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Centrifugal affinity chromatography

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ABSTRACT

A new technique termed centrifugal affinity chromatography (CAC) is presented in this paper. CAC combines a high flow-rate, created by centrifugal force, with the specificity of affinity chromatography. This technique has been used for the purification of human immunoglobulin G. Furthermore this technique has been used to remove human albumin from serum and the effect of centrifugal force, ionic strength and pH has been studied. A test for determining the percentage of glycosylated hemoglobin in hemolysates has also been developed. This test, employing centrifugal chromatography, is more than three times faster than commonly used gravity flow methods.

INTRODUCTION

We have designed a simple and rapid technique, centrifugal affinity chromatography (CAC), which is an extension of centrifugal chromatography. Several authors have described the use of centrifugal chromatography for ion exchange and gel permeation[1] but a centrifugal force has never been used before for performing affinity chromatography processes.

High-performance liquid affinity chromatography (HPLAC) has been widely used to analyse the protein content of various samples. HPLAC is best utilized for the routine analysis of multiples of the same, or similar, samples utilizing the same column and HPLAC requires considerable technological expertise. The cost of HPLAC is a serious draw-back for a laboratory where only occasional applications are needed. Conversely, affinity chromatography, based on gravity flow, can be very time consuming when many different columns must be screened. CAC can be a good alternative for these techniques. The rapid flow-rate created by centrifugal force saves time compared with gravity flow chromatography. CAC is extremely easy to perform and requires little training of the users.

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As an initial example, we employed CAC to purify human immunoglobulin G (IgG) on Protein A columns. The second system we studied was the specific removal of human albumin from human serum using Blue-Trisacryl® gel[2–5]. The system was optimized with regard to the centrifugal force, binding-time, ionic strength and pH.

Finally, CAC was adapted to a test for the determination of the percentage of glycosylated hemoglobin and was found to be superior to gravitiy flow methods[6,7].

EXPERIMENTAL

Materials

Centrex® columns, Affinica® gel and Affinica equilibration, elution and regeneration buffers were the kind gift of Schleicher & Schuell (Keene, NH, U.S.A.). the 5-ml columns contained a supporting cellulose acetate membrane (pore-size: 0.2 mm in experiments with human albumin experiments; pore-size: 0.45 mm in case of experiments utilizing human IgG and diabetic hemoglobin) and the columns themselves were made of polypropylene.

The centrifuge used was the IEC Model HN-SII (Intl. Equipment, Needham Heights, MA, U.S.A.). The centrifugal force was determined from the radius, at the membrane-level, of each of the centrifugal columns.

In the human IgG purification experiments the Centrex columns were filled with 1-cm settled bed-height of Affinica Protein A gel.

Blue-Trisacryl gel from IBF (Reactifs IBF Soc. Chim. Pointet Girard, Villeneuve la Gareneu, France) was used to remove human albumin from blood serum (1 cm settled bed-height was used in experiments with purified human albumin, 2 cm settled bed-height in experiments with whole serum). The maximum binding capacity of this gel, as described by the manufacturer, was 15 mg of human serum albumin/ml settled gel. Experiments were performed at saturating amounts unless otherwise specified. To compare CAC analysis of glycosylated hemoglobin with the gravity flow system, the Pierce Glyco Test, utilizing the instructions from the manufacturer, was used as the comparative standard.

For the determination of glycosylated hemoglobin, the wash buffer consisted of 0.25 *M* ammonium acetate and 0.05 *M* $MgCl_2$, adjusted to pH 8.5 with hydrogen chloride. The elution buffer contained 0.1 *M* Tris · HCl and 0.2 *M* sorbitol, adjusted to pH 8.5. Columns were regenerated by washing with 0.05 *M* acetic acid. All buffers contained 0.02% sodium azide.

Human IgC was purified from serum by ammonium sulfate precipitation and ion-exchange chromatography[8] and stored in 5 m*M* phosphate buffer pH 6.8 and 0.02% sodium azide. Purfied human albumin (25% solution) was the kind gift from the Waco Chapter of the American Red Cross. For hemolysis, human blood (0.1 ml) was mixed with 0.9 ml sodium phosphate KCN buffer (pH 6.7) and incubated for 10 min with gentle shaking.

As standards we used glycosylated hemoglobin controls from Pierce (Rockford, IL, U.S.A.).

Methods

In the purification of human IgG, Protein A affinity columns were centrifuged between each step for 1 min at 600 *g*. Initially the columns were equilibrated with

Affinica equilibration buffer (5 × 1 ml). Subsequently, 4 ml, containing about 4 mg of human IgG, was applied to the column, after which the columns were centrifugally washed with the same buffer. In the last step, human IgG was eluted with 2 × 3 ml elution buffer.

To prepare Blue-Trisacryl centrifugal columns for the removal of albumin from human serum, they were first treated with 6 *M* guanidine hydrochloride (3 × 1 ml). This treatment removed any excess dye which may have been physically absorbed to the column and permitted a contamination-free product to be obtained. Between each column application, the columns were centrifuged for 2 min at 600 *g*. After the first treatment, the columns were equilibrated in 0.025 *M* citrate (pH 4) or 0.025 *M* phosphate buffers adjusted to various ionic strengths with sodium chloride and to various pH values (pH 5.2, 7.2, 9). Subsequently, 0.5 ml of a 25% pure human albumin solution diluted eight fold with the same buffer was added to the columns (whole blood serum was 10 times diluted). After this, columns were washed twice with the same buffer used to equilibrate the columns (2 × 3.5 ml). Finally the human serum albumin was eluted with 0.5 *M* KSCN (2 × 2.5 ml).

Electrophoretic analysis was done in a Protean cell (BioRad Laboratories, Richardson, CA, U.S.A.) on a 10% polyacrylamide sodium dodecyl sulphate (SDS) denaturing gel by the method of Laemmli[9].

Before analyzing the elution fractions by electrophoresis they were concentrated eight fold utilizing the Centricon 10 micro concentrator (Amicon, MA, U.S.A.). A 10% acrylamide SDS gel was used to analyze the completeness of the removal of human serum albumin from the samples[8]. Staining was performed with 0.1% Coomassie Blue dissolved in 5% glacial acetic acid containing 10% methanol.

Determination of glycosylated hemoglobin was performed by a modification of the Pierce gravity-flow glycosylated hemoglobin procedure. Centrifugal columns containing Pierce Glycogel B were initially equilibrated with 2 ml of wash buffer and subsequently 50 ml of hemolysate was applied to each column. After this, the columns were washed twice with wash buffer and the non-bound fraction was collected. Between each wash the columns were spun at 160 *g* for 3 min. The receiver tubes were then changed and the bound glycosylated hemoglobin fraction was eluted with 3 ml of elution buffer for each column. The amount of glycosylated and non-glycosylated hemoglobin was spectroscopically measured at 414 nm.

RESULTS AND DISCUSSION

Human IgG was the first material to be purified in our laboratory using CAC. At least 85% of human IgG was bound to the Affinica gel columns when an amount of human IgG comparable with the maximum capacity (15 mg human IgG/ml settled gel as determined by the manufacturer) was applied. The residence time was altered by changing the incubation time for the human IgG sample in the column before centrifugation. No influence of the binding time was detected. The elution time was varied by changing the length of time for the incubation of the Affinica gel columns with elution buffer prior to centrifugation. Such alterations had no significant influence on the amount of human IgG recovered in the elution fractions. The minimum achievable residence time was 1 min and 20 s. This time consisted of 20 s for fluid application and 1 min for centrifugation and braking.

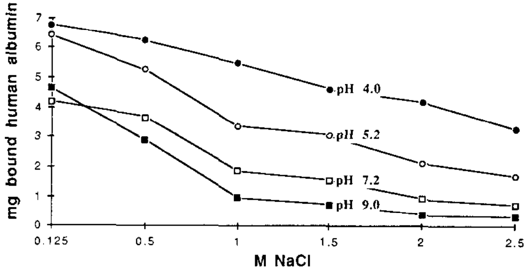

Fig. 1. The binding of purified human albumin to Blue Trisacryl during centrifugal affinity chromatography (600 *g*) at various pH values and ionic strengths.

The removal of human serum albumin from blood serum was the second example of CAC which we investigated. To study the influence of ionic strength and pH on the binding of human serum albumin to Blue-Trisacryl gel during CAC we applied purified human serum albumin to these columns. More human serum albumin was bound to the Blue-Trisacryl gels at lower ionic strength and pH (see Fig. 1). The same experiment was performed with whole serum. At pH 5.2 and 0.125 *M* sodium chloride, all serum proteins seemed to bind to the column. In order to get a more specific separation of human serum albumin from other blood proteins, a higher pH and salt concentration had to be employed. The best selective removal of human serum albumin from other blood proteins was achieved at pH 7.2 and 1 *M* sodium chloride (Fig. 2, lane 1). It is easily seen from Fig. 2 that it is possible to use CAC to completely remove human serum albumin from blood serum. The electrophoresis of concentrated whole serum is precluded by the presence of enormous quantities of human serum albumin which creates a long smear pattern on the surface of the electrophoresis gel and obliterates many other serum components. However, after CAC-based removal of human serum albumin from serum, the samples can be considerably concentrated prior to electrophoresis. This permits the detection of protein bands which would be otherwise obliterated.

Changing the flow-rate by increasing the applied centrifugal force from 350 to 600 *g* had no significant influence on the binding of purified human albumin to the columns. Alternatively, the flow-rate through the column can be changed by changing the membrane pore-size. Varying the membrane pore-size from a molecular-weight cut-off from 1 000 000 to 100 000 dalton did not significantly alter the binding of human serum albumin to Blue-Trisacryl columns. These data show that the flow-rates utilized in these experiments permit maximum binding of human serum albumin to the Blue-Trisacryl gel. It can also be noticed that some cracking of Blue-Trisacryl gel occurred occasionally.

To provide another example of the application of CAC we developed a test to determine the percentage of glycosylated hemoglobin in hemolysates utilizing a centrifugal column filled with boronate gel. Fig. 3 shows the profile of non-glycosylated and glycosylated hemoglobin in this CAC-based test. This suggests the intriguing possibility that CAC can be used for monitoring diabetes mellitus in clinical laboratories since the results obtained with the CAC-based test and the gravity based test were similar (Fig. 4), but the centrifugal method was three times faster. The resulting

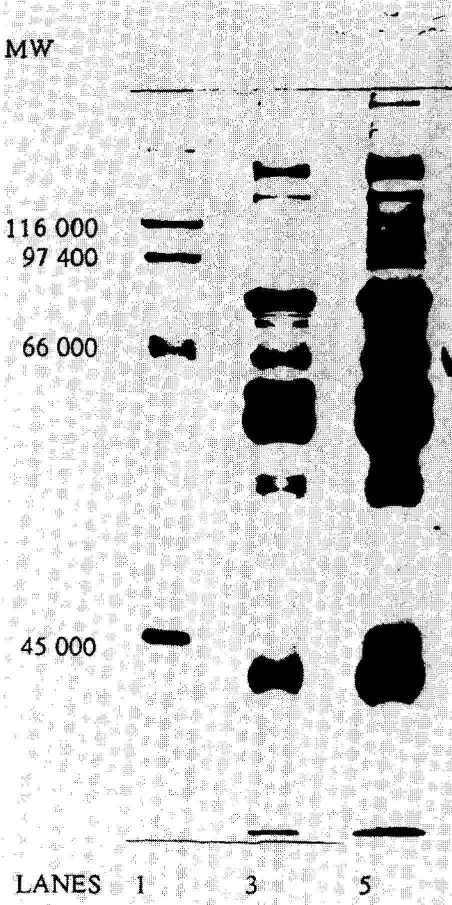

Fig. 2. The removal of human albumin from whole serum using centrifugal affinity chromatography (pH 7.2, 1*M* sodium chloride Blue-Trisacryl gel). Lane 1 contains markers, lane 3 human serum from which albumin has been removed prior to an eight-fold concentration and lane 5 contains whole serum. [Markers were: ovalbumin, molecular weight (MW) = 45 000; bovine serum albumin, MW = 66 000; phosphorylase B, MW = 97 400; and β-galactosidase (*E. coli*), MW = 116 000.]

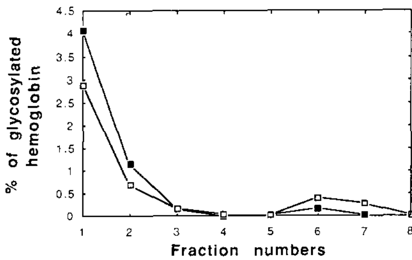

Fig. 3. The profiles of non-glycosylated (1–5) and glycosylated (fractions 6–8) hemoglobin of diabetic (□) and non-diabetic (■) hemolysates.

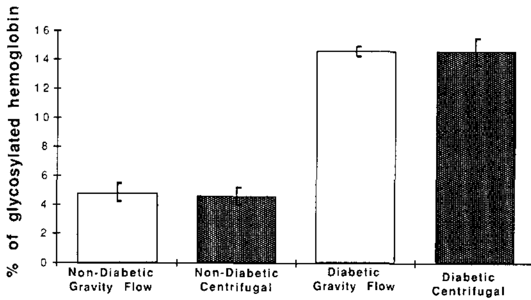

Fig. 4. Comparison of the centrifugal affinity chromatography based test for the detection of the percentage glycosylated hemoglobin in standard hemolysates with a gravity based method. The percent of glycosylated hemoglobin was determined using normal and diabetic standards (Pierce) as described in the text.

reduction of work time could reduce the labor costs significantly and, therefore, the centrifugal based method would be less expensive than the commonly used gravity based method.

In the above described experiments some cracking of the gel was noticed. Scouten and Elhardt[10] described cracking during gel permeation chromatography. They selected shrinking and cracking resistant matrices for gel permeation chromatography to prevent this problem. During CAC, however, cracking may even be an advantage. Cracking can explain the low binding and elution residence time needed in our experiments. When all of the interstitial water, and some of the intrastitial water, is removed from the beads, they shrink and finally the gel cracks. When a sample is subsequently applied to this column, an intimate absorption and contact between the sample and the column matrix occurs and, therefore, the effective local concentration is higher than in case of a normal chromatography column.

The advantage of CAC compared with gravity chromatography is not only the speed of analysis but also the fact that higher final product concentration can be obtained. During CAC, samples become less diluted since most of the interstitial, and even some of the intrastitial, water is removed. Furthermore, CAC is a very useful technique, unlike HPLAC, for the screening of different types of matrices, in that, using the HN-SII centrifuge, 18 different columns can be analysed simultaneously. Other centrifuges with other configurations may permit even larger number of column types, possibly hundreds, to be investigated simultaneously.

We have also noticed that the type of matrix and the applied centrifugal speed can be very important. The choice of matrix and the applied centrifugal speed are influenced by the hardness and the hydrophobicity of these beads. A matrix which is too rigid and/or too hydrophobic may have all of the fluid removed from the internal pores by the centrifugal process. Since it is difficult to rewet these types of matrices, some loss of capacity may occur. Conversely, poor yields may also be seen on elution due to the fact that some internal pore area will not be accessible to the elution buffer. Such matrices can be used but have to be employed at an appropriate, decreased centrifugal speed. Centrifuging such matrices until they lose all their interstitial fluid causes the development of air bubbles in the openings of such pores and subsequently they are inaccessible to exchange with the surrounding buffers. Conversely, a matrix which is excessively soft might collapse under the centrifugal force applied in the process and clog the membrane at the bottom of the column.

CONCLUSIONS

CAC has been demonstrated to be a very useful technique for many varied applications. The potential of this technique has been demonstrated in terms of the speed of analysis, ease of operation and costs of analysis. The purification of human IgG is an example of preparative application of CAC. The removal of human albumin from serum and the determination of the percentrage of glycosylated hemoglobin in hemolysates are both clinical applications. It is the authors' belief that CAC can be useful for many other applications, including the purification of nucleic acids, hydrophobic chromatography and dye chromatography. Unlike HPLAC, which is very useful for screening many samples on the same column, CAC is very useful for screening the same type of sample on many different columns, an obvious first step when one is trying to optimise an affinity chromatography system.

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