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RAPID PURIFICATION OF MONOCLONAL ANTIBODIES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Three different high-performance liquid chromatographic (HPLC) techniques, *i.e.*, ion-exchange, hydrophobic interaction and hydroxyapatite chromatography, have been used to purify monoclonal antibodies from ascites fluid. The monoclonal antibodies were raised against coagulation factor VIII. Precipitation of the antibodies by ammonium sulphate prior to HPLC made it possible to purify the antibody in one chromatographic step. In sodium dodecyl sulphate-polyacrylamide gel electrophoresis this highly pure preparation revealed only one extra polypeptide besides the light- and heavy-chain immunoglobulin polypeptides. Attempts were also made to purify the antibody without prior ammonium sulphate precipitation. A combination of ion-exchange and hydrophobic interaction chromatography resulted in an antibody preparation comparable in purity to the one obtained from ammonium sulphate-precipitated immunoglobulin. The rapid HPLC techniques were found to be very useful for purification of monoclonal antibodies on a preparative scale, where sample loadings of up to 25 mg of ascites protein were fully resolved in satisfactory yields.

INTRODUCTION

Since the development of techniques to produce monoclonal antibodies (mAb), several reports of their isolation have appeared. Usually the first step is precipitation with ammonium sulphate, which is followed by DEAE-cellulose anion-exchange chromatography. In some cases the precipitation step has been shown to reduce the antibody activity[1]. Purification of mAb has been performed successfully using Protein A-Sepharose affinity chromatography[2], but one subclass of the mouse immunoglobulin (IgG_1) was reported to bind only weakly and another (IgG_3) not at all[2,3]. Anion-exchange chromatography of mouse ascites fluid on DEAE Affi-Gel Blue is as lengthy as the Protein A-Sepharose technique, and the isolated immunoglobulin fractions were shown to be contaminated with other proteins[3].

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Recent developments in high-performance liquid chromatography (HPLC), anion-exchange, hydrophobic interaction and hydroxyapatite columns, have opened up new possibilities for the separation of mAb from mouse ascites fluid[4,5]. The aim of this study was to test three different chromatographic techniques, ion-exchange, hydrophobic interaction and hydroxyapatite chromatography, and to study the possibility of using a chromatographic procedure consisting of only one or two steps, to isolate mAb rapidly from ascites fluid, free from contaminating proteins and in a functionally active state. The pros and cons of precipitating the IgG with ammonium sulphate prior to chromatography in relation to the final purity of the mAb preparation were also examined.

MATERIALS AND METHODS

The ascitic fluid containing antibodies against factor VIII was prepared by injecting *ca.* $5 \cdot 10^6$ antibody-producing mouse hybridoma cells into the peritoneal cavity of congenic mice (Balb/C), pretreated with 0.5 ml pristane. After 7–10 days, ascitic fluid containing mAb could be collected from the intraperitoneal cavity. It was centrifuged at 2500 *g* for 10 min to remove cells and other large particles and was subsequently diluted (1:1) in 0.15 mol/l sodium chloride. The diluted ascites fluid was stored at −20°C. In this study, a commercially available mAb against factor VIII, purchased from IIC (Institutional Immunology Corporation, U.S.A.) (Batch No. Anti-Factor VIII:C 10004), was also used.

Ammonium sulphate precipitation was performed at +4°C. Equal volumes of diluted ascites fluid and saturated ammonium sulphate were mixed by slow addition of the ammonium sulphate solution during gentle stirring. On the next day, this material was centrifuged (10 000 *g* for 20 min) and washed twice with 50% ammonium sulphate solution. The precipitate was dissolved in distilled water and dialyzed against phosphate-buffered saline (PBS) (0.14 mol/l sodium chloride, 0.003 mol/l potassium chloride, 0.010 mol/l phosphate buffer, pH 7.4). Solid polyvinylpyrrolidone (PVP) was added (to 3%, w/v) to the sample and stirred at 4°C for 4 h. After centrifugation at 17 000 *g* and filtration through a Millipore filter Millex-HV (Millipore Corp., Bedford, MA, U.S.A.), the clear supernatant was applied to the column.

All experiments were performed on HPLC instrumentation consisting of a Model 100A solvent-delivery system and Model 421 gradient module (Beckman, Berkeley, CA, U.S.A.), combined with a Model HP-1040A detection system, HP-85 computer, HP-9135 A Winchester drive and HP-7470A plotter (Hewlett-Packard, Waldbronn, F.R.G.). The fractions were collected every minute in a Model 201 fraction collector (Gilson Medical Electronics, France) in the Time program mode.

Ion-exchange chromatography was performed on a Mono Q HR 5/5 anion-exchange column (50 mm × 5 mm I.D.) (Pharmacia Fine Chemicals, Uppsala, Sweden) with starting buffer A, 0.020 mol/l Tris–HCl (pH 8.0), and final buffer B, 0.020 mol/l Tris–HCl (pH 8.0) containing 1.5 mol/l sodium chloride. The gradient was generated over 50 min at a flow-rate of 1 ml/min. The sample load was between 0.5 and 25 mg protein.

Hydrophobic interaction chromatography was performed on a TSK gel Phenyl 5 PW column (75 mm × 7.5 mm I.D.) (Toya Soda, Japan). Proteins were loaded on the column in solutions of high ionic strength, *e.g.*, (buffer A) 0.030 mol/l Tris–HCl,

1.0 mol/l sodium sulphate (pH 7.5). Elution was accomplished by reducing the ionic strength and the polarity of the mobile phase, *e.g.*, (buffer B) 0.030 mol/l Tris–HCl, containing 5% 2-propanol. This polarity-reducing step was performed by using a linear gradient for 80 min at a flow-rate of 1 ml/min. The sample load was between 0.3 and 2.5 mg protein.

Hydroxyapatite chromatography was performed on a Bio-Gel HPHT column (100 mm × 7.8 mm I.D.) (Bio-Rad, Richmond, CA, U.S.A.) with buffer A, 0.01 mol/l sodium phosphate, 0.3 mmol/l calcium chloride (pH 6.8),and final buffer B, 0.35 mol/l sodium phosphate, 0.01 mmol/l calcium chloride (pH 6.8). The gradient was generated over 70 min at a flow-rate of 0.5 ml/min. The sample load was between 4 and 5 mg protein.

All samples used in this study were first treated with PVP[6]. This treatment was found to be a good clarifying step, and no loss of mAb activity was noticed. The samples for ion-exchange and hydroxyapatite chromatography were diluted and filtered through a 0.45-μm Millipore filter (Millex-HV) to reduce the salt concentration and to remove small particles, respectively. For hydrophobic interaction chromatography, solid sodium sulphate was added to a concentration of 1 mol/l in order to increase the ionic strength and make it possible to adsorb the IgG on the column matrix. The filtration step was used here also. Protein determinations were made by the Lowry method[7], using serum albumin as a standard.

The activity of monoclonal antibodies was determined by a radioimmunoassay (RIA) method. The fractions to be analysed were diluted in phosphate buffered saline (PBS), containing 1% bovine serum albumin (BSA). A 0.1-ml aliquot of each diluted sample was incubated with 0.05 ml of ^{125}I-labelled antigen, which was diluted to contain *ca.* 10 000 cpm per 0.05 ml. After 5 h, 0.1 ml of a donkey-antimouse IgG-coupled cellulose suspension (Sac-Cel; Wellcome Reagents Ltd., Beckham, U.K.) was added for precipitation and 30 min later the tubes were washed with 2 ml of PBS. The resulting pellets were then counted in a gamma counter. The purity of various mAb fractions was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)[8] of reduced samples. The total polyacrylamide concentration in the separating gel was 12%. Silver staining was used to visualize the protein bands[9]. As a standard, the low-molecular-weight (LMW) reference from Pharmacia was used: phosphorylase *b*, MW 94 000; albumin, MW 67 000; ovalbumin, MW 43 000; carbonic anhydrase, MW 30 000; trypsin inhibitor, MW 20 1000 and alpha-lactalbumin, MW 14 400).

RESULTS AND DISCUSSION

HPLC methods have been found to have great potential for analytical and small-scale purification of proteins. Therefore, these techniques were tested for their ability to purify mAb. In some instances, it is necessary to obtain small amounts of purified mAb, *e.g.*, for analytical tests with labelled antibodies. The purpose of this study was to investigate the purification of mAb by ion-exchange, hydrophobic interaction and hydroxyapatite columns separately or in combination.

In a first set of experiments, ascites fluid containing mAb against factor VIII was chromatographed. Before chromatography, ammonium sulphate precipitation was used as a first purification step. The results for the three different columns are

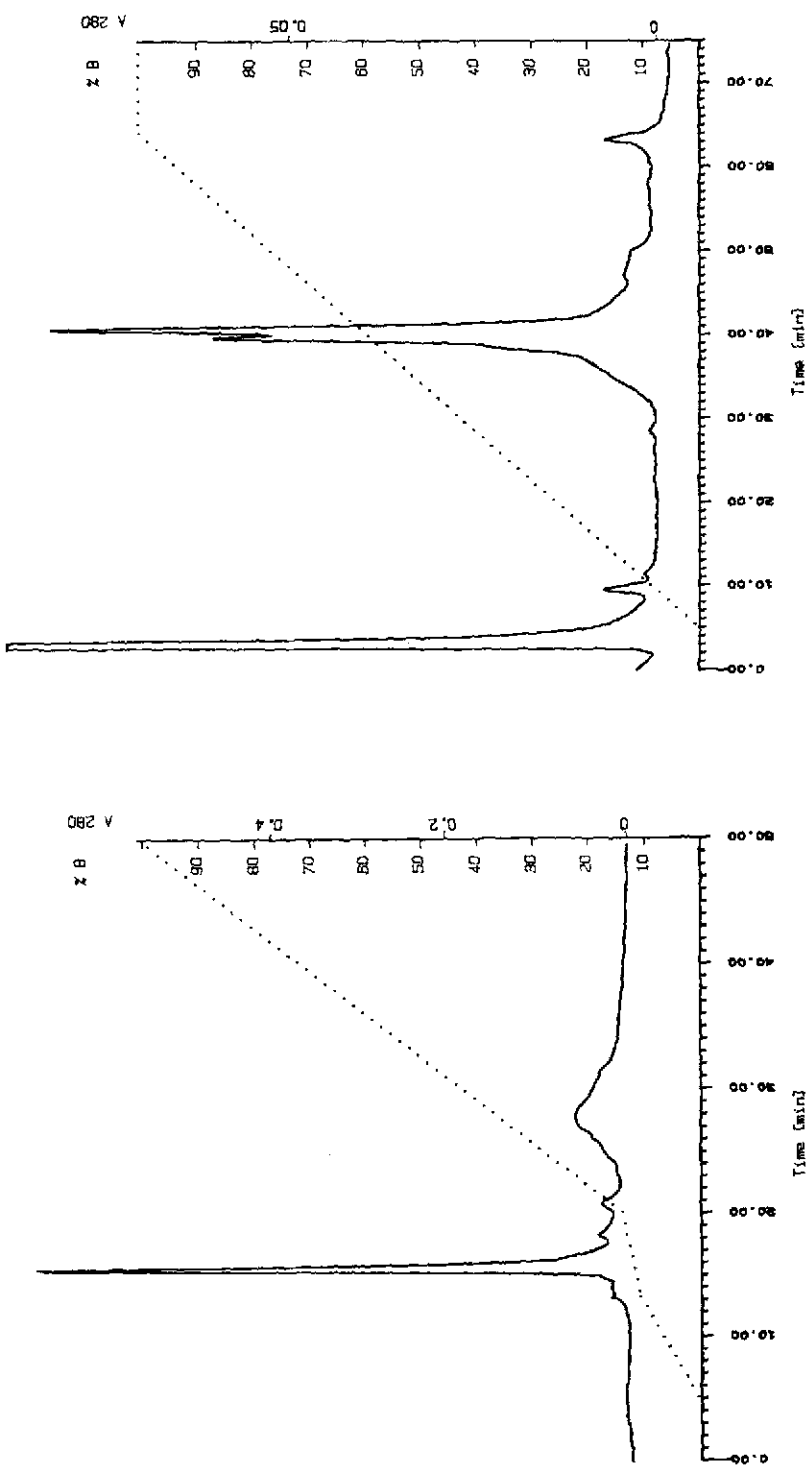

Fig. 1. Chromatographic pattern for monoclonal antibodies (against factor VIII) obtained by ion-exchange chromatography. Sample load: 4 mg protein. The solid line indicates detection by UV (280 nm) and the broken line the salt gradient.

Fig. 2. Hydrophobic interaction chromatography of monoclonal antibodies. Sample load: 2.5 mg protein. Other details as in Fig. 1.

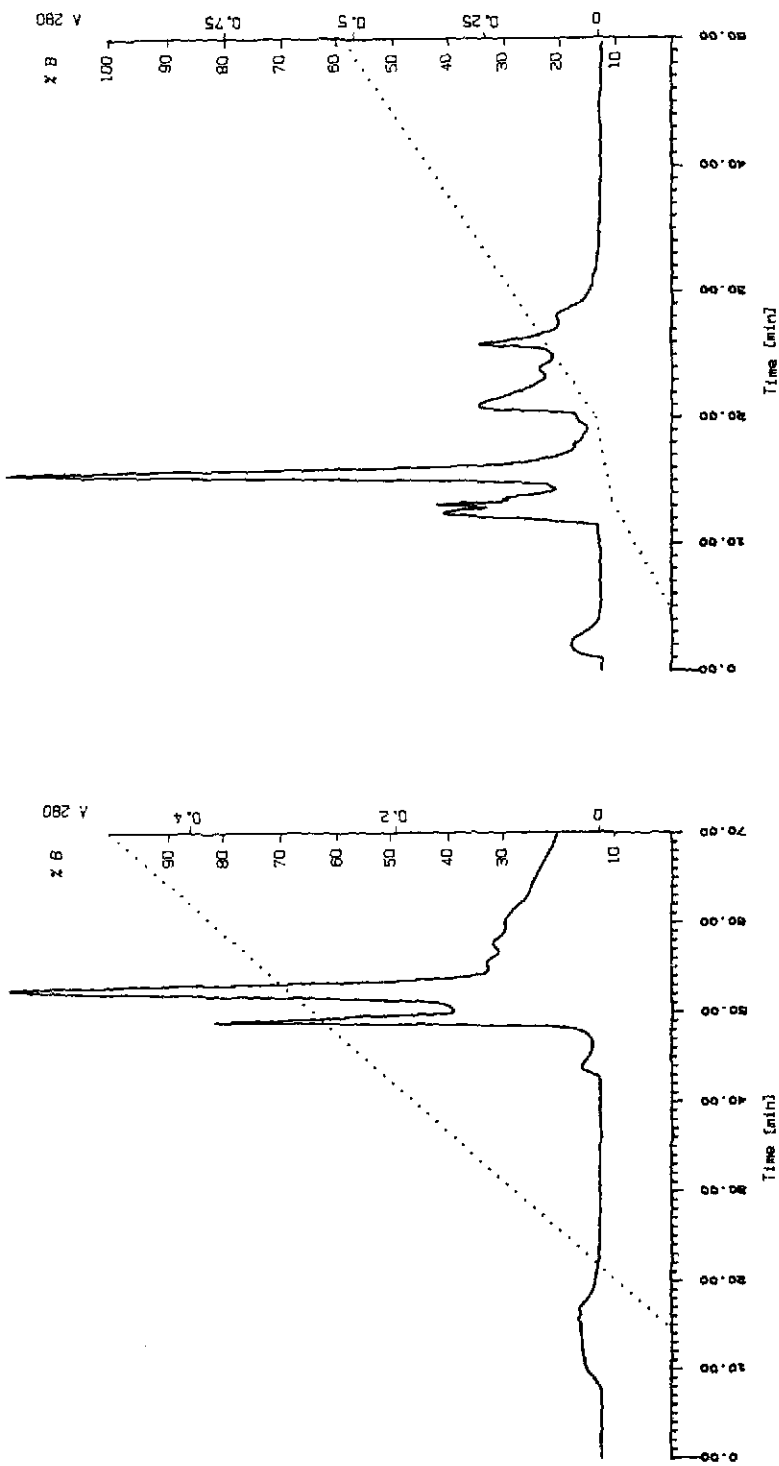

Fig. 3. Hydroxyapatite chromatography of monoclonal antibodies. Sample load: 4 mg protein. Details as in Fig. 1.

Fig. 4. Chromatographic pattern for monoclonal antibodies (against factor VIII) obtained by ion-exchange chromatography. Sample load: 0.5 mg. Other details as in Fig. 1.

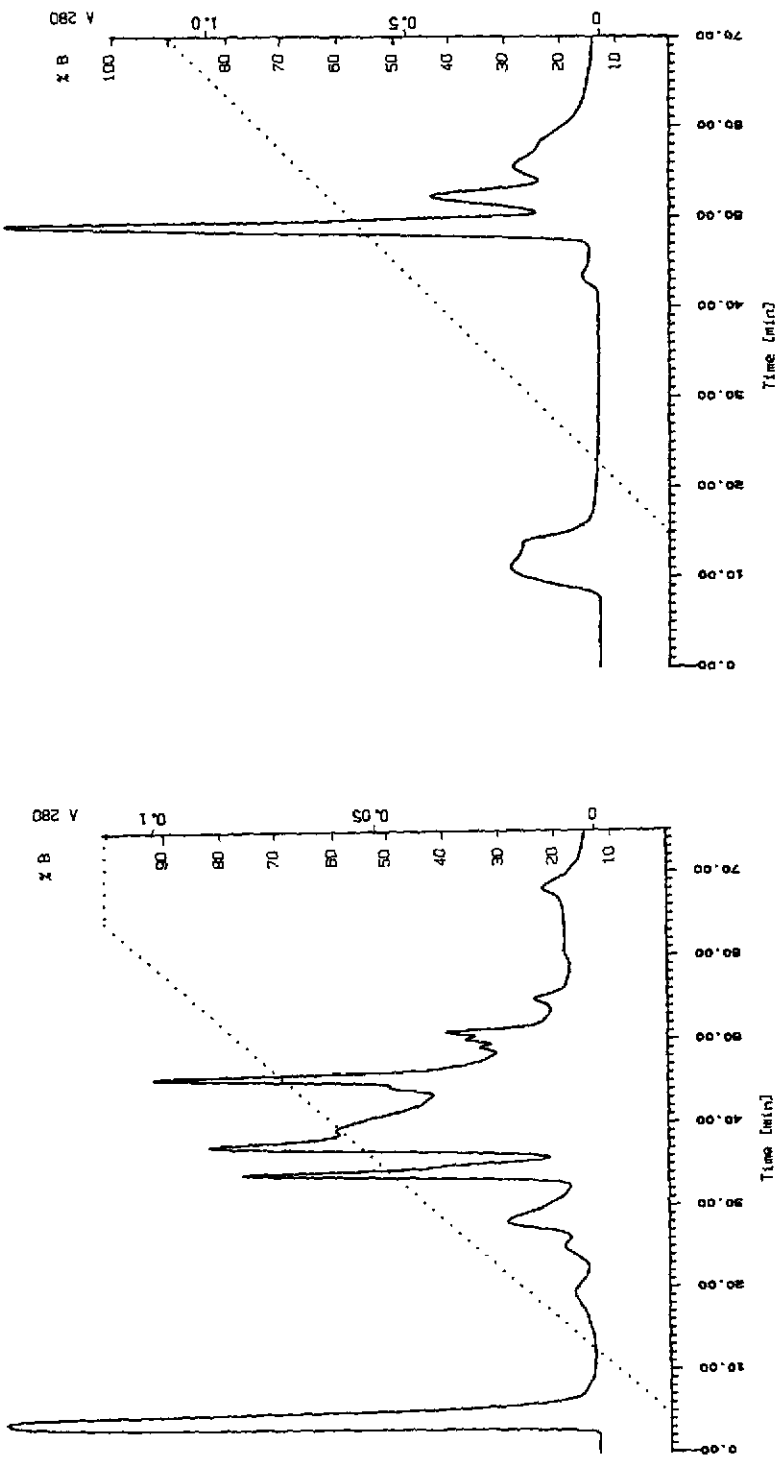

Fig. 5. Hydrophobic interaction chromatography of monoclonal antibodies. Sample load: 0.5 mg. Details as in Fig. 4.

Fig. 6. Hydroxyapatite chromatography of monoclonal antibodies. Sample load: 5 mg protein. Other details as in Fig. 1.

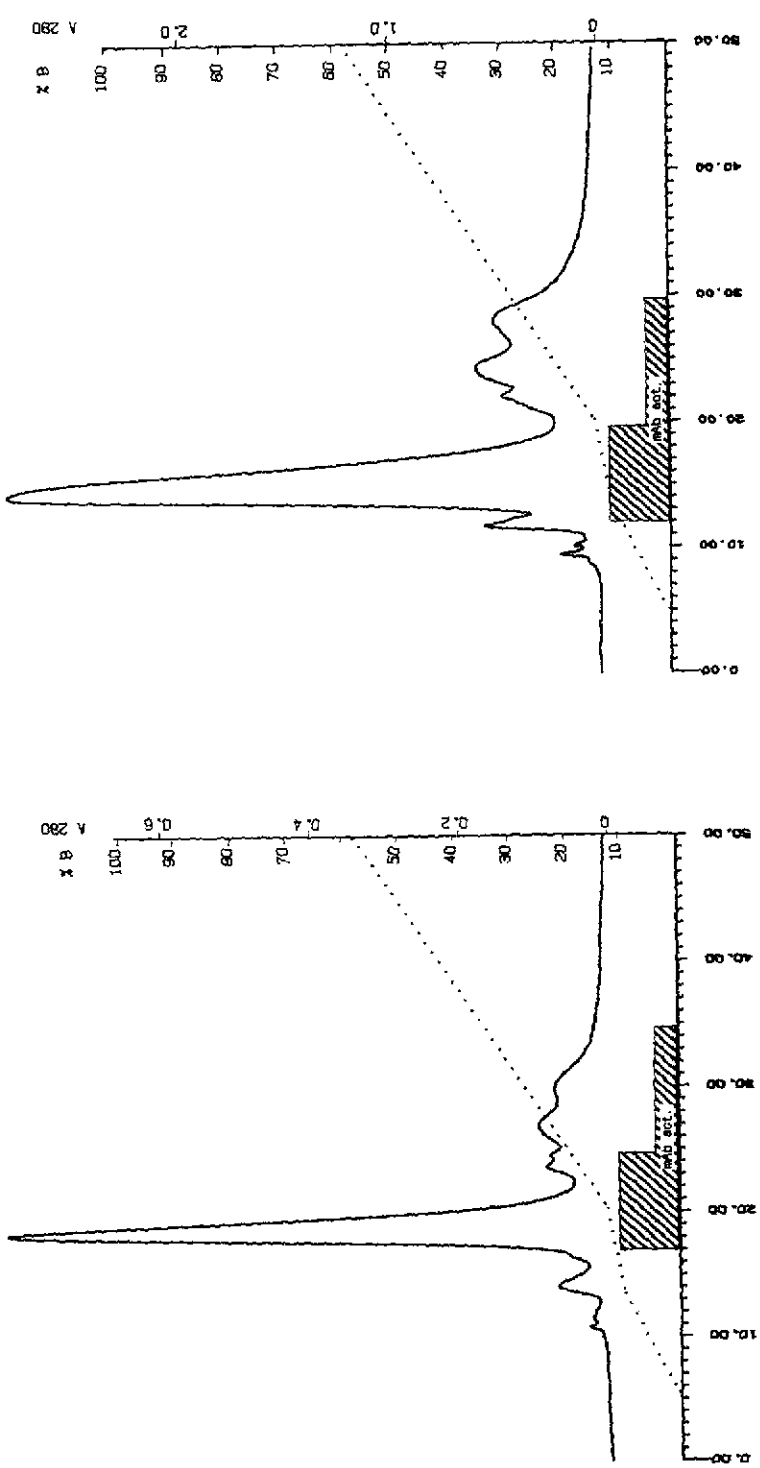

Fig. 7. Ion-exchange chromatography of low sample load (3.1 mg protein) of ammonium sulphate-precipitated ascites fluid, containing antibodies against factor VIII. The hatched area indicates the antibody activity. Other details as in Fig. 1.

Fig. 8. Ion-exchange chromatography of high sample load (25 mg protein) of ammonium sulphate-precipitated ascites fluid. Details as in Fig. 7.

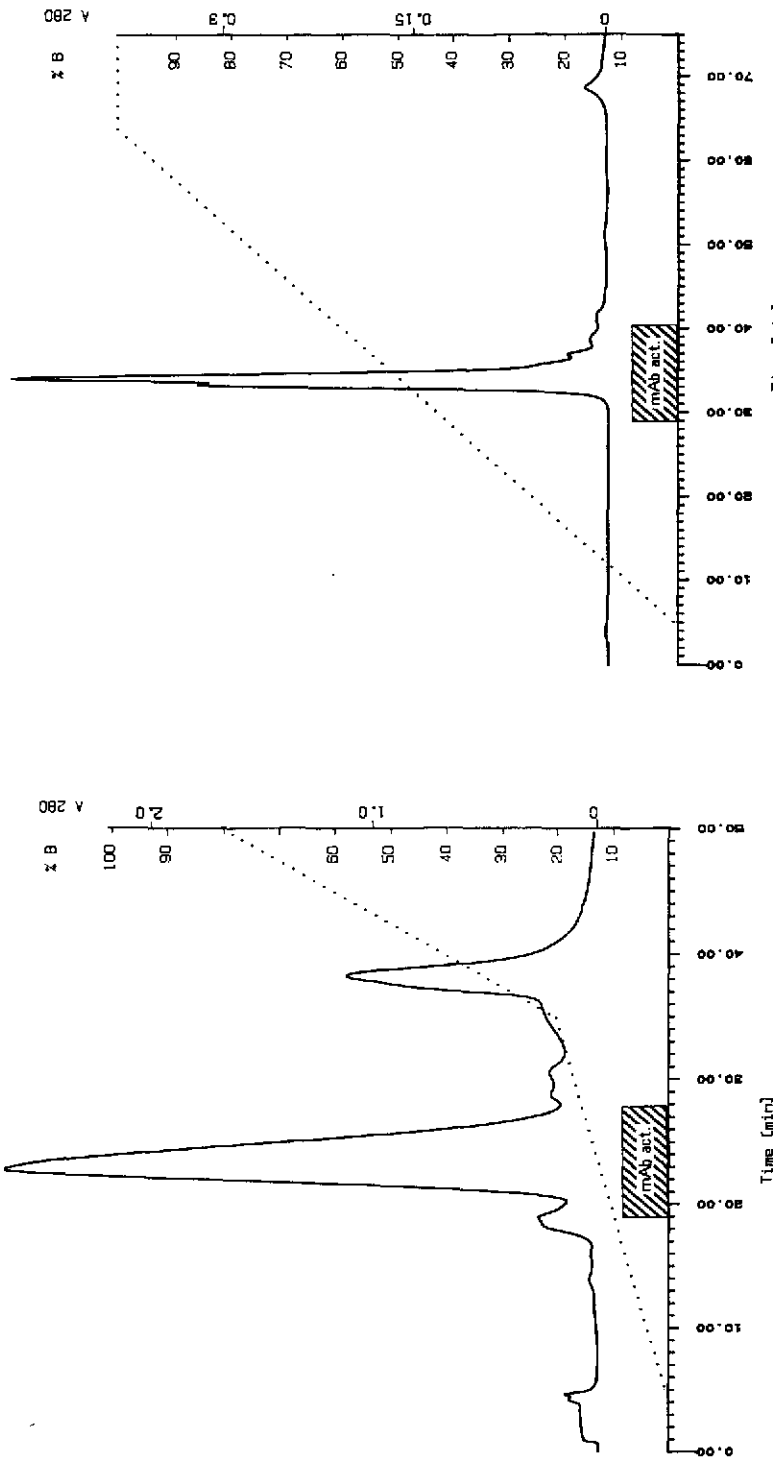

Fig. 9. Two-step chromatographic procedure (see Fig. 10), performed after ammonium sulphate precipitation of sample. The first step is ion-exchange chromatography. Sample load: 25 mg protein. Other details as in Fig. 7.

Fig. 10. The second step hydrophobic interaction chromatography. An aliquot of fractions 21–27 from the first step was applied. Sample load: about 0.3 mg protein. Other details as in Fig. 7.

shown in Figs. 1–3. By ion-exchange chromatography (Fig. 1) one main peak was obtained and by hydroxyapatite chromatography (Fig. 3) the main peak was further divided into two peaks. Hydrophobic interaction chromatography (Fig. 2) gave partial separation of two components and one large unretarded peak which, from its spectrum, was found to be of non-protein origin. In a similar experiment, a commercially available crude mAb preparation against factor VIII was tested (Figs. 4–6). In these experiments, the hydroxyapatite column (Fig. 6) gave a result similar to that in the previous experiment (Fig. 3). Both ion-exchange (Fig. 4) and hydrophobic interaction chromatography (Fig. 5) gave a more complex picture than that in Figs. 1 and 2. This indicates that both ion-exchange and hydrophobic interaction chromatography give higher resolution than hydroxyapatite chromatography.

It is often desired to isolate mAb in large quantities. The binding capacity of the ion-exchange column is *ca.* 25 mg of protein, according to the manufacturer. The next set of experiments was undertaken with increased protein loadings (Figs. 7 and 8). The higher protein loading (25 mg) was separated almost as well as the small sample loading (3.7 mg). In order to improve the separation at the high protein loading, a different gradient was used (Fig. 9). The separation was significantly improved. The main peak was collected and applied to the hydrophobic interaction column (Fig. 10). An almost indentical chromatographic pattern was obtained as in Fig. 2, indicating that the main mAb peak from Fig. 9 (high loading) does not contain

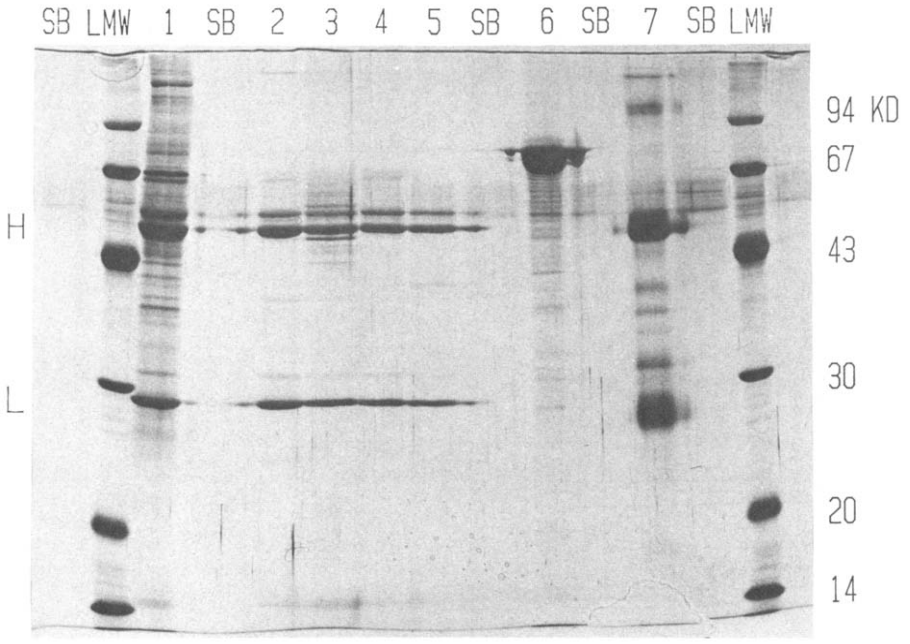

Fig. 11. SDS-PAGE of 2-mercaptoethanol-reduced samples from various purification steps. Samples: SB = sample buffer (see ref. 10); LMW = molecular weight reference; 1 = sample used in Figs. 7–9 (ammonium sulphate-precipitated ascites fluid); 2 = fractions 18–20 of Fig. 7; 3 = fractions 13–15 of Fig. 8; 4 = fractions 21–27 of Fig. 9; 5 = fractions 32–39 of Fig. 10; 6 = human transferrin; 7 = human IgG. H and L are heavy and light chain of IgG, respectively.

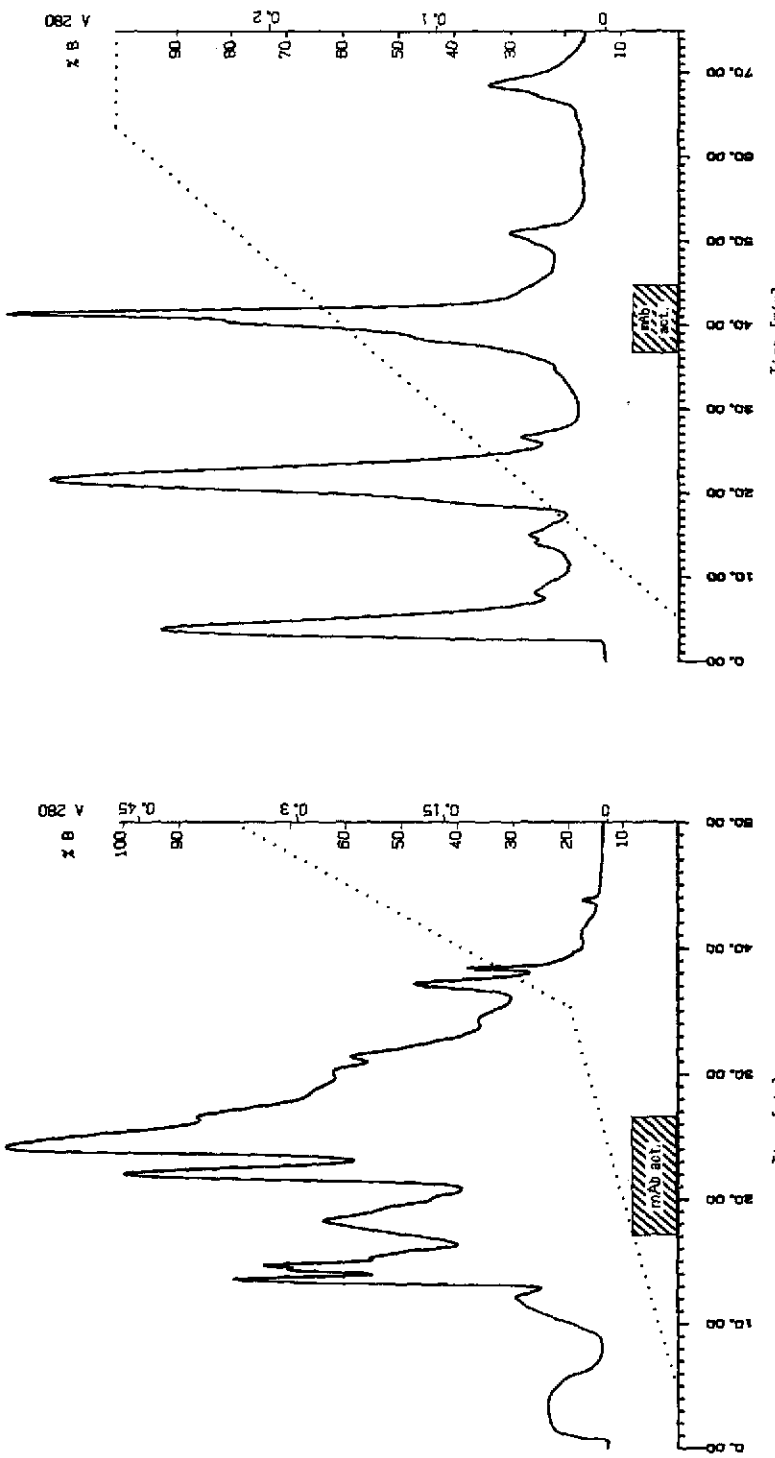

Fig. 12. Two-step chromatographic procedure (see Fig. 13) performed with unfractionated ascites fluid sample (no ammonium sulphate precipitation). The sample (25 mg protein) was applied to an ion-exchange column. Other details as in Fig. 7.

Fig. 13. An aliquot of fractions 18–24 from Fig. 12 was applied to the hydrophobic interaction column. Sample load: about 0.3 mg protein. Other details as in Fig. 7.

any major impurities. This suggests that the column can be used preparatively (25 mg) to isolate mAb without loss in purity of factor VIII mAb.

The composition and purity of the chromatographic fractions were tested by SDS-PAGE (Fig. 11). The ammonium sulphate-precipitated ascites sample contains, in addition to the heavy and light chains of IgG, several contaminating proteins (lane 1). These proteins can be efficiently removed by ion-exchange chromatography (lane 2), except for one polypeptide, which migrates somewhat more slowly than the IgG heavy chain. When 25 mg of ammonium sulphate-precipitated ascites fluid were chromatographed on the ion-exchange column, the main IgG peak was only slightly more contaminated (lane 3), but the extra polypeptides were efficiently removed by changing the gradient profile for ion-exchange chromatography (lane 4). The extra polypeptide band still remaining in the highly purified mAb preparation could not be separated from the antibody by hydrophobic interaction chromatography, indicating a high degree of structural similarity of this protein with the IgG molecule (lane 5). Transferrin (lane 6) and human IgG (lane 7) were chromatographed alongside as standards.

It has been reported[1] that the antibody activity can be partially lost during ammonium sulphate precipitation. Therefore, it was of interest to investigate whether it was possible to obtain for the monoclonal antibody the same purity without prior ammonium sulphate precipitation. For ion-exchange chromatography the same con-

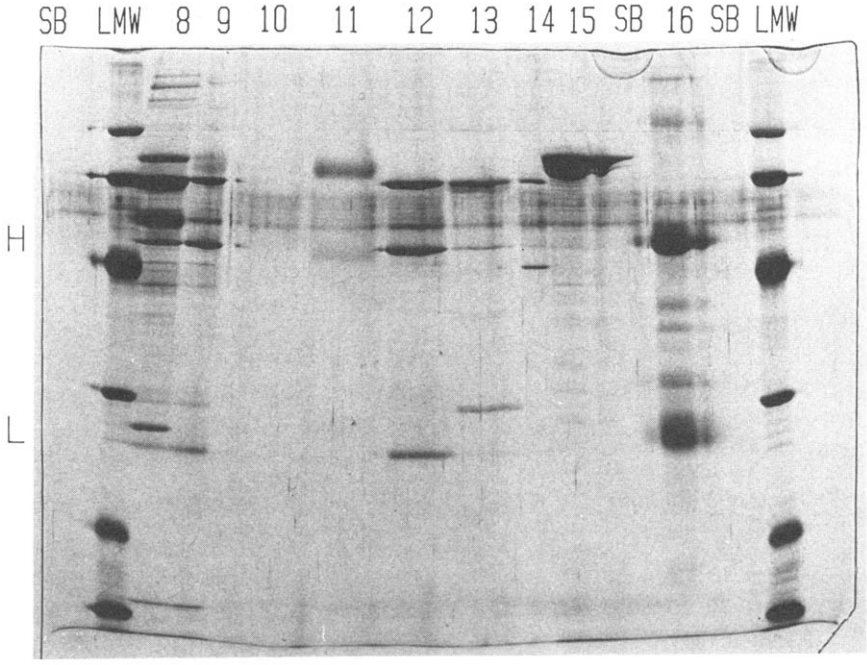

Fig. 14. SDS-PAGE of 2-mercaptoethanol-reduced samples from various steps. Samples: SB = sample buffer (see ref. 10); LMW = molecular weight reference; 8 = sample used for Fig. 12 (no ammonium sulphate precipitation); 9 = fractions 18–24 of Fig. 12; 10 = fraction 5 of Fig. 13; 11 = fraction 22 of Fig. 13; 12 = fraction 42 of Fig. 13; 13 = fraction 51 of Fig. 13; 14 = fraction 69 of Fig. 13; 15 = human transferrin; 16 = human IgG.

ditions were used as in Fig. 9. The result is shown in Fig. 12. Here a much more complex picture is obtained, showing that the monoclonal antibody is not the main component of unfractionated ascites fluid. This is also clearly seen after SDS-PAGE of ascites fluid (Fig. 14, lane 8). The area of activity from the ion-exchange column was pooled and again applied to the hydrophobic interaction column as described above. This step generated three main peaks, the monoclonal antibody activity being associated with only one peak.

The purity of the various chromatographic fractions was analysed by SDS-PAGE (Fig. 14). Lane 9 contains the pooled fraction from Fig. 12. Several discrete polypeptide bands are missing from this fraction relative to the ascites fluid (lane 8). The peaks eluted from the hydrophobic interaction column (Fig. 13) were also analysed by SDS-PAGE. The peak containing the mAb activity was the only one that contained the heavy and light IgG polypeptides (lane 12). This highly purified fraction still contained two contaminating polypeptide bands. However, it was only slightly less pure than the fraction obtained by ion-exchange chromatography from ammonium sulphate-precipitated ascites fluid (see Fig. 11, lanes 2 and 4). From these results it is obvious that the ammonium sulphate step gives a final mAb preparation of slightly higher purity than the one obtained if it is omitted.

The yield of mAb by ion-exchange chromatography was found to be about 90%. From various experiments with the hydrophobic interaction column the protein recoveries were estimated to be about 75% in our experiments.

CONCLUSION

Our results show that the HPLC techniques are efficient for purification of mAb and that the analytical columns used in this study can be utilized for preparative-scale purification. Use of larger columns will, of course, improve the capacity. If ammonium sulphate can be used as a first step, a single chromatographic procedure is sufficient for obtaining a highly pure mAb preparation. However, if ammonium sulphate precipitations must be avoided, ascites fluid can be used directly as starting material to isolate highly pure mAb, by a two-step HPLC chromatographic procedure.

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