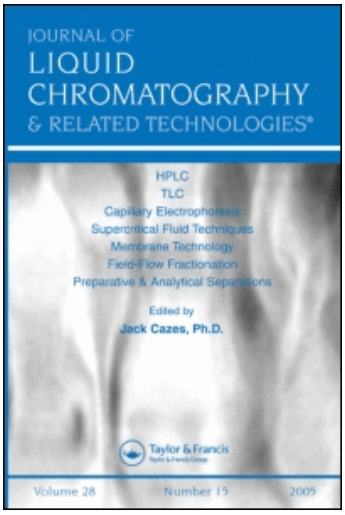

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New Design in Centrifugal Precipitation Chromatography for the Preparative Separation of Proteins

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New Design in Centrifugal Precipitation Chromatography for the Preparative Separation of Proteins

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Abstract: Centrifugal Precipitation Chromatography (CPC) utilizes a moving solvent gradient to separate proteins and macromolecules by repetitive steps of precipitation and dissolution in a column. Proteins fractionate at locations that depend on their solubility in differing ammonium sulfate concentrations. Prior to the current study, construction of a CPC instrument has been difficult due to the complicated design of the separation column and its connections. The current manuscript introduces an innovation that replaces the custom designed column with a commercial one. This change still results in efficient separations of human albumin and γ-globulin in a mixture, as well as in human serum.

Keywords: Centrifugal precipitation chromatography, Ammonium sulfate, Protein fractionation, Protein separation, Preparative separation

INTRODUCTION

Centrifugal Precipitation Chromatography (CPC) aims to separate proteins in a preparative manner without the necessity of a solid support. In 2000, Y. Ito described a prototype CPC instrument that employs an ammonium sulfate (AS) gradient for protein fractionation.[1] Since the initial introduction, we have attempted to improve column capacity, resolution, and ease of

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construction. The current model allows easier duplication while maintaining the separation efficiencies of past designs. It specifically addresses the issue of reproducibility, which has been an obstacle to integrating CPC as a useful method for separating proteins.

At its core, CPC serves to eliminate the tedious and inefficient method of protein separation by manual stepwise precipitation. Secondly, CPC removes the need for a solid support in the column, as required by more accepted methods of protein separation, including high performance liquid chromatography (HPLC). This is a well-known cause of adsorptive loss and deactivation of sample. The column capacity is also far greater than that of HPLC, enabling the accommodation of sample sizes up to 0.5 g or 2.0 mL.

Currently, a commercially available column from General Electric Healthcare (USA) allows easy assembly of the instrument. More importantly, the new design does not sacrifice the advantages of conventional CPC, including flexible manipulation of AS gradient, elimination of impurities, and high efficiency of separation.[2] The present manuscript describes the principle, design, and preliminary results of the newest prototype of the instrument.

EXPERIMENTAL

Instrument Design

In the single-cartridge system, one 110 cm long MidGee Hoop Cross Flow Filter (MGHCFF) cartridge (Fig. 1a) functions as the separation column, while another one is used to balance the rotating plate (Fig. 2). For the dual-cartridge system, two cartridges are connected in series. Each cartridge (Fig. 1b) is composed of two narrow-bore hollow fiber dialysis tubes (0.5 mm lumen each) inside a larger polysulfone shell (3 mm diameter). The size of the pores of the inner tubes is 10 kDa. The two ends of the

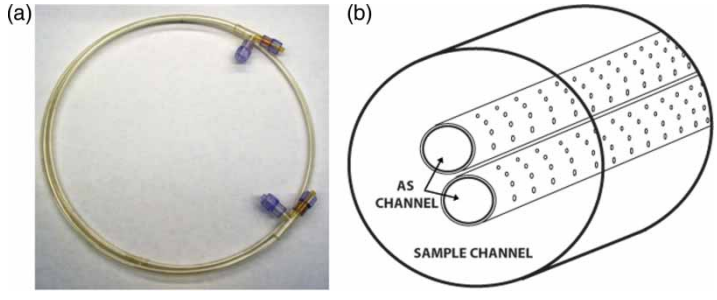

Figure 1. (a) MidGee Hoop Cross Flow filter cartridge which serves as a separation column; (b) Cross-sectional view.

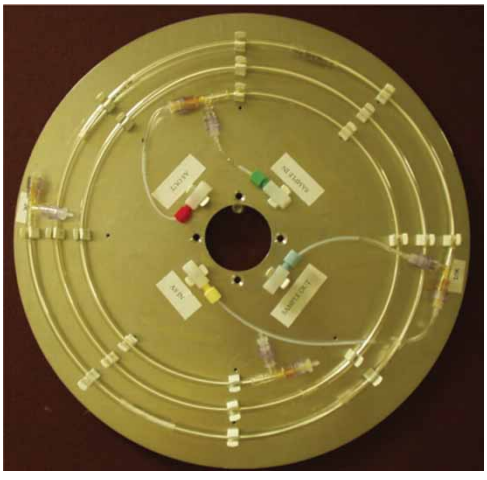

Figure 2. Two cartridges of a single-cartridge system with one cartridge used as the separation column and the other is used to balance the rotating plate.

cartridge each contain two Luer-Lok fittings, which allow the sample and solvent to be injected and eluted in a reverse directional pattern. Ten kDa is an allowable size for the diffusion of the solute across the inner tube membrane to the lumen of the outer tubing, creating a gradient that allows for the separation.

The cartridges are connected together with tubing and clamped in spiral configurations to a metal plate (Fig. 2), which is rotating at various speeds, subjecting the sample to centrifugal force. The spinning metal plate is screwed to a centrifuge with a control unit built by Pharma-Tech Research Corporation (Baltimore, MD, USA).

The sample is pumped through the internal end of the spiral while the solvent is pumped concurrently in the same direction, also from the internal end. The sample elution and leftover AS solution are collected from the external end. The solvent is injected into the column using a Shimadzu dual pump, which allows a varying gradient range over a set period of time. The sample is injected manually, and the tubing is connected to a pump which injects a buffer solution following the sample.

A fraction collector allows the separated sample to be saved in test tubes according to time corresponding to the solvent gradient. A wavelength strip-chart recorder is used to detect the content of each tube of elution.

Principle

In the column, an AS concentration gradient forms in the space between the two inner dialysis tubes and the outer polysulfone shell. Initially, the sample is

subjected to a high AS concentration which precipitates the sample. Centrifugal force keeps the precipitated sample in the column while the AS concentration gradient establishes itself. As the AS gradient changes from high to low, the protein content of the sample that is associated with a specific AS concentration is dissolved and eluted. Additionally, the dual pump gradually lowers the solvent concentration range over a time period after the initial base gradient is set up. The effect is a constant change and advancement of the gradient within the cartridge, which causes the sample to be dissolved and precipitated numerous times. The result is an efficient separation.

Reagents

The solvent consists of 95% saturated AS solution and 5% 1 M K_2HPO_4 solution. The sample buffer solution consists of a 50 mM solution of 2:1 molar ratio between K_2HPO_4 and KH_2PO_4. The protein standards sample consists of human serum albumin and human γ-globulin that were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human serum used in this experiment was obtained from the National Institutes of Health Blood Bank (Bethesda, MD, USA).

Standard Protocol

The stock solution is made by combining 95% saturated AS solution with 5% 1 M K_2HPO_4 solution. This mixture will protect the pump from being damaged by AS precipitation. The sample column is initially filled with 80% solvent (76% AS) and 20% buffer solution before the sample is injected. Once in the column, the sample is subjected to 60 minutes of 76% AS to allow precipitation before the AS concentration gradient is lowered. Afterwards, the solvent gradient is gradually lowered from 80% to 20% over a time period with a consistent flow rate. The rotational speed was 800 RPM.

RESULTS

Single-Cartridge Column

The varying parameters tested included column size, sample/buffer flow rate, solvent flow rate, and solvent gradient time. During initial trials, the separation column consisted of a single MGHCFF cartridge. The flow rate parameters tested were 0.025–0.1 mL/min (sample) and 0.5–2.0 mL/min (solvent). Additionally, solvent gradients of 420, 800, and 1,200 min were also tested (Table 1). Overall, the optimal conditions, as defined by the best separation efficiency for the separation of human albumin and γ-globulin, was a

Table 1. Outline of gradient time for single-cartridge tests

Time (min)	Solvent (conc.)
0	80%
60	80%
420	20%
2000	0%

sample flow rate of 0.025 mL/min, solvent flow rate of 1.75 mL/min, and gradient period of 420 min. The results are shown in Fig. 3.

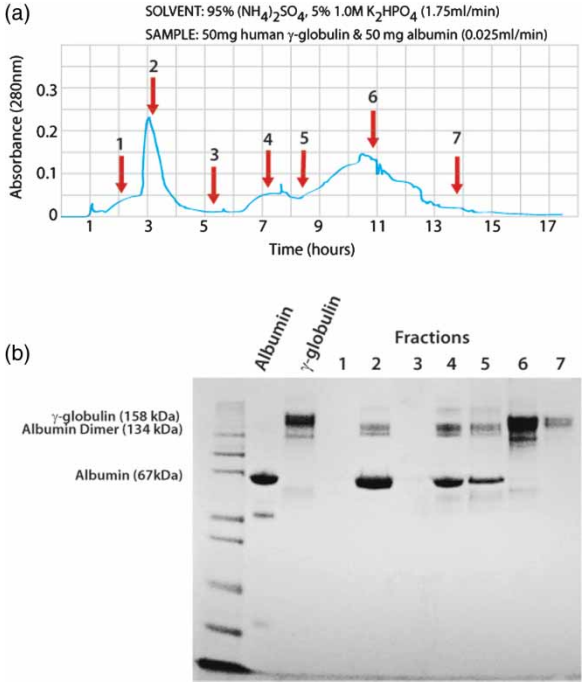

Figure 3. Separation of human albumin and γ-globulin using one MidGee Hoop Cross Flow filter cartridge as column. (a) Experimental conditions: solvent flow rate, 1.75 mL/min; sample flow rate, 0.025 mL/min; gradient period, 80–20%/420 min; fraction collection every 25 min. (b) SDS-PAGE analysis using precast tri-glycine 12% gel at 160 V for 65 min, followed by Coomassie blue staining.

Dual-Cartridge Column

Second, a dual-cartridge system was tested to determine the impact of column capacity increase on separation efficiency. Two identical MGHCFF cartridges

Table 2. Outline of gradient time for dual-cartridge tests

Time (min)	Solvent (conc.)
0	80%
60	80%
1200	20%
2000	0%

are attached in series and arranged in similar spiral patterns. The same variants in experimental conditions as in the single-cartridge column tests were examined. The optimal specifications were a sample flow rate of 0.05 mL/min, solvent flow rate of 1.0 mL/min, and gradient period of 1,200 min. The results are shown in Fig. 4.

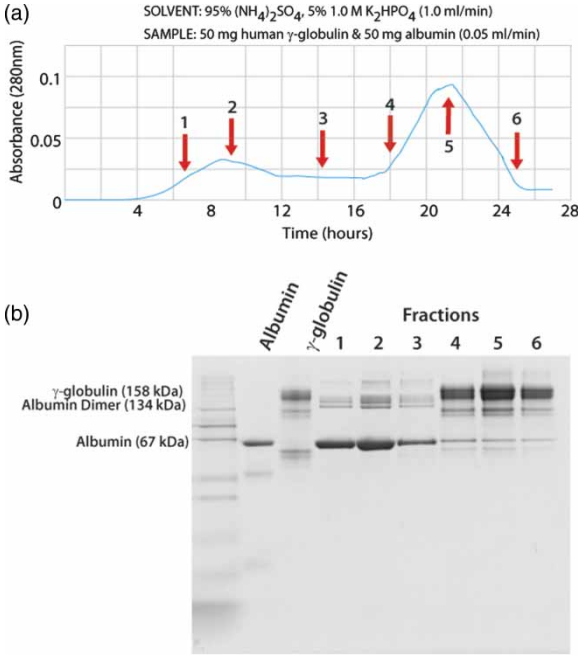

Figure 4. Separation of human albumin and γ-globulin using dual-cartridge system. (a) Experimental conditions: solvent flow rate, 1.0 mL/min; sample flow rate, 0.05 mL/min; gradient period, 80–20%/1,200 min; fraction collection every 50 min. (b) SDS-PAGE analysis using precast tri-glycine 12% gel at 160 V for 65 min, followed by Coomassie blue staining.

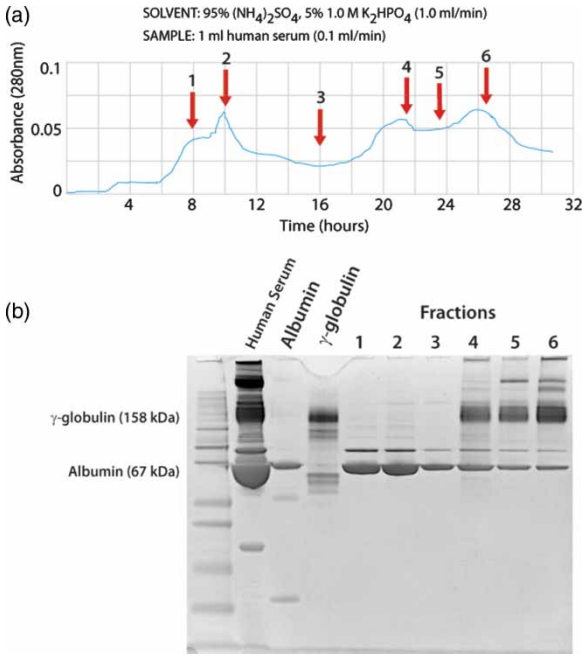

Figure 5. Separation of proteins in human serum using dual-cartridge system. (a) Experimental conditions: solvent flow rate, 1.0 mL/min; sample flow rate, 0.1 mL/min; gradient period, 80–20%/1,200 min; fraction collection every 50 min. (b) SDS-PAGE analysis using precast tri-glycine 12% gel at 160 V for 65 min, followed by Coomassie blue staining.

Human Serum Separation

Lastly, proteins in human serum were separated using the dual-cartridge system. One mL of the human serum sample obtained from the NIH Blood Bank was injected into the column. The solvent gradient was kept the same as the one used to separate albumin and globulin (Table 2). The specifications were a sample flow rate of 0.1 mL/min, solvent flow rate of 1.0 mL/min, and gradient period of 1,200 min. The results are shown in Fig. 5.

DISCUSSION

Variable Parameters and their Implications

The three adjustable parameters examined were sample flow rate, solvent flow rate, and solvent gradient. The three variables factored together to produce the resolution. An appropriate sample flow rate will allow the sample to stay

within the column so it can be exposed to the AS gradient. A sample flow rate that is too slow will allow the solvent gradient to catch up before the appropriate dissolved protein can be eluted out. For example, protein A dissolves in 60% AS while protein B dissolves in 45%. If the sample flow rate is too slow, the dissolved protein A will not be forced out of the column before the solvent gradient reaches 45%. Thus, protein B will dissolve as well in the column, causing a mixture of the two dissolved proteins to recombine. On the other hand, if the sample flow rate is too fast, the sample will be forced out of the column before the proteins have a chance to precipitate and dissolve again.

Just as importantly, the solvent flow rate influences separation. A solvent flow rate that is too slow will result in less AS diffusing through the pores of the inner tube surface, preventing the optimal AS concentration. A solvent flow rate that is optimal will allow the AS to diffuse across the membrane while also supplying fresh solvent with the appropriate level of AS. A solvent flow rate that is too fast will skew the natural osmotic pressure inside the column by forcing water through the pores into the outer space (sample channel).

In theory, the longer the solvent gradient, the better the resolution because a longer gradient allows more exposure of the proteins in a sample to the level of AS in which it precipitates and dissolves. However, there is a point when the extra time in the gradient period is unnecessary because the performance of the instrument has reached an optimum. Of course, the gradient range can be adjusted to accommodate the specific proteins that need to be separated.

One vs. Two Cartridges

The results indicated that a dual-cartridge system produced better separation efficiencies than a single-cartridge system, regardless of the solvent gradient. The conditions that generated the best separation (Fig. 3) were a sample flow rate of 0.05 mL/min, a solvent flow rate of 1.0 mL/min, and solvent gradient period of 1,200 min. In this trial, the sample traveled slowly enough in the column to be subjected to the developing AS gradient. Centrifugal force held the precipitated proteins in the column until the appropriate level of AS came along. Once dissolved, the protein traveled out of the column with the mobile phase in the sample channel. Based on a comparison between dual-cartridge and single-cartridge systems, human serum was separated using a dual-cartridge system.

The advantages of CPC have implication for its widespread use as a first degree purification strategy. It has not escaped our attention that the method should also be applicable to the separation of other macromolecules,[3] and the easy construction of the columnar system described above should greatly facilitate such experiments.

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REFERENCES

1. Ito, Y. Centrifugal precipitation chromatography: principle, apparatus, and optimization of key parameters for protein fractionation by ammonium sulfate precipitation. Anal. Biochem. **2000**, *277*, 143–153.
2. Yu, H.; Ito, Y. Preparative separation of proteins using centrifugal precipitation chromatography based on solubility in ammonium sulfate solution. Prep. Biochem. Biotechnol. **2004**, *34* (1), 1–12.
3. Panarat, T.; Hsu, J.T.; Ito, Y. Fractionation of protein, rna, and plasmid dna in centrifugal precipitation chromatography using cationic surfactant ctab containing inorganic salts NaCl and NH_4Cl. Biotechnol. Bioeng. **2004**, *88* (1), 52–59.

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