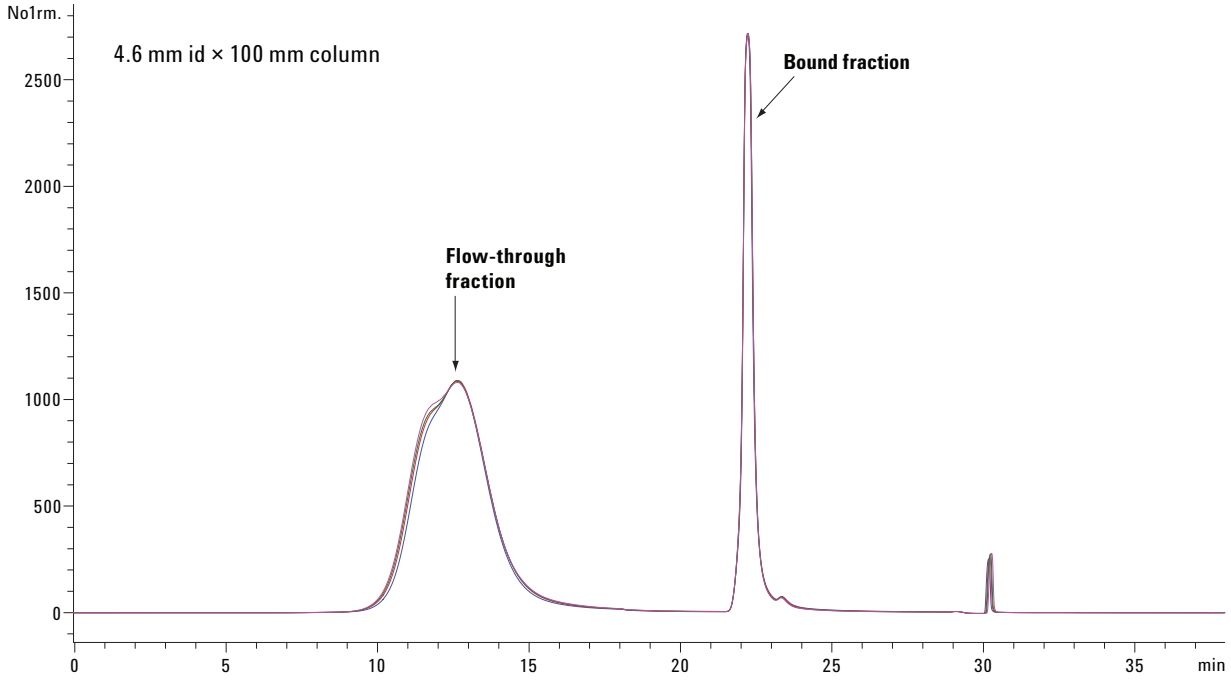


Figure 2. Overlay of chromatograms from runs 1, 50, 100, 150, and 200 on the Human 14 column.

Figure 3 shows 1D gel electrophoretic analysis data for the starting material (crude plasma and serum) and the resultant flow-through and bound fractions. The gel separation clearly indicates the unmasking effect of the immunodepletion column, the absence of the high-abundant protein bands in the flow-through fraction (Lanes 3 and 7), and the appearance of the low-abundant proteins bands previously not seen in the crude sample lanes (Lanes 2 and 6).

Much development went into the loading (Buffer A) and elution (Buffer B) buffers in order to provide efficient binding, washing, solubilization, and desorption of the targeted plasma proteins. This is required to ensure the efficient binding and releasing of the 14 high-abundant proteins and the long life of the columns as these two are closely related in an affinity device. Run-to-run reproducibility of depletion is crucial for proteomic studies when comparing control and diseased samples. We have tested depletion reproducibility by repeated injection of plasma samples on a single column. As demonstrated by the chromatograms (Figure 2), the column remained stable and performed reproducibly during 200 runs. SDS-PAGE analysis (Figure 4) of the flow-through fractions during 200 runs confirms the repeatability of depletion and agrees with the chromatographic data. Also important for proteomics studies is a lack of carry-over or cross-contamination between runs. This was tested and confirmed by the injection of blanks between sample runs.

To positively impact downstream analysis, the amount of affinity removal needs to be significant. We measured that removing the 14 abundant proteins results in a significant depletion of protein mass – approximately 94%. To determine the overall depletion with MARS Human 14, the flow-through fractions were analyzed for total protein content and compared to the starting serum and plasma samples. The results show that with human plasma there is approximately 92% depletion of total protein content and approximately 94% depletion with human serum. Compared to the Human 6 column, this is approximately 10% greater removal of total protein content.

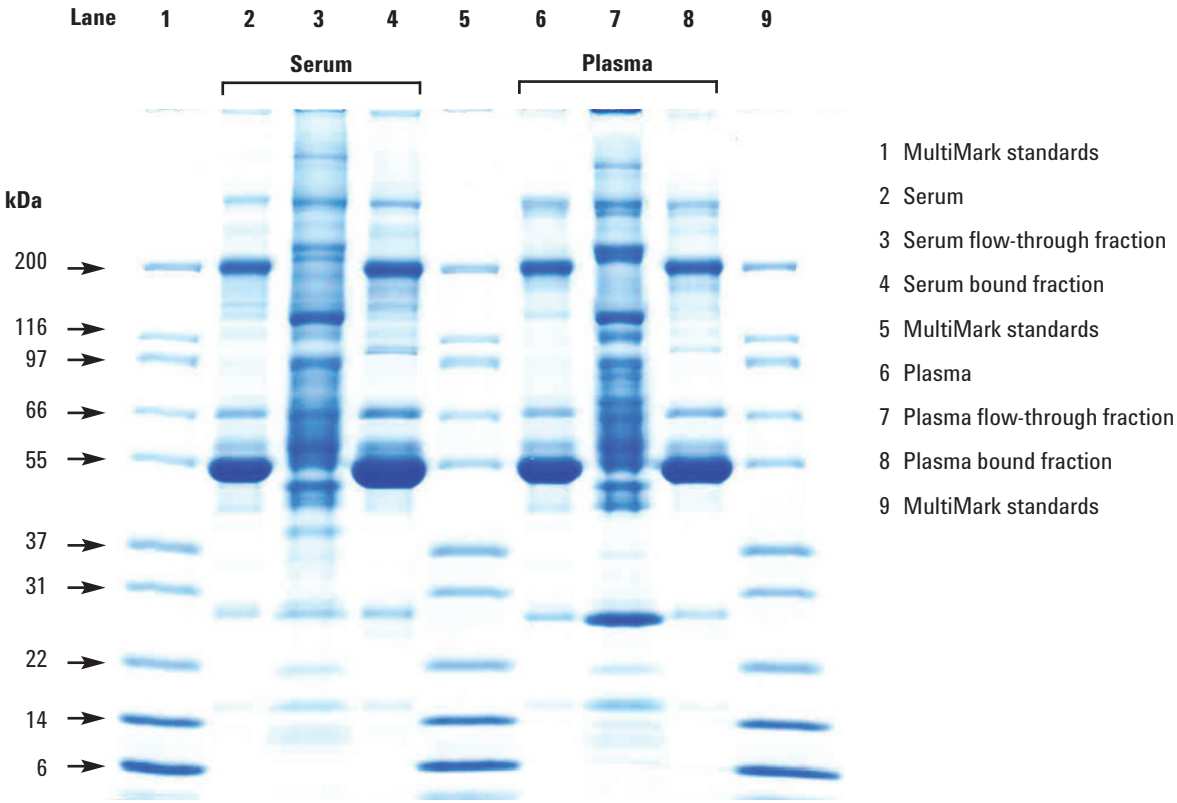


Figure 3. SDS-PAGE analysis of human serum and plasma protein fractions from the Human 14 column. An equal amount (10 µg) of crude serum (Lane 2), serum flow-through (Lane 3), serum bound fraction (Lane 4), crude plasma (Lane 6), plasma flow-through (Lane 7), and plasma bound fraction (Lane 8) were separated on 4 to 20% SDS-PAGE gel under nonreducing conditions. Lanes 1, 5, and 9 are the molecular weight standards (MultiMark 12) from Invitrogen. The proteins were stained with Coomassie Blue dye.

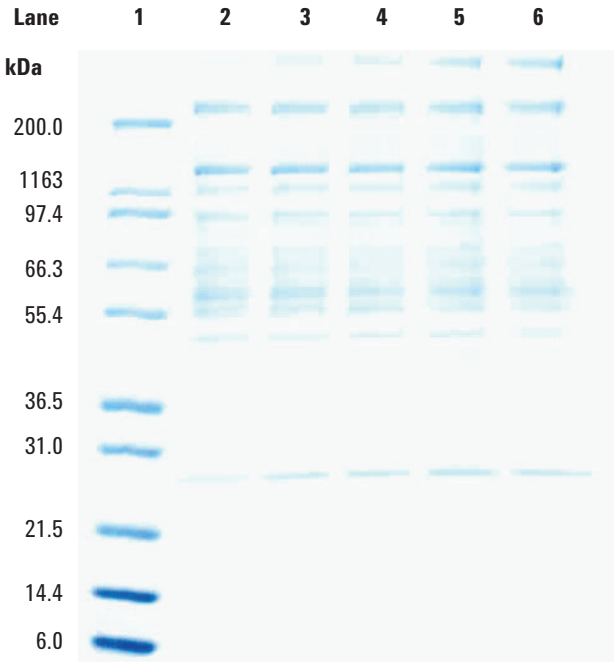


Figure 4. SDS-PAGE analysis of the flow-through fractions from multiple runs on the Human 14 column. Analysis of column performance during 200 runs as visualized using SDS-PAGE separation: Lane 1 - Multi-Mark Standard; Lanes 2 through 6 - Flow-through from runs 1 to 200, every 50th run shown. A total of 8 µg of protein was loaded into each well.

Table 1. Depletion Efficiency of High-Abundant Proteins from Human Serum and Human Plasma on the Human 14 Column

Sandwich ELISA immunoassays described in the Experimental section were used to measure the concentration of high-abundant proteins in flow-through fraction after 200 runs of immunodepletion.

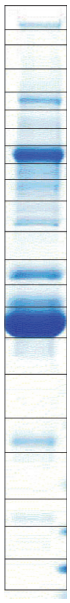
Target protein	Depletion in serum (%)	Depletion in plasma (%)
Albumin	99.9	99.9
Haptoglobin	99.0	98.9
Transferrin	99.7	99.9
IgG	99.9	99.9
IgA	99.9	99.9
a1- Antitrypsin	99.7	99.5
a2-Macroglobulin	99.6	99.3
a1-Acid glycoprotein	99.0	99.0
Apolipoprotein AI	99.2	98.5
Apolipoprotein AII	98.0	98.0
Complement C3	99.0	99.0
IgM	99.7	99.5
Transthyretin	98.3	97.5
Fibrinogen	N/A	97.6

Another important aspect of affinity removal is the completeness of depletion of each of the targeted proteins. This was determined by comparing standard sandwich ELISA analysis of the flow-through fractions with the starting serum and plasma samples. The reported efficiency of depletion is measured after 200 runs, not when the column is new. The results of the depletion efficiency of both serum and plasma for the 14 individual proteins are shown in Table 1.

The specificity of immunoaffinity depletion is a critical parameter for meaningful downstream analysis of proteomics samples. This requires a device that exhibits minimal unspecific binding of nontargeted proteins to the column media. To determine unspecific binding of proteins, we analyzed the bound fraction on a 1D SDS-PAGE gel. The entire gel lane was sliced into 24 sections, and after the in-gel trypsin digestion procedure, the peptides were identified by HPLC-Chip LC/MS/MS. The data indicated a very limited number of nonspecifically bound proteins with all of the targeted proteins identified (see Table 2). It is important to note that these nontargeted proteins were also identified in the flow-through fraction with much higher MS intensity, indicating that only a small amount of these proteins bind nonspecifically to the immunodepletion column.

Table 2. Proteins Identified in the Serum Bound Fraction by LC/MS/MS

Human serum was depleted on the Human 14 column according to the procedure described in the Experimental section. Proteins retained on the column were eluted and resolved on an SDS-PAGE (Fig. 2, Lane 4). The entire gel lane was sliced into 24 gel sections and processed for identification by tryptic digestion and LC/MS/MS analysis. Proteins in bold type (the first 13) were targeted for removal by the Human 14 column.



1	**Human serum albumin**
2	**IgG**
3	**IgM**
4	**IgA**
5	**Haptoglobin**
6	**Transferrin**
7	**Alpha1-antitrypsin**
8	**Alpha2-macroglobulin**
9	**Complement C3**
10	**Alpha1-acid glycoprotein**
11	**Apolipoprotein AI**
12	**Transthyretin (prealbumin)**
13	**Apolipoprotein AII**
14*	Apolipoprotein B-100
15*	Plasma protease C1 inhibitor
16*	Zinc-alpha-2-glycoprotein
17*	Apolipoprotein L1

*Untargeted proteins not quantitatively removed

When ELISA and LC/MS/MS results are viewed collectively, it is clear that the Human 14 column offers comprehensive depletion of targeted high-abundance proteins, while allowing lower abundance proteins to be collected for enriched downstream analysis.

Conclusions

The Agilent MARS Human 14 can reproducibly and reliably deplete the top 14 abundant proteins from human serum, plasma, CSF, and other biological fluids. This allows for the enrichment of low-abundant proteins of interest for further analysis, such as protein fractionation, electrophoretic analysis, and LC/MS. The column is extremely robust and performs reproducibly for over 200 runs.

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