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REVIEW

APPLICATIONS OF PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE SEPARATION AND PURIFICATION OF PEPTIDES AND PROTEINS

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LIST OF ABBREVIATIONS

AFC (Biospecific) affinity chromatography
CCC Counter-current chromatography

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HIC Hydrophobic interaction chromatography
HPLC High-performance liquid chromatography
IEC Ion-exchange chromatography
M_r Relative molecular mass
RPC Reversed-phase chromatography
SEC Size exclusion chromatography

1 INTRODUCTION

Chromatography has played a major role in the recent advances made in our understanding of the mechanisms involved in the synthesis and metabolism of proteins. This role has always had two faces On the one hand, liquid chromatography provides a rapid, sensitive and very selective method of analysis of proteins [1] On the other hand, it permits the isolation or the purification of most protein samples in the range of production most convenient for biochemistry or pharmaceutical studies, between a few nanograms and a few kilograms [1–15] This part has always been very important, because in vivo assays are often required to ascertain that the proper protein has been identified and is analyzed

Recent breakthroughs in genetic engineering and the rapid development of the biotechnologies have made possible the synthesis and recovery of the specific genes coding for a given protein and the cloning of these genes in microorganisms, mainly bacterias, in order to engineer enzymes and other proteins in host organisms in which they do not normally exist Unfortunately, these hosts are most generally unable to excrete the proteins they are programmed to manufacture and they store them inside their cells, from which they have to be freed and recovered [16–18] The cells can be harvested from the cultures and lyzed A complex mixture containing fragments of cell membranes and organites, together with the host cell natural proteins and nucleic material and the coveted proteins is recovered Performing separations and purifications of biopolymers from these crude products on a process scale is one of the greatest challenges facing the biotechnologists who are attempting to bring the laboratory results to fruition in the world of practical applications [8,19]

The task is made all the more complicated by the severe requirements of the regulatory agencies who demand extremely low trace levels of the residual material of bacterial origin Furthermore, defective protein molecules originating from malfunctions of the overloaded cell machinery must be eliminated also Finally, there are no two proteins alike Some are highly hydrophilic, other hydrophobic, some are basic, other acidic, some are extremely stable, other very sensitive to minor changes in their environment Some are individual molecules, other agglomerates Their molecular mass span nearly two orders of magnitude Accordingly, methods which give satisfactory results with some proteins can absolutely not be used with others Thus, very powerful separa-

tion methods must be used, and these methods must be easily adapted to changes in the nature of the product involved [20–23]. Liquid chromatography has long been used for the analysis of protein mixtures [24]. It provides the separation power and the flexibility required, but suffers from a serious drawback which must constantly be kept in mind when developing new application methods.

The biological activity of a protein molecule characterizes one specific conformer and disappears completely for apparently minor changes in the conformation It depends essentially on the integrity of its three-dimensional structure, which, itself, is dependent on the chemical composition and the physical properties of its environment (such as the possible presence of an organic solvent and its nature, the types of buffer solution, ionic strength, pH, etc) Protein molecules are usually tightly coiled chains, kept stable by bridges and interactions between side-groups. The structure tends to minimize the hydrophobicity of the groups or parts of the molecule at the outside (i e., in contact with a saline solution) and to maximize the interactions between the hydrophobic groups placed inside the molecule. Physico-chemical interactions between a protein molecule and other chemical entities, especially the solid surface of a stationary phase, whether adsorption is the main retention mechanism or not, is susceptible to trigger an isomerization process which minimizes the free energy of the system When the protein molecule returns in solution, it is not in the same conformation as when it was injected and has suffered total or near total loss of its biological activity. This change can be spontaneously reversible, in which case the separation scientist does not care. If it is not, and if the transformation of the inactive conformer into the active one, by proper refolding, does not take place or is too slow, the separation process investigated cannot be used for preparative purposes [25]. So-called "soft" interactions, including the use of stationary phases exhibiting surfaces whose chemical properties are closely similar to that of the protein exterior surface or to water (peptides, polyether chains, polyols, sugars or polysaccharides, etc.), of polar solvents (water, light alcohols) and of soft ions is therefore recommended. Because of the variety and complexity of the problems which may arise, and because of the huge differences in nature and behavior between proteins, no one separation method or combination of techniques may be expected to work well with all biopolymers. At any rate, an ideal method of biopolymer isolation should be able to give high-purity material with a high production rate and a good recovery yield, which includes total conservation of their biological activity

High-performance liquid chromatography (HPLC) is the only separation method which meets all these different requirements It is rapid and efficient and it has been possible to develop phase systems which do not cause excessive degradation of proteins. It has been widely used for analytical separations Its preparative and process applications are undergoing rapid development for the isolation and purification of peptides [1,26–30] and proteins [31–36]

The intense pace of the current development of the applications of prepa-

TABLE 1

COMPARISON BETWEEN THE THREE CHROMATOGRAPHIC DEVELOPMENT MODES IN PREPARATIVE HPLC

Mode	Column size	Flow velocity	Dilution degree	Production rate	Need to regenerate column	Experimental conditions	Recovery yield
Displacement	Usually analytical (30–50×0 4–0 6 cm I D)	Low	Can concentrate minor components	High	Yes	Very different from analytical	<100%
Overloaded elution	Usually large size (30–50×2–10 cm I D)	High	Dilute slightly	High	No	Similar to analytical	Adjustable, most often <100%
Linear elution	Usually large size (30–50×2–10 cm I D)	High	Dilute strongly	Low	No	Similar to analytical	Adjustable, most often ca 100%

TABLE 2

EXAMPLES OF THE USE OF DISPLACEMENT HPLC FOR THE PURIFICATION OF PROTEINS AND PEPTIDES

Compound	Type	Stationary phase	Particle size (μm)	Mobile phase	Displacer	Flow-rate (ml/min)	Column size (mm×mm)	Sample size (mg)	Recovery yield[a] (%)	Ref
β-Lactoglobulin A and B	IEC	DEAE-5PW, Synchropak, AX-300, DEAE Spherogel-TSK	10	0 2 *M* KH_2PO_4 (pH 6 0)	1% Carboxymethyl dextran (CMD)	0 1	45×2 1 45×2 1 45×2 1	3 2 6 4 12 8	N A N A N A	62
β-Lactoglobulin A and B	IEC	TSK gel, DEAE-5PW	10	0 025 *M* Phosphate buffer (pH 7 0)	10 ml/ml Chondroitin sulfate	0 1	75×7 5	60–100	95	57
Ovalbumin, α-lactalbumin, soybean trypsin inhibitor	IEC	DEAE-5PW	10	0 02 *M* Tris-phosphate (pH 7 0)	1% CMD in carrier	0 5	75×7 5	25	N A	61
Peptides Met-NH_2 Benzoyl-Arg Benzoyl-Arg-OEt Benzoyl-Arg-Met-NH_3	RPC	Amicon-ODS	10	0 1 *M* H_3PO_4 buffer (pH 2 2)	40 g/l Butoxyethoxy-ethanol in carrier	0 1	250×4 6	13 ml of 45 m*M* 1 4 m*M* 3 6 m*M* 45 m*M*	 100 87 86 93	60

[a]N A = not available

TABLE 3

COMPARISON BETWEEN DIFFERENT TECHNIQUES USED FOR PREPARATIVE HPLC OF BIOPOLYMERS

Method	Retention mechanism	Popularity	Cost	Specificity
IEC	Electrostatic interactions between functional groups in the molecules and on the surface of the ion exchangers	+ + + +	High	Intermediate
RPC	Strong hydrophobic interactions between molecules and alkyl groups on the surface of reversed-phase packings	+ +	Intermediate	Intermediate
HIC	Soft hydrophobic interactions between molecules and hydrophilic groups bound to the surface of adsorbent packings, under salt gradient condition	+ + +	High	High
SEC	Difference of dynamic volume or size of molecules	+ +	Intermediate	Intermediate
AFC	Biospecific interactions between sample molecules and affinity ligand bonded on a support	+	Very high	Very high
Chromato-focusing	Difference in isoelectric point of biopolymer molecules on ion exchangers	+	High	High
CCC	Difference in solubility of sample components in two immiscible solutions	+	Low	Low

rative HPLC in biotechnology is demonstrated by a tremendous increase of the amount of literature published A computer search has indicated that around 1000 papers dealing with all aspects of preparative HPLC were published during the period of 1967–1988 Of them, 800 have appeared since 1980 This huge amount of information is difficult to review properly This paper does not attempt to cover comprehensively all aspects of the preparative applications of HPLC to the isolation and the purification of peptides and proteins, but to survey the main trends in current research

Several books discussing both the theory and the practice of preparative HPLC have been published recently [37–39] They give a good survey of the general literature on preparative HPLC Verzele and Dewaele [37] have written an excellent review of the field. Wankat [38] described and discussed large-scale separation and purification from the chemical engineering viewpoint It is excellent reading for the study of scaling-up problems, but contains little material directly related to protein preparation The third book [39] contains much practical information, most of which has, unfortunately, become obsolete because of the rapid development of the field

An excellent review on the various retention mechanisms available for protein separations, although written with analytical applications in mind, can be

used for the selection of the best phase systems to be investigated further for the solution of a new problem [25]. Many review articles [40–45] discussing the applications of preparative liquid chromatography to the separation of biopolymers have appeared in the scientific literature

In the following, we discuss first the advantages of the elution and displacement modes (see also Tables 1 and 2) and present briefly some results regarding the optimization of the experimental conditions of a separation Then, according to their retention mechanism, we describe the techniques used in the preparative HPLC of peptides and proteins (see Table 3) Those are ion-exchange, reversed-phase, hydrophobic interaction, size exclusion and biospecific affinity chromatography A few other methods are also discussed. Table 4 summarizes the main features of the applications selected from the literature to illustrate the most important approaches available

2 SEPARATION MODES IN PREPARATIVE HPLC OF PROTEINS

There are three modes of chromatography. frontal analysis, elution and displacement The three have been used for preparative applications In some cases, two of them have been combined After a frontal analysis or a displacement run, however, the column must be cleaned and initial conditions restored before another run can be started, which is not required in isocratic, isorheic elution. For this reason, frontal analysis is rarely used, and never used alone. The two modes of chromatography of practical importance for the development of preparative chromatographic processes of peptides and proteins are elution and displacement

In elution chromatography, a sample pulse is injected in a stream of mobile phase, at the column inlet The pulse duration can be a fraction of a second, as it is in analytical chromatography, or longer, but it is much smaller than the retention time of the compounds investigated Otherwise, it would be frontal analysis Experimentalists try to make the pulse profile as close to a rectangle as possible, but axial dispersion relaxes the steep front and rear of the pulse, which are more commonly exponential decays The elution mode can be further subdivided into isocratic and gradient elution and into linear and non-linear (or overloaded) types The first classification is based upon the nature of the mobile phase stream at the column inlet If the mobile phase composition remains constant, it is isocratic elution In gradient elution, the concentration of one of several components of the mobile phase varies progressively during the run The retention times of the analytes depend on the rate of variation of the mobile phase composition The second classification depends whether, with the loading amount of sample used, the column operates in the linear or in the non-linear region of the equilibrium isotherm corresponding to the retention mechanism used.

Production rate in elution mode increases with increasing volume (volume

overload) and/or concentration (concentration overload) of the sample injected in the column Because the band width of all the eluates increases in both cases, there is a limit to the sample size which can effectively be used When the sample size increases from the very low values typically used in analytical applications, the recovery yield remains constant and the production rate increases linearly with increasing sample size [37]. This takes place as long as the bands are separated. When the band of the compound of interest touches its neighbor, the recovery yield starts decreasing with increasing sample size, since the wings of the elution band must be clipped to eliminate contamination The production rate keeps increasing but does so more and more slowly. Eventually, a maximum value of the production rate is reached. However, simple determination of the optimum conditions through the extrapolation of the Gaussian profiles and the use of the classical procedures of analytical chromatography is not possible as soon as the elution is carried out under non-linear conditions [46]

When the sample size increases, the concentration of an eluate in the stationary phase at equilibrium does not increase in proportion to its concentration in the mobile phase. It is a more complex function of the concentration of this compound and of all the others. As a consequence, there is a velocity associated to each concentration, which is a function of the local concentrations of all the eluates [47] The elution profiles of a pair of compounds, the influence of the experimental conditions on the production rate and yield and the optimization of these conditions have been discussed [46–54].

Displacement is an alternative, non-linear mode using the same retention mechanism as elution, but where the sample is pushed out of the column by a stream of a solution of a compound which is more retained than all the sample components. Under such conditions, the components of the sample are eluted as a train of rectangular bands On the contrary of what is observed in elution, the retention time of a band depends on the concentration of the compound in the feed, while the heights of these bands depends on the nature of the corresponding compound. By choosing suitable experimental conditions, it is possible to collect fractions which contain at least some of the mixture components at higher concentrations than they are in the original sample [55–57] Large throughputs and high yields can be achieved by adjusting the various experimental parameters, such as the displacer concentration, the mobile phase flow velocity, the column length, the amount of feed injected, etc [58] There has been no study published yet on the optimization of the mobile phase velocity, the sample size and the column length in displacement chromatography It has been shown, however, that accurate predictions of band profiles can be made, that, because a certain degree of overlap between the bands of the various components of the feed at their exit from the column does take place, it is impossible to achieve a 100% yield in displacement chromatography and that the production rate can be calculated [59].

The distinct advantage of displacement over elution is the possibility to collect enriched fractions of certain components, at a concentration larger than they are in the feed. This is interesting only for minor components, for which interference with neighbor zones will be serious Then, it will be impossible to achieve a good recovery yield and a multi-step process will become necessary. A related advantage is the very limited extent of the dilution of the main components of the mixture during the separation On the other hand, the shortcomings of the method are the relatively long time needed for the regeneration of the column, which reduces the production rate and the great difficulties often encountered in finding a suitable displacer for a given multi-component mixture, especially in the case of biopolymers, which increases the development costs Moreover, most chromatographers involved in the development of preparative HPLC applications are more familiar with elution than with displacement mode. It is also simple and tempting to scale-up a separation which has been successful at the analytical level. A convincing comparison between elution and displacement chromatography has never been made The advantages and drawbacks of both are rather well known. Some are quantitative, other qualitative and it is difficult to peg a dollar figure on some of them. Calculations of the optimum production rate for a certain component to be extracted from a feed of given composition, using either mode, have never been carried out

All these problems explain why up to now most major applications of HPLC to the purification of peptides and proteins have used elution rather than displacement However, high-performance displacement chromatography has been recently employed for the isolation of peptides [60], proteins [57,61,62] and other compounds [55,56,58]

3 OPTIMIZATION OF EXPERIMENTAL CONDITIONS IN ELUTION CHROMATOGRAPHY

Many papers dealing with the optimization of the experimental parameters of a chromatographic separation have been published. These parameters include the column dimensions, the particle size of the packing material, the mobile phase velocity and the sample size. In many cases, it is not possible to carry out the desired purification in a single step The impurities to be eliminated are often too numerous and a single retention mode cannot afford the desired separation Most studies are essentially empirical and tend to extrapolate observations beyond their range of validity [63,64] A limited number of them only is reported here More information is reported elsewhere [46,65]

Optimization procedures for preparative liquid chromatography of proteins with emphasis on increasing the selectivity of the process rather than the column efficiency have also been reported [44,65–67] An economic analysis of preparative HPLC of proteins takes into account the feed properties, the char-

acteristics of the fractionation, the required product purity, the desired throughput and the operating costs [68] Comparing conventional chromatography and HPLC, Berkowitz et al. [69] suggested a general strategy for the solution of all protein purification problems, involving a three-step approach and the use of silica-based packing materials. During the first step of the process no product is actually collected but necessary information is acquired. Short columns, packed with 3–5 μm particles are used to determine rapidly the optimum experimental conditions for scaling-up the chromatographic separation The second step utilizes a wide-bore preparative column, packed with 40 μm particles, to treat large volumes of crude samples and to collect concentrated fractions containing the interesting species. The final step employs high-efficiency columns, packed with 3–15 μm particles, to purify the components of interest and achieve the high purity requested

Two theoretical approaches based on the theory of non-linear chromatography have been described They give results which are in excellent agreement [49,51,54] The first approach uses exact numerical solutions of the non-ideal model of chromatography This model is based on the numerical solution of the system of partial differential equations made of the mass balance equations of the compounds involved. It permits (among other calculations) the prediction of the band profile of a pure compound eluted by a pure mobile phase or by a binary mixture. Experimental results are in excellent agreement with the calculated profiles [53] The prediction of the profiles of the elution bands of a mixture is also possible. The exact competitive equilibrium isotherms of the compounds involved must be known, however, which has prevented up to now the exact comparison with experimental results. Qualitative comparisons between experimental and calculated profiles show the best possible agreement [53] Using this calculation procedure, and a Simplex optimization routine, it is possible to determine the experimental conditions for which the production rate is maximum [49] Some additional constraints, such as the required purity of the product or a minimum recovery yield, can be introduced.

The second approach is derived from the exact solution we have given to the ideal model of chromatography, for a rectangular pulse of a binary mixture [50]. The ideal model assumes the column efficiency to be infinite. A correction can be made to take into account the finite efficiency of real columns. The advantage of this second approach is that it gives algebraic relationships between the production rate or the recovery yield and the parameters characterizing the separation problem investigated [51] Thus, trends can be securely predicted and optimum localized Exact values can be calculated from the solution of the exact semi-ideal model, by numerical integration The agreement between the results of the two approaches demonstrates the validity of the correction made for the effects of the finite column efficiency [49,51].

The main conclusions of this work are the following [51] The production rate of a purified fraction of a certain compound, at constant degree of purity

and from the same feed, increases as the square of $(\alpha-1)$, where α is the relative retention of the prepared compound and its closest impurity (i.e., the ratio of the origin slopes of their equilibrium isotherms). The production rate increases rapidly with increasing inlet pressure, if the optimum column is used in each case. There is an optimum value for the ratio dp^2/L, of the square of the packing particle diameter to the column length: long columns packed with coarse particles accommodate larger sample sizes, but give longer cycle times than short columns packed with fine particles. This optimum value depends on the characteristics of the separation problem and on the maximum inlet pressure available. Columns should be operated at very high flow velocities. The more efficient column permits a larger production rate because its efficiency may be traded for a shorter cycle time. This is possible only if the pressure available at the column inlet is sufficient to operate the column at the high mobile phase velocity required for maximum production rate. Calculations show that the column efficiency at the flow velocity which gives the maximum production rate is low. With a very small, analytical-scale sample size, this efficiency permits a resolution between the bands of the main compound and its closest impurity which is almost always smaller than unity.

The most efficient columns, i.e., those which have the smallest constants A and C of the traditional plate height equation, are those which give the highest production rate. In fact the most efficient column would always give the highest production rate, provided only that it can be operated at a high enough mobile phase velocity, and thus that the equipment can be set at the required pressure. This conclusion, which is in agreement with some previous experimental results, is at the opposite of "common wisdom" in the matter. Some have long submitted that low-efficiency columns are better in preparative chromatography than high-efficiency ones, because their efficiency remains constant in a much larger range of sample sizes [39]. This phenomenon is simple to explain, but has nothing to do with the optimization of production rate. Chromatographic bands broaden with increasing sample sizes and the column efficiency, as it is conventionally measured in analytical chromatography, decreases. This is not due to a kinetic problem, however, but to a thermodynamic one.

The bands broaden at high column loading because the equilibrium isotherm is not linear and, consequently, the velocity associated to a concentration of the compound investigated does not remain constant but varies with the concentration. The resistances to mass transfers, which are responsible for the finite efficiency of the column, have no reason to change as indicated by the band broadening. As a matter of fact, for low or moderate molecular masses [below ca. 1000 relative molecular mass (M_r)], the molecular diffusion coefficient does not vary much in the concentration range 0–5%, which is the most common in preparative liquid chromatography. The contribution of the mass transfer kinetics to band broadening does not vary much in this concentration

range, except for polymers, proteins included. This problem of the concentration dependence of the mass transfer kinetics on protein concentration should be investigated further

The production rate depends on the cycle time and the throughput per cycle The combination between the requirements for a large sample size, a short cycle time and a high recovery yield results in the need to operate a rather efficient column at high velocity This explains the importance of the maximum inlet pressure available These results apply to proteins as well as to other compounds Because of the very low values of the diffusion coefficients of these compounds, high reduced velocities are associated to much lower values of the actual mobile phase velocity than for more conventional, low-molecular-weight compounds This permits the use of small-particle packing materials with reasonable inlet pressures

4 ION-EXCHANGE CHROMATOGRAPHY

For more than three decades, ion-exchange chromatography (IEC) has been an integral part of all the processes of isolation, purification and identification of proteins Classical ion exchangers tend to exhibit low rates of mass transfers for protein molecules They were weakly cross-linked resins, swelling in contact with the mobile phase, and their swollen volume is often influenced by changes of the experimental conditions, e g , depends on the type of buffer used, the ionic strength and the pH of the mobile phase, etc Finally, they were unable to withstand the high flow velocities and pressures used in HPLC Accordingly, the column efficiencies were low and the quality of the separation poor The principles of HPLC (columns packed with small-particle materials and operated at high flow velocities) could not be used to improve them

Recently, new, high-performance ion exchangers have been developed, which are rigid, remain stable under high pressure, do not swell markedly in the presence of various salt buffers and, nevertheless, have good ion-exchange capacity These ion exchangers can be made into small particles and be used at high flow-rate They permit the rapid isolation of proteins, the use of large sample volumes and can be operated under mild conditions [70] As a consequence, high-performance IEC still constitutes by far the most popular technique for achieving separations and purifications of biopolymers It has become widely used in preparative applications

This popularity is also explained by some of the properties of the IEC retention mechanism These properties are very different from those of the various modes of adsorption chromatography [hydrophobic interaction chromatography (HIC), reversed phase chromatography (RPC) etc] which are also widely used for the separation and purification of proteins The number of electrical charges carried by a protein molecule depends on its isoelectric point and on the pH of the solution Thus, the retention in IEC which depends on

the charge of the molecules of eluate can be modified by adjusting properly the pH of the mobile phase Furthermore, the Coulombian forces which are responsible for the retention in IEC decrease as the square of the reverse of the distance between the charge carriers Since proteins carry only a rather small number of charges, and these charges are rather remote from each other, the interaction energy in IEC remains of the same order of magnitude as with low-molecular-mass organic ions. Admittedly, in IEC of proteins, mixed retention mechanisms take place Some molecular interactions, similar to the adsorption interactions observed in HIC or RPC, take place, as demonstrated by the fact that the retention of at least some proteins does not become negligibly small when the pH is such that they are neutral. Nevertheless, IEC is [with size exclusion chromatography (SEC) for another reason] the only type of chromatography with which proteins may be eluted under isocratic conditions This makes IEC a very simple separation method to develop when a new problem arises.

The use of IEC for the purification of biomolecules, e.g., insulin, monoclonal antibodies, animal growth hormones, enzymes, etc., has been reviewed [71,72]. Hupe and Lauer [73] have studied preparative applications of IEC for the purification of proteins, with emphasis on column design and on the properties of packing materials. Rounds et al. [74] have discussed the influence of the physical properties of silica-based anion exchangers (including their pore size distribution, specific surface area and surface density of charged sites) on the intrinsic loading capacity of the stationary phase in preparative protein chromatography. They have found that anion exchangers having wide pores, a high specific surface area and a high density of positively charged sites provide the largest loading capacity, and that the protein-binding capacity depends on the accessible surface area of the anion exchanger, not on its total surface area.

Peptide mapping is a very important analytical problem encountered in the control of the synthesis of proteins. A combination of IEC and of thin-layer chromatography has been applied to analytical and preparative peptide mapping by Aromatorio et al. [75,76] Both analytical mapping and the preparative separation of the peptides from trypsin digests of proteins were performed by high-performance IEC on a macroreticular anion exchanger [77].

In most cases, the use of ion-exchange packing materials having an average particle size between 10 and 30 μm is advocated for preparative scale HPLC [78]. The rationale for this recommendation is that the resolution obtained with small-particle packing materials decreases rapidly when the column is overloaded [39]. As explained in the previous section, this erroneous suggestion results from a fundamental misconception regarding the nature of mass transfer kinetics (which are measured by the column efficiency at infinite dilution of the eluate) and the nature of the phenomena responsible for band broadening at large sample size (which originate in the non-linear behavior of the equilibrium isotherm). Rejecting the use of small particles on the ground

TABLE 4

ION-EXCHANGE CHROMATOGRAPHY EXAMPLES OF PREPARATIVE APPLICATIONS TO THE PURIFICATION OF PROTEINS AND PEPTIDES

N A = not available

Compound	Stationary phase	Particle size (μm)	Mobile phase[a]	Flow-rate (ml/min)	Elution mode	Column size (mm)	Sample size (mg)	Recovery yield (%)	Ref
Human and ovine prolactin Bovine thyroid stimulating hormone	Mono-Q-anion exchanger	10	(a) 20 m*M* Piperazine (pH 9 6) (b) 300 m*M* NaCl in (a)	1	Step gradient	50×5	0 05–20	84	80
Human growth hormone	DEAE-5PW	20	(a) 20 m*M* Glycine (pH 8 0) + 75 m*M* NaCl (b) 0 5 *M* NaCl in (a)	30	Linear gradient	200×55	240	N A	86
Human growth hormone	SP-5PW	13	(a) 20 m*M* Glycine (pH 8 0) + 75 m*M* NaCl (b) 20 m*M* Glycine (pH 8 0) + 0 5 *M* NaCl	4	Linear gradient	150×21 5	40	N A	86
Thyroid stimulating hormone, Luteinizing hormone	DEAE-Sepharose	N A	(a) 5 m*M* Na glycinate (pH 9 6) (b) 50 m*M* Na glycinate (pH 9 6) (c) 0 2 *M* Na glycinate (pH 9 6) (d) 0 1 *M* NaCl in (c) (e) 0 4 *M* NaCl in (c) (f) 1 *M* NaCl in (c)	1 6	Step gradient	350×16	350	92 93	
Ovalbumin	SynChroprep AX-200	30–50	(a) 20 m*M* Tris (pH 7 0) (b) 0 5 *M* NaCl in (a)	9	Gradient	250×21	3000	N A	81

Bovine serum albumin	Synchroprep AX-300	30	(a) 20 m*M* Tris-HCl (pH 7 0) (b) 500 m*M* NaAc in (a)	1 5	Linear gradient	250×25	1000	N A	84
Human adult serum	SynChropak AX-300	30	(a) 20 m*M* Tris-HAc (pH 7 0) (b) 0 5 *M* NaCl in (a)	3 2	Linear gradient	100×25	1000	N A	84
IgG	S Fast-flow Sepharose cation exchanger	N A	(a) 10 m*M* Na phosphate (pH 6 5) + 10 m*M* NaCl (b) 10 m*M* Na phosphate (pH 6 5) in 0 1 *M* NaCl	450	Step gradient	100×11 5	6500	N A	
IgG1	Mixed-bed SynChropak Q-300 and S 300 (4/1)	30	10 m*M* NaH_2PO_4 (pH 7 5)	20	Isocratic	250×25	1170	95	79
IgG	MA7P WAX (PEI[b]), MA7C WCX (carboxyl)	7	(a) 16 m*M* Tris (pH 8 5) (b) 18 m*M* Tris + 0 5 *M* NaCl (pH 8 5)	0 7	Linear gradient	30×4 6	0 3–0 5	91–92	89
Monoclonal antibodies	Mono-Q HR 5/5 anion exchanger	10	(a) 20 m*M* Tris-HCl (pH 8) (b) 1 5 *M* NaCl in (a)	1	Gradient	50×5	0 5–25	90	82
Monoclonal antibodies	Mono-Q HR 5/5 anion exchanger	10	(a) 20 m*M* TEA-HCl (pH 7 7) (b) 350 m*M* NaCl in (a)	1	Linear gradient	50×5	10	N A	88
Antigen binding fragments	Mono-Q HR 5/5 anion exchanger	10	(a) 5 m*M* Na_2HPO_4 (pH 8 0) (b) 0 3 *M* Na_2PO_4 (pH 8 0)	1	Linear gradient	50×5	10	N A	88
Plasma membrane proteins	TSK-DEAE-5PW	10	(a) 10 m*M* HEPES and 0 05% CHAPS (pH 6 1) (b) 1 *M* NaCl in (a)	1	Step gradient	75×7 5	5	90	93
Membrane-bound proteins	Protein-Pak DEAE 5PW, alkaline anion exchanger	10	(a) 25 m*M* Tris-HCl + 0 1% Brij-35 (pH 7 5) (b) 1 *M* NaCl in (a)	1	Linear gradient	75×7 5	13	72	90
Membrane flavoproteins	Mono-Q HR 10/10 anion exchanger	10	(a) 5 0 m*M* Tris-HAc (pH 7 5) (b) 1 *M* NaCl + (a)	1	Linear gradient	100×10	48	89 2	91

(Continued on p 446)

TABLE 4 (continued)

Compound	Stationary phase	Particle size (μm)	Mobile phase[a]	Flow-rate (ml/min)	Elution mode	Column size (mm)	Sample size (mg)	Recovery yield (%)	Ref
Superoxide dismutase	DEAE-5PW	20	(a) 20 m*M* Tris–HCl (pH 7 5) (b) 0 3 *M* NaCl in (a)	30	Linear gradient	200×55	300	67	86
Superoxide dismutase	DEAE-5PW	13	(a) 20 m*M* Tris–HCl (pH 7 5) (b) 0 3 *M* NaCl in (a)	4	Linear gradient	150×21 5	60	90	86
Bovine carbonic anhydrases	Cellulose Whatman DE-23	N A	(a) 10 m*M* Tris-HCl (pH 8 7) + 0 2 *M* NaCl (b) 0 6 *M* NaCl in (a)	N A	Gradient	N A	1200	85	83
Ribonuclease A, chymotrypsin, lysozyme	SynChroprep CM300	30	(a) 20 m*M* Tris (pH 7 0) (b) 500 m*M* NaAc in (a)	1 5	Linear gradient	250×25	1000	N A	84
Lipoxidase	DEAE-5PW	20	(a) 20 m*M* Tris–HCl (pH 8 0) (b) 0 5 *M* NaCl in (a)	30	Linear gradient	200×55	1000	90	86
Lipoxidase	DEAE-5PW	13	(a) 20 m*M* Tris–HCl (pH 8 0) (b) 0 5 *M* NaCl in (a)	4	Linear gradient	150×21 5	200	90	86
Lipoxidase	SP-5PW	13	(a) 20 m*M* NaAc (pH 4 5) (b) 0 5 *M* Na_2SO_4 in (a)	4	Linear gradient	150×21 5	200	80	86
Lipoproteins	SynChropak AX-300	10	(a) 20 m*M* Tris–HCl in 6 *M* urea (pH 7 9) (b) 0 2 *M* Tris–HCl in 6 *M* urea (pH 7 9)	1	Linear gradient	250×9	ca 20	N A	
Aldolase	Accell CM (carboxymethyl)	37–55	(a) 50 m*M* Na phosphate (pH 6 0) (b) 1 *M* NaCl in (a)	2	Linear gradient	100×9	4 2	91	87
Glucose-6-phosphate dehydrogenase	Accell QMA (quaternary methylamine)	37–55	(a) 20 m*M* Tris (pH 8 0) (b) 380 m*M* NaCl in (a)	1 5	Linear gradient	100×9	17 5	81	87

Restriction endonucleases	Mono-Q HR	10	(a) 20 m*M* Tris-HCl (pH 7 4) and 5% glycerol (v/v)	1	Linear gradient	50×5			92
Ban II			(b) 0 4 *M* NaCl in (a)	0 63			8400 U/h	N A	
Sac I			(b) 0 7 *M* NaCl in (a)	1 33			40500 U/h	N A	
Sph I			(b) 0 4 *M* NaCl in (a)	2 30			5000 U/h	N A	
Isoenzymes of lactate dehydrogenase H4 and MH3	Mono-Q QR 5/5	10	(a) 20 m*M* Na phosphate (pH 8 0) (b) 0 8 *M* NaCl in (a)	2	Step gradient	50×5	7	25	94
Murine EGF	Brownlee AX-300	N A	(a) 10 m*M* NH_4HCO_3 (b) 500 m*M* NH_4HCO_3	0 1	Linear gradient	30×2 1	0 0017	>90	
Tryptic digest of bovine brain S-100b protein	Di-ion CDR-10	5–7	(a) Water (b) 0 25 *M* $CH_3SO_3NH_4$ (pH 2 8), 50% ACN and 25% isopropanol	0 89	Convex curve gradient	50×0 4	0 1-6	N A	
Bacterial ribosomal proteins	TSK-gel SP-5PW	13	(a) 0 1 *M* KH_2PO_4, 8 *M* urea and 0 5 m*M* dithioerythritol (pH 5 6 with methylamine) (b) 1 *M* KCl in (a) (pH 5 6 with H_3PO_4)	1 5	Step gradient	150×21 5	100 to 400	80–100	85

[a]Tris = tris(hydroxymethyl)aminomethane, Ac = acetate, TEA = triethanolamine, ACN = acetonitrile, HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate
[b]PEI = polyethyleneimine

that the column apparent efficiency is going to be low anyway when it is overloaded is tantamount to confusing kinetics and thermodynamics To achieve the maximum production rate, rather small particle sizes should be considered As explained in Section 3, there is an optimum value for the ratio d_p^2/L for each separation problem and the particle size should be chosen accordingly.

Ion-exchange packing materials having small size pores (ca. 300 Å) are suitable for the preparation of low-molecular-mass proteins (with a molecular mass up to ca $30 \cdot 10^3$ M_r), while the purification of larger-molecular-mass proteins (up to ca $100 \cdot 10^3$ M_r) and of protein aggregates, when they stable enough, requires the use of materials with larger pores (ca. 1000 Å) The pH of the mobile phase and the nature of the counter ion are two of the main factors controlling the phase selectivity in preparative IEC. The optimization of the mobile phase pH and the choice of the counter ion should be performed in order to maximize the differences between the retention of the proteins of interest This operation is usually carried out by the trial-and-error method, since little theoretical background material is available to guide in this choice.

As an example of application, a mixed-bed column, packed with a 4 1 mixture of two ion exchangers, SynChroprep-Q-300 and SynChroprep-S-300, was used for the extraction of 47 3 mg of pure immunoglobin G1 (IgG1) from 1170 mg of bovine serum in 19 min Because in this case the protein does not bind to either of the ion exchangers, the risk of denaturation is effectively eliminated [79] This is an excellent example of the best approach to high production rate of purified protein, with total conservation of biological activity If experimental conditions can be found, under which the protein of interest is not retained while the impurities are, it is possible to inject large amounts of feed solution (i.e., in frontal analysis), until the impurities begin to elute Then a rapid gradient can regenerate the column, since little attention has to be paid to the resolution between unwanted compounds

A selection of examples of applications of preparative high-performance IEC for the isolation and the purification of peptides and proteins is given in Table 4

5 REVERSED-PHASE CHROMATOGRAPHY

RPC was developed during the seventies for the analysis of polar organic compounds, using a non-polar or weakly polar stationary phase and a polar eluent In some cases, the stationary phase is a porous silica gel whose surface has been grafted with n-alkyl chains (mainly octadecyl, to a lesser extent octyl- and butyl-bonded silicas are used). The mobile phase is a solution of water and methanol or acetonitrile. A variety of other solvents has been used. Additives are also frequent. The use of salts of a bulky organic ion and a mineral counter ion (e g., tetrabutyl ammonium chloride or sodium dodecyl sulphate) is the most notable example

At the end of the seventies, RPC became the most popular method for the analysis and isolation of peptides and proteins [95–98]. Retention takes place due to hydrophobic interaction between the protein molecule and the surface of the porous packing material, chemically bonded with a hydrocarbon group Because of the strength of these interactions and the large surface area of protein molecules (a single protein may interact with tens of alkyl chains), the retention is very strong In order to free the protein, hydrophobic interactions should be weakened, by making the mobile phase more organic Due to the use of acidic eluents containing organic modifiers, as well as under the influence of the hydrophobic interactions on the stationary phase, many proteins are denaturated during the chromatographic process A given protein can exist under a huge number of conformers The original, biologically active protein is a well arranged, tightly coiled chain, with most of its polar groups oriented towards the outside, where they interact with water molecules and the non-polar groups oriented towards the inside where they interact together The outside surface of the protein is made of polar and non-polar patches This minimizes the free energy of the protein in the water solution and stabilizes the system

When the protein comes in contact with the surface, the minimum free energy would be achieved by maximizing the interactions between the non-polar surface of the adsorbent and the non-polar groups, while orienting most of the polar groups towards the polar mobile phase This corresponds to a structure which is very different from that of the biologically active conformer. Thus, the thermodynamically stable adsorbed state is an uncoiled, biologically inactive protein conformer Since the protein backbone is rather flexible, it is usually a matter of a short time before a kinetic pathway permits the transformation Therefore, RPC tends to promote protein denaturation The spontaneous recoiling in aqueous solution takes place sometimes rapidly, in which case no problem arises In other cases it does take place slowly enough to interfere with the chromatographic process and generate unbecoming band profiles In many cases, the eluted protein will never recoil into its biologically active form within an acceptable time This is of little importance for analysis but is unacceptable in preparative applications, hence the efforts to modify the implementation of the retention mechanism, which eventually lead to the development of HIC.

It is convenient to discuss separately the two closely related techniques, RPC and HIC (next section) Stationary phases used in RPC include essentially all *n*-alkyl-bonded silica, to which can be added perfluoropropyl silica and cyanoethyl silica Other bonded phases, including aminopropyl silica and the diol phases, belong to HIC Solvents used in RPC include methanol and acetonitrile, but isopropanol and acetone are the two main organic modifiers used in preparative RPC of proteins The former is suitable for dissolving proteins having small solubility in other solvents while the latter, which has a low vis-

cosity, permits the achievement of a better resolution The ionic strength of the mobile phase and its pH have a strong influence on the retention Most mobile phases contain a salt buffer Besides sodium phosphates and formate buffers, ammonium bicarbonate has been recommended in the purification of synthetic peptides because of its excellent volatility. Ammonium bicarbonate can be simply removed from the collected fractions by freeze-drying, leaving pure peptides [99]

Gabriel et al. [100] have briefly reviewed the development of preparative RPC of peptides and proteins Pearson and Regnier [101] have studied in detail the effect of the length of the *n*-alkyl chain chemically bonded to a silica support on the retention of proteins and on their separation and recovery in preparative applications. They have found that the retention times were independent of the chain length or the carbon loading, but that protein resolution and the loading capacity of the column were influenced by the chain length They concluded that short *n*-alkyl chains are better than octadecyl for protein fractionation. Rubinstein [102] published a comprehensive review dealing with the isolation of proteins in amounts up to 50 mg, and compared results obtained with two chemically bonded stationary phase, one typical of RPC, LiChrosorb RP-8, and one considered now as a HIC phase, LiChrosorb Diol.

Synthetic, biologically active peptides were purified using radially compressed cartridges (now, an obsolete technology), handpacked with Vydac C_{18}, Vydac C_4 or diphenyl-derivatized silicas of different particle sizes (10–20 μm) [103] and mobile phases of different compositions Up to gram amounts of hormone analogues and of other peptides could be prepared The use of disposable Sep-Pak C_{18} cartridges as preparative columns was reported [104] Proteins separated on the cartridge could be collected as pure fractions The use of these inexpensive columns permitted the preparation of 38 mg of a pure peptide from 150 mg of crude human endorphin [105] A Prep PAK-500 C_{18} cartridge packed with 75 μm particles was used to purify samples of 1–10 g of L-Leu(Gly)$_3$, a recovery better than 95% was reported [106]

Applications of preparative RPC also include the isolation and purification of the following peptides S1–S5 (Synthetic, see Table 5) [107], enterotoxin ST6 [108], various hepta- and octapeptides [109], ferricsocin [110], different synthetic peptides [111–117], bovine insulin and bovine serum albumin [118], human insulin and human-like growth factor II [119], ribosomal proteins [120], astroprotein [121], protein S6 [122] and parathyroid hormone antagonist [123] RPC at both the analytical (1 nmol) and the semi-preparative (1 μmol) levels was also applied for studies of peptide mapping and protein sequencing [124]

A survey of numerous applications of preparative RPC to the isolation and the purification of peptides and proteins is given in Table 5

6 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Although more recent than RPC, HIC has rapidly become more popular because of the excellent results obtained with this method in analytical applications The development of the preparative applications of HIC are a normal consequence of this early success The separation mechanism used in HIC is based on the use of the weak hydrophobic interactions which take place between the proteins and a stationary phase made by grafting weakly polar chemical groups on the surface of a porous silica gel Whereas in RPC very strong interactions take place between protein molecules in aqueous solutions and the alkyl (usually octadecyl) chains and extreme conditions (such as a solvent gradient with acetonitrile) are needed to desorb them, in HIC the molecular interactions between the protein molecules and the grafted groups are much weaker To promote them, the proteins are "salted out" the aqueous solution by a high salt buffer concentration, the proteins are then freed by a negative ionic strength gradient.

Such experimental conditions are much less aggressive than those used in RPC Therefore, the biological activity of the proteins separated by HIC is retained, because the high salt concentration used in the mobile phase does not change the protein structure during the separation In comparison with RPC, HIC is a softer and gentler technique for biopolymer separations The main disadvantage of HIC is that it requires the use of a mobile phase containing high concentrations of salt buffers, which has a high viscosity and etches stainless-steel pumps and other parts of the chromatographic hardware This can eventually cause leakage in the system, unless the chromatograph be very carefully operated and maintained The use of a titanium pump or a metal-free pump is preferable in HIC, but this is costly In preparative applications, the hardware cost for HIC is going to be much higher than with other modes of chromatography (except perhaps IEC) In order to extend the column life, a clean-up procedure was developed [126] This procedure uses a gradient elution from pure 10 m*M* phosphoric acid aqueous solution to a solution of 80% isopropanol and 20% of the 10 m*M* phosphoric acid aqueous solution It was used to remove periodically the column contaminants

The stationary phase used in HIC is made with either silica or a rigid cross-linked polymer matrix, chemically bonded with groups which are more polar than the alkyl chains used in RPC Oligomers of ethylene glycol have been used, as well as peptides, heparin, sugars, etc While for over ten years octadecyl-bonded silica was considered as about the only important stationary phase for liquid chromatography, the mid-eighties have seen a considerable renewed interest in the synthesis of chemically bonded phases and in the investigation of the chromatographic properties of these phases A considerable variety of substrates and reagents have been used In many cases, however, the study is very limited and information on many of these phases of potential interest is

TABLE 5

REVERSED-PHASE CHROMATOGRAPHY EXAMPLES OF PREPARATIVE APPLICATIONS TO THE PURIFICATION OF PROTEINS AND PEPTIDES

N A = not available

Compound	Stationary phase	Particle size (μm)	Mobile phase[a]	Flow-rate (ml/min)	Elution mode	Column size (mm)	Sample size (mg)	Recovery yield (%)	Ref
Insulin-like growth factors (IGFs)	LiChrosorb RP-18	N A	(a) 0 1% TFA in water + 10% ACN (b) 0 1% TFA in water + 50% ACN	5 5	Step gradient	500–10	20	72	119
Parathyroid hormone antagonist	μBondapak C_{18}	N A	(a) ACN–water (2 8) and 0 1% TFA (b) 70% ACN + 30% water + 0 1% TFA	4	Linear gradient	300×7 8	86 5	36	123
Plant hormone isomers	LiChrosorb RP-18	15–25	MeOH–water (70 30)	N A	Isocratic	400×80	3 5 g/h	99 5	
Bovine serum albumin and bovine insulin	Zorbax C_8-capped	12	(a) 85 mM H_3PO_4 + morpholine (pH 2 6) (b) ACN	20	Linear gradient	250×21 5	600 (7 5 mg/g)	N A	118
Ribonuclease A	Brownlee RP-300	N A	(a) 0 9% NaCl (pH 2 1) (b) ACN	0 1	Linear gradient	300×2 1	0 092	90	
EGF							0 025	90	
Cytochrome C							0 134	90	
Lysozyme							0 080	90	
Bovine serum albumin							0 140	90	
β-Lactoglobulin							0 094	90	
Myoglobin							0 079	90	
Cytochrome C	Zorbax C_8-capped	12	(a) 0 1 M H_3PO_4 + morpholine (pH 2 6) (b) ACN–water (84 64)	2	Linear gradient	250×4 6	8 mg/g	N A	118

Ferricsocin	LiChrosorb	7	ACN–water (9 1)	12	Isocratic	250×16	1000	N A	110
Enterotoxin STa (heat stable enterotoxin)	Aquapore RP-300	N A	(a) 0 1% TFA (b) 0 1% TFA in MeOH	2	Step gradient	250×7 1	5	>98	108
Synthetic peptides	Vydac C_{18}	15–20	(a) 0 1% TFA or TEAP (b) ACN	85–100	Linear gradient	300×50	1–3 g	N A	112
Synthetic peptides d-GAVP (des Gly[9]-NH_2-[Arg[8]]-vasopressin)	Nucleosil	25–40	(a) TEAF with 1% formic acid (pH 3 5)+10% ACN (b) TEAF+20% ACN	0 83	Linear gradient	400×26	1000	N A	117
Synthetic peptide fragment of human interleukin I	P/E C_{18}	N A	(a) 0 1% TFA (b) MeOH	7	Step gradient	250×22	150	70	114
Synthetic peptides	Radial-Pak C_{18}	N A	(a) 0.1 *M* $(NH_4)_2CO_3$ (pH 7 7) (b) i-PrOH–ACN–(a) (30 30 40) or i-PrOH–(a) (80 20)	1	Linear gradient	N A	0.35–25	>85	99
L-Leu(Gly)	Prep Pak-500 C_{18}	75	(a) Water–MeOH–TFA (95 5 0 05) pH 2 3	100	Isocratic	30×5 7	1–10 g	>95	106
Peptides S1–S5	Vydac C_{18}, SynChropak C_{18}	15–20 20–30	(a) 0 05% TFA (b) 0 05% TFA in ACN	5	Linear gradient	250×10	225	>97	107
Synthetic octadecapeptide	Radial-Pak C_{18}	N A	(a) 1 5 m*M* TEAP (pH 3 2) (b) i-PrOH–ACN–7 5 m*M* TEAP (40 40 20)	1	Linear gradient	N A	5	N A	125
Octa- and heptapeptides	Spherisorb C_{18}	5	(a) TEA (0 1 *M* formic acid, pH 3)–EtOH (72 28), counter ion *d*-10-camphorsulphonate (0 05 *M*)	4	Isocratic	250×10	10	90	109
Synthetic peptides	Vydac C_{18}, C_4 and diphenyl	10–20	See reference	75–125	Step gradient	300×50	0 1–3 g	99	103

[a]TFA = trifluoroacetic acid, TEAP = triethylammonium phosphate, ACN = acetonitrile, Me = methyl, Et = ethyl, i-Pr = isopropyl

limited to work originating from a single laboratory where it has been carried out on only one batch of the material, may be on a single column

In HIC, the pH and the nature and concentration of the salt(s) contained in the mobile phase are the most important factors affecting the chromatographic behavior of biopolymers and especially their retention Ammonium and sodium sulfate are two of the most common and most effective salts used in HIC However, the sodium sulfate concentration must be adjusted carefully, because of the limited solubility of this salt which may precipitate in the chromatographic system, and especially in the pump cylinder, when the pressure is raised This reduces the life time of valves and piston seals Optimization of the mobile phase pH for maximum selectivity may be achieved by using the method of pH retention mapping [127]

The following are typical examples of the use of HIC for the extraction and/or the purification of proteins With a column packed with TSK Phenyl-5PW, it has been possible to purify in a single run, by preparative HIC, 50–200 mg of lipoxidase, phosphoglucose isomerase and lactate dehydrogenase and to recover these proteins with a biological activity of 86, 70 and 93%, respectively Substantially larger amounts, ca 1 g of crude sample could be treated in a single day, by repeated injections [128] A 150 mm×21 5 mm column, packed with 15–20 μm particles of Vydac silica-based ether-bonded packing, was used for the purification of up to 344 mg of a mixture of four proteins, while the same column permitted also the purification of 667 mg of a crude soybean trypsin inhibitor [126] HIC, combined with other chromatographic techniques, has been employed for the separation of the enzymes contained in a ca 200-mg sample of crude materials on a glass column [94]

Table 6 summarizes detailed experimental data on the isolation or purification of biopolymers by preparative HIC

7 SIZE EXCLUSION CHROMATOGRAPHY

In principle, size exclusion (also called gel filtration) chromatography allows the separation of the components of a sample simply according to the size or hydrodynamic volume of their molecules in a given solvent, used as mobile phase It is specially useful as a preliminary isolation procedure for concentrating the components of interest from crude materials and/or as a final step for the separation of pure proteins from their aggregates The column packing is a porous solid (silica gel or rigid organic polymer or resin) The surface of the pores must have chemical properties very similar to those of the solvent or rather to the layer of solvent in direct contact with the proteins, in order to avoid any adsorption Ideally, the compounds injected should have no retention at all on the column The pore size distribution should be such that most components of the mixture have access to part of the pores Separation is based on the fraction of the total pore volume to which the various components have

access The exact retention mechanism is discussed in the book by Yau et al [136] The absence of retention is a hard condition to fulfill and in many practical cases there is some extent of mixed mechanism

The advantages of the method result from the retention mechanism The entire sample is eluted within one column volume No compound should be more retained The elution is rapid and complete Separation is based on molecular size, i e , on a property very different from those used in IEC, RPC or HIC, providing for the possibility to resolve interferences occurring on columns of these types Finally, since the molecules of the compound separated do not come into contact with a surface, there is no opportunity for conformation changes to take place, the method is extremely soft and retains very well the biological activity of the proteins

Conversely, SEC has two important disadvantages First, it has a low peak capacity, only compounds which have a molecular size between two rather close thresholds can be separated on a given column Those smaller than the lower limit have access to the entire pore volume and are eluted with the solvent, while those larger than the upper limit have no access to any pore and are eluted together, with a retention volume equal to the column external porosity The peak capacity of the method depends on the ratio of the column internal and external porosity, a value which does not exceed 1 2 with the best column packing materials available, except with the new Aligned Fiber Columns, whose efficiency is still not sufficient in practice [137,138] Secondly, silica and organic polymers, which are the two most widely used packing materials for high-performance SEC, tend to adsorb proteins readily through ionic and hydrophobic interactions, respectively In other words, it is very difficult to prevent any of various molecular interactions to take place between proteins and the surface of the packing materials This adsorption interferes with an ideal size exclusion mechanism In spite of various surface treatments, it is difficult to avoid adsorption of some compounds In this case, these compounds may be eluted later than the solvent peak and the determination of an approximate molecular weight becomes impossible These deficiencies influence to some extent the applications of SEC to the purification of proteins

In practice, another experimental problem, which does not exist in analytical applications, has often to be solved for preparative applications The solubility of the sample, or at least of its main components, in the mobile phase is often limited On the other hand, rather concentrated solutions are injected in preparative chromatography in order to maximize the production rate Guanidinium hydrochloride, urea, Brij-35 and organic acids are commonly used as solubilizing agents of proteins when needed There is not much information available on the production rate in preparative SEC, nor on the relationship between sample size, concentration and band broadening

Among other applications, SEC is useful for the isolation of plasma lipoproteins, due to their wide molecular mass distribution The enzymes β-galacto-

TABLE 6

HYDROPHOBIC INTERACTION CHROMATOGRAPHY EXAMPLES OF PREPARATIVE APPLICATIONS TO THE PURIFICATION OR PROTEINS AND PEPTIDES

N A = not available

Compound[a]	Stationary phase	Particle size (μm)	Mobile phase	Flow-rate (ml/min)	Elution mode	Column size (mm)	Sample size (mg)	Recovery yield (%)	Ref
Human serum γ-globulin	Bio-Gel TSK phenyl-5PW	13	(a) 0 1 *M* NaH_2PO_4, 1 7 *M* $(NH_4)_2SO_4$ (pH 7 0) (b) 0 1 *M* NaH_2PO_4	1	Linear gradient	75×7 5	1 5–2	N A	129
Human serum albumin	TSK-gel ether-5PW	13	(a) 0 1 *M* NaH_2PO_4, 1 7 *M* $(NH_4)_2SO_4$ (pH 7 0) (b) 0 1 *M* NaH_2PO_4, 0 68 *M* $(NH_4)_2SO_4$ (pH 7 0)	40	Linear gradient	200×55	75	92	130
Monoclonal antibodies	TSK-gel phenyl-5PW	13	(a) 30 m*M* Tris–HCl, 1 *M* Na_2SO_4 (pH 7 5) (b) 30 m*M* Tris–HCl (pH 7 5) + 5% 2-isopropanol	1	Linear gradient	75×7 5	0 3–2 5	75	82
Ovalbumin α-Chymotrypsinogen A	Synchroprep propyl	30	(a) 0 1 *M* KH_2PO_4, 2 *M* $(NH_4)_2PO_4$ (pH 6 8) (b) 0 1 *M* KH_2PO_4	9	Linear gradient	300×25 4	1000 150	N A	132
Ovalbumin, RNase, lysozyme, α-chymotrypsin, α-chymotrypsinogen A	Synchroprep propyl	30	(a) 0 1 *M* KH_2PO_4, 2 *M* $(NH_4)_2PO_4$ (pH 6 8) (b) 0 1 *M* KH_2PO_4	1 5	Step gradient	250×10	4–13	N A	133
Lipoxidase, phosphoglucose isomerase, lactate dehydrogenase	TSK-gel phenyl-5PW	13	(a) 0 1 *M* NaH_2PO_4, 1 8 *M* $(NH_4)_2SO_4$ (pH 7 0) (b) 0 1 *M* phosphate (pH 7 0)	4	Linear gradient	150×21 5	200 100 54	86 70 93	128

α-Amylase	TSK-gel ether-5PW	13	(a) 0 1 M NaH_2PO_4, 0 66 M Na_2SO_4 (pH 7 0) (b) 0 1 M phosphate (pH 7 0)	40	Linear gradient	200×55	1500	90	130
β-Amylase, α-chymotrypsin, lysozyme	TSK-gel phenyl-5PW	10	(a) 0 1 M NaH_2PO_4, 1 5 M $(NH_4)_2PO_4$ (pH 7 0) (b) 0 1 M NaH_2PO_4 (pH 7 0) + 5% isopropanol	1	Linear gradient	75×7 5	1 3 0 4 0 2 0 05	80 92 90 90	132
Lipoxidase, ferredoxin, NADP reductase	TSK-gel phenyl-5PW	10	(a) 0 1 M NaH_2PO_4, 1 5 M $(NH_4)_2SO_4$ (pH 7 0) (b) 0 1 M NaH_2PO_4 (pH 7 0)	0 5	Linear gradient	75×7 5	1 0 3 0	89 100	131
Lipoxidase Phosphoglucose Isomerase	TSK-gel phenyl-5PW	15	(a) 1 5 M $(NH_4)_2SO_4$, 0 1 M NaH_2PO_4 (pH 7 0) (b) 0 1 M NaH_2PO_4 (pH 7 0)	40	Linear gradient	200×55	1000 500	84 99	134
Isoenzymes of lactate dehydrogenase	Spheron-Micro	12	(a) 0 1 M NaH_2PO_4 (pH 7 0) + 30% $(NH_4)_2SO_4$ saturated sol (b) 20 mM NaH_2PO_4 (pH 7 0)	5	Step gradient	120×12	200	38	94
Lactate dehydrogenase	TSK-gel phenyl-5PW	15	(a) 1 8 M $(NH_4)_2PO_4$, 0 1 M NaH_2PO_4 (pH 7 0) (b) 0 1 M Na phosphate (pH 7 0)	40	Linear gradient	200×55	200	82	134
Malate dehydrogenase	Spheron-Micro 300	12	(a) 0 1 M NaH_2PO_4 (pH 7 0) + 30% saturated $(NH_4)_2SO_4$ (b) 0 1 M NaH_2PO_4 (pH 7 0)	4	Linear gradient	12×120	270	70	135
Cytochrome C Lysozyme CHT and CHTG Ribonuclease STI	Ether bonded Vydac silica (300A) HIC support	15–20	(a) 3 0 M $(NH_4)_2SO_4$ + 0 5 M NH_4Ac (pH 6 0) (b) 0 5 M NH_4Ac (pH 6 0)	21	Linear gradient	150×21 2	60 60 60 144 667	N A	126

[a]CHT = α-chymotrypsin, CHTG = α-Chymotrypsinogen A, STI = soybean trypsin inhibitor

sidase and urease have been extracted and purified from crude preparations on a TSK-3000 SW column, the recovery of enzymatic activity was nearly quantitative and samples up to 100 mg could be injected [139] TSK-3000 SW columns were also used for the isolation of IgM and IgG [140], of human serum apolipoproteins [141] and of isoenzymes of lactate dehydrogenase [94] Applications of TSK-5000 SW and of other types of TSK size exclusion packing materials for the purification of human factor III C [142], of sea worm chlorocruorin and of other proteins [143] have also been reported The applications of SEC to protein separations include also albumin and IgG on GF-250 column [118], antibody conjugates on GF-250XL and GF-450XL [144], membrane-bound alkaline phosphatase on Protein-PAK 300 SW [90] and other proteins on Lichrosorb Diol [44] A combination of SEC with other chromatographic methods for the isolation of lactate dehydrogenase has been discussed by Skabrahova et al [94] The purification of proteolipid protein [145] and other proteins [146] by SEC has also been reported

Other examples of the applications of SEC to the extraction and separation of proteins are described briefly in Table 7

8 BIOSPECIFIC AFFINITY CHROMATOGRAPHY

Biospecific affinity chromatography (AFC) is a type of chromatography for which the retention mechanism is based on the formation of a specific complex Ideally, only one protein in the sample can make this complex with the stationary phase This compound is very strongly retained, almost indefinitely Other compounds, including proteins, are more or less retained by a combination of possible retention mechanisms, such as IEC, RPC and HIC Their retention is weak, at least in comparison, and they can be eluted, possibly with a solvent or an ionic strength gradient which has no effect on the stability of the specific complex Then, when the column is cleaned of all foreign material, the bonded protein is freed by washing the column with a solution of a reagent which dissociates the complex and the protein is eluted and recovered pure This mechanism has some obvious advantages but its implementation requires the satisfactory solution of several difficult problems [147]

AFC is a powerful tool for the separation and isolation of proteins, provided that a column has been designed which contains a high density of bonded specific complexation reagent [148–152] For this reason, it is unique among the various modes of liquid chromatography It is the most discriminating of all isolation procedures and, in principle, permits the selective extraction, the concentration and purification of a single protein, provided a specific antigene has been prepared previously For this reason it is the dream of many biochemists The high column loadabilities and recovery yields which can potentially be achieved, combined with the extreme selectivity of the mechanism, have

rendered AFC the most powerful and most promising purification method available [41,153–159]

On the other hand, some major disadvantages exclude AFC from wide applications in the extraction, separation and purification of proteins, especially at the preparative scale First, the extreme selectivity means that a different stationary phase must be used for almost every different separation. This requires the synthesis of a new stationary phase and the design and packing of a new column every time a new protein must be prepared Considerable inventory problems are rapidly generated Secondly, there is a serious lack of suitable support media for the immobilization of these ligands Packing materials which are commercially available are too expensive and are unstable under a variety of potentially useful experimental conditions This prevents them from being widely and routinely used in preparative and large-scale applications, where cost and the need for skilled chemists is a much more serious obstacle to the development of a new technology than in analytical applications.

Thirdly, the selectivity is not always as unique as expected Proteins having the same active or binding site but different side-chains are not separated Proteins are also strongly retained on AFC stationary phases by hydrophobic interactions and can be very difficult to extract without dissociation of the complex Finally, the kinetics of dissociation of the selective complex formed may be very slow In his study of AFC for the extraction of concanavalin, Muller and Carr [160a] have shown the dissociation reaction between the protein and the substrate to be much slower in the chromatographic column, where the substrate is bound to the stationary phase, than when the two compounds coexist in solution In some cases, the yield and the concentration of the prepared protein are considerably improved by filling the column with the reagent used for the dissociation of the complex and letting it stay overnight with no mobile phase flow than by washing the column under a constant stream

An empirical approach to the prediction of the biospecific affinity chromatographic behavior of various proteins in both traditional and high-performance liquid chromatography has been discussed Good agreement between the experimental data and the theoretical predictions has been reported [154] Sada et al [160b] have studied the effect of the pH, the ionic strength, the nature and charge of the anion species and the antibody concentration on the adsorption equilibrium in immuno-affinity chromatography with monoclonal and polyclonal antibodies It has been found that the characteristics of the binding affinity is heterogeneity for polyclonal antibodies and homogeneity for monoclonal antibodies non-linear and linear Scatchard plots have been obtained for the former and the latter, respectively A review of all the aspects of AFC has been published [157], and also papers describing the purification of rabbit muscle lactate dehydrogenase on a Procion Blue MX-R affinity column [161], the isolation of membrane flavoproteins on a 5′-ADP-agarose HR 10/

TABLE 7

SIZE EXCLUSION CHROMATOGRAPHY EXAMPLES OF PREPARATIVE APPLICATIONS TO THE PURIFICATION OF PROTEINS AND PEPTIDES

N A = not available

Compound	Stationary phase	Particle size (μm)	Mobile phase	Flow-rate (ml/min)	Column size (mm)	Sample size (mg)	Recovery yield (%)	Ref
IgG IgM	SK-3000 SW	N A	0 1 *M* NaH_2PO_4, + 0 2 *M* NaCl (pH 7 0)	2	600×21 5	N A	> 75	140
Albumin IgG	GF-250	N A	0 2 *M* Phosphate buffer (pH 7 5)	5	250×21 2	5 4 0 6	50	118
Human serum apolipoprotein	Micro Pak TSK 3000SW	N A	50 m*M* Tris-HCl buffer (pH 7 0) and 6 *M* urea or 6 *M* guanidium chloride	8	300×22	10-50	> 80	135
Human factor VIII C	HPSEC TSK 5000 PW	18	50 m*M* imidazole (pH 7 0) + 0 15 *M* NaCl	8 5	600×25	4 g/10 ml	80	142
Antibody conjugate	GF-250XL GF-450XL	N A	0 2 *M* Phosphate buffer (pH 7 0)	3-6	250×21 2	1-3 ml	N A	144
Albumin, chymotrypsin A, lysozyme	LiChrosorb Diol	5	8 m*M* K_2HPO_4, 42 m*M* Na_2HPO_4, 0 1 *M* NaCl (pH 7 5)	21	250×23 5	75	N A	44
Membrane-bound alkaline phosphate	Protein-Pak 300SW	N A	0 25 m*M* Tris-HCl, 0 2 *M* NaCl, 0 1% Brij-35 (pH 7 5)	0 5	250×7 5	0 36	70	90
Isoenzymes of lactate dehydrogenase H4 and MH3	Ultropac TSK 3000 SWG	N A	20 m*M* Phosphate buffer (pH 7 0)	6	600×21 5	50	29	94
β-Galactosidase Urease	TSK-gel G3000 SW	N A	0 2 *M* Phosphate buffer (pH 6 7)	5	600×21 5	75 90	93 100	138
Sea worm chlorocruorin and other proteins	TSK-gel G5000 PW	18	10 m*M* KH_2PO_4-100 m*M* KCl buffer (pH 7 0)	0 96	600×21 5	0 75	N A	143

TABLE 8

AFFINITY CHROMATOGRAPHY EXAMPLES OF PREPARATIVE APPLICATIONS TO THE PURIFICATION OF PROTEINS AND PEPTIDES

N A = not available

Compound	Stationary phase	Particle size (μm)	Mobile phase[a]	Flow-rate (ml/min)	Elution mode	Column size (mm)	Sample size (mg/g)	Recovery yield (%)	Ref
Rabbit muscle lactate dehydrogenase	Procion Blue MX-R chemically bonded silica	40-63	2 mM NADH in 10 mM HEPES (pH 7 0)	10	Isocratic	300×25	0 33	80	161
Pig heart nucleoside diphosphate kinase	Cibacron Blue 350 3G-A immobilized on beaded, cross-linked agar	N A	(a) 50 mM Tris-HCl (pH 8 0) + 2 mM EDTA (b) 0 25 M NaCl (c) 1 mM ATP	3	Isocratic	100×15	N A	N A	167
Membrane flavoprotein	5'-ADP-agarose HR 10/10	N A	(a) 20 mM Tris-Ac buffer (pH 7 4) + 1 mM EDTA (b) 2 mM ADP in (a)	N A	Isocratic	100×10	N A	42	91

[a]HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

TABLE 9

HYDROXYAPATITE COLUMNS EXAMPLES OF APPLICATIONS TO THE PURIFICATION OF PROTEINS AND PEPTIDES

N A = not available

Compound	Stationary phase	Particle size (μm)	Mobile phase[a]	Flow-rate (ml/min)	Elution mode	Column size (mm)	Sample size (mg)	Recovery yield (%)	Ref
Macrophage colony-stimulating factor (M-CSF)	HCAA-7610	N A	(a) 0 1% SDS, 0 05 *M* NaH_2PO_4 (b) 0 1% SDS + 0 5 *M* NaH_2PO_4	N A	Linear gradient	100×7 6	4 5	ca 100	171
Monoclonal antibodies	Bio-gel HPHT	N A	(a) 0 01 *M* NaH_2PO_4, 0 03 m*M* $CaCl_2$ (pH 6 8) (b) 0 35 *M* NaH_2PO_4 + 0 01 m*M* $CaCl_2$ (pH 6 8)	0 5	Linear gradient	100×7 8	4–5	N A	82

[a] SDS = sodium dodecyl sulphate

10 column [91], the separation of plant hormones [162], of human serum proteins [163] and of other proteins [164–166]

A few selected examples of the use of AFC for preparative applications are described in Table 8

9 OTHER CHROMATOGRAPHIC METHODS

Besides the techniques discussed above, which are all classical and rather widely used, a few other chromatographic methods or methods related to chromatography have been used for the purification of peptides and proteins [168–172] We cite here HPLC with hydroxyapatite as a stationary phase, which is a type of adsorption chromatography, chromatofocusing, which is a mode of using an IEC column, and counter-current chromatography (CCC), which is an implementation of liquid–liquid chromatography using no inert support material for the stationary liquid

HPLC columns packed with hydroxyapatite particles have been employed for the isolation of antibodies, with a good recovery yield [82,173] The selectivity of this adsorbent is different from that of all other modes of chromatography A much shorter life time than for other columns, however, is a big problem and limits the applicability of these columns Nau [174] reported a unique packing material of this type, used effectively for the rapid purification of antibodies Other data are reported in Table 9

CCC was applied to isolation of synthetic peptides and plant hormone, as reported by Ito and co-workers [175,176] Recently a series of papers have been published by Knight et al [177] on the application of the method to the extraction and purification of peptides The use of multi-layer coil planet centrifuge and compact horizontal flow-through coil planet centrifuge for the purification of [N-Ac, Ala < 2 < 9]cholecystokinin 26–23 and of bovin insulin was discussed With this approach up to 1 g polypeptides can be purified in single run Putterman et al [178] have used both CCC and HPLC to separate synthetic nona- and hexapeptides The former gives a preliminary purification of the crude samples while the latter completes the final isolation of pure compounds without any risk of damaging the column A 100-mg sample amount has been treated in one run One of the major advantages of the method is the virtually total absence of solid surface on which the protein molecules could be adsorbed and isomerized The main drawback is the rather low column efficiency achieved in CCC and the difficulties encountered in attempts made at scaling-up the process, due to the slowness of the radial mass transfer by diffusion As a consequence, the length of a transfer unit increases rapidly with increasing column diameter

Chromatofocusing is another mode of elution chromatography used for preparative separation of proteins on ion exchangers, according to their different isoelectric points [179] A stream of mobile phase with a pH gradient is

flown into the column after the sample has been injected The nature of the stationary phase and the direction of the gradient are arranged so that each protein moves at its isoelectric point, i e , the molecules of each protein concentrate in the column at the point where their isoelectric point is equal to the pH and move with this point in the gradient The zones are concentrated and do not broaden as they do in classical chromatography The molecules which tend to migrate faster than the mass center of the band enter a pH range where they are ionized with a charge such that they are now retained by the column Hence they are slowed down and they stay with the pack Conversely, the molecules which tend to move more slowly than the mass center of the band, i e , the isoelectric point of the protein, get the opposite charge and tend to migrate faster This explains why the process is focusing

10 CONCLUSION

The applications of the various modes of preparative HPLC to the extraction, isolation and purification of peptides, enzymes, hormones and proteins is generating great interest within the scientific and industrial communities The strict purity requirements imposed on the biotechnology industry have made preparative HPLC an essential separation process, almost always involved somewhere in the long production processes of proteins Various techniques of preparative HPLC have already been employed successfully for the achievement of high-purity peptides and proteins

It should be mentioned, however, that the classical methods of liquid chromatography still play an important role in this area [180], especially at the beginning of the protein separation process, due to their ability to treat large amounts of crude materials Besides, the requirements of the purchase of huge amounts of expensive packing materials for the packing of preparative columns and of the use of a sophisticated technique for column operation and maintenance still exclude preparative HPLC from major utilizations in industrial purifications

At present, preparative HPLC and classical column chromatography supplement each other, and none of them can completely replace the other Nevertheless, the reading of the many successful studies being presently published on the development of new packing materials suggests that it is only a matter of time until new high-efficiency and low-cost packing materials for preparative HPLC be commercially available and preparative HPLC becomes applied routinely to the industrial isolations and purifications of many peptides, proteins and other biopolymers This will contribute greatly to the development of new and better drugs, as well as to a deeper understanding of the mechanisms through which cells and organs operate

11 SUMMARY

The basic differences between the problems encountered in the development of the analytical and preparative applications of high-performance liquid chromatography are analyzed and the principles of optimization of a preparative separation outlined The application of these principles to the purification of peptides and proteins are discussed with emphasis on the specific problems raised by the separation of high-molecular-mass compounds The relative advantages and inconveniences of displacement and overloaded elution are presented The applications of the various retention modes of liquid chromatography (ion exchange, reversed-phase adsorption, hydrophobic interactions, size exclusion and bioaffinity) to the separation of various peptide and protein mixtures and/or to the extraction and purification of some of these compounds are reviewed Separation performance reported in the literature are summarized in table form

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