*Journal of Chromatography*, 603 (1992) 273–278
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 242

# Short Communication

# Multiple ligand applications in high-performance immunoaffinity chromatography

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(First received August 19th, 1991; revised manuscript received April 3rd, 1992)

ABSTRACT

A mixture of antibodies specific for albumin and transferrin, immobilized onto a single support, was used for the simultaneous extraction of albumin and transferrin from immunoglobulin G by high-performance immunoaffinity chromatography. The affinity column was coupled to a strong cation exchanger in order to monitor the succes of the extraction and to demonstrate the compatibility of the two chromatographic modes. Coupling of non-affinity chromatography with multiple ligand affinity chromatography is discussed as an alternative to positive affinity chromatography for protein purification.

## INTRODUCTION

The chromatographic purification of a protein from a complex sample matrix often poses a difficult challenge. The bulk of the extraneous components in a mixture can usually be removed using non-affinity chromatographic modes such as ion-exchange, hydrophobic interaction and size-exclusion chromatography [1,2]. However, complex mixtures frequently contain components whose chromatographic behavior closely resembles that of the protein of interest. When this is the case purification through non-affinity methods alone is very difficult, and an affinity step is often included in the procedure. Immunoaffinity chromatography is recognized as a powerful tool in protein purification [1,3,4]. It is characterized by extremely specific selectivity and is particularly valuable for separations involving components of such similar physical characteristics as to preclude resolution through other chromatographic modes. Good examples of such problematic separations include the resolution of the immunoglobulin fraction of serum according to class, or the purification of an antigen-specific antibody from the immunoglobulin fraction.

Immunoaffinity chromatography is usually associated with "positive" affinity methods in which the specificity of the immobilized affinity ligand is directed toward the compound of interest. When the sample is applied to an affinity column using this approach, the compound of interest is extracted through complexation with the affinity ligand and the other components in the sample pass through, unretained. The product is released from the affinity ligand by eluting in low-pH buffer or high concentrations of chaotropic salt solutions. An alternative to the positive displacement approach relies on the direct removal of contaminants from the compound

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0021-9673/92/$05.00 

of interest by using immobilized affinity ligands specific for the contaminants instead of the product. This technique, which can be called negative affinity chromatography, is often used as a clean-up step in the final stages of a purification [5,6]. The contaminant, using this approach, is retained on the column and the product is collected in the unretained fraction.

Negative affinity chromatography can be used to extract multiple contaminants in a single step. By mixing the required antibody populations, each bearing a specificity for one of the contaminants, and immobilizing the mixture as a whole, a multiple ligand affinity column is prepared posessing a multiple specificity for the antigens against which the individual antibodies were raised. In this study multiple ligand high performance affinity chromatography is demonstrated for the single step removal of two proteins, human albumin and transferrin, from a third protein, human immunoglobulin G (IgG). This was achieved by passing a mixture of the three proteins through an affinity column containing purified anti-albumin and anti-transferrin antibodies immobilized onto the surface of a silica-based affinity support. In order to monitor the success of the purification, the affinity column was positioned as a pre-column immediately preceding and coupled to a high-performance cation-exchange column for which a method had been developed for the separation of the three proteins. The compatibility of the conditions for the affinity and cation-exchange modes suggests the possibility of single-step performance of negative-affinity and cation-exchange chromatography. Furthermore, the concept can be extended to include affinity chromatography coupled to anion-exchange, size-exclusion and hydrophobic interaction chromatographic modes. The purification of the antibodies used in the study by positive affinity methods is also described.

## EXPERIMENTAL

### *Materials*

Proteins and antisera were obtained from Sigma (St. Louis, MO, USA). Human transferrin (Sigma T 3400) was stated by the manufacturer to be 98% pure and human albumin (Sigma A 8763) was stated to be globulin free and to have been purified from 96–99% albumin. Human IgG (Sigma I 4506) was described as at least 95% pure. Antisera to transferrin (Sigma T 6225) and albumin (Sigma A 1151) consisted of the $\gamma$-globulin fraction of whole goat antiserum and were tested by the manufacturer for antigen specificity. Other chemicals, all reagent grade, were from Baker.

### *Apparatus*

Chromatography and affinity purifications were performed using Rainin (Woburn, MA, USA) (Model HPX) HPLC pumps and Knaur Model 71 and Model 87 UV detectors from Rainin. Contro' of pumps, HPLC methods, data acquisition and treatment were accomplished with the Macintosh-based Dynamax HPLC Method Manager from Rainin. Spectrophotometric measurements were made on a Hitachi (San Jose, CA, USA) Model U-200 scanning spectrophotometer.

Hydropore-EP and Hydropore-5-SCX were obtained from Rainin. Hydropore-EP served as the affinity support in this study and is based on spherical silica (12 $\mu$m, 300 Å), the surface of which is chemically modified and possesses an epoxide functional group which reacts with proteins resulting in their covalent immobilization onto the support surface. Hydropore-5-SCX, based on spherical silica (5 $\mu$m, 300 Å), is a strong cation exchanger possessing a sulfopropyl functional group. Hydropore-EP and Hydropore-5-SCX were packed into 15 mm × 4.6 mm I.D. and 100 mm × 4.6 mm I.D. stainless-steel column modules, respectively. The column modules were coupled using Dynamax end fittings and analytical guard column hardware from Rainin.

### *Purification of anti-albumin and anti-transferrin antibodies from goat antiserum*

Antibodies specific for human albumin and transferrin were isolated from the $\gamma$-globulin fraction of goat antiserum according to procedures similar to those described in [7]. This was accomplished by passing the antiserum through a 100 × 4.6 mm I.D. column possessing the appropriate immobilized antigen, either albumin or transferrin. After washing the column in 20 m*M* potassium phosphate containing 150 m*M* sodium chloride at a pH of 6.9 (PBS loading buffer) to remove unretained and weakly associated components, the retained,

antigen-specific antibodies were eluted from the column by switching the mobile phase to the elution buffer, 50 m*M* PBS, pH 2.5. The retained fraction was collected, quickly neutralized with 5% sodium hydroxide, and the absorbance was measured at 280 nm. The amount of protein was estimated by using a 0.1% extinction coefficient of 1.3. The antigen columns had been previously prepared by recirculating a solution of albumin or transferrin dissolved in 1.0 *M* potassium phosphate, pH 7.0, through a column packed with Hydropore-EP for about 18 h.

Fig. 1 shows a chromatogram obtained from an injection of the γ-globulin fraction of goat anti-human transferrin onto the transferrin column. The first peak corresponds to unretained protein while the second peak represents the retained fraction corresponding to anti-transferrin antibodies. The chromatogram from an injection of anti-albumin antiserum onto the albumin column was very similar to the one shown in Fig. 1. Injections of the purified transferrin and albumin antibody preparations onto their respective antigen columns, using the chromatographic conditions described in Fig. 1, showed a retained band and almost no unretained protein, indicating highly pure and specific antibody populations.

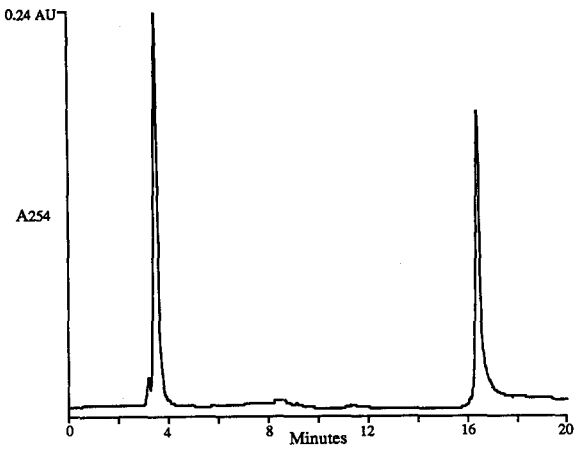


Fig. 1. Goat anti-human transferrin (γ-globulin fraction) injected onto the transferrin antigen column. Transferrin antibodies are found in the retained band. Sample: goat anti-human transferrin antiserum, 24.5 μg in 20 μl of load buffer; column: human transferrin immobilized on Hydropore-EP 100 × 4.6 mm I.D.; load buffer: 150 m*M* sodium chloride in 20 m*M* potassium phosphate, pH 6.9; elution buffer: 150 m*M* sodium chloride in 50 m*M* potassium phosphate, pH 2.5; flow-rate: load and elute at 0.5 ml/min, wash at 3.0 ml/min; detection at 254 nm, 0.24 AUFS; method: step gradient from loading to elution buffer.

*Preparation of the multiple ligand affinity column: immobilization of anti-transferrin and anti-albumin antibodies*

To combined solutions of purified antibodies containing equal amounts of anti-transferrin and anti-albumin antibodies was added an equal volume of saturated ammonium sulfate solution resulting in the precipitation of the antibodies. The precipitated protein was then isolated by centrifugation and reconstituted in a minimal volume of water. The anti(albumin,transferrin) affinity column was produced by introducing this solution, containing about 2.7 mg of protein, into a short cartridge packed with Hydropore-EP. The dimensions of the bed were 15 mm × 4.6 mm I.D. After 18 h at room temperature the affinity cartridge was washed several times with PBS, alternating between pH 6.9 and 2.5, after which the cartridge was ready to use. The capacity of the affinity cartridge was measured and found to be 330 μg and 375 μg, respectively, for transferrin and albumin when each was loaded separately. Previous studies indicate that the maximum albumin binding capacity for a cartridge of this size, at high densities of immobilized antibodies, would be 2.5–3 mg.

*Chromatography*

A method was developed for the separation of transferrin, albumin and IgG on the SCX stationary phase, and peaks for the individual components in the mixture were assigned by injection of the individual species. Mixtures of either (1) albumin and transferrin or (2) albumin, transferrin and IgG were injected onto either the SCX column alone or onto the SCX column coupled to the anti-(albumin, transferrin) affinity cartridge with the affinity cartridge positioned in the manner of a guard column. The chromatographic conditions, including protein concentrations, were identical for all injections and are described in Fig. 2. The proteins were eluted using a linear salt gradient formed from two pumps and a high pressure mixer; the volume of the mixer was 1.4 ml.

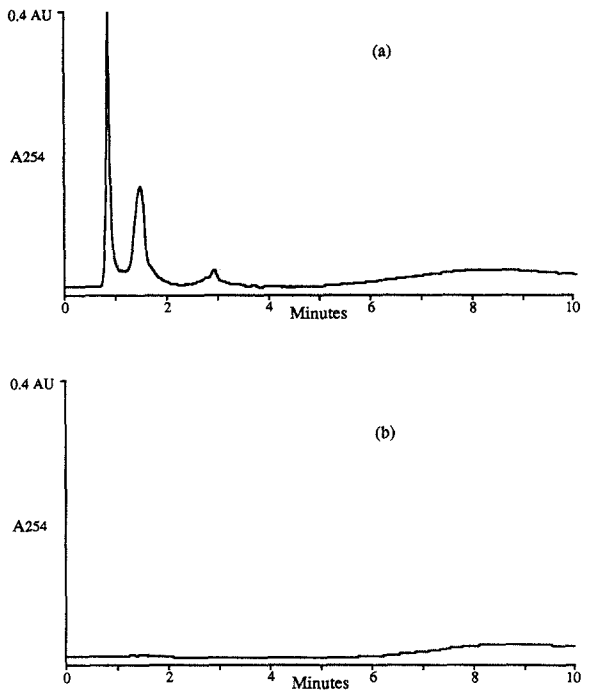


Fig. 2. (a) Mixture of albumin and transferrin injected onto the SCX column. Sample: human albumin (13.3 μg) and human transferrin (13.3 μg) in 20 μl buffer A; column: Hydropore-5-SCX, 100 × 4.6 mm I.D.; buffer A: 20 m*M* sodium acetate, pH 5.8; buffer B: 1.0 *M* sodium chloride in A; flow-rate: 1.0 ml/min; detection at 254 nm, 0.4 AUFS; method: 0–5 min, 0–70% B; 5–10 min, 70% B. (b) Same sample as in (a) injected on an anti-(albumin,transferrin) affinity column (15 × 4.6 mm I.D.) coupled to and preceding the SCX column. The conditions were identical to (a).

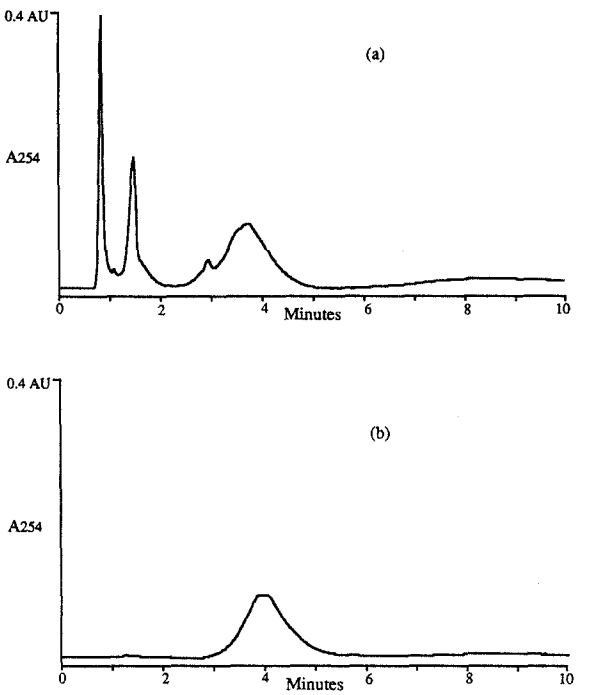


Fig. 3. (a) Mixture of albumin, transferrin and IgG injected onto the SCX column. Sample: human albumin (13.3 μg), human transferrin (13.3 μg) and human IgG (20.0 μg) in 20 μl buffer A; column: Hydropore-5-SCX, 100 × 4.6 mm I.D. The conditions were identical to those described in Fig. 2. (b) Same sample as in (a) injected onto the anti-(albumin,transferrin) affinity column (15 × 4.6 mm I.D.) coupled to and preceding the SCX column. The conditions were identical to (a).

## RESULTS AND DISCUSSION

The chromatogram shown in Fig. 2a is from the injection of a mixture of albumin and transferrin onto the SCX column. Albumin is represented by the peak appearing at 0.8 min and transferrin appears as a major peak at 1.5 min and also as a minor peak at about 2.9 min. In Fig. 2b a chromatogram is shown from the injection of the same mixture onto the anti-(albumin,transferrin) affinity cartridge coupled to and immediately preceding the SCX column. A comparison of the two chromatograms indicates the absence of albumin and transferrin in the chromatogram obtained from the coupled system as a result of their capture by the affinity pre-column. The chromatogram in Fig. 3a was obtained from the injection of a mixture of albumin, transferrin and IgG onto the SCX column. Albumin and transferrin appear as in Fig. 2a and IgG appears as a broad band at about 3.7 min. Fig. 3b shows a chromatogram for the injection of the same mixture onto the coupled affinity-SCX columns, obtained under the same conditions. Conspicuously absent in the second chromatogram are the peaks corresponding to albumin and transferrin which were extracted from the mixture by the affinity pre-column. The retention of IgG in the second chromatogram, seen as a broad band at 4.0 min, was delayed by about 0.3 min relative to that seen in Fig. 3a as a result of the additional dead volume contributed by the affinity pre-column. The areas of the two IgG peaks in Fig. 3a and b are nearly the

same indicating that very little, if any, IgG was lost in its passage over the anti-(albumin,transferrin) affinity column.

The integrity of the antigen–antibody complexes formed between albumin, transferrin and their antibodies in the affinity pre-column was apparently unperturbed by application of the salt gradient, as indicated by the absence of these proteins in the chromatograms shown in Figs. 2b and 3b. The stability of the complexes under these conditions allows the extraction of components from a mixture through the affinity mode while at the same time applying the conditions required for achieving separations in either a cation-exchange or anion-exchange mode. The compatibility of these conditions thus permits the two modes to be directly coupled thereby combining their respective selectivities in a manner analogous to that which has been described for other multi-dimensional chromatographic systems [8–11]. In addition to ion-exchange chromatography, these conditions would also be compatible with size exclusion chromatography and, in some cases, hydrophobic interaction chromatography.

For a mixture of IgG, albumin and transferrin, the application of both affinity and SCX modes is unnecessary for purifying IgG from the other two components. In this mixture only two impurities are present which can be easily removed with the multiple ligand affinity cartridge alone. Even without the affinity mode, major resolution of the three components was achieved in the SCX mode, as seen in Fig. 3. In this study the non-affinity (SCX) mode was coupled to the affinity cartridge solely for the purpose of illustrating the compatibility of the two modes and for monitoring the success of the affinity extraction. A coupled system, analogous to that described in this study, would be most useful for more complex sample mixtures for which either separation mode alone would be insufficient for purification of the product. For such a hypothetical sample mixture most of the extraneous components could be separated from the compound of interest through a non-affinity mode, yielding a product in a predominantly pure state but containing a limited number of minor, but difficult to remove, contaminants. To this mode would be coupled a multiple ligand affinity column possessing immobilized antibodies with specificities corresponding to the contaminants co-eluting with the product in the non-affinity mode. The affinity column would thus extract the components from the mixture which, in the non-affinity mode, could not be separated from the product. For a purification scheme consisting of several non-affinity steps the affinity column would be coupled to the last mode in the sequence. The affinity cartridge in this study possessed specificities for only two proteins, but the principle can be readily extended for multi-component extraction, according to the number of contaminants present.

In this study a very short affinity pre-column (1.5 cm) was coupled directly to the SCX column, and the IgG peak eluting from the coupled column arrangement showed only slight broadening in comparison to the peak obtained from injection of the sample on the SCX column alone (Fig. 3b and a, respectively). However, for some processes direct coupling of the affinity column could result in significant losses of resolution in the non-affinity mode thereby introducing unnecessary contaminants overlapping with the product band. Undesirable interference from the coupled affinity column would arise from contributions of the affinity column to band broadening and from tailing due to weak associations between sample components and the affinity stationary phase. This would be particularly true for longer affinity columns and for complex, concentrated samples.

While the coupling arrangement used in this study might suffice for many applications, the problems described above could be avoided by the installation of one or more multiple port switching valves to control the path of the sample and mobile phase. In such an arrangement the sample would initially pass through the non-affinity column with the valves positioned such that the effluent containing any early eluting bands would by-pass the affinity column. Immediately before elution of the band containing the product, the valve configuration would be switched to re-direct the effluent from the first column through the affinity column. The product and any co-eluting impurities would then pass from the first column directly onto the affinity column which would be designed to extract the impurities co-eluting with the product. If the separation of the mixture on the first column subsequent to product elution were also of interest (for example, if it were desirable to collect more than one product

from the separation) the valve(s) could be returned to the initial configuration, once again by-passing the affinity column and preventing its interference in the final stages of the separation. Several authors have recently published papers describing system configurations designed to coordinate processes involving coupled columns [9,10,12] which could be adapted for the purposes described here.

Immunoaffinity chromatography continues to be an expensive process, and is even more so for applications in which the necessary antibodies are unavailable and must be custom produced. Purification of a product by immunoaffinity extraction of the contaminants requires antibodies specific for the contaminants. In many cases the identity of the contaminants may be either unknown or, if known, their antibodies may be unavailable. As the number of impurities increases this problem is compounded. Thus, for many processes the multiple ligand-negative affinity approach would prove impractical, and a positive affinity approach would be indicated.

For some processes, however, the amount of antibody required for the positive affinity approach could exceed by many times that needed for the removal of a few percent impurity. In these cases the reduction in antibody requirements achieved through the negative affinity approach could justify the effort to isolate the impurities and produce their purified antibodies. Whether this would be worthwhile would depend on the scale of the process, the difficulty in isolating the impurities, and on the level of in-laboratory expertise.

Another consideration which can make the positive affinity approach less attractive are factors that are associated with positive affinity chromatography but are less important in the negative affinity approach. These include the use of denaturing eluents and the non-immunospecific binding of unwanted sample components to the affinity phase along with the product [13–15]. As pointed out in ref. 14, non-specific binding of contaminants, in particular, is a common problem in positive affinity chromatography. This results in the elution of the non-specifically bound contaminants along with the product, and may necessitate additional chromatographic steps in order to reach the desired level of product purity. In negative affinity chromatography, since the product appears in the unretained fraction this is less of a problem.

In conclusion, the compatibility of the conditions used in negative affinity chromatography with other separation modes suggests that this technique could be readily coupled with other kinds of stationary phases. In addition antibody populations of multiple specificity can be immobilized onto a single support thereby permitting single step removal of multiple impurities. Many non-affinity modes are themselves compatible and amenable to on-line coupling, as reviewed by Majors [11], so that in some cases an affinity column could be incorporated into a pre-existing coupling scheme. Currently available automation enables precise coordination of system devices and events within a coupled system through method programming, thereby providing the potential for reducing the number of steps in a complex purification scheme.

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