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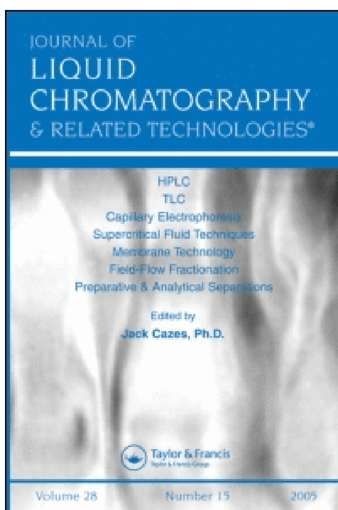

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Use of New HPLC Resins to Solve Old Problems

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USE OF NEW HPLC RESINS TO SOLVE OLD PROBLEMS

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ABSTRACT

A summation of recent experiences from this laboratory is described in this report. In the case of isolating peptides for eventual use in protein sequence analysis, an argument is made for the return to multistep chromatography in order to simplify complex mixtures of peptide fragments. Making use of new resins for ion exchange and reverse phase chromatography, yield of material even after three chromatographic steps is in excess of 90%. A partial enzymatic digest yielding multiple overlapping peptides can readily be sorted by this approach. For the isolation of monoclonal antibodies, the use of carboxymethyl derivatized synthetic resins allows for rapid isolation and concentration of antibodies which can then be highly purified on diethylaminoethyl polymeric based resins.

INTRODUCTION

High performance liquid chromatography (HPLC) is currently utilized by scientists in virtually every discipline of

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medicine and biology. Instrument manufacturers have made a bewildering number and variety of hardware components available to consumers and, more to the point of this report, manufacturers of resins have presented a virtually unlimited choice of sizes of columns and types of packing materials. This manuscript will concern itself with two specific separations: 1) peptides generated after enzymatic hydrolysis of proteins for amino acid sequence analysis and 2) and monoclonal antibodies isolated from either ascites fluids or tissue culture supernates. These are two areas in which growing numbers of investigators have become interested and for which new families of resins are fast becoming commercially available.

Historically, HPLC was best suited for characterization of small molecules and depended upon reverse phase resins which consisted of irregular shaped silica particles to which were bonded various functional groups. The efficiency of these resins was far greater than that of dextran based resins and since they could withstand high pressures, contact time between material and resin could be significantly reduced and ultimate yield of product was greatly enhanced. Improvements in resin technology have included use of sized, spherical silica particles to reduce back pressure, the development of various sizes and shapes of columns, and the availability of many functional groups and improved capping procedures to continue

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to improve yields of separated materials. Two direct results of these improvements have been an increase in "theoretical plates" for these columns and the ability to separate larger compounds such as peptides and some proteins. Two limitations of silica based resins have been the inability to elute large protein molecules in a dependable and consistent way and the inability to utilize solvents above pH 8.0. These problems stem directly from the relatively small pore sizes in typical silica resins (50-120 A) and the fact that silica hydrolyses at alkaline pH. Newer reverse phase resins which utilize organic polymer materials as their backbones and are available with larger pore sizes (300-1000 A) have done a great deal to eliminate both the problem with separating high molecular weight peptides and the restrictions of pH of the mobile phases.

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Immunoglobulins have traditionally been isolated using DEAE resins since they became available in the late 1950's (1). This resin was chosen because at a slightly alkaline pH, gamma globulin containing antibody activity comes straight through a column packed with this material. If large volumes of serum need to be processed, a large column can be utilized. Although it was appreciated that immunoglobulin and antibody activity actually could be found throughout the DEAE profile, the bulk of activity was isolated in the gamma globulin fraction and was free of albumin and other major contaminants (2). Monoclonal

antibody technology has, unfortunately, radically changed DEAE separations. Since any given monoclonal antibody is a homogeneous population and will have its own unique electrophoretic charge, there is little chance of predicting where it will elute in the electrophoretic profile without testing a variety of elution conditions. There are examples of monoclonal IgG antibodies eluting anywhere from the gamma globulin region all the way out to the albumin region. There are several sorts of DEAE resins currently available which are specifically tailored for use in the HPLC and consist of a strong anion exchanger bonded to either silica, coated silica or one of several polymeric materials. Silica ion exchange resins suffer from the same problems which plague silica reverse phase resins, i.e. generally small pore sizes and degradation at alkaline pH ranges. More recent silica resins have become available with larger pores (500 -1000 A) and coating the silica with either polyacrylamide or other protective layers greatly inhibits alkaline hydrolysis. In addition, there are now several polymer based ion exchange resins such as polyviton, polystyrene/divinyl benzene, and other proprietary formulations. Although these resins are available, there is still a fundamental problem which relates to the unpredictable nature with which monoclonal antibodies elute from anion exchangers. In addition, when it is desired to isolate monoclonal antibodies from tissue culture supernates

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instead of from ascites fluids, there is a serious problem with phenol red, the most common pH indicator present in tissue culture medium, adsorbing to the ion exchanger and eluting very slowly over a long period of time. The advent of polymer based resins with cationic functionalities encouraged us to try a different philosophical approach to isolating monoclonal antibodies and the results of that approach will form part of the basis of this report.

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MATERIALS AND METHODS

RESINS. Microbondapak C18 (Waters Associates, Milford, Mass) in a 4.6 X 150 cm steel column was compared with a column obtained from Polymer Laboratories (Stow, Ohio) which was also 4.6 X 150 cm packed in a steel column. The PLRP-S resin is a polymer with 300 A pores and is capable of operating at any pH range. Ion exchange resins were Fractogel DEAE superfine and Fractogel CM superfine obtained from EM Science (Gibbstown, NJ) and were poured into a variety of column configurations as indicated in the manuscript. In most cases, these columns were run using a simple peristaltic pump since they have such low backpressures and do not require HPLC pumps.

BUFFERS. Buffers for the Fractogel CM ion exchanger were 0.01M sodium acetate, pH 5.5 as starting buffer and the same buffer

but containing 0.5M sodium chloride was the limit buffer. Buffers for the Fractogel DEAE ion exchanger were 0.015M Tris, pH 8.4 as starting buffer and the same buffer with 0.5M sodium chloride was the limit buffer. Peptide isolations performed on the Fractogel DEAE ion exchanger utilized a volatile buffer system of pyridine and N ethyl morpholine (1 ml of each/ 500 ml water, pH 8.0 with formic acid) as starting buffer and the same constituents except 5 ml of each/ 500 ml of water, pH 4 as the limit buffer. Reverse phase HPLC was performed using 0.025% trifluoroacetic acid (TFA) in water and acetonitrile with .025% TFA as the developing eluent.

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SAMPLES. The peptides followed through size separation, ion exchange separation, and reverse phase chromatography were from a tryptic digest of extensively reduced and alkylated (iodoacetic acid) immunoglobulin kappa light chain. The monoclonal antibody separations are from tissue culture supernates of murine hybridoma cell lines propagated in this laboratory. The fusion partner for the cell lines utilized here is an IgG2b secretor so that the active antibody containing fraction is mixed in with a series of inactive immunoglobulins.

INSTRUMENTATION. Reverse phase chromatography was performed on a Waters Associates HPLC consisting of two M6000 pumps, WISP

autoinjector, 680 gradient controller, 440 photometer with an extended wavelength module at 214nm, and a strip chart recorder. An ISCO (Lincoln, NE) fraction collector was used to collect column effluents and is wired into the HPLC strip chart recorder to deliver a signal each time a tube is advanced. For ion exchange chromatography, an ISCO UA5 monitor was used to detect peaks at 280nm in the effluent.

RESULTS

SEPARATION OF TRYPTIC FRAGMENTS OF HUMAN IMMUNOGLOBULIN LIGHT CHAIN. Ten mg of extensively reduced and radioalkylated human κ chain were digested with 1% w/w TPCK trypsin (Worthington Biochemicals, Freehold, NJ) at 37 C for 4 hours and a crude peptide separation was performed on Sephadex G50 (Pharmacia) in a column 1.6 X 20 cm equilibrated in 0.1M ammonium hydroxide. This separation takes less than 2 hours and the recoveries are in excess of 90%. Typically, there are 4 or 5 fairly discreet "clusters" of peptide material which can be pooled independently and lyophilized. Only on very rare occasions can any pure peptide be isolated at this stage and Figure 1 shows the profiles of a small aliquot of 2 of these pools compared on a silica based C18 reverse phase resin (B) and the C18 reverse phase polymer based resin (A). It is clear that for the material chosen for this example which represents the pool of large peptides from the size separation, there are some clear

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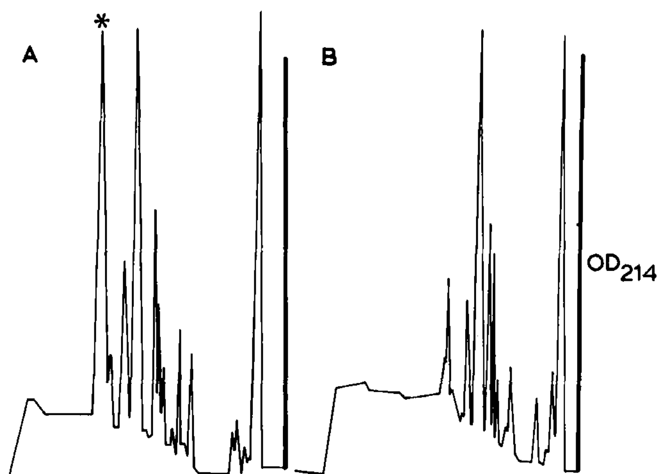

Figure 1. Elution profile of large (> 35 amino acids) peptides from a silica based (panel B) and polymer based (panel A) C18 reverse phase column. Flow rate was 0.6 ml/min, starting buffer was 10% acetonitrile, and a 30 min linear gradient to 50% acetonitrile was used. Injection to injection time was 40 minutes. Full scale on the 214 nm photometer was 0.2 absorbance units. The asterisk (*) depicts the major peak which eluted from the PLRP-S resin and has never been eluted from the silica resin. Elution is from right to left.

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differences in recovery of material eluted from these columns. There are a series of peptides which are quite comparable, but there is also a major peptide which elutes late in the profile from the polymer based resin and is simply not eluted from the silica based resin. It should be pointed out that neither profile represents any optimization of separation - simply a comparison under identical conditions. Although it is possible to slow the gradient and spread out the peaks by increasing

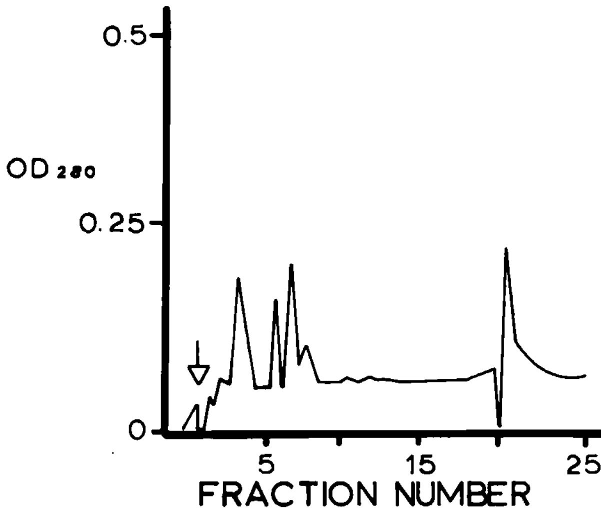

Figure 2. Elution profile from DEAE - Fractogel (0.6 x 12 cm in a pyridine/N-ethyl morpholine gradient system of peptides (10-30 amino acids in length) from an enzymatic digest of immunoglobulin L chain. Flow rate was 1.2 ml/min and the mixture had approximately 12 major peptides as judged by direct reverse phase analysis of the mixture before ion exchange chromatography.

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total run time, peaks clustered as seen here would prove to be difficult to isolate to purity in this one step. Therefore, the bulk of the material isolated after size separation is typically fractionated on a DEAE ion exchange resin to further simplify the mixture. Figure 2 depicts the elution profile from DEAE Fractogel (0.6 X 12 cm) of another of the size fractions from Sephadex G50. A typical column run (100 ml of buffer, 1.2 ml/min) takes less than 2 hours and recovery of radiolabel is in excess of 90%. This profile was

monitored at 280nm on an ISCO UA5 monitor and the buffer flows from the pump through the reference side of the detector, through the column, and then through the sample side of the detector. This route automatically "subtracts" the increasing OD280 due to increasing pyridine concentration (3). Each group of eluted peptides, not necessarily purified at this stage either, is lyophilized in a SpeedVac apparatus (Savant, Hicksville, NY). Figure 3 shows the elution profile from the polymer based reverse phase HPLC resin of two of the DEAE "peaks" and it is apparent that the limited mixture of major peptides in this fraction will be quite simple to separate into purified materials which are ready for amino acid sequence analysis.

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ISOLATION OF MONOCLONAL ANTIBODIES. Three different column configurations were used in these studies. 100 ml of cell culture supernate were concentrated by positive pressure to approximately 20 ml and dialyzed overnight against 0.015 M Tris pH 8.4. The material which still had considerable phenol red was loaded onto a 1.6 X 18 cm column of Fractogel DEAE and eluted with a linear salt gradient. Figure 4 depicts the elution profile and the fractions which contain the monoclonal antibody as determined by an enzyme linked immunoadsorbent assay (ELISA). The column has separated the tissue culture supernate into a series of fractions which correspond to

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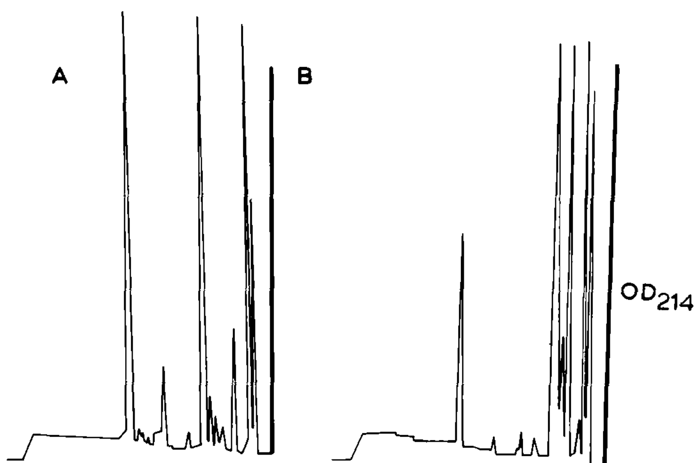

Figure 3. Elution profiles from PLRP-S analytical column of an aliquot (8%) of material from fraction 3 (A) and 6 (B) from figure 2. Column conditions were identical to those described in figure 1.

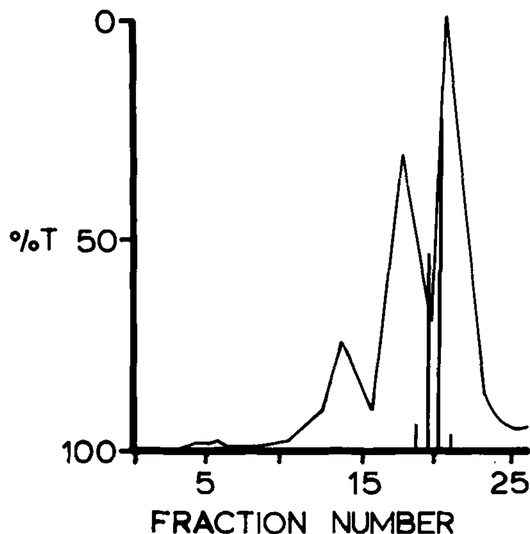

Figure 4. Elution profile from DEAE - Fractogel (1.6 x 18 cm) in an aqueous TRIS system at pH 8.4 of a concentrated (100 ml reduced to 20 ml), dialyzed sample of monoclonal antibody in tissue culture supernate. Flow rate was 1.2 ml/min. Antibody activity (black bars) was monitored by an enzyme linked immunosorbant assay (ELISA).

increasing negative charge with albumin being the last major protein to elute.

Although the bulk of gamma globulin elutes early, this particular monoclonal antibody (which is present in such low concentration (approx. 1 mg) so as not to be identified by optical density in the profile) elutes relatively late in the profile and therefore has not been purified even to the point where it can be used to coat ELISA plates. In addition to this lack of separation, the column becomes seriously contaminated with phenol red and will have to be eluted with high salt containing buffers for many hours. Note that for 1 mg of antibody, a relatively large column was necessary since the majority of the resin is committed to binding albumin, the most predominant protein in either ascites or cell culture supernates. Figure 5, on the other hand was the result of simply diluting 100 ml of tissue culture supernate with 200 ml of 0.01M sodium acetate, pH 5.5, filtering the diluted material through Whatman 1 filter paper (4.5 cm circle in a Buchner funnel) and pumping it onto a column of Fractogel CM (1.6 X 6 cm) at a flow rate of 5.8 ml/min. The profile shows an enormous "fall-through" followed by material eluted by means of a salt gradient. This run was completed in less than 2 hours and the total linear salt gradient volume was 200 ml. The "fall-through" fractions consist of albumin and other proteins while the salt eluate contains transferrin (pink) and

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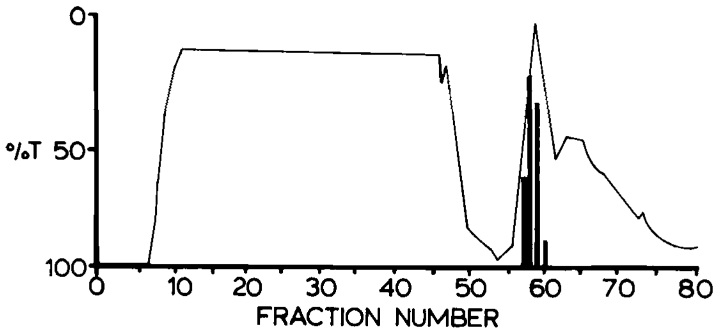

Figure 5. Elution profile from CM - Fractogel separation of 100 ml of monoclonal antibody cell culture supernate which has been diluted with pH 5.5, 0.01 M sodium acetate. Flow rate was 5.8 ml/min, column dimensions were 1.6 x 6 cm, and a linear gradient (200 ml total volume) from 0 - 0.5 M NaCl was used. Antibody activity (black bars) was monitored by ELISA.

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gamma globulin. What has not been depicted here is the fact that all of the phenol red is also in the "fall-through" fractions - it does not seem to adsorb to this resin under these conditions at all. The monoclonal antibody activity is seen to reside entirely in the gamma globulin containing fraction eluted in a small volume after the start of the gradient. Figure 6 depicts the separation which occurs if the gamma globulin fraction from the material isolated in Figure 5 is diluted three-fold in 0.015M TRIS pH 8.4 to reduce the sodium chloride concentration and loaded onto a small (0.4 X 8 cm) column of Fractogel DEAE in the Tris system. The pink (transferrin) material is separated from the antibody activity

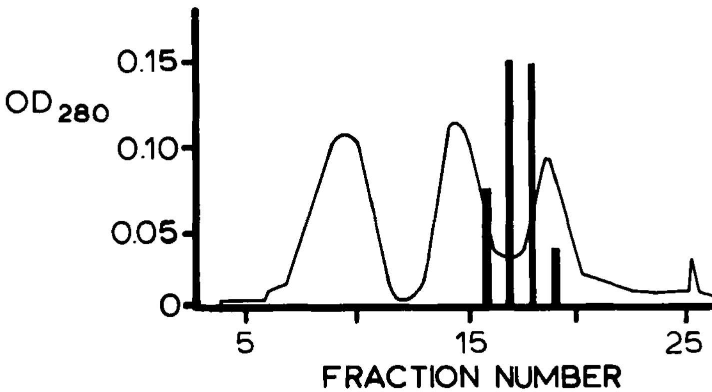

Figure 6. Elution profile from DEAE - Fractogel separation of material with antibody activity in figure 5 after dilution with pH 8.4, 0.015 M TRIS. Flow rate was 1.2 ml/min, column dimensions were 0.4 x 8 cm, and a linear gradient (80 ml total) from 0 - 0.5 M NaCl was used. Antibody activity (black bars) was monitored by ELISA. The early eluting peak was identified as transferrin by polyacrylamide electrophoresis and the remaining peaks are immunoglobulins as judged by the same analysis. They presumably originate from tissue culture serum constituents and the production of hybrid molecules with no antibody activity by the producing hybridoma fusion line utilized in these studies.

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and the antibody activity is separated from the bulk of gamma globulin resulting in a preparation of virtually pure monoclonal antibody which can be used for any and all subsequent assay systems. During this entire process which starts with a CM chromatographic step, no dialysis is required and all 100 ml is processed to pure monoclonal antibody in less than 4 hours with recovery estimated from ELISA assays to be in excess of 90%.

DISCUSSION

These studies attempt to demonstrate several points of interest to chromatographers. The most general and overriding concept is that with currently available resins, in particular polymer based, large pore resins, yields of materials are so high as to make it feasible to pass a sample over several columns without suffering significant losses. The benefits of that approach are that mixtures of materials (peptides or proteins) can be simplified so that in the end, it is not necessary to depend upon the ability to separate a dozen or more peptides as they elute from a column. Various columns flow at different rates and rather exacting calculations need to be made to determine the lag time from detector to fraction collector. When several peaks elute from a column within seconds of each other, it is difficult to collect each in a pure state. So, although large numbers of "theoretical plates" are useful for analytical mapping and comparing elution profiles, the requirements for isolating preparative amounts of materials (whatever the scale) are quite different and are not easily solved by a single pass through a single column under one set of conditions. In a practical sense, protein chemists have been using the approach described here for many years - the difference is simply that the resins available today are much better than anything previously available. For peptide and protein separations,

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this manuscript has attempted to demonstrate with several examples that a 300 A polymer based C18 column outperforms a comparable silica based column with smaller pores, both in terms of the ability to recover materials and the potential of using mobile phases spanning the entire pH range. Recovery of material is so outstanding on these columns, that it is not usually necessary to run test chromatograms at each separation step to see if the elution profile can be modified in some way to ensure purification of at least a few peptides at each step. Rather, an enzymatic digest is routinely subjected to molecular sieving, ion exchange in a volatile buffer of each of the 4 to 5 pools, and reverse phase chromatography of each pool from ion exchange. On rare occasions, it is necessary to rechromatograph material after the C18 step and that is typically performed on a reverse phase column with a cyano functional group.

The isolation of monoclonal antibodies has become sufficiently important that a number of dedicated systems and resins are currently available. Looking through the literature, it is possible to find 4 popular methods to purify monoclonal antibodies used by most investigators. Affinity purification of antibodies (4) using an appropriate immobilized antigen gives rise to pure antibody, but it often proves difficult to isolate large amounts of antibody, the activity of the antibody is sometimes affected by the conditions necessary

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to elute the antibody from the antigen, and sometimes it is impractical to isolate the relevant antigen for immobilization. Another popular technique involves use of specialized affinity resins designed to adsorb specific serum components, typically albumin. These affinity dyes (5) are capable of partially clearing serum, but are not terribly efficient. A more recent innovation is the use of hydroxyapatite (6). This seems to work well, but is somewhat limited by the fact that large amounts of serum or tissue culture supernate are difficult to work with and conditions of elution of monoclonal antibodies need to be modified for each monoclonal. The fourth method, probably the most popular, and certainly historically the one which immunologists have relied upon for 25 years is the use of DEAE and other strong anion exchangers (2). Although DEAE has come a long way since it was available bound only to cellulose (7), there are a number of philosophical problems associated with its use for the separation of monoclonal antibodies. In the first place, the very best DEAE resins available for HPLC technology are very expensive. These resins, polymeric and proprietary in nature, are 1000 A pore size resins capable of withstanding several thousand pounds of pressure, with high capacities, and therefore amenable to pumping large volumes of solvent (at virtually any pH) quickly. Although they are high capacity resins, there is no question but that albumin binds to them better than any other major serum protein and they are

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simply not capable of being loaded with more than a few milliliters of ascites or tissue culture fluid. In addition, since each monoclonal antibody has a unique net charge at a given pH, there is again, no predicting where the antibody will elute in the gradient and each monoclonal must be screened and the elution conditions adjusted. Although it is possible to perform large numbers of test runs with modified elution protocols using little material and at high speed, eventually one must find out where the antibody has eluted and the assay for that may take a day or two. So the HPLC part is rapid, but the answer comes slowly.

Because of these problems with the four most popular methods of monoclonal antibody isolation, and especially because this laboratory generates far more tissue culture fluid than ascites fluid which complicates the albumin problem exponentially, a general technique has been developed which allows large (or small) volumes of ascites or tissue culture supernate to be processed and relatively pure antibody isolated in a matter of hours from start to finish. The approach takes advantage of the fact that large-pore (500 and 1000 A) polymer based cation exchangers also exist. At slightly acidic pH values (5.5 in the example given here) these exchangers do not bind albumin, phenol red, and a host of other alpha and beta mobility proteins found in serum. They do bind immunoglobulins and "slow" beta proteins such as transferrin. Just as is the

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case for monoclonal antibodies with DEAE (where it is not exactly clear why many antibodies elute so late in the elution profile) in all cases examined in our laboratory to date, all monoclonal antibodies bind to the cation exchanger. Even antibodies which upon subsequent elution from DEAE turn out to elute quite late in the profile bind well to the CM column. The obvious immediate advantage of this approach is that large volumes of ascites or tissue culture supernate can be passed over a very small column of CM Fractogel since only the antibody fraction will adsorb and the bulk of serum proteins pass through. In addition, the sample need only be diluted to reduce the salt concentration and adjust the pH - no concentration or dialysis is necessary. Further fractionation of the salt eluted antibody containing fraction can be performed on very small DEAE columns since most of the other serum proteins are gone and again, no dialysis is necessary - simply diluting the antibody containing eluate approximately 3 fold with DEAE salt-free starting buffer is enough to optimize the salt concentration and pH.

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SUMMARY

This manuscript attempts to sum up the observations of this laboratory over the past six months with respect to approaches to the isolation of peptides for amino acid sequence analysis and the isolation of monoclonal antibodies for

immunoassays. The general conclusions reached here are that it no longer seems necessary to go to a great deal of trouble optimizing chromatographic separations so as to be able to isolate materials in a single pass through a single column. Rather, it would appear that with the advent of very powerful resin technology yielding polymer based resins which operate at a wide range of pH's and pressures and yielding recoveries well in excess of 90%, a strong case can be made for the return to multiple steps of isolation thereby simplifying peptide mixtures and making the ultimate preparative recovery of useful materials much more predictable.

The isolation of monoclonal antibodies from large volumes of tissue culture supernates can be performed in a matter of hours with no dialysis or concentration steps by first using CM functional groups to concentrate and purify the antibody containing fraction with subsequent DEAE chromatography of that material. Both types of separation take advantage of new, medium pressure polymeric wide-pore resins described in this report.

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view of the authors' experiences rather than serve as a formal source of reference to the literature. The authors make no claim as to originality of approaches or unique findings presented herein.

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