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A fast and reproducible method for albumin isolation and depletion from serum and cerebrospinal fluid

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Abstract

Analysis of serum and plasma proteomes is a common approach for biomarker discovery, and the removal of high abundant proteins, such as albumin and immunoglobins, is usually the first step in the analysis of serum and plasma proteomes. However, albumin binds peptides and proteins, which raises concerns as to how the removal of albumin could impact the outcome of the biomarker study while ignoring the possibility that this could be a biomarker sub-proteome itself. The first goal of this study was to test a new commercially available affinity capture reagent from Protea Biosciences and to compare the efficiency and reproducibility to 4 other commercially available albumin depletion methods. The second goal of this study was to determine if there is a highly efficient albumin depletion/isolation system that minimizes sample handling and would be suitable for large numbers of samples. Two of the methods tested (Sigma and ProteaPrep) showed an albumin depletion efficiency of 97% or greater for both serum and cerebrospinal fluid (CSF). Isolated serum and CSF albuminomes from ProteaPrep spin columns were analyzed directly by LC-MS/MS, identifying 128 serum (45 not previously reported) and 94 CSF albuminome proteins (17 unique to the CSF albuminome). Serum albuminome was also isolated using Vivapure anti-HSA columns for comparison, identifying 105 proteins, 81 of which overlapped with the ProteaPrep method.

Keywords

albuminome; cerebrospinal fluid; depletion; mass spectrometry; serum

1 INTRODUCTION

Human serum albumin (HSA) is a negatively charged, highly soluble protein with well-known ligand-binding and transport properties [1] and it is the most abundant protein in human serum, comprising approximately 50% of the total serum protein concentration [2].

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CONFLICT OF INTEREST

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HSA binds both exogenous and endogenous compounds, such as drugs (e.g. ibuprofen and diazepam), metal ions, peptides (e.g. bradykinin, interferons, insulin) and proteins (e.g. serum amyloid A and Streptococcal G) [3, 4]. Recent studies have focused on characterizing the “albuminome” (peptides and proteins bound to HSA) and have shown that the albuminome is a serum and plasma sub-proteome [5–9].

The depletion of HSA and other high abundant proteins (e.g. IgG, transferrin, and haptoglobin) is often the initial step in serum and plasma based proteomics studies where the discovery of potential protein biomarkers, which are often in low abundance, is the goal. However, depletion of HSA from serum samples results in the removal of this potentially important sub-proteome, which is often ignored even though it can influence the analysis/outcome of the depleted samples and has the potential itself to be used as a biomarker source. For example, studies have shown that HSA undergoes a conformational change at the N-terminus as a specific result of myocardial ischemia [10–12]. This led to the development of the albumin bound cobalt (ABC or IMA (ischemic modified albumin)) test, which measures the ability of HSA to bind cobalt and is the only FDA approved test to rule out myocardial ischemia [13–16]. In addition, HSA bound proteins and peptides have potential use as cancer biomarkers [9, 17], suggesting that detailed characterization of this sub-proteome could be exploited in many disease settings.

Depletion or enrichment of HSA from serum or plasma is commonly achieved through the use of antibody- or dye-based spin or liquid chromatographic (LC) columns (e.g. Seppro® IgY14, Sigma Aldrich), or chemical based methods (e.g. NaCl/EtOH [18, 19]). The latter is based on substantial modification of the Cohn purification schema [20] that has been used to purify HSA since the 1940s. Affinity-based technologies are available as 96 well plates and spin columns and are fast methods for isolating HSA and are able to handle small sample volumes [21–23], or as HPLC columns capable of partitioning larger quantities. All devices are vulnerable to non-specific binding of proteins/peptides and issues with recovery and carryover can arise if used for successive runs [18, 21–25].

Various mass spectrometry-based approaches have been used to examine the albuminome. Our laboratory [6] compared albuminomes from both the bound fraction obtained from anti-HSA antibody affinity chromatography and chemical based NaCl/EtOH method. The HSA purified fraction was further separated by size-exclusion chromatography (SEC) to isolate HSA-binding protein-HSA complexes. Additional protein separation was carried out by either 1-D SDS-PAGE or RP-HPLC of the SEC fractions and intact mass analysis was carried out by MALDI-TOF and protein identification by LC-MS/MS analysis following tryptic digestion of these fractions. More recently, Gay *et al* examined the proteome of commercially available HSA solutions [5], in which the HSA had already been purified using the traditional Cohn method [20], followed by strong anion exchange chromatography and SDS-PAGE to fractionate the HSA bound proteins prior to digestion and 2DLC-ESI-MS/MS. Unfortunately, this group did not distinguish between those proteins which were bound to HSA versus total proteins present in the HSA enriched sample. In a similar approach, Zhou *et al* [8] identified 126 albuminome proteins using an antibody-based and also dye-based affinity method for albuminome isolation followed by trypsin digestion, strong cation exchange chromatography for a subset of samples, and LC-MS/MS but limited

their analysis to species under 30 kDa. Lowenthal *et al* [9] also used antibody-affinity based methods for albuminome isolation from cancer patients followed by 1-D SDS-PAGE and LC-MS/MS for protein identification and reported 1208 albuminome proteins across all disease settings. Finally, Scumaci *et al* [7] used an ad hoc method in which they immobilized purified HSA to a solid activated immunoaffinity support, effectively creating an “HSA column”. The HSA conjugated to the column was used as a bait to bind proteins from a plasma sample passed over the HSA column providing a view of the potential albuminome. They analyzed the proteins bound to the column by 2D-LC-MS/MS and reported 63 potential plasma albuminome proteins, but this was an *in vitro* method and may or may not be indicative of the *in vivo* albuminome. Although these studies characterized the albuminome, each employed multiple steps to isolate and fractionate the HSA bound peptides and proteins, but what is required is a single step method with minimal fractionation steps. A quick method would be optimal especially when processing large numbers of samples for verification or validation of potential biomarker candidates found in the albuminome. Of course, HSA is also a dominant protein in other body fluids including cerebral spinal fluid (CSF). Analysis of CSF proteins has the potential to lead to discovery of biomarkers of neurological diseases or conditions [26]. Yet the CSF albuminome has yet to be investigated, even though the ratio of CSF HSA/serum HSA is currently used as an indicator of the integrity of the blood-brain barrier [27–29]. Thus, the approach of exploiting the albuminome as a means of identifying potential biomarkers is also relevant in this context. The goal is to find a protocol for the isolation of the albuminome that is generally adaptable for multiple body fluids.

The first goal of this study was to test the effectiveness of a new affinity-based method, utilizing ProteaPrep spin columns (Protea Biosciences), which employs a proprietary non-antibody based ligand-ligand interaction for capture of HSA and IgG. This commercial affinity matrix (<https://proteabio.com>) has not yet been investigated as a method of albuminome depletion/isolation from serum and was compared to four other commonly used depletion methods for depletion/capture efficiency of HSA and IgG. In addition we tested the effectiveness of two methods (ProteaPrep spin columns and Sigma anti-HSA/IgG spin columns) for depletion of HSA from CSF. Although the HSA (and total protein) concentration in CSF is ~100 fold lower than that in serum (the upper range of HSA concentration is approximately 0.5 mg/mL [30] compared to ~50 mg/mL in serum [2]) the relative abundance of HSA and other high abundant proteins in CSF is still high enough to potentially cause masking of low abundant proteins when searching for biomarkers of neurological disorders and recent studies have employed depletion of high abundant proteins in CSF to better characterize the CSF proteome [31–33]. The second goal of this study was to characterize the serum and CSF albuminomes isolated using selected methods from the first study and to determine the effectiveness and reproducibility of these methods and to compare the results between the methods used in this study, as well as to those previously published. The ProteaPrep and Vivapure spin columns were the only methods available with an HSA only option and these were selected for albuminome isolation and analysis from serum and the ProteaPrep column was selected for albuminome isolation and analysis from CSF. Part of our goal was to develop a method for albuminome isolation and analysis that required no fractionation and could be used to quickly process large numbers of samples.

We show that the albuminome proteins identified can vary depending upon the isolation method, although there is good overlap (81 proteins) for the serum albuminome isolated from both ProteaPrep and Vivapure spin columns.

2 MATERIALS AND METHODS

2.1 Materials and reagents used can be found in the Supplemental Material and Methods

2.2 Depletion of HSA and/or IgG from human serum and CSF using multiple depletion methods

Albumin and IgG were depleted from pooled human serum using seven different methods: 1) ProteaPrep HSA spin columns, 2) ProteaPrep HSA 96-well plate, 3) ProteaPrep HSA/IgG depletion column, 4) Protein G-NaCl/EtOH depletion method, 5) Vivapure anti-HSA/IgG depletion kit, 6) Sigma Blue HSA/IgG depletion kit, and 7) Sigma anti-HSA/IgG depletion columns. HSA was depleted from pooled human CSF using ProteaPrep HSA and Sigma anti-HSA/IgG spin columns. Depletion was carried out for all methods according to the manufacturer's protocol with slight modifications in some instances. Protein G-NaCl/EtOH method was carried out as previously described [19]. Control samples (no depletion) were prepared by mixing 10 μ L of serum and 100 μ L of CSF with 390 μ L and 300 μ L of 10 mM PBS, respectively. Protein concentration was determined by the BCA method for each depleted sample and samples were qualitatively analyzed by 1D-SDS-PAGE and immunoblot. HSA depletion efficiency was quantitatively analyzed by multiple reaction monitoring (MRM) for the three best HSA depletion methods from serum (ProteaPrep HSA/IgG, Protein G/NaCl, Sigma anti-HSA/IgG) and the two methods used for HSA depletion from CSF. A detailed description of the protocols for each depletion method and for SDS-PAGE and immunoblot analysis can be found in the Supplemental Material and Methods.

2.3 Isolation and LC-MS/MS analysis of serum and CSF albuminomes

Serum and CSF albuminomes isolated from ProteaPrep HSA columns and serum albuminome isolated from Vivapure anti-HSA columns were trypsin digested and analyzed in triplicate on an EASY-nLC 1000 connected to an Orbitrap Elite (Thermo) equipped with a nanoelectrospray ion source. The data is available in [34] PRIDE through the ProteomeXchange (<http://proteomecentral.proteomexchange.org/cgi/GetDataset>) accession number PXD000036. Detailed protocols for albuminome isolation as well as LC-MS/MS conditions and database search parameters can be found in the Supplemental Material and Methods.

3 RESULTS AND DISCUSSION

The “albuminome” refers to the proteome of albumin and its peptide and protein binding partners. It should be noted that observation of a protein in the albuminome does not necessarily mean that the protein is directly bound to HSA, as its presence could be due to an indirect association with another protein that is directly bound to HSA, for instance complexes that are associated with HSA in which one protein of the complex has direct contact with HSA but the other protein may not. Additionally, some proteins may be bound non-specifically to the capture resins used. However, we cannot distinguish these proteins

from the proteins directly bound to albumin. Therefore, reference to a protein being an albuminome protein refers to any protein that was eluted from capture resin.

3.1 Depletion of HSA from serum and CSF using ProteaPrep spin columns and ProteaPrep depletion plate and comparison of multiple HSA/IgG depletion methods

To determine the efficiency of HSA depletion using serum and CSF, the HSA depleted serum and CSF eluted from ProteaPrep spin columns and 96-well plate as well as HSA and IgG depleted serum from other methods were analyzed by SDS-PAGE and immunoblot using anti-HSA or anti-IgG antibodies (Figures 1 and 2, all methods) or using a MRM assay developed against HSA (selected methods) (Table 1). Based on the immunoblot data the ProteaPrep HSA spin column was observed to be equally effective for depletion of HSA from both serum and CSF but the ProteaPrep 96-well plate was not as effective for the depletion of HSA from serum. One probable reason for the decreased efficiency of the plate is due to the inability to mix the serum with the bead bed, which most likely affects the binding kinetics of HSA to the depletion ligand. Importantly, multiple passes of the sample through the well improved the effectiveness of the depletion to that seen with the spin columns. HSA depletion from CSF was roughly equivalent for the Sigma anti-HSA/IgG column and ProteaPrep HSA/IgG column and these two methods, in addition to the Protein G-NaCl/EtOH method, showed roughly the same effectiveness for HSA depletion from serum. Sigma Blue HSA/IgG and Vivapure anti-HSA/IgG columns showed lower depletion effectiveness. IgG depletion from serum was observed to be roughly equivalent across all methods, but this could be skewed due to the fact that the amount of IgG present will be dependent upon the effectiveness of HSA depletion, since we based our comparisons on total protein load.

MRM analysis of HSA was performed to quantitatively assess the HSA depletion efficiency of the ProteaPrep HSA column as well as the three most effective HSA/IgG methods. Percent depletion efficiency was determined as described in the methods and reproducibility was assessed for each method tested (Table 1). Sigma anti-HSA/IgG columns had the best depletion efficiency for serum at 99.8% but the ProteaPrep HSA and ProteaPrep HSA/IgG columns were only slightly behind at 98.1% and 99.1%, respectively, and the Protein G-NaCl/EtOH had the lowest efficiency at 90%. Depletion efficiency from CSF was comparable to that for serum using both the Sigma anti-HSA/IgG and ProteaPrep HSA columns (99.0% and 96.6%, respectively), indicating that albumin can be removed from multiple body fluids with equal efficiency.

The ProteaPrep HSA spin column performed the best in terms of reproducibility, with %CV of 16% for both serum and CSF. The Protein G-NaCl/EtOH method had the second lowest %CV at 27%, while the three HSA/IgG methods tested had a %CV above 40%. The spiked heavy peptide IS was used to calculate the instrument CV for each sample and was shown to be highly reproducible at the instrument level with %CV between 2.5 and 8.

3.2 Characterization of the serum and CSF albuminomes

CSF albuminome was isolated using ProteaPrep HSA columns and serum albuminome was isolated using both ProteaPrep HSA and Vivapure anti-HSA columns. Isolated albuminomes

HSA column as a method for albumin isolation when qualifying the NaCl/EtOH isolation method. Only 5 additional proteins overlapped with the current study and Zhou's study. The differences in proteins observed are potentially due to the different experimental conditions used in each study (i.e. serum source, albuminome isolation method, fractionation steps, MS instrumentation, search engines and search parameters). Zhou's study employed multiple conditions for albuminome release from a Montage Albumin Depletion Kit (Millipore) and reported that the albuminome proteins observed were dependent upon the release conditions used [8]. Additionally, Zhou *et al* limited their analysis to species smaller than 30 kDa, which most likely removed some of the larger more abundant proteins in the sample. Zhou also used multiple enzymatic stringencies, most likely resulting in different albuminome proteins observed in their study. The overall number of proteins reported in the current study is in line with the total numbers reported by Gundry and Zhou, even though the current study did not employ a fractionation step after albuminome isolation.

To date there has not been a characterization of the CSF albuminome and this study is the first to analyze this sub-proteome. Seventeen proteins identified by our investigation have not been reported in any previous study (current study included) and 67 of the 94 proteins have been shown to be expressed in brain or CSF, according to the HPRD and Uniprot databases, and some of these have known roles in neurological diseases. Amyloid beta A4 protein is known to play an integral role in the progression of dementia and Alzheimer's disease (AD) [35–39]. Only two peptides of amyloid beta A4 protein (T₅₁HPHFVIPYR₆₀ and C₆₁LVGEFVSDALLVPDK₇₆) were observed at relatively low abundance (3 and 7 spectral counts, respectively, over 9 total MS runs), suggesting that perhaps only a fragment of the protein (since both peptides are in sequence) is bound to HSA and this fragment is in low abundance. These peptides are not associated with AD, as amino acids 672–713 comprise the amyloid beta peptide (A β _{1–42}) responsible for plaque formation and symptoms of AD and dementia [40]. However, the association of amyloid beta A4 (or peptides from the protein) with HSA suggests that HSA may serve as a transport molecule for clearing degraded amyloid beta A4, perhaps rendering them unable to aggregate. Three chains of fibrinogen, alpha, beta, and gamma, were observed in the CSF albuminome with sequence coverage of 2%, 44%, and 39%, respectively, indicating that the beta and gamma chains are bound to HSA in their intact forms and the alpha chain may be bound as either peptides or a protein fragment. Given the fact that only two peptides covering 20 amino acids are observed for the alpha chain, we can postulate that peptides of the alpha chain (or possibly a fragment) are what are bound in the albuminome. Fibrinogen has been reported to interact with and induce oligomerization of A β _{1–42} [41] and fibrinogen deposition has been shown to accelerate neurovascular damage and neuroinflammation in mouse models of AD [42]. Additionally, fibrinogen gamma chain has been reported as a candidate biomarker for AD [43].

4 CONCLUSIONS

In this study we show that the ProteaPrep HSA and Vivapure anti-HSA spin columns are fast, easy, and reproducible methods for HSA capture, and hence depletion, from serum and CSF and are comparable to other methods in HSA depletion efficiency but require fewer steps for capture compared to previously published methods [6–9]. We have provided the

first study of the CSF albuminome and have identified 94 proteins bound in the CSF albuminome, 18 of which are unique to CSF and not present in the serum albuminome in either the current study or previous studies [6–9].

We show that albuminome isolated from serum or CSF can be quickly analyzed without the need for fractionation prior to MS analysis, reducing sample handling and processing time. Both the ProteaPrep HSA spin column and Vivapure anti-HSA column produced results that are consistent with previously published reports, even without fractionation prior to MS analysis, which was performed in most of the previously published studies. However, the amount of HSA present in our samples could potentially be an issue, as the high abundance of HSA can lead to saturation of the MS detector and cause some of the lower abundant proteins to be missed. In this respect it might be useful to employ a protein fractionation step prior to MS analysis. Consequently, this is a tradeoff that has to be assessed by the investigator and will depend on the biological question being asked. This study focused on analyzing a quick and easy method for albuminome analysis, one that could potentially be used to screen large numbers of samples with relative ease when searching for potential biomarkers in the albuminome or to assess whether a potential biomarker of interest is bound to albumin and in what relative abundance. Therefore, we did not carry out the fractionation step in this study given our goal was to find a method that offers fast and easy albuminome isolation, and we believe we have shown that this can be achieved using commercial spin columns. Although this study only focused on spin columns from Protea and Vivapure, it stands to reason that other commercially available methods could yield similar results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CSF	cerebrospinal fluid
HSA	human serum albumin

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Figure 1.

A) SDS-PAGE and **B)** western blot for depletion of HSA from serum and CSF using ProteaPrep spin columns and depletion of serum using ProteaPrep plate. Lane assignments: M – marker, H – HSA standard, 1 – un-depleted serum, 2 – HSA depletion from serum using ProteaPrep spin column, 3 – HSA depletion from serum using ProteaPrep depletion plate (1 pass), 4 – HSA depletion from serum using ProteaPrep depletion plate (2 passes), 5 – HSA depletion from serum using ProteaPrep depletion plate (3 passes), 6 – un-depleted CSF, 7 – HSA depletion from CSF using ProteaPrep spin columns, 8 – HSA depletion from CSF using Sigma anti-HSA/IgG spin column.

Figure 2.

Comparison of HSA/IgG depletion methods: **A)** SDS-PAGE silver stained gel, **B)** anti-HSA western blot, **C)** anti-IgG western blot. Lane assignments for each are as follows: H – HSA standard, Ig – IgG standard, 1 – non-depleted serum, 2 – ProteaPrep HSA/IgG, 3 – Protein G-NaCl/EtOH, 4 – Vivapure anti-HSA/IgG, 5 – Sigma Blue HSA/IgG, 6 – Sigma anti-HSA/IgG.

Figure 3.

A) Protein overlap for serum albuminomes isolated from ProteaPrep and Vivapure spin columns and CSF albuminome isolated from ProteaPrep columns in the current study. **(B)** Protein overlap of the 63 non-immunoglobulin serum albuminome proteins identified using both the ProteaPrep and Vivapure methods with the proteins identified in two additional studies (Gundry [6] and Zhou [8]). Gundry *et al* excluded immunoglobins from their final protein list and they were therefore excluded from the comparison. Albuminome proteins reported by Scumaci [7] and Lowenthal [9] were omitted from this comparison for reasons discussed above in the text.

Figure 4.

A) SDS-PAGE and B) anti-HSA immunoblot of serum albuminomes isolated from ProteaPrep HSA and Vivapure anti-HSA columns. M – Marker; H – HSA standard; 1 – ProteaPrep albuminome eluted in 25% ACN, 5% FA; 2 – Vivapure albuminome eluted in 100 mM Glycine/HCl pH 2.8; 3 – ProteaPrep albuminome eluted with 100 mM Glycine/HCl pH 2.8. Both methods appear to isolate the dimer and monomer forms of albumin and switching elution buffers did not appear to have an effect on the SDS-PAGE profile of the ProteaPrep albuminome.

Table 1

Assessment of HSA and IgG depletion efficiency of multiple depletion methods by MRM.

Depletion Method	Body Fluid	fmol HSA ^a	% HSA EFF ^b	% CV
Protea Spin HSA only	Serum	330	98.1	16.1
Protea Spin HSA only	CSF	560	96.6	15.9
Protea Spin HSA/IgG	Serum	150	99.1	50.0
Protein G-NaCl/EtOH	Serum	1180	90.0	27.1
Sigma anti-HSA/IgG	Serum	40	99.8	44.4
Sigma anti-HSA/IgG	CSF	160	99.0	60.0

^a 10 µg of depleted serum and CSF were digested, desalted, and eluted in 100 µL of 70% ACN, 1% FA. fmol HSA represents the amount albumin in 10 µg of depleted serum.

^b HSA depletion efficiency was measured as describe in methods. Non-depleted serum contained 17230 fmol of HSA per 10 µg of protein and non-depleted CSF contained 16460 fmol of HSA per 10 µg of protein.