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PROTEIN A, HYDROXYAPATITE AND DIETHYLAMINOETHYL: EVALUATION OF THREE PROCEDURES FOR THE PREPARATIVE PURIFICATION OF MONOCLONAL ANTIBODIES BY HIGH-PERFOMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Three rapid, reproducible and feasible monoclonal antibody purification procedures by means of high-performance liquid chromatography have been evaluated. The stationary phases employed were high-performance hydroxyapatite, high-performance Protein A and high-performance anion-exchange resin. The purity of the final immunoglobulin preparations was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions and, subsequently, by high-performance gel permeation chromatography. The immunoreactivity of purified monoclonal antibodies was determined by the radioimmunoassay method.

INTRODUCTION

Intraperitoneal injection of hybridoma cells into mice generates ascitic fluid, containing not only monoclonal antibodies (MoAbs), but also some other proteins, such as transferrin, albumin and proteases[1]. Due to their high specificity, MoAbs have found a great number of applications in the field of diagnosis, therapy and basic and applied research. The demand for large amounts of purified MoAbs for *in vitro* and *in vivo* use prompted the development of rapid, reproducible and feasible purification procedures.

Classical open-column chromatography is the most widely employed method for obtaining MoAbs on a large scale by means of anion-exchange (DEAE-cellulose)[2], affinity (Protein A)[3] and gel chromatography (Sephacryl, Sephadex)[4]. Recent improvements in high-performance liquid chromatography (HPLC) technology and the availability of new rigid polymeric resins has allowed the development of more rapid, convenient and efficient procedures for MoAbs purification. However, though several reports regarding the analytical procedure have been published[5–7], little has been done to develop preparative HPLC techniques for purification of MoAbs[16,17].

We have studied the preparative purification of MoAbs by HPLC, in order to verify the applicability of conventional purification techniques to HPLC. In this paper, we compare preparative Protein A, hydroxyapatite and anion-exchange HPLC

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focusing attention on protein recovery, purity of MoAbs and immunoreactivity. The three procedures employed provided highly purified MoAbs and a high protein recovery. The high-performance Protein A chromatography is therefore preferred for purification of immunoglobulin G (IgG). A previous chromatographic procedure to separate different MoAbs from the contaminant proteins is not required. The pH stepwise gradient elution can allow the separation of IgG subclasses[3]. Anion exchange or hydroxyapatite chromatography was employed for IgM purification.

MATERIALS AND METHODS

Materials

All the solvents employed were of HPLC reagent grade (E. Merck, Darmstadt, F.R.G.). Glycine, sodium chloride, disodium hydrogenorthophosphate, sodium dihydrogenphosphate, citric acid, tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) and 2-mercaptoethanol were from Farmitalia Carlo Erba (Milan, Italy). Bovine serum albumin (Fraction V, chemical grade) was obtained from Miles Biochemicals and Immunochemicals (Milan, Italy). Polyethylene glycol (PEG) 6000 was from Merck.

The mouse ascitic fluid, containing an IgG1 kappa light-chain antibody (EK3) against human thyrotropin hormone (hTSH) was provided by Professor Ekins (Middlesex Hospital, London, U.K.).

Chromatographic equipment

Preparative purification of MoAbs was performed with an HPLC system (MAPS Preparative System 100; Bio-Rad, Richmond, CA, U.S.A.). The system was supplied with an automated injection mechanism (50-ml injection capacity), a variable-wavelength UV detector and a conductivity monitor for gradient elution. The MAPS Preparative System also featured dual-flow paths for analytical and preparative separation and a fraction collector. For integration and peak area reports, a Model 3392A integrator (Hewlett-Packard, Vancouver, WA, U.S.A.) and a Bio-Rad recorder, respectively, were used.

An Affi-Prep Protein A preparative column (100 mm × 25 mm; 50-ml bed volume), MAPS HPHA preparative guard cartridge (25 mm × 15 mm; 7.5-ml bed volume), MAPS HPHA preparative column (50 mm × 25 mm; 25-ml bed volume) and MAPS HPHA analytical cartridge (30 mm × 4.6 mm; 0.5-ml bed volume) were obtained from Bio-Rad. A Protein Pak DEAE 5PW semipreparative column (150 mm × 21.5 mm; 55-ml bed volume) was from Waters Associates (Milford, MA, U.S.A.). A high-performance gel permeation column (Bio-Sil TSK 250, 300 mm × 7.5 mm; Bio-Rad) together with a Waters 510 pump and an U6K injection system were used for the evaluation of the purity of MoAbs. Detection was performed at 278 nm by employing a Waters 481 UV spectrophotometer. For peak area integration, a Waters 730 data module was used.

Electrophoresis system

The purity of MoAbs was also evaluated by high-performance gel permeation chromatography (HP-GPC) as well as by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, according to the method

of Laemmli[8]. SDS-PAGE was performed on the Phast system (Pharmacia, Uppsala, Sweden) which consists of integrated separation and development units. The samples were loaded onto prepacked gels (PhastGel gradient 10-15). Gels were stained with Coomassie Brilliant Blue on a Phast system development unit.

Sample preparation

Prior to purification, ascitic fluid was treated with ammonium sulphate (50% saturation) at 4°C for 1 h. The solution was then centrifuged at 4000 rpm for 20 min; the pellet was resuspended and then dialyzed overnight at 4°C against deionized and distilled water. The sample was dried in a Speed-Vac concentrator (Savant Instrument, Farmingdale, NY, U.S.A.). It was resuspended in the chromatographic starting buffers and passed through a 0.45-μm filter.

Preparative high-performance hydroxyapatite (HPHA) chromatography

The conditions for HPHA chromatography were according to a previous report on the purification of murine MoAbs[9]. In order to optimize the separation method, the sample (100 μl) diluted to 1 ml in 10 m*M* phosphate buffer at pH 6.8 (buffer A) was injected into an HPHA analytical cartridge. A linear gradient was then performed from buffer A to 300 m*M* phosphate buffer at pH 6.8 (buffer B) in 15 min at a flow-rate of 0.4 ml/min.

As shown in Fig. 1a, the MoAb peak was not separated from contaminant proteins. A second chromatographic development was carried out with the same sample (100 μl); the cartridge was conditioned with 10% buffer B. After injection of 100 μl of the sample diluted to 1 ml in buffer A containing 10% of buffer B, the

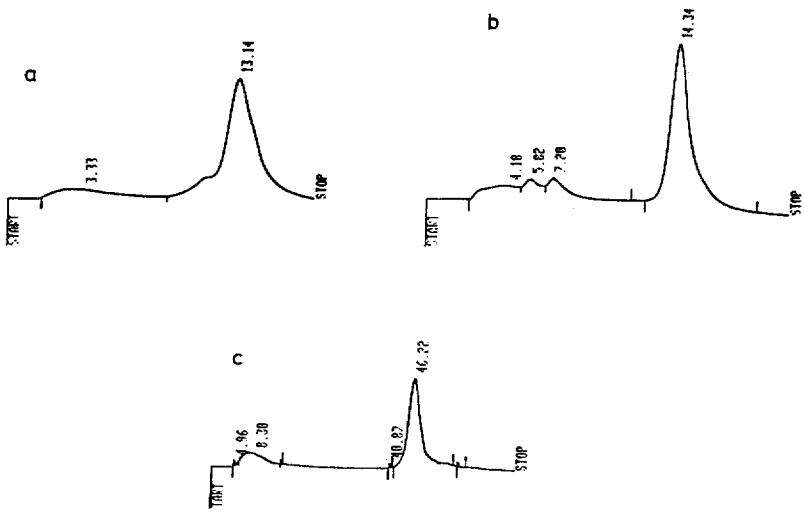

Fig. 1. Development of scale-up methodology for HPHA chromatography. (a) First chromatography on the HPHA cartridge; (b) chromatogram of the second injection into the HPHA cartridge; (c) elution of a 10-ml sample of ammonium sulphate-precipitated mouse ascites from the preparative HPHA column. Peak at 46.22 min is the IgG peak.

elution was performed with a linear gradient from 10 to 100% of buffer B in 15 min at a flow-rate of 0.4 ml/min.

Fig. 1b depicts the chromatographic pattern of the second injection. The MoAb peak was eluted at 14.34 min, while the contaminant proteins (as confirmed by SDS-PAGE) were fully eluted with 15% buffer B. The preparative HPHA column was conditioned with 15% of buffer B at a flow-rate of 4 ml/min. After injection of 10 ml (150 mg total protein) of the sample, isocratic elution with 15% buffer B for 30 min was followed by a linear gradient up to 100% buffer B in 30 min. The preparative elution profile is shown in Fig. 1c.

Preparative high-performance Protein A (HPPA) chromatography

Preparative Protein A chromatography was performed with a high-molarity binding buffer (1.5 *M* glycine, 3 *M* sodium chloride, pH 9.0) which allows efficient binding of all murine IgC subclasses and an elution buffer of low pH and ionic strength (0.1 *M* citrate buffer pH 3.0)[10]. The flow-rate was 4 ml/min. A 10-ml volume (150 mg total protein) of ammonium sulphate-treated ascitic fluid, resuspended in binding buffer, was loaded onto the column. As soon as the baseline of the unretained material returned to zero, the immunoglobulins were eluted with the acidic buffer. Fig. 2 shows the preparative purification of EK3 MoAb.

Protein Pak DEAE 5PW chromatography

The dried ascitic fluid (150 mg) was resuspended in 10 ml 20 m*M* Tris–HCl buffer (pH 8.5), and the sample was applied to the Protein Pak DEAE 5PW semipreparative column, equilibrated in the same buffer. The elution buffer was 50 m*M* Tris–HCl, 0.3 *M* NaCl (pH 7.0) and the flow-rate was 4 ml/min. The elution conditions were: isocratic for 10 min with starting buffer, then a linear gradient to 100% of elution buffer in 60 min. Fig. 3 depicts the anion-exchange chromatographic pattern.

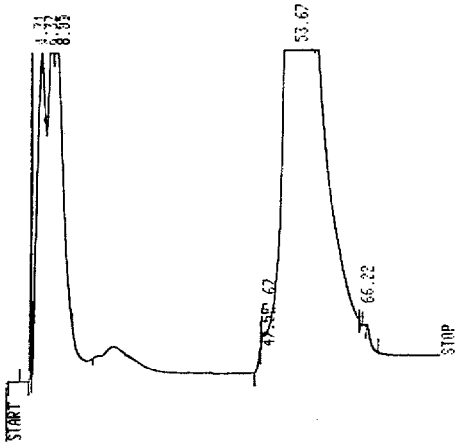

Fig. 2. High-performance Protein A chromatography of ammonium sulphate-precipitated mouse ascitic fluid (10 ml). Peak at 53.67 min is the IgG peak.

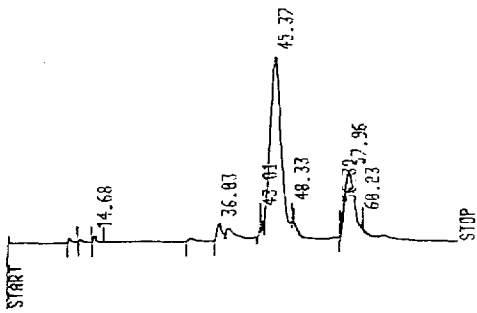

Fig. 3. Anion-exchange chromatography on a Protein Pak DEAE 5PW column of 10 ml of ammonium sulphate-precipitated ascitic fluid; flow-rate, 4 ml/min; elution for 10 min with 20 m*M* Tris–HCl (pH 8.5), then linear gradient from 20 m*M* Tris–HCl (pH 8.5) to 50 m*M* Tris–HCl, 0.3 *M* NaCl (pH 7.0) in 60 min.

Other assay methods

Protein recovery was determined by measuring the amount of proteins in ascitic fluid and in the collected peaks with the Bio-Rad protein assay kit. The immunoglobulin titration was performed with a radioimmunoassay method: 100 μl (30 000 cpm) [^{125}I]hTSH (Eurogenetics, Turin, Italy) were added to 100 μl of crude ascitic fluid or purified MoAb serial dilutions, both at an initial concentration of 1 mg/ml, and incubated overnight at room temperature. The MoAb–antigen complex was separated from unbound MoAb using 2 ml of 20% PEG 6000 (w/v in Veronal buffer, pH 8.6) in the presence of 100 μl of normal human serum. The titre was calculated as the dilution of the MoAb that binds 50% of the tracer. The inverse of this value was defined as the specific anti-hTSH activity.

RESULTS

Collected peaks from the three chromatographic methods were dialyzed against saline phosphate buffer (PBS, pH 7.2) and analyzed by reducing SDS-PAGE in order to determine the immunoglobulin peak. Fig. 4 shows the SDS-PAGE of the preparative HPHA chromatographic peaks. Lane 6, peak at 46.22 min (Fig. 1c), contains only bands corresponding to immunoglobulin heavy and light chains, while lane 5 is the unretained material (albumin and other contaminant proteins). The immunoglobulin peak (100 μl) was then passed through the HP-GPC column to establish the degree of purity. The eluent was PBS and the flow-rate was 1 ml/min. The column was first calibrated with molecular-weight standards (gel filtration calibration kits, low and high molecular weight, Pharmacia) in order to determine the immunoglobulin retention time. The immunoglubulin peak area percent was taken as the percent of purity. The peak area percent of IgG in the preparative HPHA purification was 98% (Fig. 5).

The SDS-PAGE analysis of chromatographic peaks, obtained by HPPA purification, is shown in Fig. 6. Immunoglobulin heavy and light chains (lane 3) were free from contaminants, as confirmed by HP-GPC analysis with a purity of 99% (Fig. 7). Albumin, transferrin and other contaminant proteins in ascitic fluid were found in the unretained material (lane 4).

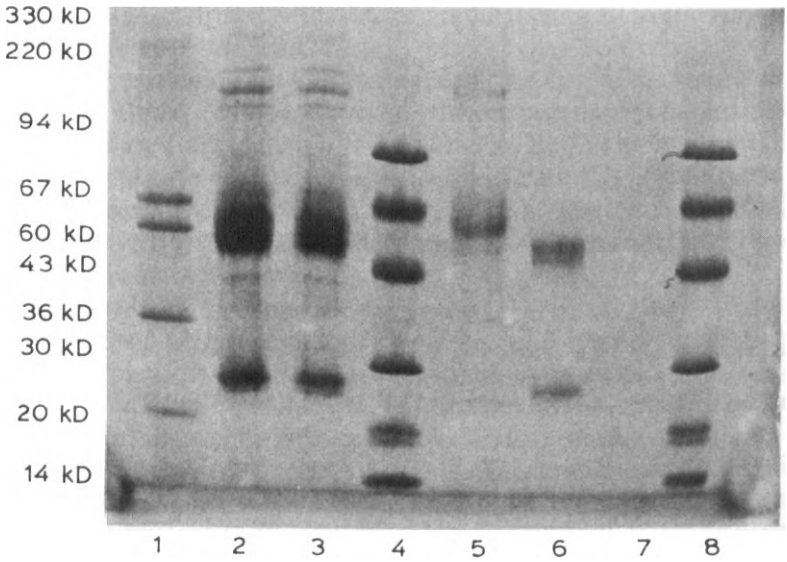

Fig. 4. Reducing SDS-PAGE of the preparative HPHT fractions. Lanes: 1, high-molecular-weight (HMW) standards; 4 and 8, low-molecular-weight (LMW) standards; 2 and 3, crude ascites; 5, peak at 8.3 min; 6, peak at 46.22 min.

In DEAE chromatography the MoAb peak (45.37 min) was well separated from the albumin peak (57.96 min), as confirmed by SDS-PAGE (lanes 3 and 6, and 4 and 7, respectively, Fig. 8). Fig. 9 shows the HP-GPC analysis of the MoAb peak (100 μl); the immunoglubulin area percentage was 99%.

The protein recovery and immunoreactivity was substantially the same in all the three chromatographic methods (Table I), and the purification factor (MoAb fraction/crude ascites anti-hTSH specific activity ratio; crude ascites anti-hTSH specific activity was 80 000) ranged from 4.8 to 4.0.

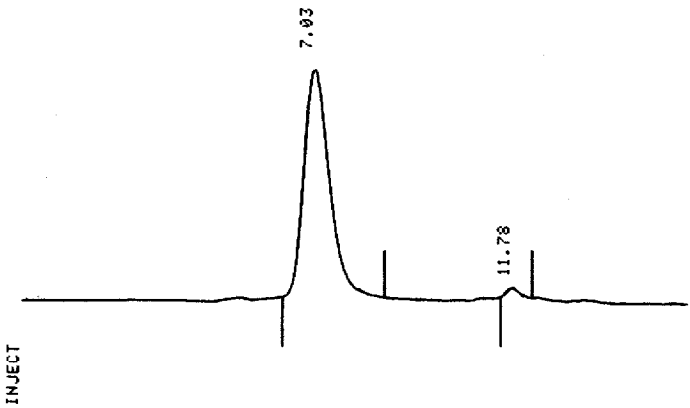

Fig. 5. HP-GPC chromatogram of the peak at 46.22 min containing immunoglobulins. The chromatogram was developed isocratically after injecting 100 μl of purified MoAb into a Bio-Sil TSK 250 column. The mobile phase was PBS. Flow-rate: 1 ml/min. Chart speed: 60 cm/h. Detection: 278 nm.

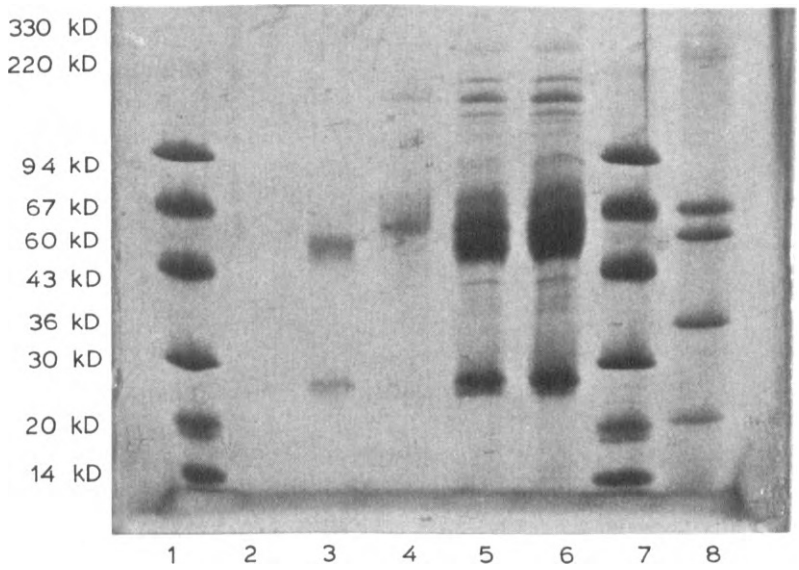

Fig. 6. Reducing SDS-PAGE of the preparative Protein A peaks from Fig. 2. Lanes: 1 and 7 LMW standards; 8, HMW standards; 3, peak at 53.67 min; 4, peak at 56.32 min from Fig. 2; 5 and 6, crude mouse ascites.

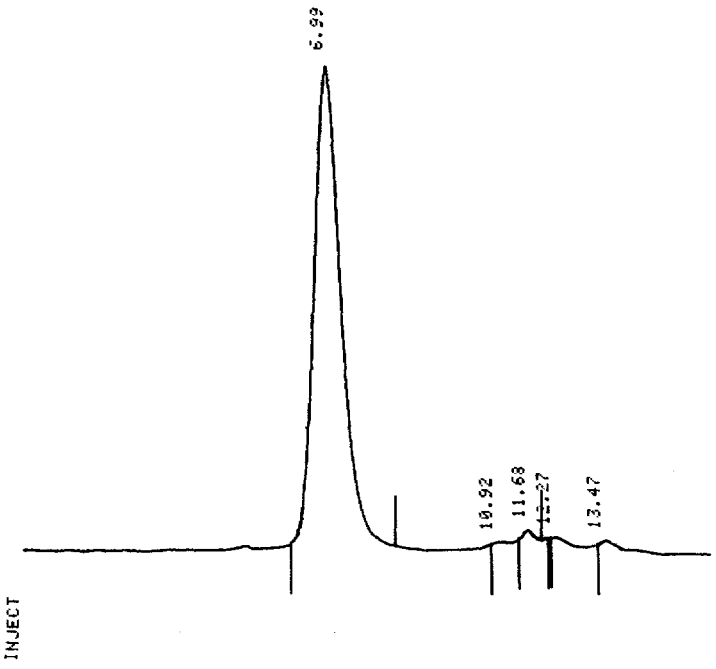

Fig. 7. HP-GPC analysis of the Protein A bound fraction. For chromatographic conditions, see Fig. 5.

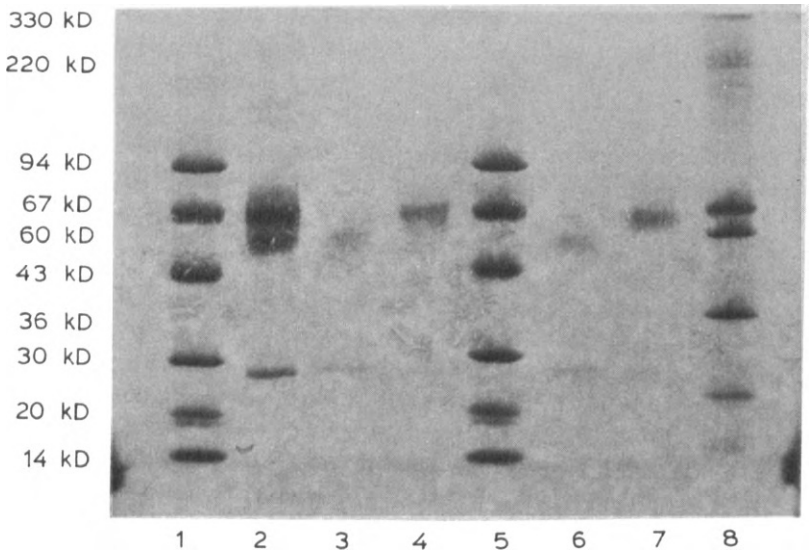

Fig. 8. Reducing SDS-PAGE after anion-exchange chromatography. Lanes: 1 and 5, LMW standards; 8, HMW standards; 2, crude mouse ascites; 3 and 6, peak at 45.37 min from Fig. 3; 4 and 7, peak at 57.96 min from Fig. 3.

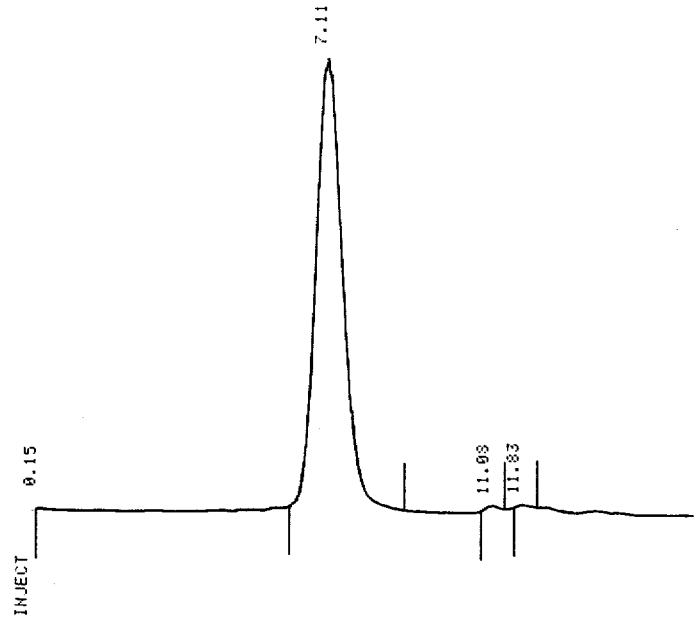

Fig. 9. HP-GPC analysis of the peak at 45.37 min from Fig. 3. For elution conditions, see Fig. 5.

TABLE 1

PURIFICATION AND YIELD OF MoAb EK3 USING HPHA, HPPA AND DEAE PURIFICATION PROCEDURES

Stationary phase	*Specific activity*	*Protein loaded (mg)*	*Protein recovery (%)*	*MoAb recovery (MG)*	*MoAb yield (%)*	*HP-GPC purity (%)*	*Purification factor*
HPHA	318 000	150	85	79.5	53	98.5	4.0
HPPA	385 000	150	90	81.0	54	99.2	4.8
DEAE	335 000	150	90	72.0	48	99.2	4.2

The maximum load capacity of the columns was also determined by charging increasing amounts of purified MoAb, obtained from previous purifications and by measuring the immunoreactivity of the void volume. As much as 250, 350 and 380 mg of MoAb were loaded in HPPA, HPHA and Protein Pak DEAE 5PW preparative columns, respectively, without found any immunoreactivity in the void volume. These quantities represent, for HPPA and HPHA, the actual column capacity, because the whole stationary phases of the columns are employed for the MoAb retention. The antibody was collected from HPHA and HPPA columns after the bulk of contaminant proteins had been eluted in the void volume. In this way the binding capacity of the matrices was employed only for antibody retention.

A further test of the Protein Pak 5PW column was performed by loading increasing amounts of MoAb and albumin in order to ascertain when the two proteins were no longer separated. Different amounts of a 1:1 mixture of pure MoAb and bovine serum albumin (BSA) were injected. The elution protocol was performed as described under Protein Pak DEAE 5PW chromatography. Resolution was effected at 250 mg of the mixture.

DISCUSSION

The advent of MoAb technology has opened up new application areas, such as diagnostics (radioimmunoimaging), therapeutics (radioimmunotherapy) and basic and applied research. In each of these areas, highly purified MoAbs are required. The methods traditionally used for purifying polyclonal antibodies (salt and polyethylene glycol precipitation, classical open-column chromatography) can often be used successfully to purify MoAbs, but these techniques usually require several hours to perform, or provide not highly purified antibodies.

Preparative HPLC chromatography provides an extremely useful alternative to conventional MoAbs purification techniques. Speed, ease and purity as well as quantitative protein recovery and large-scale production are the advantages of HPLC purification.

This paper describes three procedures for preparative purification of an IgG1 MoAb. Hydroxyapatite provides highly purified material thanks to the capacity of its crystal surface able to separate proteins with slight differences in their adsorption groups. Its calcium, phosphate and hydroxide ions adsorb oppositely charged sites of proteins or other large molecules. For this reason, the molecular tertiary structure is

very important in this separation method which does not involve the isoelectric point of proteins but an ionic interaction between molecules and the hydroxyapatite stationary phase[11,12]. Affinity chromatography on Protein A is the most widely employed method for the specific separation of immunoglobulins from contaminant ascitic fluid proteins. Unlike previously published reports of the use of Protein A[13,14] to purify MoAbs, the employment of an high molarity binding buffer at pH 9.0 provides increased capacity to bind the mouse IgG1 subclass. A total capacity of 250 mg of IgG1 was achieved using the preparative Protein A column. Furthermore, the hydrophilic rigid matrix to which Protein A is linked allows high flow-rates (up to 10 ml/min) and substantially reduced separation times. Anion-exchange chromatography has been widely used for many years in the purification of antibodies. The analytical anion-exchange column (Mono Q, Pharmacia) has been succesfully used to purify MoAbs[5]. Large-scale preparation of MoAbs on a semipreparative anion-exchange HPLC column had not been investigated, so far. The experiment performed in our laboratory demonstrates that the separation of MoAb from albumin was still possible up to 250 mg of a 1:1 mixture of the two components.

Table I lists the parameters investigated to compare the purification protocols of HPHA, HPPA and DEAE 5PW chromatography. The protein recovery, MoAb purity and immunoreactivity of the three procedures are very similar. Such purified MoAbs can be employed in all the previously mentioned applications. On the contrary, differences in the applicability of each purification method were found. The HPHA chromatography requires at least two chromatographic developments of the HPHA analytical cartridge to accomplish the MoAb purification. As reported previously[15], IgM can be purified on an HPHA column. The use of extremely mild conditions preserves the MoAb activity. HPPA chromatography is very easy to perform, and gradient elution with buffers at different pH can be employed to separate IgG subclasses. Mouse IgM cannot be quantitatively purified by Protein A chromatography, because of their low affinity for Protein A[14]. An high-molarity and high pH binding buffer not only retains mouse IgG1 more strongly on Protein A, but is also able to buffer the acidic step of MoAb elution. The MoAb bound to the matrix begins to be eluted as the pH is decreased. The first portion of the MoAb fraction contains a residual of binding buffer which maintains neutral pH for the whole MoAb fraction.

DEAE anion-exchange chromatography can be employed to purify both IgG and IgM. Mild elution conditions preserve the MoAb activity. The MoAb fraction obtained by DEAE 5PW chromatography was well separated from contaminant proteins, but, as reported by Burchiel *et al.*[5], it is also possible to find a protein (transferrin) eluted with MoAb. In this case the contaminant protein can be removed by a second purification step, using a gel permeation column able to eliminate the low-molecular-weight contaminant protein.

In our laboratory, preparative purifications of mouse IgG monoclonal antibodies are performed on HPPA columns. Affinity chromatography on Protein A was selected because it does not require further setting up of the method and thus can be employed in routine purification of different MoAbs. Furthermore, by means of the pH stepwise gradient elution, endogeneous mouse immunoglobulins can be partially removed from the MoAb fraction. Mouse IgM or antibodies of other animal species that show low affinity for Protein A are purified on HPHA or DEAE 5PW columns.

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