

Analysis of Low-Abundance Serum Proteins Using Mass Spectrometry

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ABSTRACT

In order to detect early-stage diseases, new biomarkers are needed that have adequate sensitivity and specificity to be applicable in detecting diseases in a large population. The classical approach for identifying disease-related proteins is two-dimensional polyacrylamide gel electrophoresis, but this method is labor intensive, requires large amounts of protein, and is not easily adapted into a diagnostic test. Recent studies have reported using mass spectrometry (MS) to identify new serum biomarkers for breast and ovarian cancer. Serum contains 60-80 mg protein/mL, but 57-71% of this is serum albumin, and 8-26% is γ -globulins. These large proteins must be depleted before smaller less-abundant proteins can be detected using MS. Affinity columns are commonly used to remove both serum albumin and γ -globulins, but because serum albumin is known to act as a carrier for smaller proteins, removal of these molecules using columns may result in the loss of molecules of interest. The objective of this study was to develop a reproducible method to deplete serum samples of high-abundance proteins in order to identify the less-abundant proteins present in serum. We used organic solvents to precipitate the large proteins out of solution. This also caused many smaller proteins to dissociate from their carrier molecules, allowing for better detection of a larger range of small proteins. After precipitation, the supernatant was concentrated to 0.1 μ g protein/mL and acidified with formic acid. These samples were analyzed using liquid chromatography coupled with electrospray ionization mass spectrometry (LCMS). The data were collected using BioAnalyst QS software. An analysis of a 30 minute segment of our data, from 48 patients' sera, resulted in detection of an average of 4,482 molecular species with molecular weights between 500 and 10,000 Da.

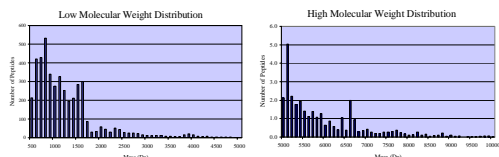
RESULTS

Figure 1: Optimization of acetonitrile precipitation.

Standard Method	New Method	Protein Precipitation Methods
Mixing after addition of Acetonitrile	Vortexing	
Gentle Mixing	Vortexing	
Chilly refrigeration	Glacier refrigeration	
higher protein concentration	lower protein concentration	
increased protein concentration	constant protein concentration	
Centrifugation		
4 min. at 12,000 rpm	10 min. at 12,000 rpm	
chilly refrigeration	chilly refrigeration	
Concentrated Volume of Supernatant		
50 μ l	200 μ l	
precipitate forms	no precipitate forms	

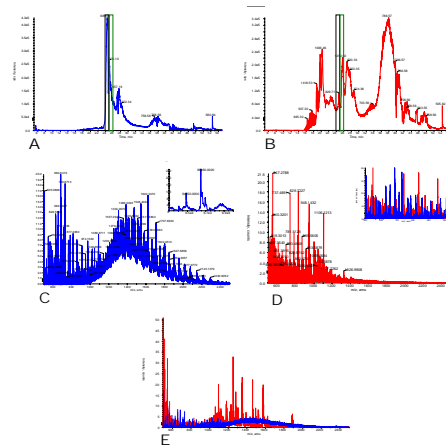
Standard method¹ involved adding 400 μ l of acetonitrile to 200 μ l of serum with gentle mixing. The mixtures were left at room temperature for 30' and then spun 4' at 12K rpm. The supernatants were removed via pipette and the volumes reduced to 50 μ l in a Speed Vac. The resulting samples were reconstituted to the original serum concentration with water. As shown in the figure on the right, the standard method varied greatly in protein yield. The table maps out the various factors tested and their respective effect on the reproducibility of protein yield. The new method involved adding 400 μ l acetonitrile to 200 μ l serum, vortexing vigorously for 5 seconds, and allowing room temperature incubation for 30' (conditions determined to be optimal). Samples were then spun for 10' at 12K rpm. The supernatant was concentrated to 200 μ l and protein assay performed. As shown in the figure, this new method results in a very reproducible protein yield. Prior to mass spectrometry, 4 μ g of protein were transferred to a new microcentrifuge tube and concentrated to near dryness. Because the precipitate that forms upon concentration is insoluble in water alone, 20 μ l 88% Formic Acid was added to the lyophilized samples and the total volume was brought to 40 μ l with HPLC water.

Figure 2: Molecular weight distribution of species observed in acetonitrile supernatant.



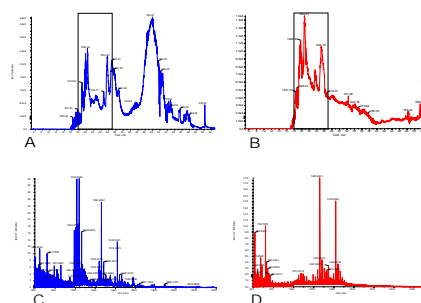
The LCMS reconstruct tool in BioAnalyst was applied to each sample run (average of 49 samples reported here) to determine the molecular weight of the species present in acetonitrile precipitated serum. The left panel shows the distribution of molecular species ranging from 500 to 5000 Daltons. The panel on the right shows the distribution of molecular species ranging from 5 to 10 kD. The majority of the species present are below 2000 Daltons. However, a significant number of species are between 2000 and 5000 Daltons.

Figure 3: Comparison of serum protein (1 μ g) before and after acetonitrile precipitation.



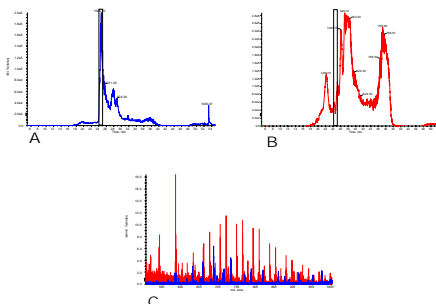
Capillary liquid chromatography, to fractionate or separate peptides and proteins, is performed using a 15 cm x 250 μ m i.d. capillary column, packed in-house using POROS R1 reversed-phase media, employing a 2.2%/min gradient to an organic concentration of 40% acetonitrile in 0.1% formic acid, followed by a 3.5%/min gradient up to a concentration of 95% acetonitrile. Chromatography uses an LC Packings Ultimate Capillary HPLC pump system, with a Famos autosampler, controlled by the mass spectrometer software (Analyst). The LC is coupled directly to the MS. Effluent from the capillary column is directed into a QSTAR Pulsar i quadrupole orthogonal time-of-flight mass spectrometer through an IonSpray source. Data is collected for m/z 500 to 2500 over the entire range of the chromatogram (58 min). Panels A and B show the total ion chromatogram (TIC) for serum protein before and after acetonitrile precipitation respectively. The averaged mass spectrum for the 1 minute retention time of 24 to 25 min of serum before ACN precipitation (Panel C) and serum after ACN precipitation (Panel D) is shown. Panel C contains an inset that shows the protein mass graph of the Bayes protein reconstruct performed on the mass spectrum from 900 m/z to 2500 m/z. The inset in panel D shows the expanded (500 to 1000 m/z) mass spectra overlay of the two samples. Panel E is an overlay of the mass spectra for the 1 minute retention time of 25 to 26 min of the two samples. Before ACN precipitation is shown in blue, after ACN precipitation is shown in red.

Figure 4: LCMS of acetonitrile supernatant compared to 30K NMW filtrate.



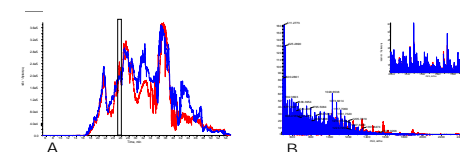
1 ml of serum was diluted with 5 ml of 25 mM ammonium bicarbonate and added to an appropriately conditioned 30 K NMWL centrifugal filter. After centrifugation, the filtrate was lyophilized to dryness and reconstituted to 40 μ l with 20 μ l 88% formic acid and 20 μ l water and the retentate was reconstituted to original 1 ml volume with HPLC grade water. 200 μ l of serum was subjected to the new method of acetonitrile precipitation outlined in Figure 1. 1 μ g of protein of each sample was loaded onto the column according to procedures outlined in Figure 3. The total ion chromatograms of ACN precipitated serum (Panel A) and reconstituted filtrate (Panel B) are shown. Panels C and D show the averaged mass spectra overlay for the 9 minute retention time of 18 to 27 minutes for the serum and the filtrate respectively. The ACN precipitated serum is shown in blue. The filtrate is shown in red. Comparison of panels C and D, shows that the ACN precipitation method is superior to the centrifugal ultrafiltration technique in both number of LMW species and intensity of peaks.

Figure 5: Examination of 30K NMW retentate before and after acetonitrile precipitation.



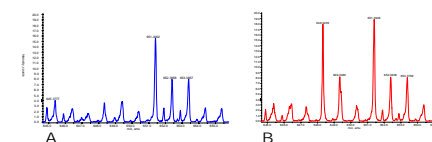
The total ion chromatograms of 1 μ g of retentate prior to ACN precipitation (Panel A) and retentate after ACN precipitation (Panel B) are shown (200 μ l of retentate was ACN precipitated as in figure 1). Panel C shows the averaged mass spectra overlay for the 1 minute retention time of 25 to 26 minutes. The retentate prior to ACN precipitation is shown in blue. The ACN precipitated retentate is shown in red. The presence of the high abundance proteins masks the information rich presence of small peptides.

Figure 6: Reproducibility of LCMS run-to-run from same sample.



The panel on the left overlays the TIC of ACN precipitated serum thawed just prior to analysis (blue) with the TIC of serum incubated at room temperature overnight (red). The panel on the right overlays the averaged mass spectrum (retention time 25-26 min) of the ACN precipitated fresh serum (blue) with the averaged mass spectrum (same retention time) of the room temperature incubated serum (red). The inset in the right panel shows the expanded mass spectrum (1000 to 1100 m/z). Even with less than careful handling of the serum, the mass spectra of the samples match closely with only slight variation.

Figure 7: Variability of LCMS from one individual to another



The same retention time and m/z range are selected from chromatographic runs of the serum of patient A and patient B. As shown, the variations in the make up of the serum from individual to individual can be detected. These differences represent a potential source of serum biomarkers.

CONCLUSION

In order to use the information-rich proteomic analysis of serum in a diagnostic manner, it is essential that the method used to prepare the sample provide reproducible results. The new method of acetonitrile precipitation described here admittedly precipitates more protein than the standard method, as evidenced by the decreased protein yield and the molecular weight distribution of species present. Analysis reveals that the majority of the visualized peptides and small proteins have a molecular weight between 500 and 5000 Daltons. However, the consistency of protein yield and reproducibility from sample to sample suggests that the treatment of the sample using the new method is more uniform and therefore a more reliable diagnostic pattern can be developed. Perhaps more importantly, the new method of acetonitrile precipitation appears to disrupt the intermolecular reaction of small proteins and peptides from the large carrier proteins providing a more complete analysis of the low molecular weight biomarkers than centrifugal ultrafiltration.²

REFERENCES

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