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ION-EXCHANGE AND HYDROPHOBIC-INTERACTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

A PRACTICAL STUDY

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SUMMARY

A number of commercially available columns for ion-exchange and hydrophobic-interaction high-performance liquid chromatography (HPLC) have been tested, ranging from very fast columns with low capacity to preparative columns. The experiments represent a selection of column applications likely to occur in a biochemical laboratory.

The use of several ion-exchange columns for separation of serum and membrane proteins has been demonstrated. Columns with a silica gel matrix and with a polymer matrix have proved to be of equal value in experiments with water-soluble proteins. In experiments with hydrophobic membrane proteins the choice of the column depends on the protein to be isolated. A combination of a cation-exchange and an anion-exchange column as a tandem as well as a mixed-bed column, were applied for the separation of serum proteins and for the isolation of a 175 000-dalton membrane glycoprotein.

One problem of hydrophobic-interaction HPLC is the poor solubility of some proteins, for example serum proteins at high salt concentrations. This difficulty can be overcome by the use of columns with higher hydrophobicity which require lower initial salt concentrations. Less hydrophobic columns have been shown to separate hydrophobic membrane proteins by a combination of salt and detergent gradients.

INTRODUCTION

Over the last five years, high-performance liquid chromatography (HPLC) has firmly established itself in biochemical laboratories as a reliable, mild, and rapid method for the separation of proteins and other biopolymers.

Almost all chromatographic methods used for protein separation on soft gels have also been adopted for HPLC separations. These include size-exclusion, ion-exchange, and reversed-phase chromatography as well as affinity chromatographic methods. Most of these methods have recently been improved considerably. Among the new achievements are the introduction of hydrophobic-interaction HPLC[1,2], the

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combination of anion-exchange and cation-exchange HPLC, in the form of tandem columns and as mixed-bed columns[3], the application of several affinity chromatographic methods for HPLC[4,5] and the use of non-porous packing materials[6,7].

This progress has prompted a response in the market where a large supply of reversed-phase, ion-exchange, and hydrophobic-interaction HPLC columns can be found. The columns differ with regard to resin characteristics as well as ligand type and density. However, the large number of available columns and the lack of uniform specifications by the manufacturers can cause confusion among purchasers.

MATERIALS AND METHODS

Reagents

The following standard proteins were used: ferritin (M_r = 440 000), immunoglobulin G (M_r = 156 000), transferrin (M_r = 76 000), bovine serum albumin (M_r = 66 000), soybean trypsin inhibitor (M_r = 21 000) and cytochrome *c* (M_r = 12 500), all from Boehringer (Mannheim, F.R.G.). Other samples containing proteins were rat serum and plasma membranes of rat liver and Morris hepatomas. In order to remove the albumin, the serum sample was purified chromatographically on an Affi-gel-blue column (Bio-Rad, Munich, F.R.G.). The serum IgG was removed by chromatography on a Protein a-Sepharose column (Pharmacia, Freiburg, F.R.G.). The plasma membranes were isolated and extracted selectively, as described previously[8,9]. All other chemicals were purchased from E. Merck (Darmstadt, F.R.G.).

HPLC

The HPLC system consisted of two pumps (Model 64, Knauer, Berlin, F.R.G.), a programmer (Model 200, Kontron Analytik, Munich, F.R.G.), a filter photometer with a deuterium lamp (Knauer) and a RH 7125 loop-injection valve (Knauer).

The following columns were used for anion-exchange HPLC: TSK-DEAE 5PW and TSK-DEAE 5SW, both 75 × 7.5 mm (Toyo Soda, Japan); SynChropak AX 300, AX 500 and AX 1000, 250 × 4.6 mm or 80 × 4.6 mm; SynChropak Q 300, 250 × 4.6 mm; SynChroprep AX 300, 250 × 8.0 mm (all from Bishhoff Analysentechnik, Leonberg, F.R.G.); Mono Q and Mono P, 50 × 5.0 mm (Pharmacia); Aquapure AX 300, 250 × 4.6 mm (Kontron); Nucleogen DEAE 500-10 (Macherey Nagel, Düren, F.R.G.); Accell QMA, 250 × 8.0 mm (Waters, Eschborn, F.R.G.); and Microanalyzer MA 7P, 30 × 4.6 mm (Bio-Rad). Preparative columns were packed with SynChroprep and Accell QMA by Knauer.

The columns used for cation-exchange HPLC were: SynChropak S 300 CM, 250 × 4.6 and 80 × 4.65 mm (Bischoff); Mono S, 50 × 5.0 mm (Pharmacia); Poly Cat-A, 250 × 4.6 mm (Molnar, Berlin); and Microanalyzer MA 7C, 30 × 4.6 mm (Bio-Rad). For the preparation of mixed-bed columns, SynChropak AX 300 anion-exchange material and SynChropak S 300 CM cation-exchange material were used. These were mixed in ratios of 3:1, 1:1, 1:3 (w/w cation exchanger–anion exchanger) and packed into 250 × 4.6 mm columns. The packings were manufactured by Bischoff.

For hydrophobic-interaction HPLC, the following columns were used: TSK-Phenyl 5PW, 75 × 7.5 mm (Toya Soda); Phenyl-Superose, 50 × 5.0 mm (Pharma-

cia); SynChropak Propyl and Hydroxypropyl, 250 × 4.6 mm and 80 × 4.6 mm (Bischoff). The Amid column, 250 × 4.0 mm, was generously provided by H. Engelhardt (Universität des Saarlandes, Saarbrücken, F.R.G.).

Other columns used were: GF-250 and GF-450, both 250 × 9.4 mm (Bio-Series for Du Pont, Bischoff) for size-exclusion HPLC, and TSK-Chelate 5PW, 75 × 7.5 mm (Toyo Soda) for metal chelate affinity chromatography.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The dialyzed and freeze-dried samples were dissolved in 62.5 m*M* Tris–HCl buffer, pH 6.8, with 3% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. In other experiments, 80–120 μl of sample was taken from the collected fractions after HPLC separation (fraction collector Super Rac, LKB, Munich, F.R.G.) and mixed with a buffer containing a five times higher concentration of the above-mentioned substances. The amount of the buffer taken for the experiments was measured in such a way as to give the original concentration after dilution by the sample. SDS-PAGE was carried out by the Laemmli method[10]. The amount of protein that was applied was between 50 and 150 μg per line.

RESULTS AND DISCUSSION

Choice of method

Fig. 1 shows different ways of employing the three HPLC methods: size-exclusion, anion-exchange, and metal-affinity HPLC. The sample in all three cases was rat serum, from which the two proteins with the highest concentrations, serum albumin and Immunoglobulin G, had been removed. The poorest resolution was usually obtained by size-exclusion HPLC (Fig. 1a). The peaks were broad, and some proteins appeared in places were they would not be expected on the basis of their molecular weights. These irregularities, which are due to the quaternary structure of the proteins, the size of the water envelope, and the interaction with the matrix, have often been discussed[11].

Separation on the basis of molecular size is particularly difficult when non-ionic detergents such as Nonidet P-40, Triton X-100, Triton X-114 and Genapol X-100 are used. However, size-exclusion HPLC is the best method, when the sample contains SDS. In this case, a shift in the range of molecular weights towards lower values is observed[11]. Because some columns are sensitive to SDS and mercaptoethanol, the column life time under these conditions will be much shorter. Fig. 1b and c show separations by anion-exchange and metal chelate affinity HPLC. These separations were much more specific, and single proteins could be effectively enriched after only two passes, *i.e.* the collection of a fraction from anion-exchange HPLC and its application to a metal chelate column. When non-ionic detergents were added, column performance was impaired, and the peaks grew broader (not shown). However, separation is still possible, even under these conditions. If samples containing SDS are to be separated, the application of size-exclusion HPLC can be highly recommended.

Because the different kinds of affinity HPLC, one of them being metal chelate HPLC, are relatively new, only a few columns are as yet available. However, prospects for these methods are very good and their use is likely to become much more

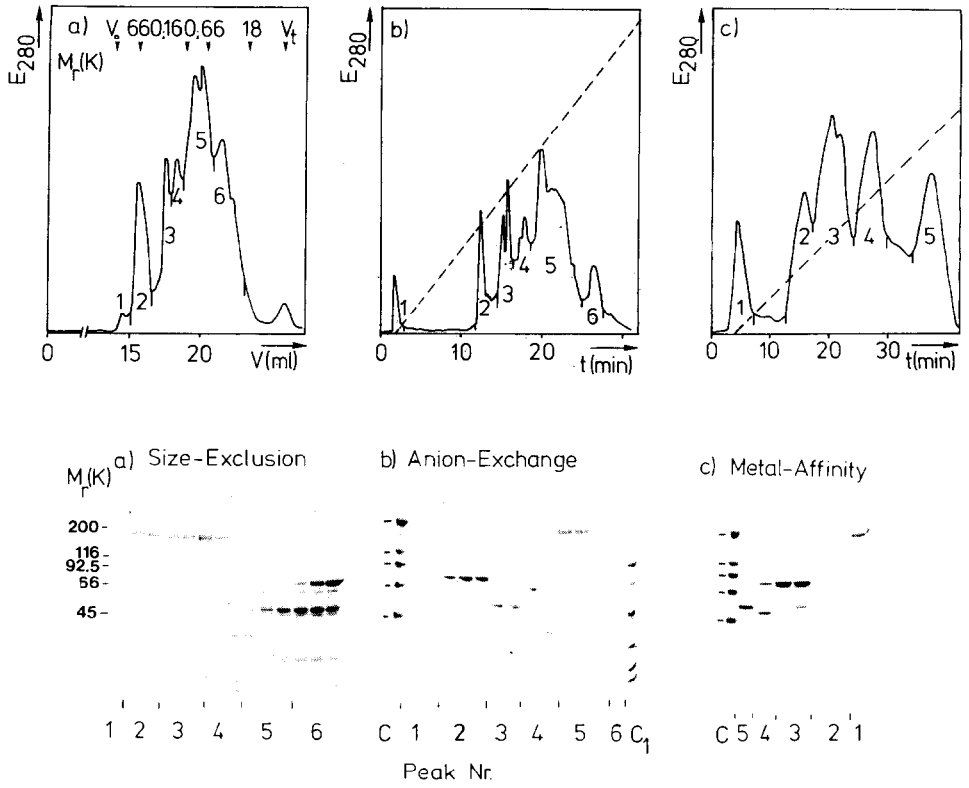

Fig. 1. Separation of rat serum proteins by three different HPLC methods. Before separation, IgG and serum albumin were removed from the sample by affinity chromatography on Protein A-Sepharose and Affi-gel-blue columns, respectively. In each case, 3 mg protein was applied in a 0.5-ml sample loop. Size-exclusion HPLC: columns, GF-250 and GF-450 in a tandem operation; mobile phase, 20 m*M* Tris–HCl buffer (pH 7.2), containing 0.15 *M* sodium chloride; flow-rate, 0.5 ml/min; pressure, 40 bar; room temperature. Anion-exchange HPLC: column, Mono Q; buffer A, 10 m*M* HEPES (pH 6.2); buffer B, 1 *M* sodiumchloride in buffer A; flow-rate, 1 ml/min; pressure, 20 bar; room temperature. The gradient is shown. Metal-affinity HPLC: column, TSK-Chelate 5PW; buffer A, 20 m*M* Tris–HCl with 0.5 *M* sodium chloride (pH 8.0); buffer B, 0.2 *M* glycine in buffer A; flow-rate, 1 ml/min; pressure, 12 bar; room temperature. Before each separation, the column was washed with 10 ml of 50 m*M* EDTA in buffer A, then the column was equilibrated with 10 ml of initial buffer, and 2 ml of 0.2 *M* zinc chloride was applied to the column by means of the sample injector (*cf.* ref. 5). The numbered peaks were collected. Their polypeptide pattern is shown by SDS-PAGE. C = calibration proteins with higher molecular weights (200, 115, 925, 66, and 45 kilodaltons); C_1 = calibration proteins with lower molecular weights (92.5, 66, 45, 31, 21.5 and 14.4 kilodaltons).

widespread. Unlike affinity HPLC, ion-exchange and hydrophobic-interaction HPLC have become more or less routine procedures in protein chemistry. The remainder of this paper will be devoted to these methods.

Ion-exchange HPLC

The subjects of our previous publications have been the anion-exchange and hydrophobic-interaction HPLC of a number of proteins[12–15]. The commercially avail-

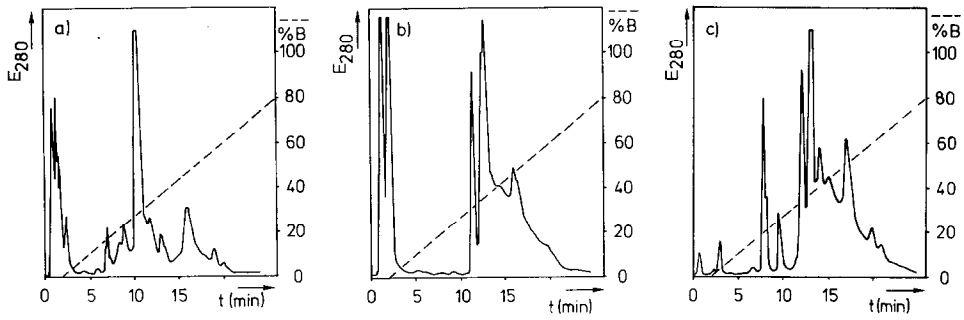

Fig. 2. Ion-exchange HPLC of rat serum, 3 mg serum protein. (a) Anion-exchange HPLC on a Mono P column. (b) Cation-exchange HPLC on a Mono S column. (c) Ion-exchange HPLC on a tandem column, consisting of a Mono S and a Mono P column. Separation conditions for all three runs: buffer A, 20 m*M* HEPES (pH 6.5); buffer B, 1 *M* sodium chloride in buffer A; flow-rate, 1 ml/min; room temperature; pressure: (a) 10 bar, (b) 12 bar, (c) 23 bar. The respective gradients are shown.

able anion-exchange columns were compared to one another and classified according to their characteristics. The results shown here should help to make full use of the columns available at present. For example, the Mono P column from Pharmacia is often used for chromatofocusing[16]. The column is very sensitive to the pH of the mobile phase. Under slightly acidic conditions it is the right tool for this kind of protein separation, as shown in Fig. 2a. The Mono S is a cation-exchange column from the same manufacturer. Under the chosen separating conditions the major part of the sample is excluded from the Mono P column. In both instances rat serum proteins were chromatographed. A minor portion, consisting of cationic proteins, was retarded and subsequently eluted by a sodium chloride gradient (Fig. 2b). Fig. 2c shows that the tandem operation of columns has certain advantages. This procedure retards almost all proteins, and improves the separation in the first part of the chromatogram. However, if the purpose of the experiment is chiefly the isolation of strongly anionic proteins, which are retarded longer, a tandem column will not improve the results, and the use of a single anion-exchange column is recommended. The available ion-exchange columns fall into two groups according to their matrices: columns with polymer matrix and with silica gel matrix. Both types have proved to be of equal value in experiments with water-soluble proteins, in our case, serum proteins[15]. In experiments with membrane proteins, which require the use of detergents in the mobile phase, either type of column was found suitable. For the isolation of the membrane-bound enzyme, dipeptidyl–peptidase IV, the silica gel column, SynChropak AX 300, showed markedly better results than the polymer columns TSK-DEAE 5PW and Mono Q. For the isolation of nicotinic acetyl-choline receptor from the electric organ of *Torpedo californica* the polymer columns were much more efficient[17]. For the separation of ferritin from cell homogenates the Mono Q column was found best[13]. Thus, as a rule, the choice of columns ultimately requires preliminary tests. The manufacturer's literature on column applications is often helpful.

Another approach to related separation problems involves columns packed with both anion-exchange and cation-exchange materials (mixed-bed columns, see ref. 3). Figs. 3–5 demonstrate the potential advantages of such columns. Fig. 3 shows the separation of standard proteins on an anion-exchange column (above) and on a

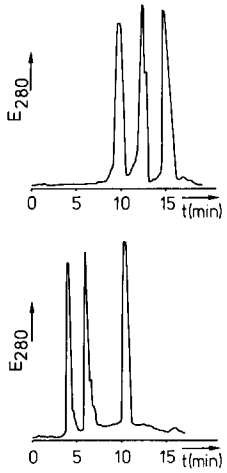

Fig. 3. Comparison between standard protein separations carried out on an anion-exchange column and on a mixed-bed column. The standard proteins used were IgG, transferrin, and bovine serum albumin, 1 mg of each. Top: separation on a Syn-Chropak AX 300 column. Bottom: separation on a column packed with a mixture of 67% (by weight) SynChropak AX 300 material and 33% SynChropak S 300 CM material. Separation conditions: buffer A, 20 m*M* HEPES (pH 6.2); buffer B, 1 *M* sodium chloride in buffer A; flow-rate, 1 ml/min; pressure, 65 bar; room temperature; gradient, 2 min at 0% B up to 100% B within 40 min. Peak order: IgG, transferrin, bovine serum albumin.

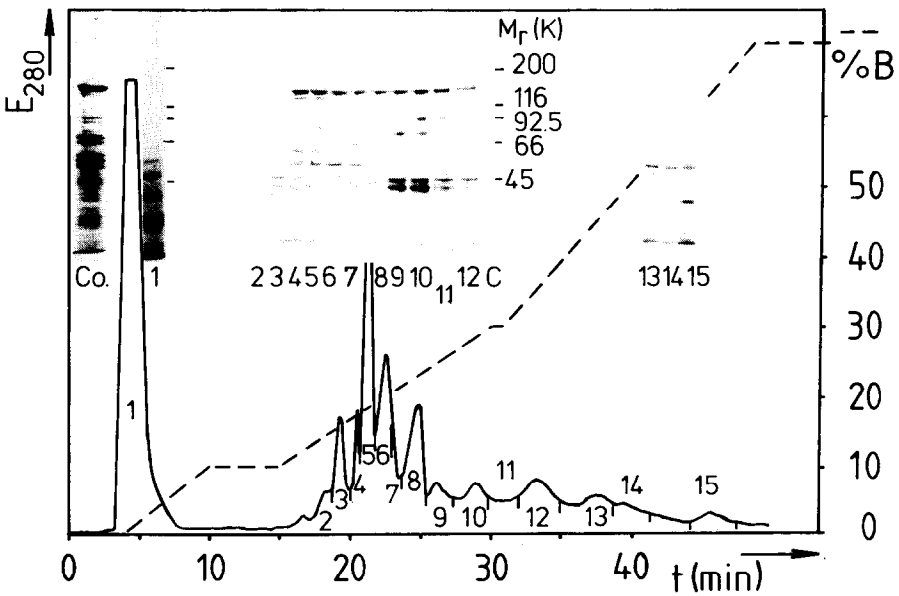

Fig. 4. Separation of liver membrane proteins on an anion-exchange column. The membrane extract (3 mg), obtained by freezing–thawing (*cf.* ref. 9) was applied to a SynChropak AX 300 column. Separation conditions as in Fig. 3, except for the gradient, which is shown. The fractions were collected and tested electrophoretically (top): Co. = complete sample (150 μg protein); C = standard proteins.

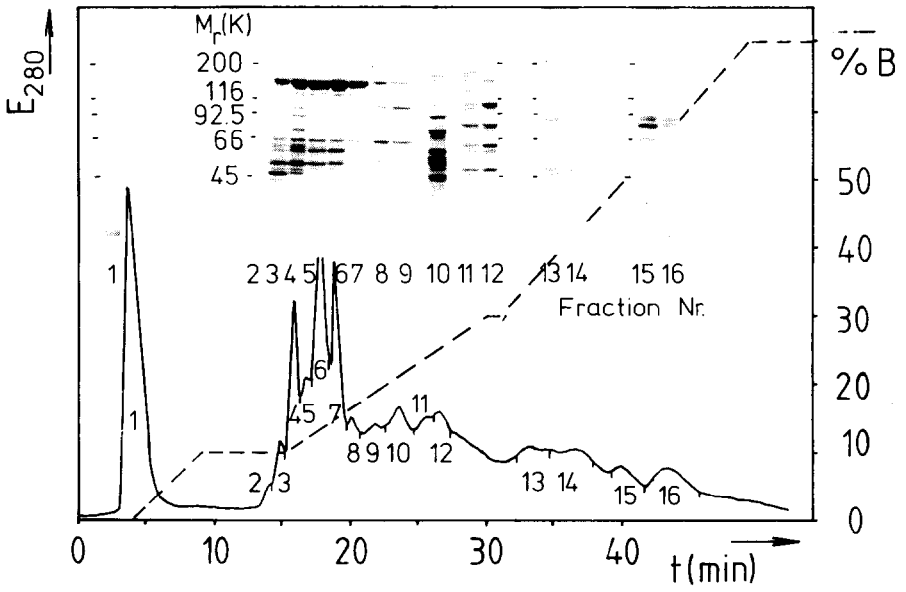

Fig. 5. Separation of liver membrane proteins (same sample as in Fig. 4) on a mixed-bed column, packed with 67% SynChropak AX 300 material (anion exchanger) and 33% SynChropak S 300 CM material (cation exchanger). For conditions, see Figs. 3 and 4.

mixed-bed column, containing 2/3 of anion-exchange material 1/3 of cation-exchange material (below). The retention time of the first two proteins, IgG and transferrin, is much shorter on the mixed-bed column. The retention time of the third protein, bovine serum albumin, is not greatly changed.

Figs. 4 and 5 demonstrate the separation of liver membrane proteins. In this experiment the use of a mixed-bed column has important advantages. The glycoproteins with the apparent molecular weight of 175 kilodaltons, which forms the major part of the sample, shows a feature unfavorable for isolation, *i.e.* microheterogeneity. When an anion-exchange column is applied, in this case an AX-300 column, a very broad peak appears (see Fig. 4). With the mixed-bed column mentioned above, the peak is narrow and further removed from the other proteins in the mixture (see Fig. 5).

The use of mixed-bed columns and columns tandems of anion-exchange and cation-exchange columns can in principle only be recommended for solving special separation problems, as illustrated in Figs. 2 and 5. Columns packed with mixed-bed resin are also commercially available. These columns packed with 5, 15 or 40-μm silica based resin are specially designed for antibody purification (ABx™ columns, Baker, see also ref. 18).

Further developments in the field of ion-exchange columns run along two different lines. On the one hand, very fast columns are available which are packed with non-porous materials. On the other hand, preparative columns are manufactured in which materials with larger particles are used. Columns of the former type, which contain ion-exchange groups bound to a non-porous support, have been commercially available for about a year. The anion-exchange and cation-exchange columns

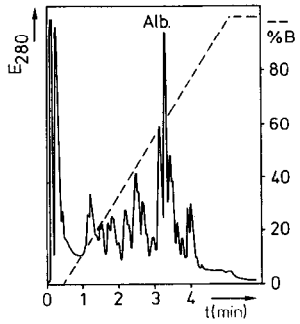

Fig. 6. Separation of rat serum (200 μg protein) on a very fast anion-exchange column, Microanalyzer MA 7P. Separation conditions: buffer A, 20 m*M* HEPES (pH 7.2); buffer B, 1 *M* sodium chloride in buffer A; flow-rate, 1 ml/min; pressure, 20 bar; room temperature. The gradient is shown.

from Bio-Rad, which we have used, give faster separations than other analytical columns. Fig. 6 shows the separation of rat serum on a Microanalyzer MA 7P anion-exchange column. A separation similar to that in Fig. 2a is achieved within 5 min, as compared with more than 20 min in the Mono P column. Fig. 7 shows that the Microanalyzer 7P column can also be applied to membrane protein separations.

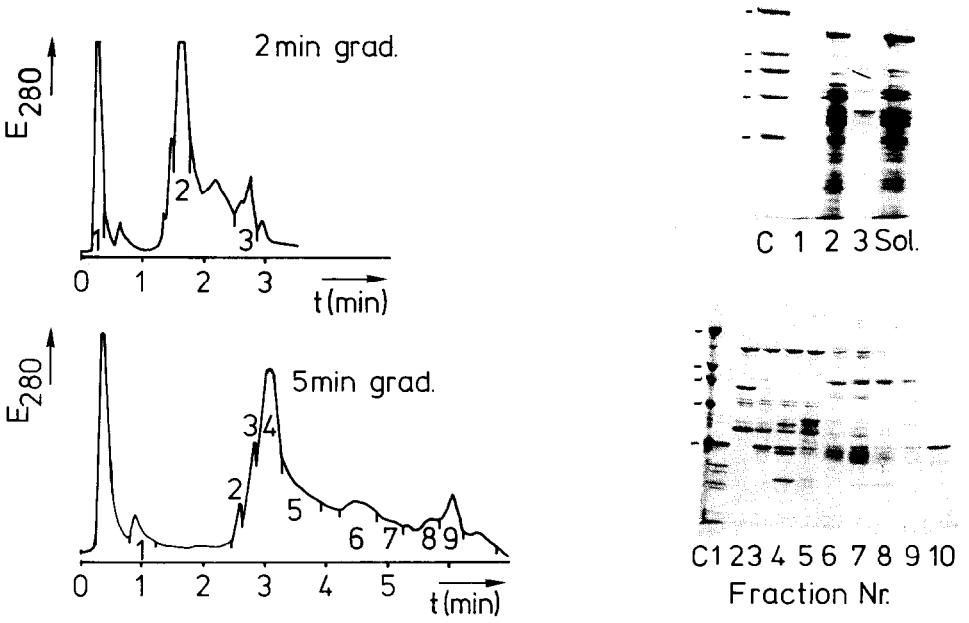

Fig. 7. Separation of liver membrane proteins on a Microanalyzer MA 7P column. Separation conditions: buffer A, 20 m*M* HEPES (pH 7.2); buffer B, 1 *M* sodium chloride in buffer A. Top: gradient, 1 min at 0% B, then up to 100% B in 2 min; about 250 μg protein was applied. Bottom, gradient, 1 min at 0% B, up to 100% B in 5 min; flow-rate in both cases, 1 ml/min; pressure, 22 bar; room temperature. The fractions were collected and tested electrophoretically: Sol. = the complete membrane extract (about 150 μg protein); C = calibration proteins [MW (downwards) 200, 116, 92.5, 66 and 45 kilodaltons].

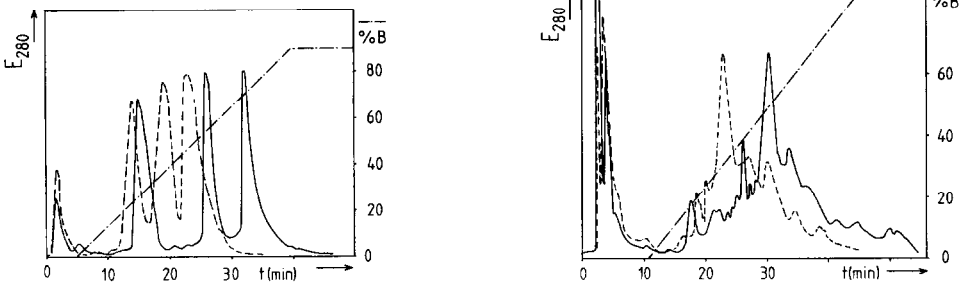

Fig. 8. Separation of standard proteins by preparative anion-exchange HPLC. Peaks represent 5 mg of the following proteins (in order): transferrin, bovine serum albumin, and soybean trypsin inhibitor. Separation conditions: buffer A, 20 m*M* HEPES (pH 6.5); buffer B, 1 *M* sodium chloride in buffer A; flow-rate, 2 ml/min; pressure, 40–50 bar; room temperature; sample loop, 2 ml; the gradient is shown; ——— SynChroprep AX 300; – – – Accell QMA.

Fig. 9. Separation of serum proteins by preparative anion-exchange HPLC. A quantity of 25 mg serum protein was applied. ——— SynChroprep AX 300 column; – – – Accell QMA column; Conditions as in Fig. 8.

In terms of separation characteristics, the MA 7P is comparable to the Mono P from Pharmacia. It is therefore rather sensitive to pH. A combination with the cation-exchange column of the same type (Microanalyzer MA 7C) yields results similar to those in Fig. 2c, but again much more rapidly (not shown). The capacity of Microanalyzer columns is smaller than that of the analytical columns (see Materials and methods and ref. 15). However, the capacity is higher than 200 μg protein, the figure given in the manufacturer's manual. We have been able to apply up to 700 μg protein in a 200-μl loop without a decrease in separating efficiency (see Fig. 7).

In the field of preparative separations the most common columns for anion-exchange HPLC are SynChroprep AX 300 and Accell QMA. These column materials can also be obtained as bulk packings. As can be seen in Figs. 8 and 9, the Accell QMA is a slightly weaker anion-exchanger than the SynChroprep. Detergents can be used with both materials, as with the "analytical" and "micro-analytical" columns in separations of hydrophobic proteins. This is shown in Fig. 10. The columns are also an adequate alternative to the rather expensive preparative TSK-DEAE SW column, which until recently had been the only available column of this type.

Hydrophobic-interaction HPLC

For several reasons our investigations concerning hydrophobic-interaction columns have not advanced as far as those of ion-exchange columns. The hydrophobic-interaction columns have only recently been introduced, and their number is still much smaller than that of ion-exchange columns. Moreover, hydrophobic-interaction HPLC has its limitations. For example, some proteins are precipitated when high salt concentrations are applied, as is necessary at the beginning of elution.

The hydrophobic-interaction columns we have used fall into three groups. The classification is shown in Fig. 11. Five standard proteins were taken for separation: cytochrome *c* (peak 1), transferrin (peak 2), bovin serum albumin (peak 3), soybean trypsin inhibitor (peak 4) and ferritin (peak 5).

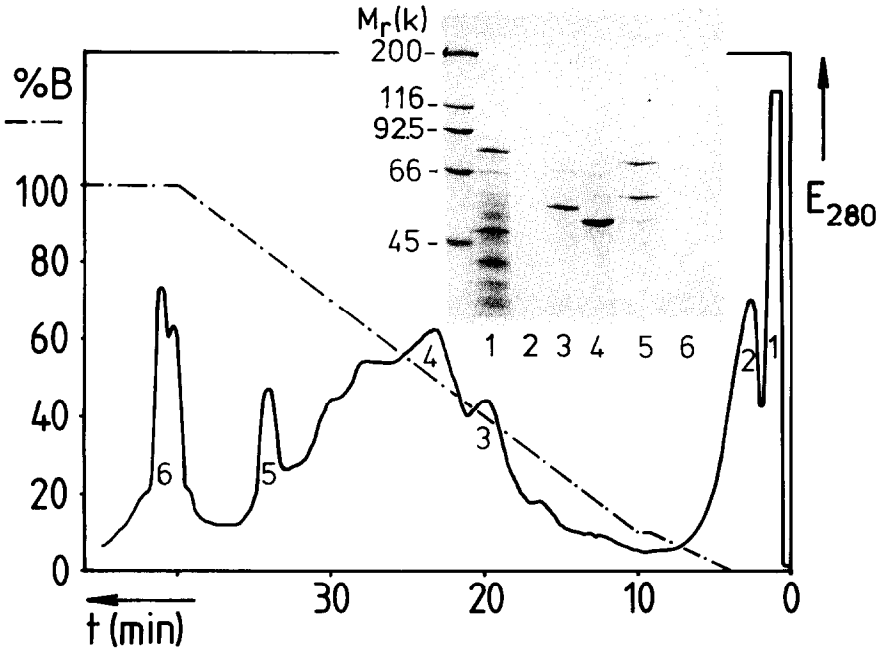

Fig. 10. Separation of membrane proteins from Morris hepatoma 9121 by anion-exchange HPLC. 15 mg protein, obtained by extraction with the non-ionic detergent Genapol X-100 (*cf.* refs. 9 and 14), was applied to a SynChroprep AX 300 column. Separation conditions: buffer A, 20 m*M* HEPES (pH 6.5) with 0.1% Genapol X-100; buffer B, 1 *M* sodium chloride in buffer A, flow-rate, 3 ml/min; pressure, 60 bar; room temperature; 5-ml sample loop; the gradient is shown. The marked fractions were collected, and an aliquot of 50–100 μg of protein was tested electrophoretically.

The first type of column is weakly hydrophobic. Among these columns are the SynChrom Hydroxypropyl and the Amid column. Retention and separation of the five proteins could only be achieved with a rather high (2 *M*) ammonium sulfate concentration. The second type is the SynChrom Propyl column, which is somewhat more hydrophobic. Consequently, a slightly lower (1.7 *M*) ammonium sulfate concentration could be used. The third type of column is even more hydrophobic, TSK-Phenyl and Phenyl-Superose. They have the advantage that the initial ammonium sulfate concentration can be lowered to 1 *M*. A disadvantage is that highly hydrophobic proteins cannot be eluted from such columns unless water or methanol is used. This is shown here with ferritin (see lower part of Fig. 13 and ref. 13). These hydrophobic columns are the best choice for the separation of serum proteins (see Fig. 12) and water-soluble cellular proteins[13]. Due to the lower ammonium sulfate concentration, the solubility of the proteins at the beginning of separation is ensured. The hydrophobic proteins can subsequently be eluted with water. Fig. 13 shows an example of the use of less hydrophobic columns for the separation of hydrophobic membrane proteins. The sample is dissolved in 0.5 *M* ammonium sulfate and the proteins are eluted selectively from the column: first with a decreasing ammonium sulfate concentration, then with water, and finally with a gradient of up to 1% detergent. In this way, a separation is achieved, parallelling the increase in hydrophobicity of sample components. Because of the complexity of the necessary operations

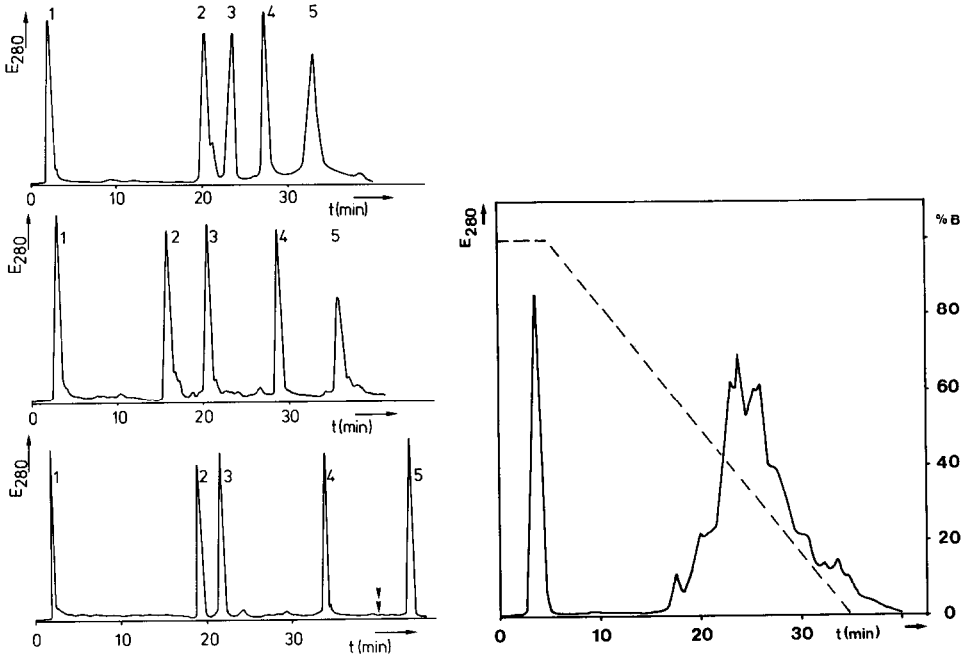

Fig. 11. Hydrophobic-interaction HPLC of standard proteins. 250 μg of cytochrome *c* (peak 1), transferrin (peak 2), bovine serum albumin (peak 3), soybean trypsin inhibitor (peak 4) and ferritin (peak 5) was applied to different hydrophobic interaction columns. Top: separation on an Amid column. Separation conditions: buffer A, 0.1 *M* sodium phosphate (pH 7.0); buffer B, 2 *M* ammonium sulfate in buffer A. Center: separation on a SynChropak Propyl column. Separation conditions: buffer A, 0.1 *M* sodium phosphate (pH 7.0); buffer B, 1.7 *M* ammonium sulfate in buffer A. Bottom: separation on a Phenyl-Superose column. Separation conditions: buffer A, 0.1 *M* sodium phosphate (pH 7.0); buffer B, 1.0 *M* ammonium sulfate in buffer A. Flow-rate in all three cases, 1 ml/min; gradient, 5 min at 100% B, then linear down to 0% B in 30 min; room temperature; pressure, 50 bar (top), 46 bar (center), 22 bar (bottom). On the Phenyl-Superose column the ferritin (peak 5) could only be eluted with water. The beginning of water elution is marked by arrows.

Fig. 12. Separation of serum proteins on a SynChropak Propyl column. 3 mg of protein was applied and eluted under the same conditions as in Fig. 11 (center). The gradient is shown.

and the strain put on the pumps by the use of detergents this method can be recommended only if other methods fail.

Publications are beginning to appear which deal with preparative separations by hydrophobic-interaction HPLC[19]. A development similar to that in the field of ion-exchange HPLC can be expected.

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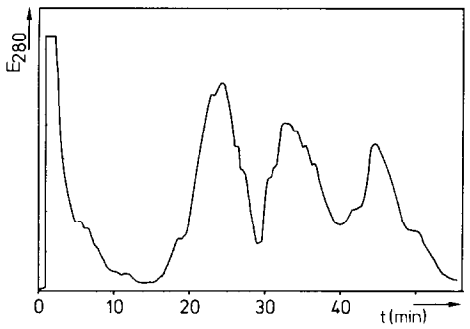

Fig. 13. Hydrophobic-interaction HPLC of liver membrane proteins. Three mg of protein from the membrane extract, obtained by freezing–thawing, was dissolved in 1 ml of 0.5 *M* ammonium sulfate containing 20 m*M* Tris–HCl (pH 7.0), and applied to a SynChropak Propyl column, 80 × 4.6 mm. The 0.5 *M* ammonium sulfate was pumped for 5 min, then a linear gradient was started and run down to 100% water in 20 min. The column was washed with water for 5 min and then a second linear gradient was started and run up to 1% Genapol X-100 (in 20 m*M* Tris–buffer, pH 7.0) in 15 min; flow-rate, 1 ml/min; pressure, 20 bar; room temperature.

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