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A fully integrated multi-column system for abundant protein depletion from serum/plasma

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ABSTRACT

This work details the transformation of a conventional HPLC system to a low back pressure liquid chromatography set-up for automated serum/plasma depletion and fractionation. A Dionex U3000 HPLC was converted to low back pressure operation (125 psi max) by replacing all narrow-bore lines to larger inner-diameter tubing. The system was configured to use two immunoaffinity columns, first for depletion of the top 14 most abundant proteins (Seppro IgY14), then for the next 200–300 proteins (Seppro SuperMix). The autosampler was dual-purposed for both injection and fraction collection. Both the flow-through and SuperMix bound proteins were collected in an automated fashion. Three samples could be depleted consecutively before the system required user intervention, and up to nine samples could be depleted within a 24 h period. This study documents the validation of the instrument performance with a 90-patient sample set, demonstrating overall CVs for 86 of the 90 samples to be within the 95% confidence intervals. Additionally, there was excellent reproducibility within the same patient (biological replicates) across days.

1. Introduction

For the past thirty years, researchers have been intently focused on delving into the depths of the human proteome. The most commonly available samples for this effort are serum and/or plasma. These media are easily acquired, are high-content sources of protein, and they are postulated to be representative of the entire proteome [1]. As the plasma proteome may contain proteins, or degradents thereof, which have leached from tissues and cells, as well as contain the circulated proteins, it is the most comprehensive sample for informing on numerous disease states [2]. The serum proteome is believed to contain millions of proteins (including isoforms) [1], and this complexity provides unique challenges to researchers. By far, one of the greatest hurdles in proteomics is the wide range of abundance levels of the proteins at any given point in time [2]. The dynamic range for the plasma proteome spans at least 12 orders of magnitude [1], and the concentration of individual proteins is variable depending on gender, age, ethnicity, disease state, etc.

Initial studies of the proteome examined the intact proteome in an attempt to deconvolute its complexity and identify its components. Those pioneering attempts relied on two-dimensional gels for separation, and the Anderson lab was the first to apply this technique to the inquiry [3]. This technique worked to separate the diversity of protein isoforms, which by 1991 tallied 727 spots corresponding to 49 different proteins [4]. The separation achieved at the time was seen as the limit for unseparated plasma due to the presence of high abundance proteins. Therefore, effort shifted to selective depletion of protein species from plasma in order to access the lower depths of the proteome. The first documented case was depletion of immunoglobulins from human serum using protein A/G prior to chromatographic separation and mass spectrometric detection [5]. The first demonstration of multi-component immunoaffinity depletion followed a year later with the use of columns to remove the top ten most abundant proteins [6].

Since this study, there have been a plethora of technologies introduced to the field to deplete single or multiple sets of the most abundant proteins. Today, there are a range of kits and columns for removing one or more of these abundant proteins. Spin columns were designed for small-scale sample preparation, and many of these were converted into continuous flow chromatography formats. All of the formats detailed below are for human sample depletion; however, there are some resources also available for selected animal models, such as mouse, rat, and bovine. The next sections provide a brief overview of the use of these technologies in the field.

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The simplest of the depletion formats available today come in the form of kits or columns that are designed to remove a specific type of protein or class of proteins. There are a number of labs that have reported on the use of technologies, the most common of which are for albumin [7–9] and immunoglobulins (IgA/G) [5,10]. This depletion format is very efficient at removing the targeted protein, or protein class; however, they are limiting when a more encompassing depletion strategy is required.

For initiating a broader depletion strategy, immunoaffinity resins with a combination of antibodies are generally used. These resins are usually available in both small-scale (spin column) and large-scale (LC column) formats. The small-scale sample preparation devices, which have now been developed by a number of companies, support depletion of serum or plasma. The spin column technology is a manual process, where each sample is depleted in series on the bench. The advantage of such columns is that the depletion is relatively fast and can be used with limited sample amounts. There are numerous publications in the literature that document the use of a variety of spin columns, such as the Agilent MARS [11], Beckman Coulter ProteomeLab IgY12 [11,12], GenWay Spin IgY12 kit [8], and the Sigma ProteoPrep 20 versions of the depletion platform [13,14]. There are a number of limitations to this technique, which include low-throughput, incompatibility with automation, and increased risk of sample-to-sample preparation errors and variability.

The other common mode of depletion is via column chromatography. The Agilent MARS depletion column has been very popular for removal of the top six most abundant proteins in plasma (albumin, transferrin, haptoglobin, alpha-1 antitrypsin, IgA, and IgG) [15–17]. Since the introduction of its first depletion column, Agilent has extended their offerings to a top-7 and top-14 depletion column, whose use are being demonstrated in the literature [18]. There are a range of other commercial and in-house columns being used in the field, including a Beckman Coulter IgY12 column that depletes the top 12 proteins [19], and a Sigma–Aldrich column that removes the top 20 proteins [20]. Our lab has also investigated the use of the Sigma Proteo20 column for use in depletion. Another, more recent technology in use is the GenWay Seppro IgY12/SuperMix columns that are designed to remove first the top 12, followed by the next top 45 abundant proteins [21]. A similar column combination, which is currently marketed by Sigma–Aldrich, is currently being used in the field [22], as well as in our laboratory. Alternative approaches include the coupling of different chromatography chemistries with depletion chromatography [23]; as well as a medium-throughput method that uses affibody molecules as affinity ligands to deplete serum followed by chromatography [24]. Finally, a newer approach couples Affi-gel blue affinity columns with an organic solvent (acetonitrile) precipitation [25].

The immunoaffinity columns, because they contain antibodies, must be operated at low pressure so that the proteins do not become denatured during use. The AKTA FPLC chromatography (GE healthcare) systems are popular choices for this type of depletion application [15,17,26], and they are recommended as the system of choice by a number of depletion column manufacturers (GenWay, Beckman). While the systems are easy to use, they are not usually equipped with auto-samplers or chilled fraction collectors. This limits the amount of automation that can be incorporated into a process, as well as requiring a significant amount of operator time to obtain the desired depleted fractions. There have been reports about using an Agilent 1100 HPLC with auto sampler for a single-column immunodepletion step [16,18,19], as well as a Hitachi Elite HPLC [27,28]. Two reports were identified where an Agilent 1100 was used with a single immunodepletion column, and incorporated automated sample injection and subsequent fraction collection [29,30]. Finally, there is a report using chromatography to link columns in series to achieve protein depletion of the glycoproteome [31].

Here, the authors present an HPLC configuration that incorporates the multi-dimensional capabilities of a Dionex U3000 HPLC for the automated injection of sample onto a two-dimensional, multi-column depletion configuration, followed by fraction collection of flow-through and bound protein components. This instrumentation overcomes the limitations of other systems by eliminating the need for manual injection of samples, provides automated fraction collection from both flow-through, as well as column-bound proteins, and allows for up to three samples to be processed as a batch before user intervention is required.

2. Materials and methods

2.1. Materials

Protein depletion columns (Seppro™ IgY14 LC10 and SuperMix LC5), stripping (1 M glycine, pH 2.5) and neutralization (1 M Tris–HCl) buffer concentrates were obtained from Sigma–Aldrich (St. Louis, MS). The elution buffer (150 mM NH_4HCO_3 pH 7.5) was prepared in-house with chemicals from Fluka (St. Louis, MS) and HPLC-grade water from an in-house milli-Q system (Barnstead, Dubuque, IA). Human patient serum was acquired from an in-house tissue repository. The repository consists of commercially acquired human samples that have been fully consented for research purposes. A total of 30 serum samples, distributed evenly (ten each) from three different cohorts (normal, breast and ovarian cancer) were obtained.

2.2. Methods

2.2.1. Sample preparation

Approximately 1 mL of each sample was divided and loaded onto two 500-μL, 0.22-μm spin filters (Millipore, Billerica, MA). The filters were spun at 13,000 rpm (GeneVac, Gardiner, NY) for 60 min. The clear serum was transferred to new glass vials, and any sample that still showed residual liquid on the top of the filter was spun for another 60 min. The filtrates were pooled and the total volume was split into 250 μL aliquots to create three "biological" replicates and one back-up sample (archive). Each replicate was diluted in an amber glass vial with 1250 μL of elution buffer (NH_4HCO_3, 150 mM, pH 7.5). Samples were stored, frozen at −80 °C until depletion.

2.2.2. Depletion

The serum depletion study was accomplished using a Dionex U3000 (currently Thermo Fisher Scientific, Sunnyvale, CA) HPLC system and the Sigma Seppro™ IgY14 LC10 and Seppro™ SuperMix LC5 columns. All samples were serially depleted through the two columns; first by the Seppro™ IgY14 LC10, then by the Seppro™ SuperMix LC5 column. Fractions were successively collected from the flow-through of both columns, and the stripping eluant from the SuperMix LC5 column.

2.2.3. Instrument design

As indicated above, a standard Dionex U3000 HPLC (consisting of FLM-3100, WPS-3000T and LPG-3600 components; calibration cartridge bypassed) was converted for use as a low back pressure serum depletion/fractionation system. The Dionex U3000 was modified to operate at low back pressure (≤125 psi) by replacing all the narrow-bore 0.005 in (0.125 μm) ID tubing (IDEX Corp, Oak Harbor, WA) with 0.013 in (0.325 μm) ID tubing (Dionex Corp, Sunnyvale, CA). This enabled high flow rate operation with a software-controlled upper pressure limit of 125 psi. The flow-rates employed using this configuration ranged from 1.0 to 2.2 mL/min.

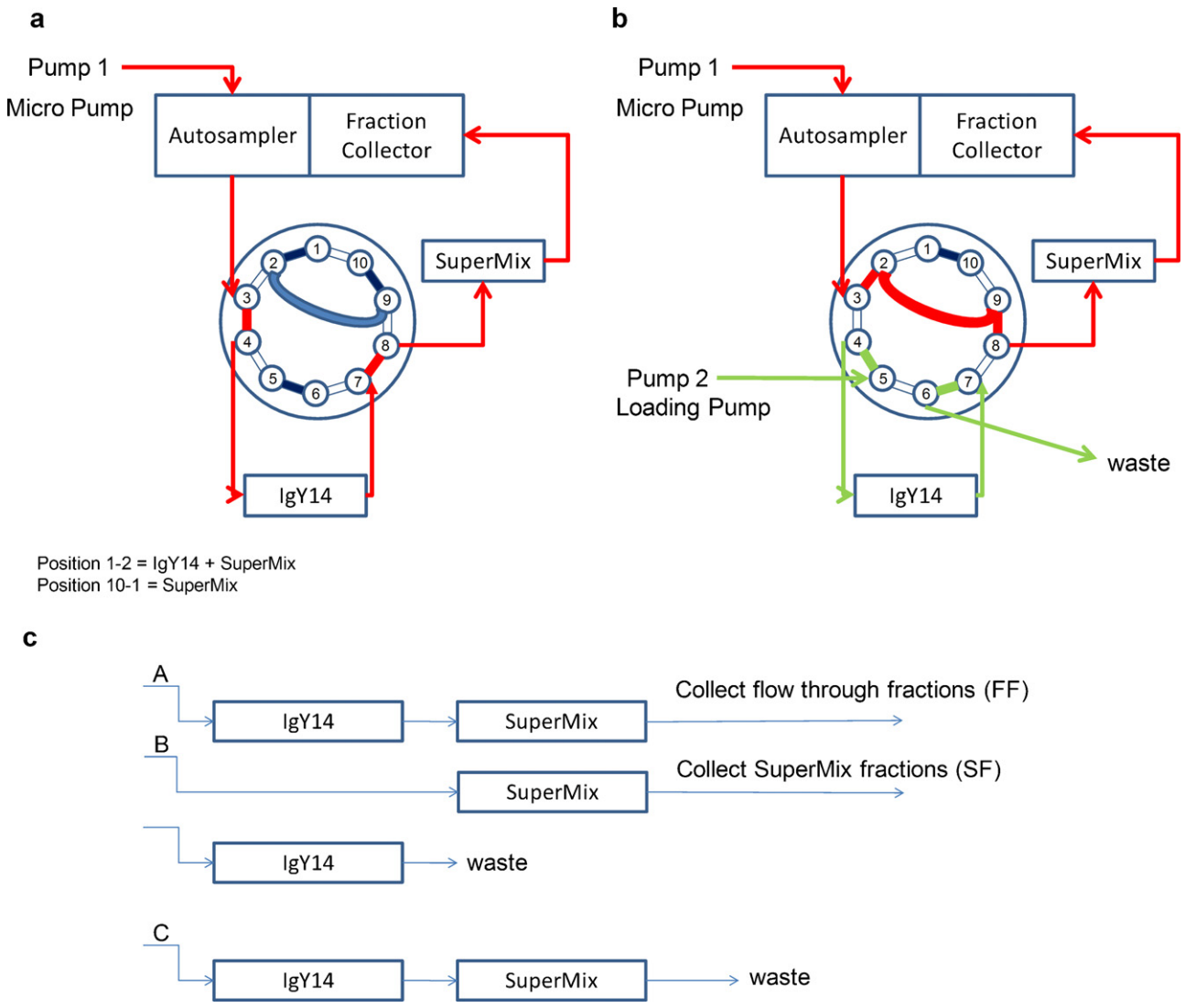

Fig. 1. The flow path design for the Dionex U3000 for serum depletion: (a) plumbing and column connections to allow collection of fractions through both columns (red pathway); (b) plumbing and column connections to allow collection of fractions through SuperMix column (red pathway) while stripping IgY14 column (green pathway); and (c) the three position and flow pathways available during depletion.

These flow rates and collection volumes were based on manufacturer's recommendations supplemented by preliminary experience with non-study sample sets. Each depletion run lasted ~75 min, allowing for as many as nine samples to be depleted in a 24 h period.

The flow path was designed to incorporate either serial or independent elution across the two depletion columns. Fig. 1 illustrates the valve set-up and flow path options through the Dionex U3000 HPLC system. In more conventional applications, the two pump heads of a U3000 are operated independently (for example, to achieve higher throughput), and one of the pumps is typically equipped with an integrated flow splitter to facilitate μL/min flow rates. In the current configuration, the splitter was removed. For convenience, the two pumps will be referred to as loading pump (typically split-less) and micro pump (usually split). The valve configuration and the flow paths are presented in Fig. 1, where the use of both micro and loading pumps, as well as the position of the two depletion columns is shown. The flow path leading to fraction collector shown in red has either both antibody affinity depletion columns in-line (IgY14 followed by the SuperMix LC5 column, valve position 1–2, Fig. 1a), or it has just the SuperMix LC5 column (valve position 10–1, Fig. 1b). Fig. 1c shows the three flow configurations the system uses throughout a depletion run. For each depletion, the serum sample was injected and loaded using buffer A (150 mM NH_4HCO_3) at 1.0 mL/min (micro pump). The serum sample was depleted across both columns and fractions were collected (Fig. 1a and c, configuration A) into 10 mL glass vials approximately every 8.5 min. The collection of the first fraction was triggered by time and each subsequent fraction was triggered by a software set maximum volume of 8.5 mL. This resulted in three flow through (FF) fractions, which were later combined to result in one low abundance protein (LAP) fraction. At approximately 30 min post-injection, the valve was switched taking the IgY14 column out of the flow path, and proteins bound to the SuperMix LC5 column were eluted and collected (SuperMix fractions SF) (Fig. 1b, red flow path). During this time, the IgY14 column was stripped (via the loading pump, green flow path), with eluant going to waste (Fig. 1b and c, configuration B). The two fractions collected from stripping of the SuperMix column (SF) were combined to give one medium abundance protein sample (MAP). The flow in both paths was 2.2 mL/min and the columns were exposed to stripping buffer (0.1 M glycine, pH 2.5) to facilitate protein elution. Finally, the two columns were put back in-line and re-equilibrated using neutralizing buffer (0.1 M Tris, pH 8.0) before the next run (Fig. 1c, configuration C). The column

Table 1
Analysis of the AUC for UV trace in the flow through fraction. The measurements are the average of three injections.

	Cohort 1			Cohort 2			Cohort 3		
	Average (mAU min)	SD (mAU min)	CV (%)	Average (mAU min)	SD (mAU min)	CV (%)	Average (mAU min)	SD (mAU min)	CV (%)
1	619.25	135.13	21.8	1164.90	99.85	8.6	795.84	59.24	7.4
2	1084.12	158.57	14.6	559.92	57.68	10.3	835.03	25.05	3.0
3	540.70	44.32	8.2	766.55	23.57	3.1	577.14	57.78	10.0
4	482.68	10.18	2.1	557.36	61.31	11.0	622.14	112.62	18.1
5	971.22	95.71	9.9	744.45	105.87	14.2	510.02	67.71	13.3
6	612.97	72.72	11.9	480.94	151.55	31.5[a]	514.17	1.10	0.2
7	1188.41	152.15	12.8	656.50	176.75	26.9	678.71	101.65	15.0
8	639.28	33.96	5.3	687.95	143.22	20.8	631.16	136.34	21.6
9	462.06	106.61	23.1	848.82	200.00	23.6	719.37	37.61	5.2
10	290.95	10.89	3.7	649.89	128.34	19.7	561.89	92.89	16.5
Min	290.95	10.18	2.1	480.94	23.57	3.1	510.02	1.10	0.2
Max	1188.41	158.57	23.1	1164.90	200.00	31.5[a]	835.03	136.34	21.6
Mean	689.17	82.02	11.3	711.73	114.81	17.0	644.55	69.20	11.0

[a] The UV detector did not autozero before start of the run.

compartment was kept at a temperature of 25 °C during depletion runs and lowered to 6 °C when not in use. This allowed for seamless operation without disconnecting and reconnecting the columns every day. The buffers were prepared fresh daily. The script for the HPLC method is listed in the Supplemental information, Fig. 1.

2.2.4. Post-depletion processing

The three flow-through fractions collected during the depletion run, with an approximate volume of 8.5 mL each, were combined and concentrated via vacuum centrifuge (miVac Duo Concentrator, GeneVac, Gardiner, NY) to a volume below 1 mL. The final volume of each sample was measured using a 200 μL pipette and the concentrations were measured using a NanoDrop ND-1000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, DE, see concentration measurement section below for details). The two SuperMix fractions (SF) collected during the depletion run, with an approximate volume of 8.5 mL each (17 mL total), were combined and dialyzed into 150 mM NH_4HCO_3 using dialysis cassettes (3500 MWCO, 12–30 mL, Thermo Scientific, Vernon Hills, IL) and two exchanges of the dialysate (4 L for 4 h, 4 L overnight). The samples were concentrated as above to a volume below 1 mL, and the volume and concentrations measured as for the FF fractions. All samples were frozen at −80 °C until further analysis.

2.2.5. Concentration measurements

Concentration measurements were performed on the serum samples before and after depletion. The measurements were obtained on a NanoDrop ND-1000 UV–vis spectrophotometer capable of full spectrum measurements (220–750 nm). For these studies, a Protein A280 method using BSA as a reference was employed, in which unknown (the sample) protein concentrations are calculated using the mass extinction coefficient of 6.7 at 280 nm for a 1% (10 mg/mL) BSA solution. Prior to every group of measurements, two BSA standards (1 and 2 mg/mL) were run in triplicate to ensure consistency of instrument performance. The concentrations were validated against a standard curve of bovine serum albumin standards (ranging from 125 to 2000 μg/mL) (Thermo Scientific, Rockford, IL). The serum samples were analyzed (in triplicate) by pipetting 2 μL of sample onto the pedestal and taking the average of the three readings. All of the concentration measurements are provided in Supplemental information, Table 1.

3. Results and discussion

This work was undertaken to try to overcome some of the limitations reported in the literature for chromatographic serum depletion of high abundance components. These include manual injection and/or fraction collection, issues with coupling multiple columns onto a single liquid chromatograph for separation, and run-to-run reproducibility. These issues were overcome using a reconfigured U3000 HPLC. The system was modified for low back pressure operations (<125 psi), while software and hardware enhancements (commercially available from Dionex), were installed to utilize the autosampler as both sampler and fraction collector. With the use of the quaternary pumps, it was possible to install and independently operate two depletion columns and automatically collect fractions from the flow-through, as well as one of the depletion columns. Finally, it was possible to run batches of three samples in series and collect all fractions of interest before user intervention was required. It was possible to deplete up to nine samples per day (with a work-day defined as 9 h), with the last three samples being set up to run overnight. This set-up allowed for 90 patient samples to be depleted in less than one month using one HPLC system and one set of columns.

To test the utility, robustness, and reproducibility of the system design, a study set comprised of serum samples was acquired and depleted. The samples consisted of serum from 30 patients, equally distributed between three different cohorts (normal, ovarian and breast cancer). The measured concentrations (after spin filtration) of the total protein in the serum samples ranged from 16 to 100 mg/mL and there was no correlation of protein concentration to cohort membership. After the samples were aliquoted and diluted as described in Section 2, the samples were given identification numbers and randomized; an effort was made to make sure no two samples from the same patient were depleted on the same day. This precaution was taken to ensure that 2/3 of any patient sample was not lost due to system failure on a given day. With the fractionation of the patient serum into three biological replicates, a total of 90 samples were depleted in this study. The total elapsed time between depletion of the first and the last sample was 29 days. The parameters were set for collection of both FF and SF fractions by time in such a way as to collect the eluting proteins completely.

Collation of the results from the depletion study yielded some interesting results. First, the depletion profiles for individual samples were distinctive, and an example of three profiles is shown in Fig. 2. While the chromatographic profiles were quite diverse in the aggregate, individual patient samples were quite reproducible from one replicate to the next, even over extended periods of time, and continued use of the columns. Fig. 3 shows a representative set of replicate analyses from a single patient. The chromatographic fidelity between replicate analyses (from depletions 16, 66 and 84) is evident in the high degree of overlap between the three profiles. For this patient, the area under the curve (AUC) for FF samples averaged 1188.41 mAU min and for SF samples 228.78 mAU min

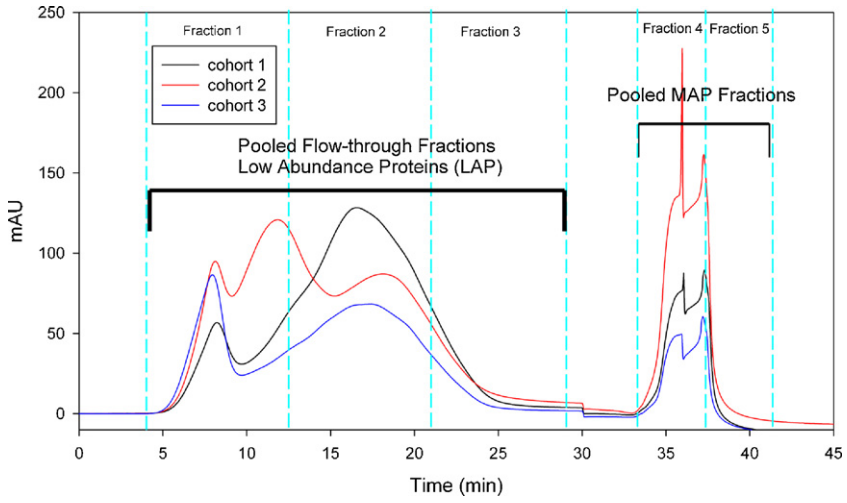

Fig. 2. Three $UV_{280\,nm}$ chromatograms corresponding to one depleted sample per cohort.

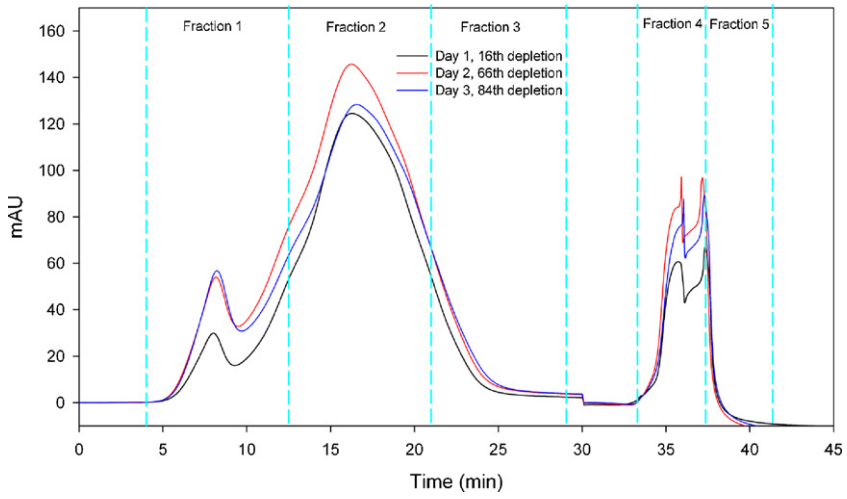

Fig. 3. Three $UV_{280\,nm}$ chromatograms corresponding to the three replicates from one patient. The depletions correspond to HPLC runs 16, 66, and 84 from the study.

with coefficients of variance (CV) of 12.8% and 16.4% respectively. A more complete assessment of the reproducibility of the method can be obtained by integration of the chromatographic profiles for all 90 samples. The AUC measurements for both the FF (Table 1) and SF (Table 2) fractions show that the mean coefficient of variance for all measurements was 13.1% for the FF samples, and for the SF samples was 15.6%. Much of the increase in the CV of the SF fractions is attributed to a single run, in which an overpressure shutdown of the system prevented complete recovery of the SF fraction. The second largest error was attributed to an erratically performing UV detector for sample P16B01. Excluding these two runs, the mean CV for the FF fractions was 12.5% and for the SF fractions was 12.9%.

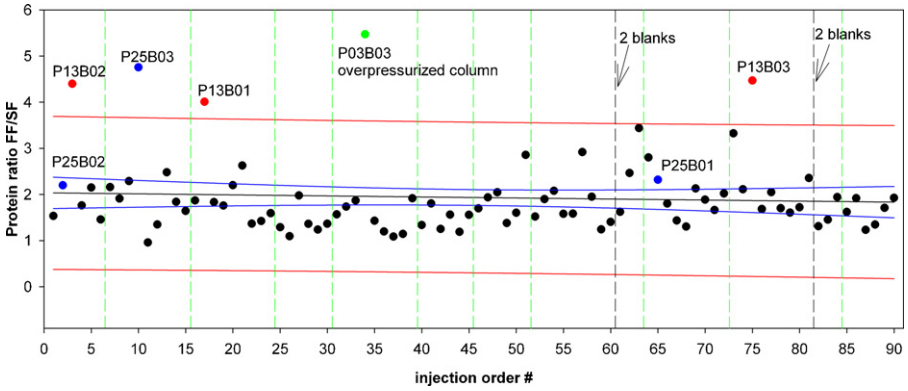

Fig. 4. The ratio of measured recovered amount of protein vs. the order of injection. Green dotted vertical lines denote each day of sample sets. The black dotted vertical lines show the place of injections of blanks. Red dots show patient P13 biological replicates, blue dots are for patient P25 replicates and the one over-pressurized sample for patient P03 is shown as green dot. The blue lines define 95% confidence intervals while red lines are 95% predicted confidence interval.

Table 2
Analysis of the AUC for UV trace in the SuperMix-bound fraction. The measurements are the average of three injections.

	Cohort 1			Cohort 2			Cohort 3		
	Average (mAU min)	SD (mAU min)	CV (%)	Average (mAU min)	SD (mAU min)	CV (%)	Average (mAU min)	SD (mAU min)	CV (%)
1	150.58	31.98	21.2	403.81	22.84	5.7	148.95	10.11	6.8
2	229.41	17.35	7.6	203.04	28.38	14.0	205.94	20.38	9.9
3	146.56	88.33	60.3[a]	87.94	12.00	13.6	160.98	19.77	12.3
4	148.70	20.96	14.1	126.67	6.03	4.8	133.34	28.52	21.4
5	230.26	39.61	17.2	222.19	20.98	9.4	87.66	18.90	21.6
6	128.69	15.42	12.0	230.58	107.75	46.7[b]	80.33	4.53	5.6
7	228.78	37.41	16.4	285.39	51.10	17.9	192.64	11.90	6.2
8	188.11	8.77	4.7	236.94	36.21	15.3	122.91	26.45	21.5
9	63.73	16.92	26.5	133.70	19.40	14.5	118.21	9.73	8.2
10	75.06	1.63	2.2	151.84	25.18	16.6	180.25	26.28	14.6
Min	63.73	1.63	2.2	87.94	6.03	4.8	80.33	4.53	5.6
Max	230.26	88.33	60.3[a]	403.81	107.75	46.7[b]	205.94	28.52	21.6
Mean	158.99	27.84	18.2	208.21	32.99	15.8	143.12	17.66	12.8

[a] The system over-pressured during switch to SF fraction.
[b] The UV detector did not autozero before start of the run.

While evaluation of the individual biological replicates provides some measure of the system performance over time, there was no individual sample or reference standard available to quantitatively establish this metric. Due to the intrinsic variability between patients, the relative efficiency of the system was monitored by calculating the ratio of the protein recovered in the FF to the SF fraction. This ratio is plotted against injection number in Fig. 4, and there are several points to comment upon. First, there is no clear trend that the system performance degrades over time, nor is there any evidence of periodicity in the data that might indicate bias in the recovery of either fraction with more or less system use in any defined interval. The vast majority of the data (85 of 90 data points) fall within the 95% predicted confidence interval defined by the red lines[2]. Three of the five outliers (shown in red) belong to the replicate analyses of the same individual, which may indicate some anomaly with the collection procedure, the sample provenance, and/or the general health/disease state of the particular patient. The fourth outlier (green point) is due to the previously described system overpressure which adversely impacted recovery of the SF fraction. Therefore, the only statistically significant point for which no obvious explanation is available is a single replicate of one patient, who's other two values fall very close to the mean (data points in blue). Blank injections were not run between regular sample depletion runs since column manufacturer cautioned about total number of injections (full cycle runs) that can be performed on the columns [32,33] and that number combined with 90 sample runs would exceed this number. The blank injections were used sparingly (prior to depletions 61 and 81) to test the system efficiency and they did not seem to alter it in any appreciable way.

Next, the impact of the sample loading on columns was tested. The original concentrations of proteins measured in starting plasma samples varied between 16 and 100 mg/mL. There is a negative correlation between the FF/SF ratio and the sample loading, as shown in Fig. 5. Using the Pearson product correlation, a correlation coefficient of −0.46 was computed with a P value of 4.8E-6 for all 90 data points. Even discounting the outliers described previously, higher sample load results in slightly reduced FF/SF ratios. Much of the variance observed at lower loadings is postulated to arise from inaccuracies in the concentration measurements, many of which were near the lower limit of quantitation for the ND-1000 instrument and method. These data may indicate some saturation or capacity limitation of the SuperMix column. However, it is also possible that the columns were not properly sized, relative to each other. If the higher loading resulted in overload of the IgY14 column, then the possibility exists that the uncaptured high abundance proteins (HAP) may have interfered with the performance of the SuperMix column. These hypotheses are pending more detailed analysis from subsequent mass spectrometry analyses that are currently underway in our laboratory. Caution is also advised in over-interpreting this data, as it does represent only a single pair of columns from specific lots, and there is no evidence in hand that would indicate that this is a systemic or recurring issue. As shown in Fig. 4, the P03B03 sample lays outside of 95% prediction band. Similarly, all replicates for patient P13 are outside of this band. The concentrations of the SF fractions for this patient were low resulting in an elevated ratio. These low measurements are in line with observed AUC for the SF fractions for this sample.

Although a high degree of automation was achieved using the converted HPLC system, user intervention is still required every few hours (three depleted samples) to remove collected fractions and place new fraction collection vials in the autosampler/fraction collector. The second limitation is the failure to collect fractions of HAP proteins stripped from IgY14 columns, which could be potentially interesting. This was a design decision, stemming from the hypothesis that there would be limited biological interest in these fractions. We have subsequently re-designed the pump configuration/programming to allow for future collection of all three sets

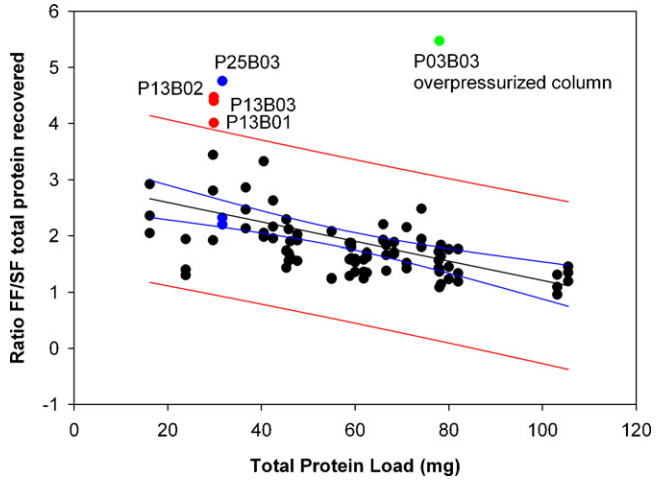

Fig. 5. The ratio of measured recovered amount of protein vs. total protein load. Sample and confidence intervals defined as in Fig. 4.

[2] Prediction confidence interval: the confidence interval for the population gives the range of variable values computed for the region containing the population from which the observations were drawn, for the specified level of confidence.

of fractions. However, this will have a modest adverse impact on throughput due to a longer gradient, and increased operator intervention for collection vial replacement.

4. Conclusion

This study has demonstrated the utility of converting a standard HPLC system for automated serum depletion. The system was tested with the depletion of 90 study samples, over the course of a month, and included the fraction collection of depleted serum as well as proteins bound to the SuperMix LC5 column. The assessment of the reproducibility of the method obtained by integration of the chromatographic profiles for all samples led to CV of 13.1% for flow-through fractions and 15.6% for SuperMix bound fractions; much of the increase in the CV was due to one overpressure error and one run with erratically performing UV detector. There was no evidence of the system performance degrading over time, nor any indication of periodicity in the recovery of neither fraction. The limitations of this current system include the number of samples that can be depleted before user intervention is required (three), as well as the inability to automatically collect the proteins bound to the IgY14 column.

A solution to the second of these issues is currently being investigated in our lab with the use of a second autosampler. With this type of arrangement, one sampler would be dedicated to injection, while the second would collect fractions. This would allow for either the same number of patients to be depleted while collecting fractions from all three sources, or for an additional patient to be depleted with the flow-through and SuperMix fractions being acquired.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.010.

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