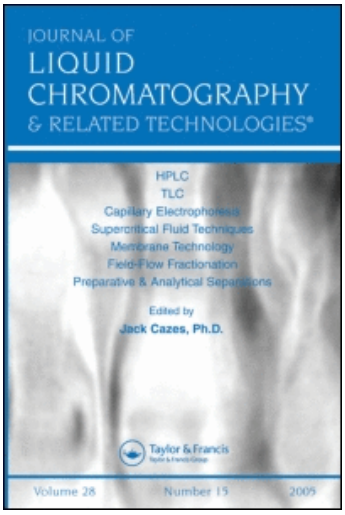

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Preparative Centrifugal Precipitation Chromatography Using Dialysis Membrane Inserted into Convoluted Tubing

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Preparative Centrifugal Precipitation Chromatography Using Dialysis Membrane Inserted into Convoluted Tubing

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Abstract: The method uses preparative centrifugal precipitation chromatography comprising a seal-free continuous-flow centrifuge machine equipped with a plastic disk and a spiral groove which accommodates convoluted tubing. A dialysis membrane is inserted into the convoluted tubing to separate proteins according to their solubility in ammonium sulfate solution. The system relies on the original model of centrifugal precipitation chromatography, which employs the centrifugal force and a concentration gradient of precipitants to perform a repetitive process of precipitation and dissolution of protein molecules. Compared to the previous model, the current model results in a higher yield of separated compounds.

Keywords: Centrifugal precipitation chromatography, Preparative separation, Protein purification and separation, Ammonium sulfate gradient precipitation, Dialysis tubing, Convoluted tubing

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INTRODUCTION

In 1999, a new method called centrifugal precipitation chromatography was introduced for protein separation in relation to the solubility of ammonium sulfate. It uses a pair of plastic disks each equipped with mutually-mirror imaged spiral grooves to sandwich a dialysis membrane to form a two identical spiral channel separated with a semi-permeable membrane. A countercurrent system between concentrated ammonium sulfate solution (AS channel) and phosphate buffer (sample channel) through these channels produces an exponential concentration gradient of ammonium sulfate in the sample channel where proteins repeat precipitation and resolution, and separate according to their solubility in the ammonium sulfate solution.[1–3]

Recently, the sample loading capacity of the method was improved by placing dialysis tubing (4 mm ID) into convoluted tubing (5.7 mm ID × 2.2 m) to increase the mass transfer area.[4] The column was fixed on a rotary plate at various points with nylon ties to form spiral configuration (Figures 1 and 2). As in the original system, protein samples injected into the column were exposed to an increasing concentration of ammonium sulfate and eluted according to their solubility.

In the present system the column is snugly accommodated into a spiral groove made in a plastic disk, so that it will not deform under high pressure allowing experiments to reproduce the same results consistently (Figure 3). This paper describes the instrumentation and results of the preparative separation of human serum proteins and human blood plasma as models.

EXPERIMENTAL

Instrumentation

Pharma-Tech Research Corporation (Baltimore, MD, USA) provided a centrifuge and control unit. The separation column used in this study was originally designed by Yu and Ito.[4] About 2.2 meters of convoluted PTFE (polytetrafluoroethylene) tubing (1 cm average I.D.) (Zeus Industrial Products, Raritan, NJ, USA) is placed into the grooves of a spiral disk that was constructed by the NIH machine shop. The spiral disk is then screwed onto the control unit. Dialysis membrane, MWCO 3,500 (ca. 4 mm I.D.) (Spectrum, San Diego, CA, USA) is placed inside the convoluted tubing and connected at both ends with PTFE tubes (8.5 mm I.D., Zeus Industrial Products) using a plastic adapter (Figure 4a). This system of the convoluted tubing and dialysis membrane allows an exchange of low molecular weight compounds between the two channels. The input of the tubing is connected to a gradient pump (SCL-10A and LC-10AD, Shimadzu Scientific Co., Columbia, MD, USA). The outlet of the tubing is emptied into a discarded waste bottle.

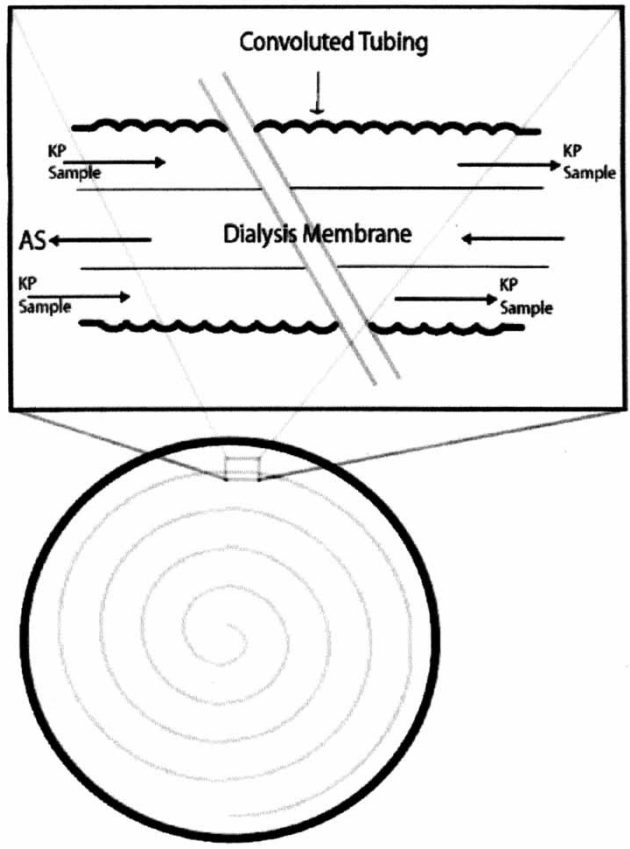

Figure 1. Dialysis membrane (MWCO 3,500) inserted into convoluted tubing. Ammonium sulfate flows in one direction in the AS channel, while potassium phosphate flows in the opposite direction in the sample channel, thus creating a countercurrent flow.

The ends of the convoluted tubing were heated and molded to fit onto the plastic adapter. The dialysis membrane is held in place by two small pieces of heat shrinkable tubing (Daburn, Northvale, NJ, USA) that enclose the ends of the membrane to the adaptor (Figure 4b). The convoluted tubing placed on top covers the Teflon tubing of the adapter. It is then secured to the adapter by a nylon tie (Figure 4c).

Reagents

Ammonium sulfate and monobasic and dibasic potassium phosphates were obtained from Fisher Scientific (Fair Lawn, NJ, USA). The protein samples,

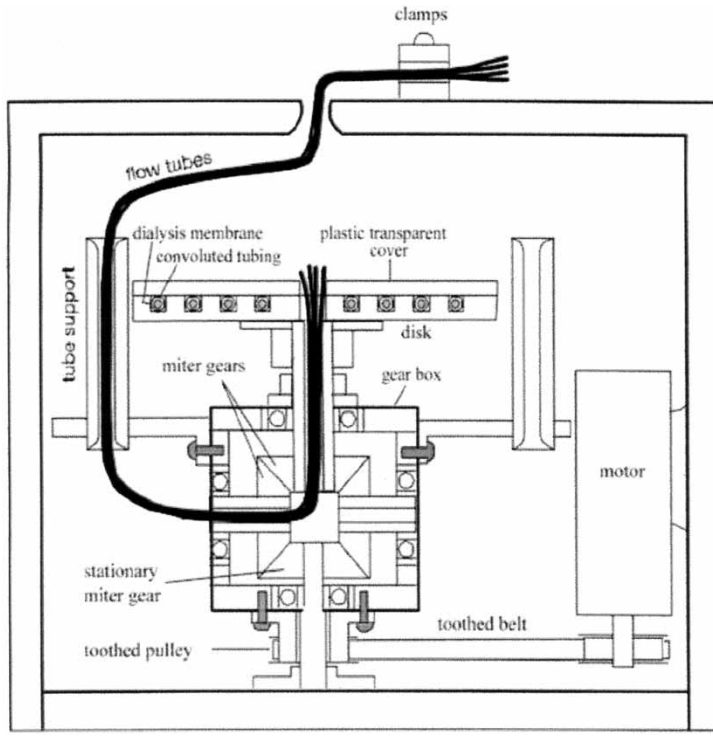

Figure 2. Cross-sectional view of the instrument shows where the convoluted tubing and dialysis membrane are placed into the grooves of the spiral disk. It is mounted on a spinning shaft of a seal-free continuous flow centrifuge.

human albumin and gamma globulins, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The human blood plasma was extracted from blood donated by the NIH blood bank (NIH, Bethesda, MD, USA).

Separation Procedure

Inside the membrane channel, 76% saturated ammonium sulfate solution (95% saturated ammonium sulfate mixed with 50 mM potassium phosphate buffer at a volume of 80:20) is pumped using the Shimadzu gradient pump. The sample channel is pumped with 50 mM of potassium phosphate while this occurs. After filling the entire column with the ammonium sulfate, the protein sample (human serum albumin and gamma globulin, each 50 mg, dissolved in 5 mL potassium phosphate buffer) is inserted into the sample channel. The column is then rotated at 800 rpm. The sample channel is pumped with 50 mM potassium phosphate (pH 6.8) at 0.5 mL/min and the membrane channel is eluted with a linear gradient of ammonium sulfate

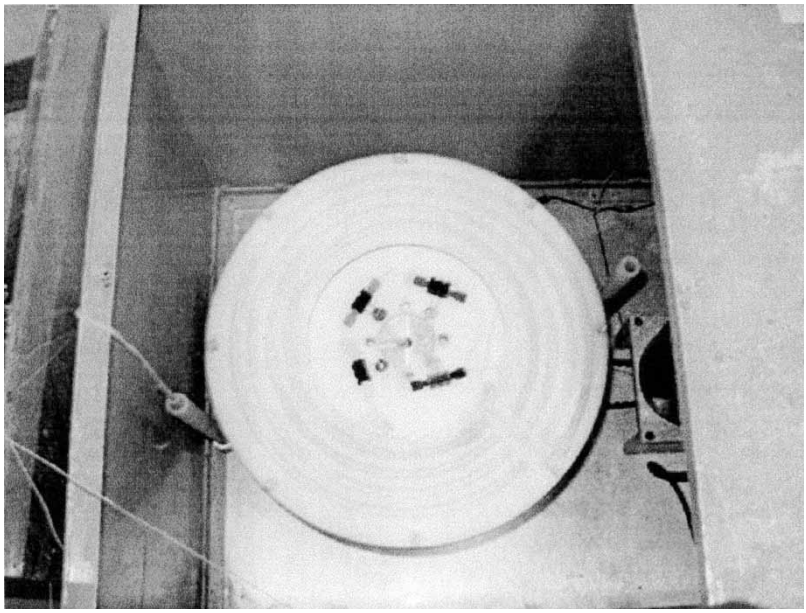

Figure 3. Photograph of the instrument from the top.

solution starting at 76% and ending at 19% for 6 hours after 1 hour of priming time of the 76% ammonium sulfate solution, at a flow rate of 2 mL/min. The results were monitored by a UV monitor (Uvicord S, LKB Instruments, Stockholm, Sweden) and fractions were taken into test tubes at 10 minute intervals.

Analysis of Protein Fractions

The protein fractions were placed in a regenerated cellulose filter (Centricon 10,000 MWCO, Amicon, Beverly, MA) and filtered by centrifugation. NuPAGE Novex Bis-Tris gel electrophoresis plates (Invitrogen, Carlsbad, CA) were used to analyze and evaluate the protein fractions. The gels were stained by a blue staining reagent (Pierce, Rodford, IL).

RESULTS AND DISCUSSION

The separations of human serum albumin and gamma globulins proved to be effective through the use of centrifugal precipitation chromatography. The chromatogram of the protein sample, using a linear gradient, shows two somewhat large peaks, the first being human serum albumin and the second

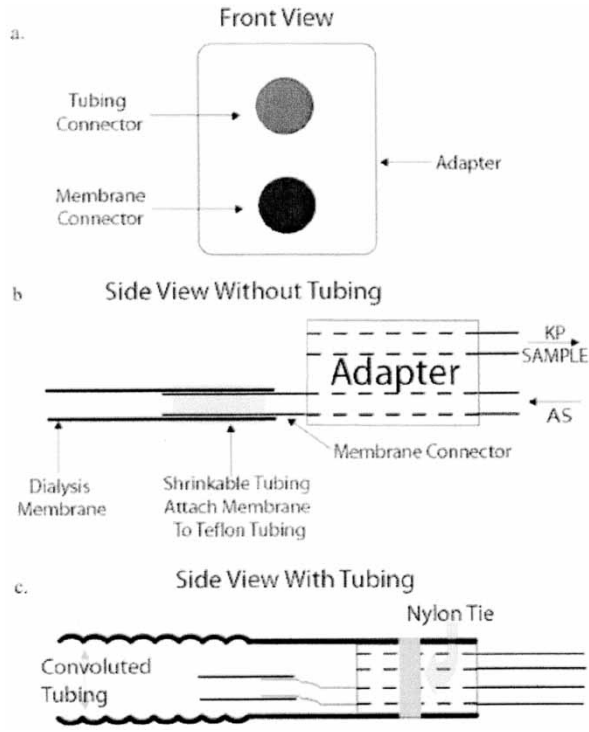

Figure 4. Adapter diagrams. (a) The plastic adapter has two holes; one hole is for the connection of the convoluted tubing, while the other is for the connection for the dialysis membrane. (b) Teflon tubing is extended from the adapter and dialysis membrane covers the tubing. To secure the membrane in place, heat-shrinkable tubing is place on top of the membrane. (c) Convoluted tubing is then secured on to the plastic adapter with a nylon tie.

peak gamma globulins (Figure 5). The linear gradient starts off at 76% ammonium sulfate from 0–60 minutes, 76% to 19% from 60 to 420 minutes, and 19% to 0% from 420 to 421 minutes. The stepwise gradient also shows two distinct peaks (Figure 6). The stepwise gradient starts at 50% ammonium sulfate, and then drops off to 0% after the first protein separation.

The results on the NuPAGE gel electrophoresis plates confirm that human serum albumin separated in the first fraction and gamma globulins separated in the third fraction (Figure 7). The second fraction was taken in between the two peaks and, as expected, no protein was observed.

The human blood plasma separation had similar results. The gel electrophoresis showed the separation of the proteins (Figure 8). The first fraction matches with the human albumin sample, while the second and third fractions complement the gamma globulins sample.

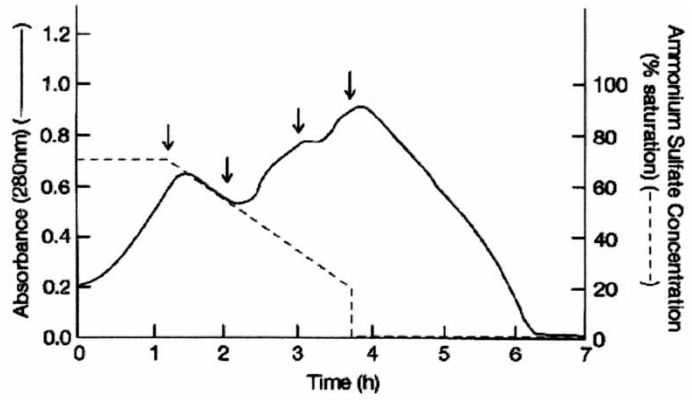

Figure 5. Linear gradient of 50 mg human albumin and 50 mg gamma globulin. After 1 hour, a linear gradient of ammonium sulfate from 76% to 19% occurs for 6 hours. It is then sharply decreased to 0% for the remainder of the experiment.

In the experiment, a stepwise gradient was used as well as a linear gradient. The stepwise weight produced nearly the same result as the linear gradient, however, it would not work as well with a higher amount of proteins in a sample. The linear gradient would be preferred because it would be more efficient and reasonable if there were multiple amounts of proteins used.

Changes were constantly being made while trying to find the most optimal and efficient way of separating the proteins. Flow rates varied of potassium phosphate in the tubing, as well as ammonium sulfate in the membrane.

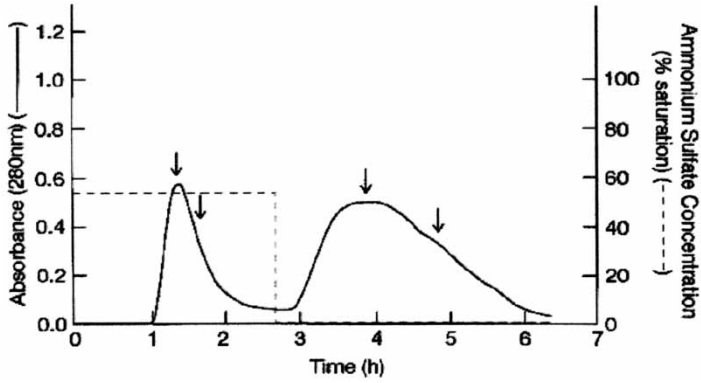

Figure 6. Stepwise gradient for 50 mg human albumin and 50 mg gamma globulin. Stepwise gradient start at 50% ammonium sulfate and is dropped to 0% after the first separation occurs. It remains at 0% for the remainder of the experiment.

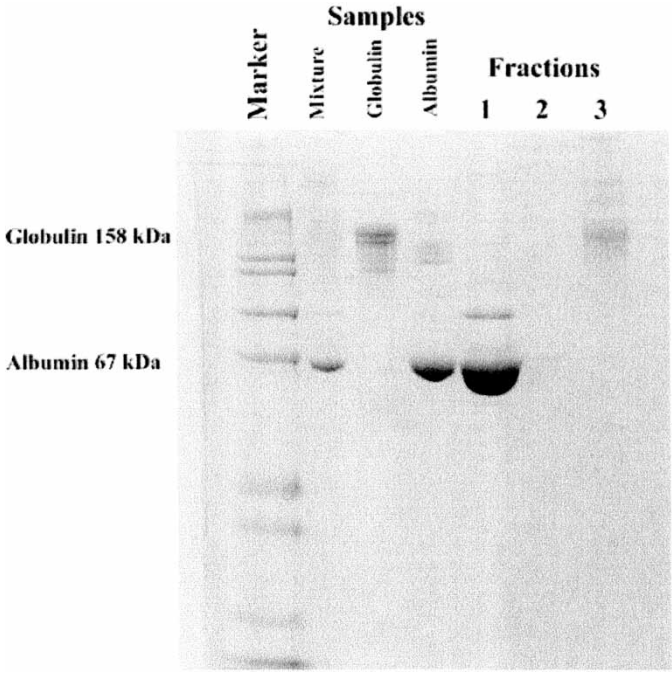

Figure 7. NuPAGE gel of the separation of 50 mg of human serum albumin and 50 mg of gamma globulin. Fraction 1 shows the separation of albumin, and fraction 3 shows the separation of globulin.

The tubing flow rates ranged from 0.1 milliliters per minute to 0.5 milliliters per minute. On the other hand, the membrane flow rates ranged from 1 milliliter per minute to 2 milliliters per minute. The optimum flow rates found after numerous experiments for the tubing and membrane were 0.5 mL/min and 2 mL/min, respectively.

Experiments were performed by placing the protein sample in the dialysis membrane as well as the convoluted tubing. Inserting the protein sample in the tubing produced better results because the convoluted tubing had a higher retention rate due to its geometric design. The protein inserted in the membrane on the other hand, tended to elute precipitated proteins without any retention. Also, the constant motion of the dialysis membrane may have caused problems, which resulted in the poor separation.

Four hour and seven hour ammonium sulfate gradients were both tested for the linear gradient. The 4-hour gradient did not show as good a separation as the 7-hour gradient, so we discovered that the 7 hour gradient was more effective.

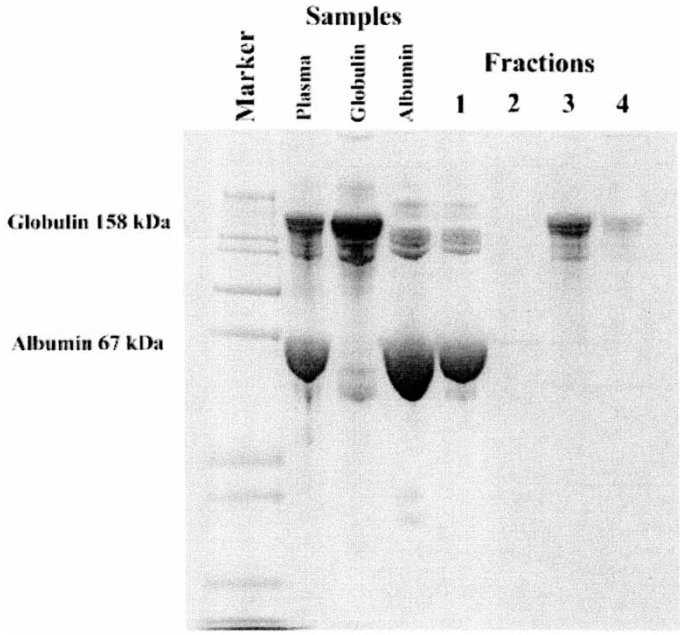

Figure 8. NuPAGE gel of the separation of proteins in 1 mL human blood plasma. Fraction 1 shows that human albumin separated white fractions 3 and 4 show gamma globulin separated.

Different sizes of dialysis membranes were briefly tested, one with a molecular weight cutting opening (MWCO) of 3,500 and the other at 8,000. However, the membrane with MWCO of 8,000 allowed the water to permeate too quickly and was difficult to control for obtaining suitable flow rates through two channels.

CONCLUSION

Countercurrent Centrifugal Precipitation Chromatography can separate large quantities of proteins according to their solubility in ammonium sulfate solution. The shallower the ammonium sulfate gradient, the better the efficiency of the separation. Also, the stepwise gradient produces nearly the same efficiency as the linear gradient when two protein components are separated. However, when multiple protein components are to be resolved, the linear gradient is preferred. This study served as a model and, thus, shows that countercurrent centrifugal precipitation chromatography can be used for preparative separation. In the future, separation in multigram quantities of proteins may also be a possibility.

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