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Protein depletion from blood plasma using a volatile buffer

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Abstract

Removal of high abundance proteins is widely used in sample processing for proteomics studies of blood plasma. Immunoaffinity (IA) depletion is currently the most specific method for performing this step. Historically, IA depletion matrices have been designed to be used with inorganic buffers. However, the presence of salts in depleted samples presents a particular problem, and these must be removed in order to make samples compatible with post-depletion processing. Desalting (dialysis, ultrafiltration, size-exclusion, etc.) usually diminishes sample integrity due to labware associated losses. Moreover, these steps require additional labor, increasing the processing time and cost of analysis. In order to avoid these problems, we have developed an IA method using a volatile buffer that can be removed from depleted samples by lyophilization. This method allows the execution of reproducible and efficient depletion of blood plasma in a semi-automated manner.
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1. Introduction

The protein profiling of blood serum and plasma is challenging due to the wide dynamic range of protein abundance that complicates the proteomic analysis. High-abundance proteins (tens to approximately 2 mg/ml) including albumin, immunoglobulins (IgG and IgA), antitrypsin, haptoglobin and transferrin mask the presence of low-abundance proteins (less than 1 mg/ml) because the loading capacity and dynamic range of the separation media and analytical instrumentation are limited [1]. The removal of these high abundance proteins depletes up to 80% of the total protein, increasing the relative concentration of low-abundance proteins and therefore improving the detection sensitivity [2,3].

Several depletion methods for specific removal of high abundance proteins have been developed. These include dye-ligands, such as Cibacron blue F3GA [4], mimetic ligands [5,6], proteins A and G [7–9] or antibodies (IA depletion)) [2,10,11]. However, IA depletion offers the most specific method for the removal of high abundance proteins [12]. Major manufacturers of IA matrices (Agilent, GE Healthcare, and GenWay) usually provide depletion protocols based on non-volatile binding buffers. Components of these binding buffers (inorganic ions) have limited compatibility with downstream steps of the sample processing (enzymatic digestion, ion-exchange chromatography, etc.). Therefore, they should be removed prior to further sample processing. The majority of salt removal methods have near quantitative sample recovery [13,14] provided that the samples consist of soluble proteins at relatively high concentrations. However, the losses increase dramatically for samples composed of low solubility proteins or for samples with low protein concentration [15].

In spite of the advantages of volatile buffers, their use for IA methods has not been extensively investigated. Only one study describing depletion using a volatile binding buffer has been reported [13]. In this case Cibacron Blue and protein G were used for the removal of albumin and IgG from cerebral spinal fluid. Therefore, one of goals of this study was to compare the efficiency of immuno-affinity depletion under volatile and non-volatile conditions.

The Multiple Affinity Removal System (MARS) developed by Agilent Technologies was used in this study. This system enables simultaneous removal of albumin, IgG, alfa-1-antitrypsin, IgA, transferrin and haptoglobin from human blood plasma. Antibodies against the targeted proteins are covalently immobilized to a matrix that is packed into standard a HPLC column. Based on gel electrophoresis (SDS-PAGE) analysis, Agilent Technologies has shown that a single multiple affinity

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depletion (MAD) column can be used for multiple (up to 200) reproducible depletion runs with the use of their proprietary non-volatile buffer [10]. Demonstrating that depletion in a volatile buffer attains the same level of reproducibility from run to run will allow sequential unattended depletion of a large number of samples. However, the number of samples in a clinical plasma proteome study is well above the single day throughput of sequential chromatography [16]. Therefore, another goal of this study was to determine whether depletion under volatile conditions can be carried out reproducibly over a period of several days. Enzyme-linked immunosorbent assays (ELISA) in addition to other analytical methods were used to evaluate the method.

2. Experimental

2.1. Chemicals and reagents

Proprietary buffers A (binding) and B (elution) for MARS were obtained from Agilent Technologies, Inc. (Palo Alto, CA, USA). SDS-gels (4–12% Bis-Tris), 2-[*N*-Morpholino] ethanesulfonic acid (MES) running buffer, Sypro Ruby stain and sample preparation reagents were from Invitrogen Canada, Inc. (Burlington, ON, Canada). Ammonium bicarbonate and hydrochloric acid (HCl) were from EMD Chemicals, Inc. (Gibbstown, NJ, USA). HPLC grade water was obtained from Fisher Scientific (Montreal, Que., Canada). EDTA-treated human plasma was obtained from Bioreclamation (Hicksville, NY, USA). Bicinchoninic acid base protein assay (BCA) reagents (Pierce, Rockford, IL, USA). BCA analysis was executed according to manufacturer's directions.

For the ELISA, human haptoglobin, human α-1-antitrypsin, mouse anti-α-1-antitrypsin and goat anti-transferrin antibodies were from VWR CanLab (Mississauga, ON, Canada). Human apo-transferrin, human serum albumin and Tris-base were from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada). Other ELISA reagents were: human IgG (Bethyl Laboratories, Montgomery, TX, USA); sheep polyclonal anti-albumin antibodies (US Biologicals, Swampscott, MA, USA); goat anti-IgG and goat anti-kappa light chain (Southern Biotechnology Associates, Birmingham, AL, USA); sheep polyclonal anti-haptoglobin antibodies (Abcam, Cambridge, MA, USA); horse radish peroxidase conjugated AffiniPure rabbit anti-sheep, anti-goat and goat anti-mouse (Jackson Immunoresearch, Inc., West Grove, PA, USA).

Unless otherwise specified, all solvents and chemical reagents were of analytical grade.

2.2. Apparatus

A human MAD column (4.6 mm × 100 mm, capacity 30–40 μl of plasma) was obtained from Agilent Technologies, Inc. (Palo Alto, CA, USA). Depletion was performed on an AKTA Explorer HPLC system (GE Healthcare, Uppsala, Sweden). A PowerWave plate reader (BioTek, Woburn, MA, USA) was used to analyze micro-well plates for the BCA assay (562 nm) and ELISA (450 nm).

2.3. Comparison of the depletion efficiency under volatile and non-volatile conditions

2.3.1. Sample preparation

Forty microliters of a thawed human plasma aliquot were mixed with either 160 μl of 150 mM ammonium bicarbonate (for the depletion in volatile buffer) or 160 μl of Agilent Buffer A (for the depletion in non-volatile buffer). Two samples were prepared for the depletion under volatile conditions, and one sample was prepared for the depletion under non-volatile conditions. Diluted samples were briefly vortexed and then spun in a microcentrifuge. All samples were prepared individually in identical time frames before an injection.

2.3.2. Chromatography

For the depletion under non-volatile conditions, 100 μl of the sample prepared in Agilent buffer A were loaded onto the pre-equilibrated MARS column in a mobile phase of Agilent buffer A at a flow rate of 0.5 ml min^{-1}. Unbound proteins (depleted plasma) were collected. Further, at 11 min, the gradient was switched to 100% of Agilent buffer B, and the flow rate was increased to 1 ml min^{-1}. After 4.5 min the gradient was switched back to Agilent buffer A, and the column was re-equilibrated for 10.5 min at a flow of 1 ml min^{-1}. The depleted plasma was dialyzed and freeze-dried.

For the depletion under volatile conditions, 100 μl of the samples prepared in ammonium bicarbonate were loaded onto the pre-equilibrated column in a mobile phase of 150 mM ammonium bicarbonate (pH 7.8) at a flow rate of 0.5 ml min^{-1}. Unbound proteins (depleted plasma) were collected. At 7 min, the flow rate was increased to 1 ml min^{-1}, and the gradient was switched to 100% Agilent buffer A for 4.5 min. Bound proteins were then eluted with Agilent buffer B for 4.5 min, and the column was washed with Agilent buffer A for 4.5 min. The column was then re-equilibrated in 150 mM ammonium bicarbonate for 5.5 min. Depleted plasma samples were either dialyzed and freeze-dried or freeze-dried twice without dialysis. The UV absorbance profiles of all runs were monitored at 280 nm.

2.3.3. Dialysis

Each of the depleted plasma samples to be dialyzed (2.25 ml in volume) was transferred into a separate dialysis cassette (Pierce, Rockford, IL, USA) and equilibrated against 4 l of water for 16 h at +4 °C with stirring. After dialysis, the contents of the cassettes were transferred into tubes and freeze-dried once.

2.3.4. Freeze-drying

Samples depleted using volatile buffer and raw plasma samples were placed in a freezer at −85 °C for at least 45 min and then dried under vacuum for 16 h. Lyophilized samples were reconstituted in 400 μl of 100 mM ammonium bicarbonate and freeze-drying was repeated.

2.4. Reproducibility of the depletion using volatile buffer

A pool of human plasma samples (different from the one used for the comparison of the depletion efficiency in volatile and

non-volatile conditions) was spun at 10,000 × g for 1 min. The supernatant was distributed into 10 aliquots of 220 μl of plasma and frozen at −85 °C. Before analysis, one frozen aliquot of plasma was thawed for 3 min in water at ambient temperature and diluted with 1100 μl of ice-cold 150 mM ammonium bicarbonate. The mix was briefly vortexed and distributed into 11 aliquots of 120 μl each. Samples were stored on ice and injected over a period of approximately 5 h with time intervals of approximately 30 min. Each aliquot was spun at 10,000 × g for 1 min immediately before injection, and 100 μl of the supernatant were depleted under volatile conditions as described above. Unbound proteins (depleted plasma) were collected and freeze-dried twice.

2.5. Enzyme-linked immunosorbent assay

ELISA was performed on duplicates of each analyzed sample following standard protocols with some modifications. Aliquots from serial dilutions of analyzed samples were transferred into wells of immulon 4HBX 96-well plates (Dynex, Chantilly, Virginia, USA) for binding. After incubation, washing and blocking, wells were incubated with primary, then with secondary antibodies. After 4 min incubation, color development was monitored at 450 nm using the PowerWave plate reader. Standard curves were constructed from readings of eight duplicated serial dilutions in the range from 12.5 to 0.098 ng of protein per well. Commercial proteins were used to build standard curves for each protein of interest.

3. Results and discussion

3.1. Comparison of the depletion efficiency using volatile and non-volatile buffers

Instructions provided by column manufacturers propose a few "polishing" runs with real samples to block the sites of irreversible binding. Therefore, the first eight injections of plasma samples were utilized in order to develop chromatography methods and obtain reproducible UV profiles. Although the maximum binding capacity of the column (according to the Agilent instructions) is 35–40 μl of blood plasma, all chromatography runs were executed at loading amounts corresponding to 20 μl of blood plasma. This loading amount ensures maximum removal of target proteins.

For the comparison of the depletion efficiency in volatile and non-volatile buffers, three runs (from 9th to 11th in the column's history) were executed in the same day. As observed from the ELISA results (Table 1), using either volatile or non-volatile buffers, the percentage of protein depletion was greater than 99% for all targeted proteins.

To evaluate the losses caused by dialysis, samples depleted under volatile conditions were processed using both freeze-drying and dialysis. In Table 1, it can be seen that the sample depleted under volatile conditions and processed without dialysis has higher protein content (228 μg) than dialyzed sample depleted under volatile (114 μg) or non-volatile (196 μg) conditions. This suggests that losses are incurred during the dialysis.

The use of volatile buffer affects neither the retention time nor the peak shape of depleted plasma proteins (Fig. 1). However, it increases the chromatography time by approximately 2 min compared to the original methodology of Agilent Technologies. This is due to the incompatibility of ammonium bicarbonate buffer with buffer B of Agilent Technologies (mixing of these two buffers causes excessive bubble production). Therefore, a plug of Agilent buffer A was always flowed between the ammonium bicarbonate buffer and Agilent buffer B.

3.2. Reproducibility of the depletion in ammonium bicarbonate

One hundred runs (from 15th to 115th in the column's history) were executed in increments of 10 injections per day.

Table 1
Comparison of the depletion efficiency under volatile and non-volatile conditions

	Amount (μg) of proteins in raw plasma[a]	Amount (μg) of protein remaining after depletion[a]			Depletion efficiency (%)[b]		
		After dialysis and freeze-drying		After freeze-drying	After dialysis and freeze-drying		After freeze-drying
		Non-volatile depletion	Volatile depletion	Volatile depletion	Non-volatile depletion	Volatile depletion	Volatile depletion
BCA results							
Total protein	1613	196	114	228	88	93	86
ELISA results							
Albumin	621	<0.1[c]	<0.1	<0.1	>99.9	>99.9	>99.9
Haptoglobin	28	<0.1	<0.1	<0.1	>99.5	>99.5	>99.5
α-1-Antitrypsin	41	<0.1	<0.1	<0.1	>99.8	>99.8	>99.8
Ig's κ-light chains	182	0.8	0.6	0.8	99.6	99.7	99.6
IgG	175	0.2	0.1	0.4	99.9	99.9	99.8
Transferrin	306	0.3	0.2	0.9	99.9	99.9	99.7

[a] Values are equivalent to 20 μl of raw plasma.
[b] Calculated as ((amount in raw plasma − amount in depleted plasma) × 100%))/(amount in raw plasma), where the protein amount in 20 μl of raw plasma is assumed to be 100%.
[c] <0.1-concentration of protein in depleted plasma was outside the linear dynamic range of ELISA.

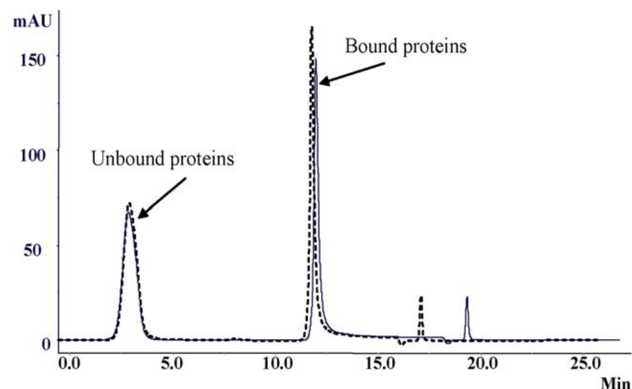

Fig. 1. UV absorbance profiles (280 nm) of plasma depletion under volatile (solid line) and non-volatile (dotted line) conditions. Unbound proteins elute at the same time under both conditions. Bound proteins elute slightly later under volatile conditions.

Table 2
Reproducibility of the depletion under volatile conditions

Day	Unbound proteins			Bound proteins		
	Average[a] (mAU)	S.D. (mAU)	CV (%)	Average[a] (mAU)	S.D. (mAU)	CV (%)
1	957	14	1.5	2044	29	1.4
2	950	6	0.6	2085	15	0.7
3	957	9	0.9	2115	18	0.8
4	941	8	0.8	2093	20	1.0
5	943	9	0.9	2077	21	1.0
6	927	13	1.4	2019	23	1.1
7	921	6	0.6	2109	16	0.8
8	894	12	1.3	2051	21	1.0
9	893	15	1.6	2075	29	1.4
10	923	7	0.8	2057	12	0.6
Study[b]	931	24	2.5	2072	30	1.4

[a] Calculation based on peak heights (at 280 nm) of 10 runs executed during a single day.
[b] Calculations based on per day average values of peak heights (at 280 nm).

The run-to-run and day-to-day reproducibility of the depletion of 100 plasma injections was monitored by UV absorbance at 280 nm. Standard deviation (S.D.) and coefficients of variation (CV) were calculated for each day and for the whole experiment (10 days) from the peak heights (280 nm) of unbound and bound proteins (Table 2). S.D. and average values were calculated using Excel Microsoft built-in functions STDEV and AVERAGE. The CV corresponds to the S.D. divided by the average value. In addition, SDS-PAGE analysis of unbound fractions reveals reproducible protein profiles from day to day (data not shown). Protein patterns were similar to those of previously published results [10].

Peak heights of unbound fractions were more reproducible from run to run than from day to day. Run-to-run reproducibility ranges from CV = 1.5% (9th day) to CV = 0.6% (second day), while the day-to-day reproducibility CV was 2.5% (Table 2). The higher CV of day-to-day peak height measurements was supported by protein quantification results obtained by BCA and ELISA analyses of the first and every subsequent 20th run (Table 3). These measurements show that the average depletion efficiency obtained on different days was 78% of the total protein content with CV = 0.9%. Relatively reproducible (CV = 10%) recovery of C3 complement protein was observed for all runs (Table 4). A negative trend of the depletion efficiency toward IgG (from 99.9 to 99.1%) and kappa light chains (from 99.3 to 97.8%) was observed (Table 3). It could not be determined whether this was related to the leakage of antibodies from the column or to a decrease in IgG depletion efficiency.

As judged by the total protein content, an approximately 8% difference in the depletion efficiency was observed between the first comparison experiment (86%, Table 1) and the reproducibility experiment (78%, Table 3). This may be explained by different protein concentrations in these samples. The sample used for the comparison experiment had higher total protein content (1460 μg per 20 μl) than the sample used for the reproducibility experiment (1290 μg per 20 μl). In addition, ELISA results show higher total amount of albumin, IgG and kappa light chains in the former experiment (977 μg per 20 μl) than in the latter (844 μg per 20 μl).

The reproducibility of the depletion process under volatile conditions is determined by several factors. These include the column stability in volatile buffer, stability of the volatile buffer, and sample stability during the analysis. The latter was one of our greatest concerns, because blood plasma contains a pool of very active proteases that can degrade a significant proportion of plasma proteins in a short time. In a separate set of experiments it was shown that the refrigeration of samples to 4 °C minimizes the intrinsic protease activity in the depleted plasma samples for a period of up to 16 h as confirmed by SDS-PAGE (Fig. 2). Thus, with a run time of 26 min, it is possible to execute up to 36 sequential depletion runs in unattended

Table 3
Reproducibility of the depletion efficiency under volatile conditions

Protein	Depletion efficiency (%)						Average (%)	CV (%)
	Run #1	Run #20	Run #40	Run #60	Run #80	Run #100		
Total protein	77	78	78	79	78	77	77.8	0.9
Albumin	>99.9[a]	>99.9	>99.9	>99.9	>99.9	>99.9	N/A	N/A
κ-Light chains of Ig's	99.3	99.2	98.9	98.2	98.3	97.8	98.6	0.6
IgG	99.6	99.4	99.3	99.3	99.4	99.1	99.4	0.2

Depletion efficiency was calculated based on BCA (total proteins) or ELISA assays as ((amount before depletion − amount after depletion) × 100%))/(amount before depletion), where the amount of proteins in 20 μl of raw plasma is assumed to be 100%.
[a] >99.9-concentration of protein in depleted plasma was outside the linear dynamic range of ELISA; N/A: calculation is not applicable.

Table 4
Reproducibility of depletion by Elisa and BCA

Protein	Amount of protein(s) (μg) in unbound fractions						Average (μg)	CV (%)
	Run #1	Run #20	Run #40	Run #60	Run #80	Run #100		
Total protein	292.3	277.1	286.8	276.2	279.4	293.2	284.2	2.7
Albumin	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	N/A	N/A
Ig's κ-light chains	1.5	1.7	2.3	2.5	3.5	4.5	2.7	42.4
IgG	0.47	0.7	0.8	0.7	0.6	0.9	0.7	22.1
C3	26.3	25.2	30.2	29.3	22.6	26.7	26.7	10.3

Total and individual protein amounts were estimated from BCA and ELISA, respectively. Plasma samples (identical for each run) loaded onto depletion column had the following protein content: total protein −1290.5 μg; albumin −532.8 μg, Ig's κ-light chains −204.3 μg, IgG-108 μg and C3 complement protein −26.3 μg. Protein amounts in raw plasma and unbound fractions (depleted plasma) were calculated with respect to the volume of raw plasma samples loaded onto depletion column.

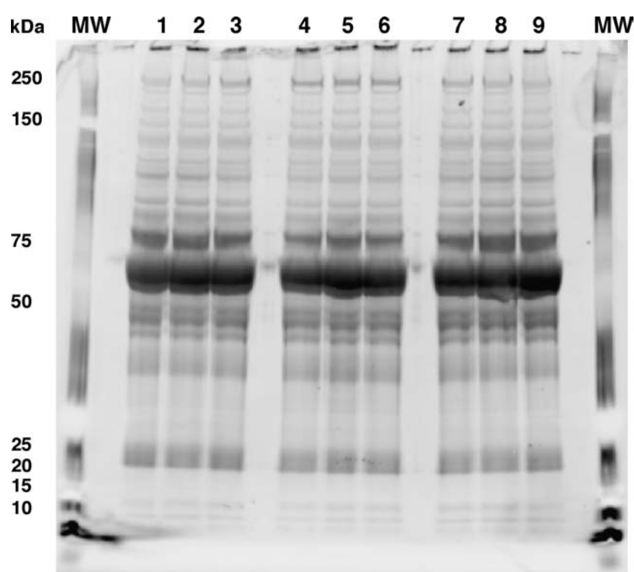

Fig. 2. Stability of raw plasma without addition of protease inhibitors at +4 °C was confirmed by SDS-PAGE analysis. Aliquots of raw plasma were diluted four-fold by buffer A without (lines 1, 4 and 7) protease inhibitors and by buffer A contained either Complete™ mixture from Roche (lines 2, 5 and 8), or home-made mixture of different protease inhibitors (lines 3, 6 and 9). All samples were incubated at +4 °C and 10 μg of each were analyzed after 0 h (1–3); 4 h (4–6) and 16 h (7–9) of incubation. Neither a degradation of bands above 10 kDa, nor accumulation of material below 10 kDa could be observed regardless of the composition of incubation buffer.

mode. The column stability is comparable using either volatile or non-volatile (Agilent) buffer systems. Furthermore, the reproducibility of the depletion in the volatile buffer system suggests that this buffer is stable over the analysis time of the present study. Therefore, the reproducibility, depletion efficiency and life span of the MARS column in ammonium bicarbonate buffer meet the criteria that determine the successful implementation of the device into an "industrial" environment of sample processing.

4. Conclusion

Immunoaffinity depletion of proteins from blood plasma using a volatile buffer is comparable in terms of reproducibility and efficiency to that of a non-volatile buffer system. The major advantage of the volatile buffer system is that there is no need to remove residual salts prior to further sample processing steps, such as proteolysis and SCX chromatography. Eliminating the sample desalting step decreases the overall sample processing time. In addition, lyophilizing the sample rather than performing dialysis minimizes sample loss.

Although the MARS column has been presented here, immunoaffinity depletion in ammonium bicarbonate buffer could be implemented with other affinity matrices. This buffer system has been successfully applied in our laboratory for the depletion of blood plasma proteins with resin-coupled protein-G, A and L. Depletion under volatile conditions should therefore be generally applicable to the preparation of samples for plasma profiling using mass spectrometry.

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