Binding Capacity Assessment and Optimization for the Multiple Affinity Removal System

Application

Bio/Proteomics

Author

Kelly Zhang
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

The Agilent Multiple Affinity Removal System combines the specificity of antibody-antigen recognition with the efficiency of standard liquid chromatography instrumentation. A ready-to-use high-abundance protein removal kit containing an affinity column and optimized mobile phases (Buffers A and B) is available for simultaneous removal of multiple proteins from human serum. The column removes albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin in a single step with high specificity and reproducibility. The buffers are optimized to minimize removal of proteins not targeted by the antibodies. Higher capacity to accommodate larger volumes of human serum per injection would, no doubt, speed up sample preparation for biomarker investigations. In this application note, we demonstrate a number of ways to optimize column binding capacities. First, two identical columns can be coupled so that the resulting tandem column can have twice as much capacity as a single column. Second, a custom column with 10 × 100-mm dimensions has a capacity about 10 times that of a 4.6 × 50-mm column. Finally, optimal binding capacity can be obtained by injecting samples at a low flow rate.

Introduction

The Agilent Multiple Affinity Removal System simultaneously removes multiple high-abundance proteins from human plasma/serum in a single sample injection. This system comprises an affinity column and optimized mobile phases (Buffers A and B). The affinity column is packed with immobilized affinity-purified polyclonal antibody resins for removing albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin with high specificity. The percentage of each antibody packing material mirrors the amount of corresponding proteins found in human serum. Therefore, passing through a single column, all six targeted proteins are captured and removed from the mixture simultaneously. Mobile phases (Buffers A and B) are optimized to minimize removal of proteins not targeted by the antibodies and to minimize binding of low-abundance proteins to albumin. The buffers were also chosen to ensure reproducibility of column performance and long column life. As a result, this technology, by removing the interferences, enables expansion of the detectable dynamic range of current protein separation methodologies, such as one-dimensional gel electrophoresis (1DGE) [1], two-dimensional gel electrophoresis (2DGE) [2], capillary electrophoresis (CE), and liquid chromatography/mass spectrometry (LC/MS).

Column capacity is defined as the injection volume of human serum from which 98%–99% of targeted high-abundance proteins are specifically removed by the affinity column. Given the high level of

albumin (>54%) in serum, human serum albumin (HSA) was used for assessment of column capacities after a Multiple Affinity Removal column is packed with a mixture of immobilized antibody resins. The column capacity for a given batch is defined and specified in the certificate of analysis that is supplied with the column. Typically, it is 15–20 µL and 30–40 µL of human serum for a 4.6 × 50-mm and 4.6 × 100-mm column, respectively.

Experimental

The Multiple Affinity Removal System for removing albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin from human serum, and HSA, as a solution of 20 mg/mL in Buffer A, are products from Agilent Technologies (Wilmington, DE). A 4.6 mm × 50-mm Multiple Affinity Removal column with binding capacity for 0.6–0.9 mg of HSA or 15–20 µL of human serum was used. A mobile phase reagent kit (Agilent Technologies) for the affinity column was used for sample loading, washing, and column regeneration (Buffer A) and for bound-protein elution (Buffer B) from the column. Injections of a diluted human plasma sample were performed under standard LC conditions with flow-through fractions collected at 1.5–4.5 min automatically into 1.5-mL plastic tubes (Sarstedt, Numbrecht, Germany). All chromatographic protein separations were performed at room temperature (22 °C) on an Agilent 1100 HPLC system with an automated sample injector and fraction collector set at 4 °C. Depleted low-abundance proteins were collected as flow-through fractions and stored at –20 °C for further analysis.

Sample Cleanup

High-abundance protein removal from crude human serum was performed according to a standard LC protocol provided with the Agilent Multiple Affinity Removal System. Briefly, crude human serum was diluted five-fold with Buffer A (20-µL serum with 80-µL Buffer A) containing protease inhibitors (COMPLETE™, Roche Diagnostic Corporation). The diluted serum was then filtered through 0.22-µm spin filters at 16,000 × g at room temperature for 1–2 min to remove particulates.

HSA Standard Solution for Checking Column Capacity

The affinity column removes six high-abundance proteins simultaneously from a given volume of human serum. Among the six high-abundance proteins targeted by the Agilent Multiple Affinity Removal System, albumin constitutes the highest in relative percentage (approximately 54%) and is therefore used for checking binding capacity of the affinity column. An HSA standard solution at 20 mg/mL in Buffer A is available for whenever there is a need for checking column capacity.

Single 4.6 × 50-mm Column

For a 4.6 × 50-mm column, a method blank was performed, and automated sample injection was set, as shown in Table 1, for injection of 50 µL of HSA in Buffer A (0%B) at a flow rate of 0.25 mL/min for 9 min. The injected HSA was retained by the affinity column as indicated by the absence of a flow-through peak. The bound fractions were eluted with Buffer B (100% B) at a flow rate of 1.0 mL/min for 3.5 min. Then, the column was regenerated by equilibrating with Buffer A (0% B) for 7.5 min with a total run cycle of 20 min. Under the same LC protocol, diluted and filtered human serum samples (equivalent to 20-µL undiluted crude serum) were injected at full capacity. See Figure 1.

Table 1. LC Timetable of a 4.6 × 50-mm Affinity Column

Line	Time	%B	Flow rate	Maximum pressure	Buffer	Description
1	0	0	0.25	120	A	Sample injection
2	7.00	0	0.25	120	A	End of washing
3	7.01	100	1.00	120	B	Start elution
4	12.5	100	1.00	120	B	Stop elution
5	12.6	0	1.00	120	A	Start regeneration
6	20	0	1.00	120	A	End run

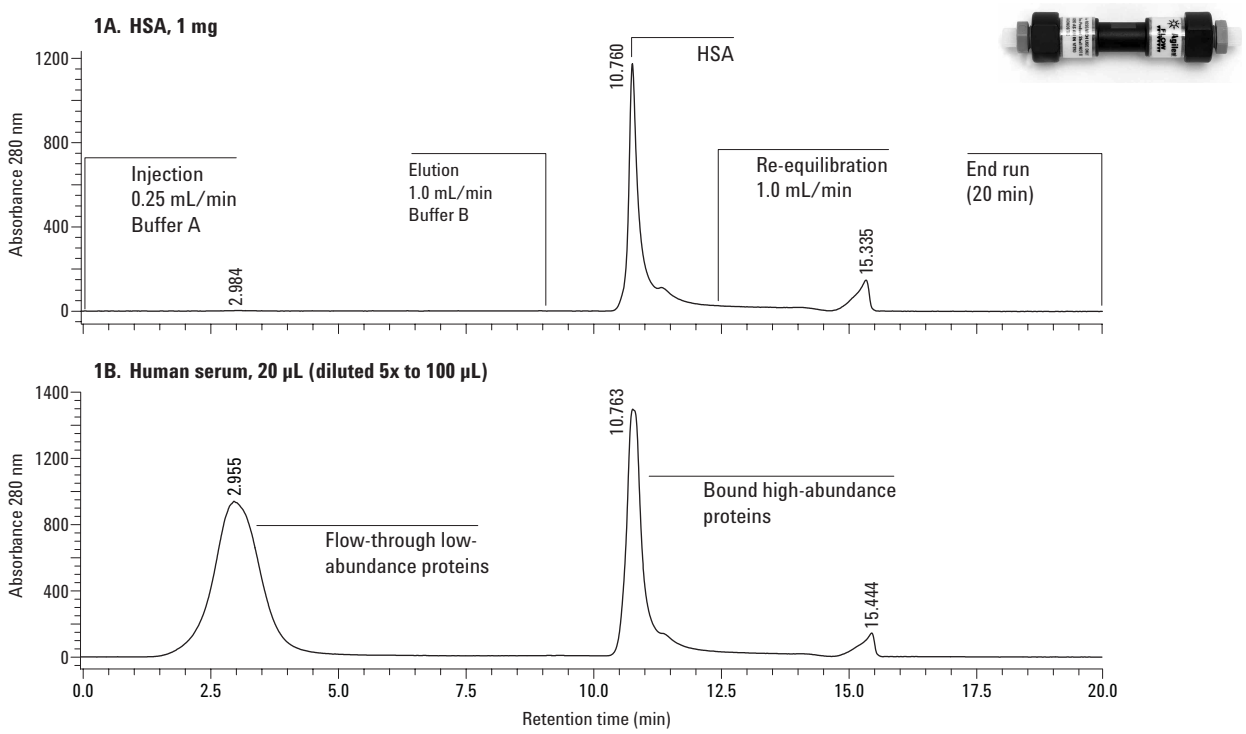

Figure 1. Chromatograms from a 4.6 × 50-mm Multiple Affinity Removal Column for injections of 50 µL of 20 mg/mL HSA (1A) and 5× diluted human serum (20 µL, diluted to 100 µL with Buffer A, 1B). Buffer A is used for sample loading, washing, and column regeneration. Buffer B is used for bound protein elution. A flow rate of 0.25 mL/min from 0 to 9 min is used for Buffer A. A flow rate of 1.0 mL/min for bound protein elution with Buffer B is used from 9 to 12.5 min. A flow rate of 1.0 mL/min for column regeneration from 12.6 to 20 min is used with Buffer A. Depleted protein fractions (flow-through) were detected from 1.5–4.5 min at 280 nm. The bound fraction was detected from 10.5 to 12 min.

Figure 1 shows the representative chromatograms obtained with a 4.6 × 50-mm affinity column. The column capacity was 1.0 mg of HSA (in Figure 1A) or equivalent to 20 µL of undiluted human serum (Figure 1B). The difference between these two chromatograms was the absence of a flow-through peak in Figure 1A when all the injected HSA was retained by the column. Human serum, however, contains thousands of proteins that do not have affinity to the immobilized antibodies and flow through the column, unretained.

Coupled 4.6 × 50-mm Columns

To check the binding capacity of two 4.6 × 50-mm columns connected with a column coupler, 100 µL of HSA solution, at 20 mg/mL was injected using Buffer A (0%B) at a flow rate of 0.5 mL/min for 10 min. See Table 2 and Figure 2. The injected HSA was retained by the affinity column as indicated by the absence of a flow-through peak. The retained fractions were eluted with Buffer B (100% B) at a flow rate of 1.0 mL/min for 3.5 min. Then, the column was regenerated by equilibrating with Buffer A (0% B) for 11.0 min with a total run cycle of 28 min. Under the same LC protocol, 200 µL diluted and filtered human serum samples (equivalent to 40-µL undiluted crude serum) were injected at full capacity.

Figure 2 shows the increased column capacity by connecting two columns of the same size together. The conjoined columns have doubled the capacity of an individual column. As measured with an HSA standard solution, the capacity was increased from 1.0 mg (Fig. 1A) to 2.0 mg (Fig. 2A). Similarly, an increased volume of injection in human serum from 20 µL (Fig. 1B) to 40 µL (Fig. 2B) was confirmed. The timetable for running the columns individually or when conjoined is different due to the different lengths of the two columns. Reproducibility of the chromatograms for the conjoined columns was confirmed by at least 10 sample injections.

Table 2. LC Timetable of 2x (4.6 × 50 mm) Affinity Columns

Line	Time	%B	Flow rate	Maximum pressure	Buffer	Description
1	0	0	0.5	120	A	Sample injection
2	10.00	0	0.5	120	A	End of washing
3	10.01	100	1.00	120	B	Start elution
4	17.00	100	1.00	120	B	Stop elution
5	17.01	0	1.00	120	A	Start regeneration
6	28.00	0	1.00	120	A	End run

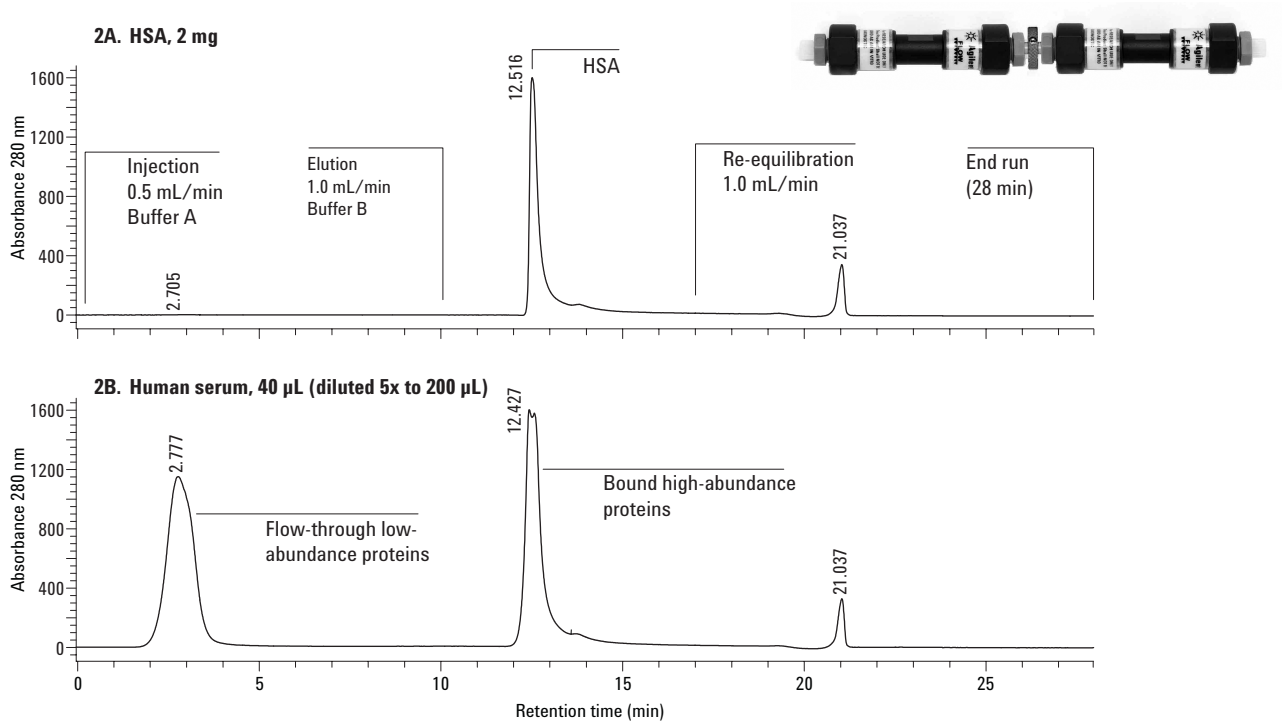

Figure 2. Chromatograms of conjoined 2× (4.6 × 50 mm) Multiple Affinity Removal columns. Injections of 100 µL of 20-mg/mL HSA (Figure 2A) and 5× diluted human serum (40 µL, diluted to 200 µL with Buffer A, Figure 2B). Buffer A is used for sample loading, washing, and column regeneration. Buffer B is used for bound protein elution. A flow rate of 0.5 mL/min from 0 to 10 min is used for Buffer A. A flow rate of 1.0 mL/min for bound protein elution with Buffer B is used from 10 to 17 min. A flow rate of 1.0 mL/min for column regeneration from 17 to 28 min is used with Buffer A. Depleted protein fractions (flow-through) were detected from 2.0–4.0 min at 280 nm. The bound fraction was detected from 13 to 14 min.

Single 10 × 100-mm Column

Another way to increase binding capacity is to increase the column diameter. This is demonstrated using a single custom 10 × 100-mm affinity column. See the parameters in Table 3. The resulting chromatogram is shown in Figure 3. Note that Figure 3, for a 10-mg HSA sample, exhibits excellent binding capacity similar to Figure 1A, for a 1-mg HSA sample, indicating a 10-fold relative increase in binding capacity.

Table 3. LC Timetable of 10 × 100-mm Affinity Columns

Line	Time	%B	Flow rate	Maximum pressure	Buffer	Description
1	0	0	0.5	120	A	Sample injection
2	20.00	0	0.5	120	A	End of washing
3	20.01	100	3.00	120	B	Start elution
4	27.00	100	3.00	120	B	Stop elution
5	27.01	0	3.00	120	A	Start regeneration
6	35.00	0	3.00	120	A	End run

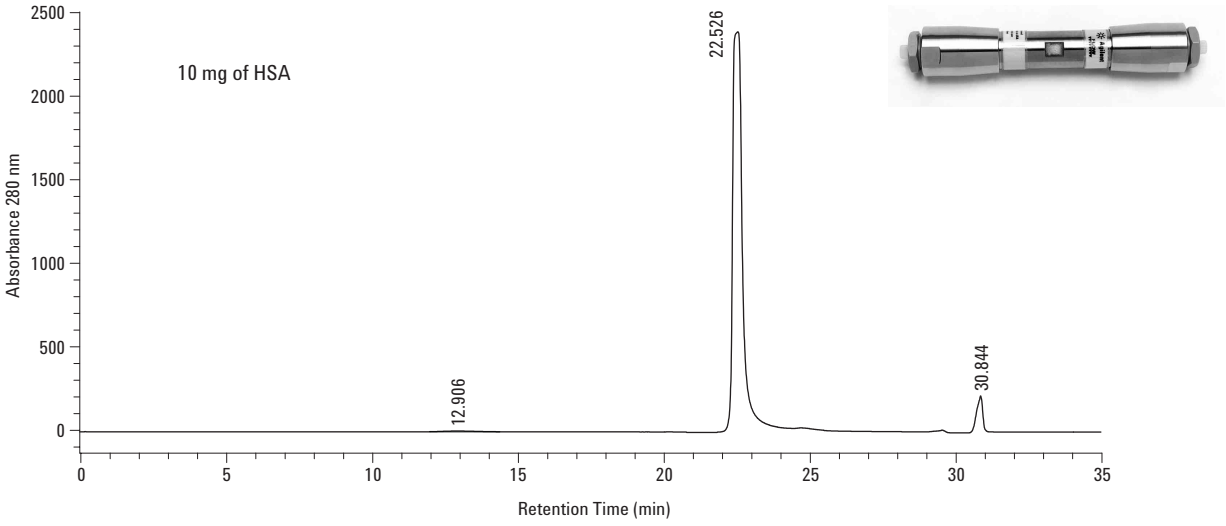

Figure 3. Chromatogram from a custom 10 × 100-mm Multiple Affinity Removal Column and a 10-mg HSA sample.

Decrease Flow Rate to Improve Binding Capacity

It has been shown that Multiple Affinity Removal columns are reusable for over 200 injections of serum under proper conditions [1]. After 200 runs, the potential exists for affinity capacity to decrease as the columns age. To help improve binding capacity when this occurs (or at any point in the column lifetime), use a slower flow rate during sample loading in Buffer A (for example, 0.125 mL/min for a 4.6 × 50 mm-column instead of 0.25 mL/min). As with any affinity-based separation, using the slowest flow rate during sample loading will almost always improve binding efficiency.

Summary Table

The affinity column options and their chromatographic characteristics are compared in Table 4.

Table 4. Protein Binding Capacity of Multiple Affinity Removal Columns

Affinity column (mm)	HSA (mg)	Human serum, undiluted (µL)	Flow rate (mL/min)	Run time (min)	Maximum pressure (Bar)
4.6 × 50	1.0	15–20	0.25	20	120
4.6 × 100	2.0	30–40	0.5	28	120
2x (4.6 × 50)	2.0	30–40	0.5	23	120
10 × 100	10.0	150–200	0.5	35	120

Conclusion

Biomarker identification requires a thorough search of proteins present in human serum from different physiologic conditions based on differences in protein levels and/or post-translational modifications. Higher amounts of proteins available for loading onto gels or mass spectrometry (MS) instrumentation can result in higher visibility of proteins and in turn, more identifiable biomarkers, using popular separation methodologies such as 1DGE, 2DGE, and LC/MS.

Several ways to increase the amount of protein loading for high-abundance protein removal were described in this application note. In one, two 4.6 × 50-mm affinity columns were connected using a low-dead volume column coupler, thereby doubling capacity from 20 to 40 µL of serum volume per injection, with total run time increasing slightly, from 20 to 28 min. Second, dramatic binding capacity increase (10×) was demonstrated using a larger volume affinity column (10 × 100 mm). Third, it was noted that capacity increase can be achieved by simply reducing the flow rate during sample loading.

Taken together, these results demonstrate the flexibility of the Agilent Multiple Affinity Removal System as a reliable tool for up-front sample fractionation in proteomics research. The Agilent Multiple Affinity Removal System possesses the required features, as the method of choice, for removal of multiple high-abundance proteins for proteomics sample preparation.

References

1. K. Zhang et al. Agilent Multiple Affinity Removal System for the depletion of high-abundant proteins from human serum. Agilent Technologies, publication 5988-9813EN. www.agilent.com/chem/affinity
2. K. Zhang et al. Removal of multiple high-abundant proteins from human serum for proteomics sample preparation. Agilent Technologies, publication 5989-0265EN. www.agilent.com/chem/affinity

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

Visit www.agilent.com/chem/affinity for more details. Email technical inquiries to affinity_removal@agilent.com.

Printed in the USA
February 10, 2004
5989-0601EN