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A Novel High-Capacity Affinity Column for the Removal of the Top Seven Abundant Proteins from Human Plasma

Authors

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Abstract

Recent HUPO studies indicate that plasma is becoming the preferred sample for proteome analysis over serum. Plasma is a unique sample, possessing proteins that span the entire human proteome. Analysis of plasma can provide valuable information for the discovery of new biomarkers and novel drug targets. However, the tremendous complexity of the plasma proteome presents extreme analytical challenges in proteome characterization.

High-abundant proteins such as albumin, IgG, transferrin, haptoglobin, IgA, anti-trypsin and fibrinogen comprise up to 90% of the total protein mass in plasma. These high-abundant protein components interfere with the identification and characterization of important low-abundant proteins by limiting the dynamic range of mass spectral and electrophoretic analyses.

Here we present data on extending this approach to a plasma sample to remove the top 7 high-abundant proteins in plasma. The novel affinity-based resin possesses a high capacity to deplete HSA, transferrin, haptoglobin, IgA, IgG, anti-trypsin and fibrinogen from human plasma. Analysis of the flowthrough fractions during multiple runs indicates reproducibility and robustness of the depletion process.

For the direct analysis of lower abundant proteins, the flowthrough fraction was simultaneously desalted and fractionated on the Agilent macroporous Reversed Phase C18 (mRP-C18) protein separation column. The combination of immuno-affinity depletion with high recovery RP-HPLC fractionation and subsequent peptide analysis by HPLC-Chip/MS expanded the dynamic range of proteomic analysis and allowed the identification of low-abundant proteins.

Introduction

Human plasma continues to serve as the preferred sample for the identification of marker proteins in drug development and disease analysis. Due to the large dynamic range of the plasma proteome, identification of low abundance proteins is a difficult task. Historically, one to two diagnostic protein markers have been identified from plasma samples each year, however, this number has actually declined to near zero in recent years due to the limitations imposed by sample analysis. In spite of the ease of solubility for most plasma proteins, the vast number of associated proteins coupled with the enormous dynamic range makes identification of low abundance targets virtually unachievable using traditional methods. Approximately half of the total protein mass in plasma is a single protein (albumin), with 10 additional proteins comprising 90% of the total.

Previous reports have addressed the effectiveness of using a high-capacity, multiple affinity removal column to deplete both plasma and serum samples of interfering high abundance proteins (1, 2, 3). This study examined a significant enhancement in the current affinity column technology, allowing plasma to be depleted of the top 7 high abundance proteins. The new column had the same specificity, reproducibility, and high capacity as the industry standard Top-6 column for serum, depleting >98–99% of the seven targeted proteins reproducibly for over 200 runs. The variability in plasma samples, such as lyophilized versus fresh frozen plasma, results in >95–99% removal of fibrinogen compared to the remaining six proteins.

The removal of high abundance proteins improved the loading capacity for 2DGE and LC/MS analyses, simplifying a complex system for biomarker discovery. LC/MS/MS analyses confirmed that the Plasma-7 column was superior to current affinity depletion technologies, yielding highly specific protein depletion. In addition, use of the column enabled a more uniform comparison of plasma and serum samples, post-depletion.

The enhanced depletion protocol resulted in an improved dynamic range for proteomic analysis, thus facilitating the detection and characterization of target marker proteins. Agilent's Multiple Affinity Removal approach with the Plasma-7 column provides a valuable commodity in the search for novel biomarkers and drug development.

Specific removal of seven high-abundant proteins depletes approximately 85–90% of total protein mass from human plasma. The low-abundant proteins in the flowthrough fractions can be studied. Removal of high-abundant proteins enables improved resolution and dynamic range for one-dimensional gel electrophoresis (1DGE), two-dimensional gel electrophoresis (2DGE) and liquid chromatography/mass spectrometry (LC/MS). The collected flowthrough fractions may need to be concentrated depending on the downstream applications

Experimental

Affinity column

The newly developed column is an extension and improvement on the Agilent Multiple Affinity Removal approach described previously (1–3). Column contains newly developed affibody ligands (Affibody, Sweden) specific for human fibrinogen. The dimeric design of the Affibody® molecule is coupled with Agilent's proprietary methods to create the highest capacity multiple depletion system. The column requires a two-buffer system for operation. Buffers A and B are optimized to minimize co-adsorption of non-targeted proteins and to ensure reproducibility of column performance and long column lifetime. Buffer A—a salt-containing neutral buffer, pH 7.4, used for loading, washing and re-equilibrating the column and Buffer B—a low pH urea buffer used for eluting the bound high-abundant proteins from the column.

Sample preparation

Before injection onto a high capacity multiple affinity column, lyophilized, citrated human plasma (Sigma Cat. No. P9523) was diluted four fold with Buffer A. The sample was transferred to a 0.22 µm spin tube and centrifuged for 1 min. at 16,000 x g to remove particulates. Diluted plasma was kept at 4°C.

ELISA analysis of the flowthrough fraction

Standard sandwich enzyme-linked immunosorbent assays (ELISA) were used to determine the completeness of removal of targeted proteins from human plasma.

Briefly, assay plates were coated with 100 µl of flowthrough fraction proteins or plasma samples diluted 10x in buffer A. After an overnight incubation at 4°C, plates were washed with PBS and the nonspecific binding sites were blocked with 200 µl of blocker solution (Bio-Rad) for 2 hours. After washing plates with PBS, 100 µl of affinity-purified rabbit anti-human antigen antibodies were added in blocker solution. Plates were incubated for 2 hours, washed, a secondary antibody HRP-conjugated goat anti-rabbit IgG (Sigma) was added for 1 hour. After washing with PBS, liquid substrate (TMB) was added and the absorbance was measured at 655 nm.

SDS-PAGE

Flowthrough fraction proteins were loaded on the SDS-PAGE without manipulations. Bound fractions were pH adjusted and loaded onto gels. Protein concentrations were analyzed using a BCA protein assay kit (Pierce). Samples were stored at –70°C until analysis. SDS-PAGE analysis was carried out using Invitrogen Tris-Glycine pre-cast gels (4–20% acrylamide, 10 wells, 1 mm). Proteins were visualized by Coomassie Blue staining with GelCode Blue (Pierce).

mRP protein fractionation

The flowthrough fraction was directly separated on a macroporous Reversed Phase (mRP-C18) protein column (Agilent) after denaturation with urea (4). 24 fractions were collected and dried with a Speed-Vac concentrator.

LC/MS/MS analysis

To analyze the specificity of the immunodepletion, the bound fraction was resolved by SDS-PAGE. Coomassie-stained protein bands were cut and processed with the Agilent Technologies Protein In-gel Tryptic Digestion Kit. Peptides were analyzed by LC/MS/MS on an Agilent HPLC-Chip/MS system interfaced with an Agilent XCT Ultra Trap LC/MS. Results were processed by Agilent Spectrum Mill software.

The dried fractions from the mRP column were digested using a trifluoroethanol-based protocol (5). Peptides were analyzed by 2D nanoflow LC/MS/MS on an Agilent HPLC-Chip/MS system interfaced with an Agilent XCT Ultra Trap LC/MS.

- Salt Steps: sample (0), 20, 50, 100, 150, 200, 250, 350, 500, 700, 1000, 2000 mM ammonium acetate.
- RP Mobile Phase: H_2O w/0.1% FA, 3% ACN & ACN w/ 0.1% FA – 500 nL/min
- Enrichment Mobile Phase: H_2O w/0.1% FA, 0.05% TFA & 3% ACN – 4 µL/min
- RP Gradient: 3% B @ 0 min., 10% B @ 2 min., 35% B @ 20 min., 50% B @ 22 min., 95% B @ 22.5 min.,95% B @ 23.5 min., 3% B @ 24 min.

Results and Discussion

The Agilent Plasma-7 Multiple Affinity Removal Column presents significant advantages over previous methods of low abundance protein analysis by enabling the easy removal of the top seven proteins (transferrin, antitrypsin, IgA, IgG, haptoglobin, and fibrinogen) from crude samples of human plasma. The removal of highly abundant proteins reduces the depth of dynamic range within plasma samples, thus enabling an increased loading capacity for proteins found in much lower abundance for PAGE and LC/MS/MS analyses.

To determine loading capacity limits for the Plasma-7 column, sequential injections of increasing plasma volumes were made and the flowthrough fractions were separated using PAGE and Coomassie Blue staining. Results show that even at the highest plasma injection volume (80 µL), there was no breakthrough of targeted proteins evidenced by staining (Figure 1; Lanes 3–5). The absence of breakthrough proteins from an injection of 80 µL of crude plasma demonstrates the dramatic improvement in column loading capacity for this system as compared to other existing high-affinity columns. In addition, subsequent ELISA analysis reported a 99.4% depletion of fibrinogen from all three plasma loads, confirming the effectiveness of target protein depletion (data not shown).

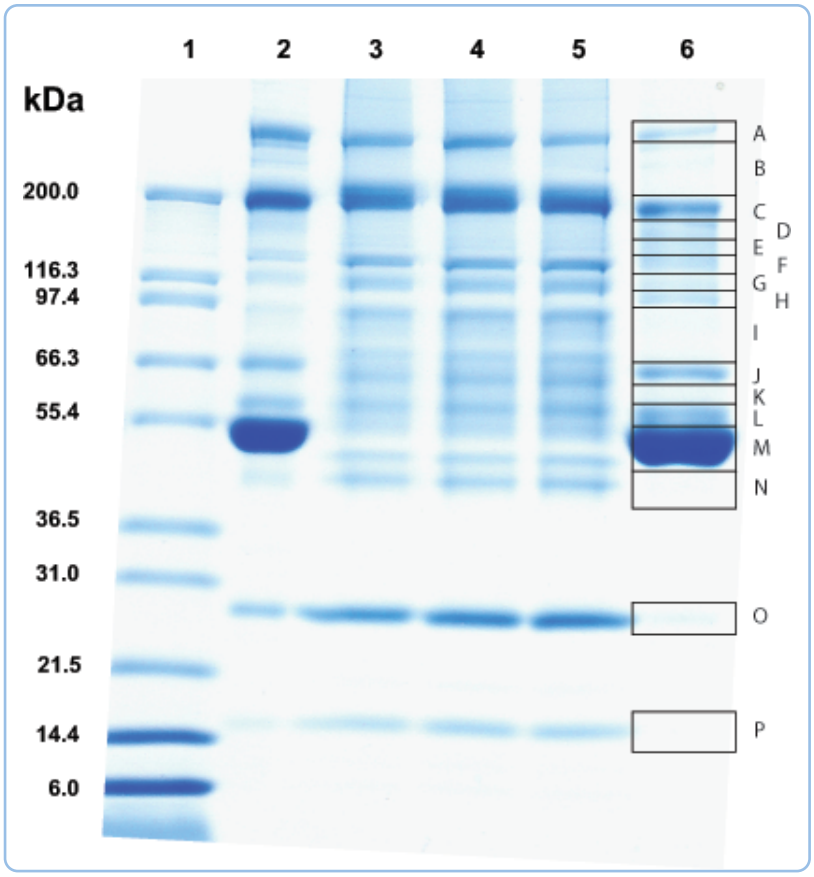

Figure 1. 1D-gel of bound and flowthrough fractions from a 4.6 x 100 mm Plasma-7 Multiple Affinity Removal Column. Lane 1 – Mark12 Standard; Lane 2 – Human Plasma; Lane 3 – Flowthrough (60 mL); Lane 4 – Flowthrough (70 mL); Lane 5 - Flowthrough (80 mL); Lane 6 – Bound fraction (10 mg). Boxed bands show respective regions of the gel (Gel bands A–P) that were excised and used in the identification of bound proteins.

To identify the proteins that were removed from plasma, proteins representing the bound fraction from the Plasma-7 Multiple Affinity Removal Column were separated by PAGE, extracted using in-gel trypsin digestion, and analyzed by LC/MS/MS (see Lane 6 of Figure 1). Of the 16 gel bands analyzed for the bound protein fraction, 13 contained proteins targeted for removal (Table 1). Other proteins removed included Complement C3 and B, two apolipoproteins, and the hemoglobin alpha chain. These results confirm the high specificity of the Plasma-7 column for removal of targeted proteins. When ELISA and LC/MS/MS results are viewed in unison, it is obvious that the Plasma-7 column offers comprehensive depletion of targeted high abundance proteins, while allowing lower abundance proteins to be collected for separate downstream analysis.

Table 1. Protein identification based upon LC/MS/MS analysis of excised, bound fraction gel bands

Gel band	Protein identified*
A	Fibrinogen
B	Fibrinogen, Haptoglobin, HSA
C	IgG, IgA, *Complement C3*
D	IgG, HSA, IgA
E	IgG, HSA
F	IgG, HSA, *Ceruloplasmin*
G	HSA, Haptoglobin, IgG
H	HSA, *Complement B pre*
I	IgG, HSA
J	Transferrin, HSA
K	HSA, Transferrin, *alpha-1-antichymotrypsin*
L	Alpha-1-antitrypsin, HSA
M	HSA
N	*Apolipoprotein H*
O	*Apolipoprotein*
P	*Hemoglobin alpha chain*

*Items in italics are untargeted proteins removed non-quantitatively.

In addition to ensuring complete separation of proteins, the Human Plasma-7 column is extremely robust, performing reproducibly over 200 runs. When column chromatograms were compared over 200 runs (at checkpoints every 40 runs), there was very little change in peak dimensions (Figure 2). The consistency of separation and repeatability over hundreds of runs guarantees high quality results for low abundance targets.

The reproducibility of the Human Plasma-7 column is demonstrated in further SDS-PAGE analysis of the flowthrough fractions (Figure 3). SDS-PAGE analysis of the flowthrough fractions from 200 runs on this column shows consistent results from beginning to end. Flowthrough samples were equally loaded based on protein concentration (10 µg per well) and exhibited no change in protein banding pattern for any of the 200 runs.

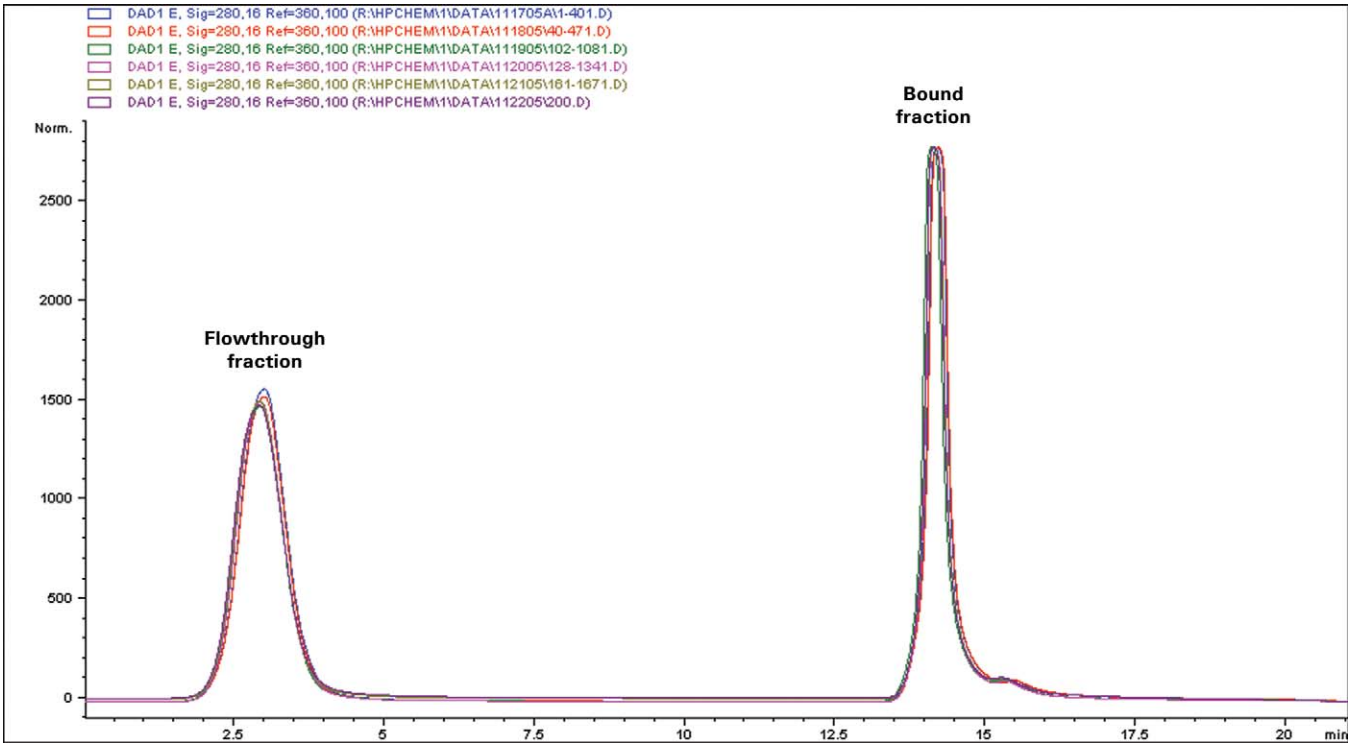

Figure 2. LC chromatograms during 200 runs of affinity removal using the Plasma-7 column for high abundance protein removal from crude plasma samples.

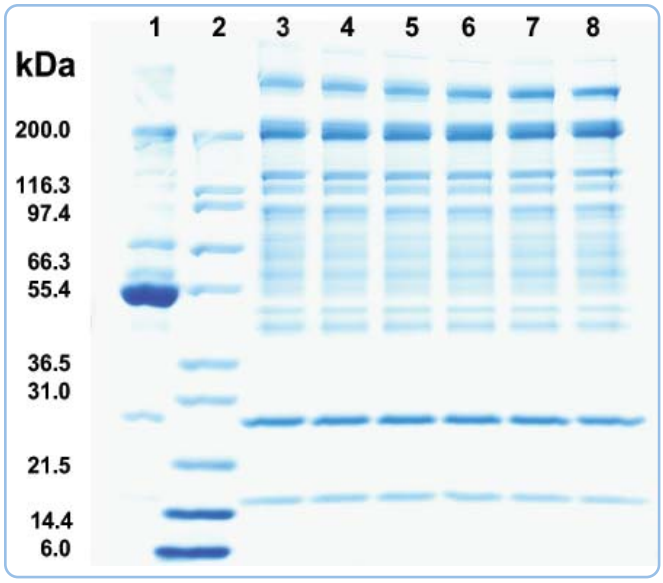

Figure 3. Analysis of column performance during 200 runs as visualized using SDS-PAGE separation. Lane 1 – Human Plasma; Lane 2 – Mark12 Standard; Lane 3 – Flowthrough (Run 1); Lane 4 – Flowthrough (Run 40); Lane 5 – Flowthrough (Run 80); Lane 6 – Flowthrough (Run 120); Lane 7 – Flowthrough (Run 160); Lane 8 – Flowthrough (Run 200). A total of 10 µg of protein was loaded into each well.

A partial representation of the numerous low-abundant proteins found in the flowthrough fraction as analyzed by LC/MS/MS is shown in Table 2. Multiple unique proteins were identified from the flowthrough fraction; many proteins were important precursors to signal transduction pathway components. Others included potential protein regulators such as nuclear receptor corepressors, serine/threonine-protein kinases, and protein kinase C-like structures.

Conclusions

The Human Plasma-7 column from Agilent offers a robust and consistent tool, particularly useful to those requiring heightened sensitivity for proteomic discovery through the removal of high abundance proteins. In addition, the combination of immunoaffinity chromatography with high recovery RP-HPLC fractionation and subsequent HPLC-Chip/MS peptide analysis expands the dynamic range by enabling the identification of low abundance plasma proteins. As one of the highest capacity columns available, the Human Plasma-7 offers an accurate and dependable high-throughput method for protein separation.

Table 2. Partial representation of the numerous low abundance proteins identified using direct 4D LC/MS analysis of the flowthrough fraction

Protein	Accession No.	No. distinct peptides
Hepatocyte growth factor-like protein precursor (ng/mL)	P26927	7
Properdin precursor (Factor P)	P27918	6
Fetuin-B precursor	Q9UGM5	7
Hepatocyte growth factor activator precursor	Q04756	6
L-selectin precursor	P14151	6
Insulin-like growth factor binding protein 3 precursor (ng/mL)	P17936	5
Selenoprotein P precursor	P49908	5
Monocyte differentiation antigen CD14 precursor	P08571	4
Insulin-like growth factor II precursor (ng/mL)	P07456	3
Platelet basic protein precursor (ng/mL)	P02775	3
Vascular endothelial-cadherin precursor (ng/mL)	P33151	3
Lipopolysaccharide-binding protein precursor	P18428	3
Neutrophil defensin 3 precursor	P59666	3
Serum amyloid A protein precursor	P02735	3
Intercellular adhesion molecule-2 precursor	P13598	2
Ryanodine receptor 1	P11716	3
EGF-containing fibulin-like extracellular matrix protein 1 precursor	Q12805	2
CD44 antigen precursor	P16070	2
Serine/threonine-protein kinase	Q00536	2
Glutamate receptor 1 precursor	P42261	2
Sentrin-specific protease 2	Q9HC62	2
Nesprin 2	Q8WXH0	2
Nuclear receptor corepressor 1	Q60974	2
Protein kinase C-like 2	Q16513	2

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