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Use of Reversed-Phase Liquid Chromatography in Biopharmaceutical Development

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INTRODUCTION

Reversed-phase liquid chromatography (RPLC) is an essential tool for analytical and preparative separations of biopharmaceuticals. Its ubiquitous application in the discovery and development of protein-based drugs arises from its high resolving power, the robustness of the separation technique, and its success in handling a wide range of separation problems. The favorable kinetics provided by hydrocarbonaceous ligands bonded to microparticulate packings typically generate column efficiencies in excess of 25,000 plates/m. Silica- and polymer-based supports are stable over a wide range of solvent chemistries and operating pressures, and the hydrophobic stationary phases are quite robust compared to other chromatographic ligands. Reversed-phase columns are available in a wide range of dimensions for applications ranging from nanoscale analytical separations to process-scale chromatography, allowing RPLC methods to be scaled during product development. The technique usually employs volatile solvents, enabling easy solvent removal in preparative applications and allowing direct coupling to mass spectrometric detectors for analytical LC-MS. Most important, a vast array of mobile-phase chemistries and commercially available RPLC columns can be brought to bear on a separation problem. However, in practice, the success of a few generic methods for proteins and peptides usually simplifies the method-development task.

Together, these characteristics of RPLC make it the preferred analytical technique for assessing protein purity and for elucidating protein structure. Reversed-phase high-performance liquid chromatography (HPLC) can also be used alone or in concert with other chromatographic techniques for purification of proteins. For the separation of synthetic polypeptides or peptides derived by proteolysis of proteins, RPLC has no equal. Reversed-phase peptide mapping is the technique of choice for confirming protein structure and identity, for determining the sites and nature of posttranslational modifications, and for characterizing protein modifications and degradation products. More recently, it has played a central role in proteomic studies that are anticipated to generate targets and leads in the drug discovery environment. Here, reversed-phase chromatography is used as a complement to or replacement for two-dimensional (2-D) gel electrophoresis as the front-end separation for LC-MS-MS identification of proteins.

A fundamental limitation of RPLC is the denaturing properties of the hydrophobic stationary phases and eluting solvents. In preparative applications, this can limit the recovery of bioactive species. In analytical applications, reversed-phase separation conditions can perturb the conformational state of the protein, causing analytes to appear as broad or asymmetric peaks, and in extreme cases to be resolved as individual conformers. In such cases, the analyst must find conditions to minimize this behavior or resort to another separation technique. For preparative work, hydrophobic interaction chromatography (HIC) may be preferred over reversed-phase chromatography. The salt gradients used for HIC elution favor maintenance of native conformations and biological activity. Because HIC is based on hydrophobic interactions, it can provide a selectivity similar to RPLC without the strongly denaturing conditions.

THE MECHANISM OF REVERSED-PHASE CHROMATOGRAPHY

Reversed-phase chromatography employs a nonpolar stationary phase and a polar aqueous–organic mobile phase. The stationary phase may be a nonpolar ligand, such as an alkyl hydrocarbon, bonded to a support matrix such as microparticulate silica, or it may be a microparticulate polymeric resin such as cross-linked polystyrene-divinylbenzene. The mobile phase is typically a binary mixture of a weak solvent, such as water or an aqueous buffer, and a strong solvent such as acetonitrile or a short-chain alcohol. Retention is modulated by changing the relative proportion of the weak and strong solvents. Additives may be incorporated into the mobile phase to modulate chromatographic selectivity, to suppress undesirable interactions of the analyte with the matrix, or to promote analyte solubility or stability.

The mechanism of reversed-phase chromatography arises from the tendency of water molecules in the aqueous–organic mobile phase to self-associate by hydrogen bonding. This ordering is perturbed by the presence of nonpolar solute molecules. As a result, solute molecules tend to be excluded from the mobile phase and are bound by the hydrophobic stationary phase. This solvophobic

exclusion provides the thermodynamic driving force for retention in RPLC. In contrast to other modes of chromatography, in which retention relies upon affinity of the solute for the stationary phase, it is the increased entropy of water in the mobile phase accompanying the transfer of a solute molecule from the mobile phase to the nonpolar stationary phase that promotes retention. Thus, retention is favored by the hydrophobic contact area of the solute and reduced by dipolar or hydrogen-bonding interaction of the solute with the mobile phase. Solvophobic theory[1] predicts and experimental observations confirm that retention (as expressed by the capacity factor, k) decreases with a decrease in surface tension and that a linear relationship exists between the logarithm of k and the volume percent of organic modifier in the mobile phase. Protein retention is thought to occur by adsorption to the hydrophobic stationary phase according to the solvophobic effect or to a sorbed layer of the nonpolar solvent component extracted from the bulk mobile phase.

An understanding of the phenomenology of protein retention in reversed-phase chromatography requires consideration of the forces involved in defining the three-dimensional (3-D) conformation of the protein. Small polypeptides (comprising a dozen amino acid residues or so) exist in solution as random coils, and their chromatographic retention in reversed-phase systems can be predicted from the summed hydrophobic properties of the individual amino acids. These can be derived from hydrophobic indices determined by water: octanol partition measurements[2] or chromatographic retention coefficients obtained from model peptides.[3–5] An example of the latter approach is represented in the retention studies of Guo et al.[6,7] These workers synthesized a family of model octapeptides with each of the 20 protein amino acids represented in tandem within the peptide sequence. Retention coefficients for each amino acid were obtained from the relative retention of each model peptide in a defined reversed-phase chromatographic system. The experimentally derived retention coefficients were used to predict the retention of 58 peptides ranging from 2 to 16 residues in length. The observed retention times in this study correlated well with the predicted values (Figure 4.1).

However, in a later study employing polymeric peptides of 5 to 50 residues assembled from block sequences, significant deviations of observed retention values from the predicted values were found for polypeptides larger than about 10 residues. These deviations for larger polypeptides were correctly interpreted as conformational effects.

Peptides larger than 10 to 20 residues adopt conformations in solution through the interplay of hydrogen bonding, electrostatic and hydrophobic interactions, positioning of polar residues on the solvated surface of the polypeptide, and sequestering of hydrophobic residues in the nonpolar interior. Protein shape is dynamic, changing continuously in response to the solvent environment. The retention process in RPLC is initiated as the protein approaches the stationary-phase surface. Structured water associated at the phase surface and adjacent to hydrophobic contact surfaces on the polypeptide is released into the bulk mobile

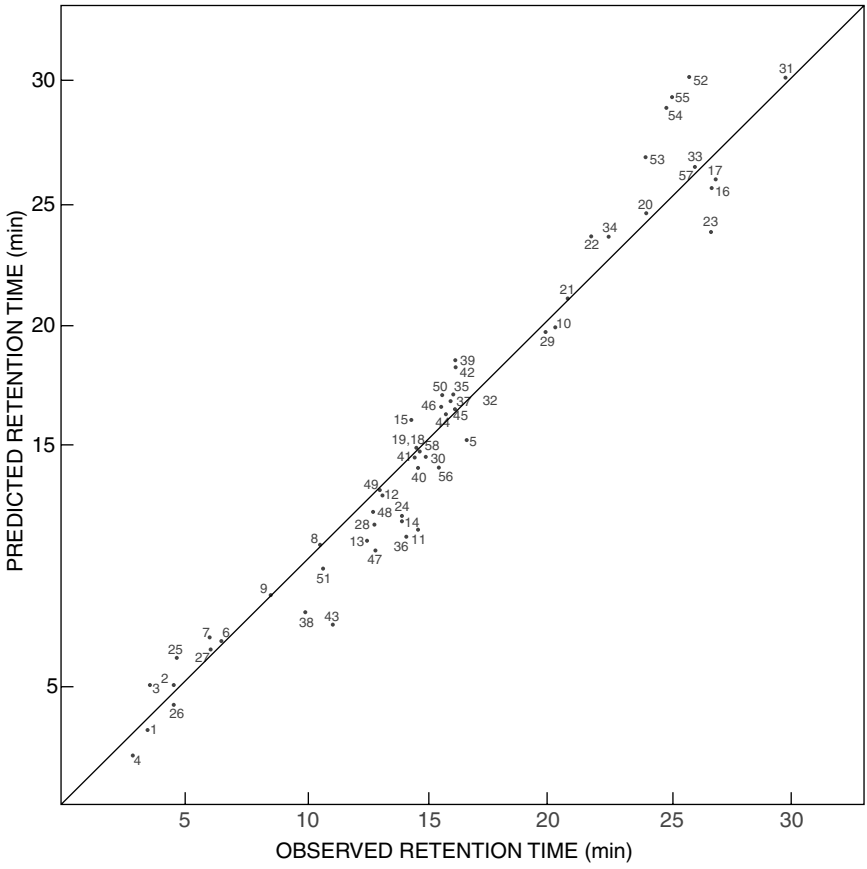

Figure 4.1 Correlation of predicted and observed retention times in reversed-phase chromatography. The predicted retention times for 58 peptides of 2 to 16 residues in length were obtained by summation of retention coefficients for each residue in the peptide. Retention coefficients were determined from the retention of model synthetic peptides with the structure Ac-Gly-XX-$(Leu)_3$-$(Lys)_2$-amide, where X was substituted by the 20 protein amino acids. (Reproduced from D. Guo, C.T. Mant, A.K. Taneja, and R.S. Hodges, *J. Chromatogr., 359:* 519 [1986]. With permission from Elsevier Science.)

phase, and this process of solvent exclusion is the driving force for protein binding to the hydrocarbonaceous phase. A protein in equilibrium between multiple conformational states may present different hydrophobic contact areas for binding (Figure 4.2). If the interconversion rate is fast (e.g., in the millisecond domain), the observed peak represents the average of the rapidly interconverting species. If the interconversion rate is slow (seconds to minutes), varying contact areas of the different conformational states may be manifested by broadened peaks or chromatographic resolution of discrete peaks.

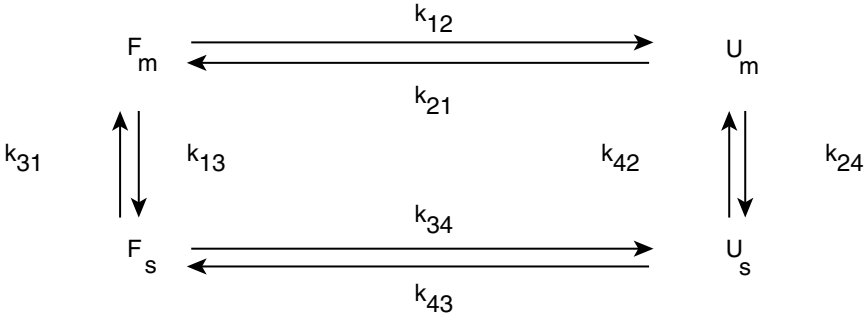

Figure 4.2 Protein transformations in reversed-phase chromatography for a two-state model. The native folded state can exist in either the mobile phase (F_m) or the stationary phase (F_s), as can the unfolded state (U_m, U_s). The equilibrium constants (k) for interconversions of the four species are indicated. (Reproduced from X.M. Lu, K. Benedek, and B.L. Karger, *J. Chromatogr., 359:* 19 [1986]. With permission from Elsevier Science.)

The molecular forces that are involved in maintaining the folded state of a protein are substantially the same as those involved in retention and elution. When the magnitude of the interactions involved in protein binding exceeds that of folding, the chromatographic process can induce conformation changes that expose hydrophobic groups and create new contact areas. As a result, the fully folded protein may undergo transitions to partially or fully denatured conformations. A globular protein may display excessive retention, conversion to chromatographically resolved folding intermediates, and loss of biological activity. For this reason, reversed-phase conditions are often considered sufficiently harsh to disallow the technique from being used as a preparative tool. In point of fact, RPLC has often proven successful for recovery of functional proteins. Small to medium mass proteins may be refractory to the hydrophobic environment, or can easily refold when returned to benign environments. Retention of biological activity will be favored by chromatographic conditions that minimize protein unfolding, e.g., more polar organic modifiers, less hydrophobic stationary-phase ligands, higher mobile-phase pH, reduced temperature, lower-capacity columns, and short column-residency times.

The characteristics that discourage the use of RPLC for preparative isolation of bioactive proteins favor its use as an analytical tool for studying protein conformation. Chromatographic profiles can provide information on conformational stability of a protein and the kinetics of folding and unfolding processes. Information about solvent exposure of certain amino acid residues (e.g., tryptophan) as a function of the folding state can be obtained by on-line spectral analysis using diode array UV-vis detection or fluorescence detection.

A common feature of protein retention in reversed-phase and other interactive chromatographic modes such as ion exchange and hydrophobic interaction

is the participation of multiple sites in the binding process. In the reversed phase, which is more likely to induce conformation changes and create new contact surfaces, cooperativity in binding may play a role in retention. The consequence of multisite binding is a profound dependence of elution on solvent strength. From a practical standpoint, gradient elution is almost universally required for separation of peptides and proteins.

A final consideration in applying chromatographic techniques to the separation of proteins is their inherently low molecular diffusion rates. This limits their rate of mass transfer in the mobile and stationary phase, and most particularly in the stagnant mobile phase within the pore systems of microparticulate packings. When conventional HPLC packings are used for protein separations, significant peak broadening will be a consequence of unfavorable mass-transfer rates. Strategies for minimizing this problem will be discussed in the context of column selection and operation.

REVERSED-PHASE SEPARATION CONDITIONS

Support Matrix Composition

The ideal chromatographic support matrix should be mechanically stable under several hundred atmospheres of pressure, chemically stable in the presence of typical reversed-phase solvents, should possess a high surface area for good chromatographic capacity, be available in a range of particle sizes for analytical and preparative applications, be able to serve as an anchor for attaching chromatographic ligands, and should possess an inert surface with no potential for interactions with peptides. Silica has all of these qualities save two, and therefore it is the most widely used matrix for reversed-phase packings. Porous microparticulate silicas have large surface areas within their pore systems and can be manufactured in particle diameters of 2 to 10 μm for analytical applications and 15 to 30 μm for preparative work. The surface silanols on silica can serve as sites for covalent attachment of ligands.

A major limitation of silica as a matrix for chromatography of peptides and proteins is the potential for interaction of basic amino acid side chains with residual silanol groups on the silica surface. These can participate in secondary retention mechanisms through hydrogen bonding or ion exchange. The presence of highly active silanol sites can cause peak tailing. If the characteristics of the underlying silica change from one batch of column packing to another, columns may exhibit variations in peak symmetry and selectivity. It is advisable to use columns with high surface coverage of the reversed-phase ligand and columns that have been prepared with high-quality silica. The presence of metal ion contaminants in the silica can increase the activity of surface silanols, promoting their ability to participate in secondary interactions. Sol-gel techniques for preparing silicas reduce the level of metal contamination, and these high-purity

silicas (sometimes termed "third-generation" or type B silicas) are the preferred matrix for columns to be used for polypeptide separations. Type B silicas also exhibit lower levels of isolated silanols (which are more likely to participate in unwanted interactions than vicinal or geminal silanols), producing a more inert surface.

A second limitation of silica is its solubility under alkaline conditions (pH >7). This problem is exacerbated under conditions of elevated temperature. This will be a concern if high-pH mobile phases are required to achieve the desired separation, or if exposure to alkaline conditions is required to clean the column after preparative applications. In the former situation, hybrid silicas are available that have extended lifetimes under conditions of elevated pH and temperature. Hybrid silicas are composed of a mixture of inorganic silica and alkyl silica. A caution in their use is the potential for selectivity changes relative to packings prepared with conventional silica. For preparative and process-scale applications in which alkaline cleaning regimes are anticipated, the use of a polymeric matrix will be preferred. Polymeric resins composed of polystyrene-divinylbenzene, polymethacrylate, or polyvinylalcohol are chemically stable at pH extremes (pH <2 and >10) and at elevated temperatures. Porous microparticulate resins of all three types are commercially available. The disadvantage of polymeric resins is their lower efficiency compared to silica-based packings.

Particle Size and Shape

Band spreading, a necessary evil in the chromatographic process, arises from the presence of multiple flow paths between particles and from resistance to analyte mass transfer in the flowing mobile phase and in the stagnant mobile phase within the particle pore system. These effects can be reduced by using particles that are spherical in shape, have a narrow distribution of particle sizes, and have reduced particle diameters. Spherical particles can be packed to form a homogeneous bed with reduced band broadening and higher porosity (and hence lower operating pressure). The advantage of small particle diameters for achieving high efficiency and resolution has long been appreciated, and HPLC packings have evolved from 10 μm in the 1970s to the 5-μm particles that are the workhorse materials in current usage. Particles of smaller diameters (e.g., 2.5 to 4 μm) can be used to obtain greater efficiency, but at the cost of increased operating pressure (note that the column pressure varies as the inverse square of the particle diameter). A useful characteristic of small particles is their reduced degradation of performance with increasing mobile-phase flow velocities. This is apparent in their shallower slopes in plots of plate height vs. flow (Figure 4.3). Because of this characteristic, short columns packed with sub-5-μm particles can be operated at high flow rates to achieve satisfactory resolution and still be within the pressure limits of the system. This strategy is being used in discovery environments in which heavy sample loads require high-throughput analytical techniques.

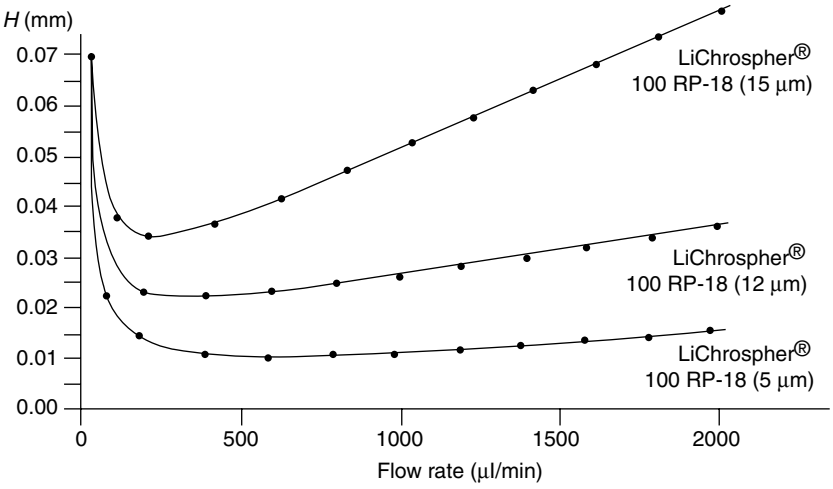

Figure 4.3 Effect of particle diameter on plate height. (Reproduced from Lichrospher & Lichroprep Sorbents Tailored for Cost Effective Chromatography, EM Separations, Gibbstown. With permission from Merck kGaA, Darmstadt, Germany, and EMD Chemicals, Inc.)

Table 4.1 Relationship Between Molecular Weight (M) and Stokes Radius (r)

Protein	M	r(Å)
Serum albumin (globular, solid spheres)	66,000	29.8
Catalase (globular)	225,000	39.8
Myosin (rodlike)	403,000	486
DNA (rodlike)	4×10^6	1170
Bushy stunt virus (globular)	10.6×10^6	120
Tobacco mosaic virus (rigid rod)	39×10^6	924

Pore Diameter

Microparticulate HPLC packings manufactured for chromatography of small molecules have pore diameters of 5 to 10 nm and surface areas greater than 200 m^2/g. These materials may be satisfactory for chromatography of peptides, but globular proteins above 10 kDa will have difficulty in accessing the internal surface of the pore system. Hence, the effective capacity of the packing will be limited to the external surface of the packing, amounting to a few m^2/g. Ideally, the pore should be at least threefold larger than the hydrodynamic diameter or Stoke's radius of the protein to allow permeation into the pore system (Table 4.1). A variety

Table 4.2 Relationship Between Pore Diameter and Surface Area

Pore Diameter (nm)	Surface Area (m^2/g)
10	250
30	100
100	20
400	5–10

of large-pore packings are commercially available for chromatography of proteins with pore diameters from 25 to 400 nm. Of these, particles with 30-nm pores have become the most popular and permit permeation of globular proteins up to 1000 kDa and random coil molecules up to 100 kDa. For larger proteins, macroporous packings may be preferred. However, increasing the particle porosity will compromise surface area (Table 4.2) and the mechanical stability of the particle. Operation of macroporous packings at elevated flow rates or pressures may not be recommended.

Alternatives to Porous Microparticulate Silicas

Restricted diffusion of a protein within a pore system can have two negative consequences for chromatography. The first is a greater dependence of efficiency on flow velocity such that resolution is compromised at high flow rates. This is particularly a limitation in preparative applications where high flow rates are desirable to achieve adequate throughput. This limitation of porous supports is shared by all modes of protein chromatography. A second problem encountered with porous materials is more characteristic of reversed-phase chromatography. Because reversed-phase conditions tend to be denaturing, there is the possibility that a folded species with facile permeation into the pore system can unfold to a partial or fully random coil configuration that will exhibit restricted diffusion within the pore. This can cause band broadening or, in worst case, can result in entrapment of the unfolded protein within the pore. This occurrence may account for the phenomenon of "ghosting" in reversed-phase gradient elution, that is, the appearance of peaks in a blank gradient following an analytical injection.

Three strategies have been employed to circumvent the problems of porous particles in protein chromatography. One strategy is to expand the pore diameter sufficiently to eliminate restricted diffusion. Of course, the consequences of this strategy are loss of mechanical stability and reduction in interactive surface area. A variation on this strategy that attempts to regain capacity through a bimodal pore system is represented by perfusion chromatography.[10] A perfusion particle contains a primary pore system of large "throughpores" that are wide enough to allow protein transport with little restriction. A secondary system of "diffusive" pores provides added surface area for capacity, but these are sufficiently shallow to minimize stagnant mobile-phase effects.

The simplest way to eliminate the problems of porous particles is to eliminate the pores. Nonporous particles of 10 μm diameter with a thin layer of stationary phase on their surfaces were in fact introduced early in the evolution of HPLC packings as a solution to the pore problem, but achieved little popularity because of their low capacity. Recently, nonporous materials have returned to the marketplace in the form of very small particles with diameters of 1.5 to 2.5 μm. The use of small particles compensates to some extent for the loss in capacity.[11] However, because of the high flow resistance of microparticulate nonporous packings, they are generally packed in short lengths and often operated at elevated temperatures.

The third strategy is the replacement of the microparticulate-packed bed with a monolith. Monolith columns contain a continuous interconnected skeleton with throughpores for transport of mobile phase and analytes. The large throughpores provide high permeability, thereby lowering the operating pressure. A secondary pore structure of shallow diffusive mesopores provides additional surface area for chromatographic capacity. Both polymer-based and silica monoliths are commercially available. Silica monolith rods[12] contain a skeleton with mesopores of approximately 13 nm diameter and macropores of about 2 μm. To minimize wall effects, the rod is "shrink wrapped" in PEEK. The mesopores provide a high surface area of 300 m^2/g. The silica monolith matrix has a porosity of 80%, which is higher than the typical 65% porosity of microparticulate-packed columns. This allows monoliths to be operated at high flow rates with modest pressures, with little loss of performance in terms of band broadening. Polymethacrylate and polystyrene-divinylbenzene monoliths are commercially available in both column and disk formats for analytical and preparative chromatography. These materials also contain a bimodal pore structure of macro- and mesopores.

Stationary-Phase Ligand

Silica-based reversed phases are prepared by reacting the silica matrix with a silane reagent carrying the desired hydrophobic ligand. Monomeric phases are synthesized using a monofunctional silane, LR_2SiX, where L is the chromatographic ligand, R is a protective group (e.g., methyl), and X is the leaving group (e.g., chlorine or alkoxy group). The resulting siloxane bond (Si-O-Si) is chemically stable above pH 2. The product of this reaction is a monolayer that exhibits favorable kinetics in the chromatographic binding of polypeptides and so displays high efficiency. An advantage of this attachment chemistry is its convenience and reproducibility. Its limitations are the presence of residual silanols following the bonding process and the susceptibility of the phase to hydrolysis. In the bonding process, the accumulation of hydrophobic ligands blocks access to all of the active silanols by steric hindrance. These residual silanols are, however, accessible to small, polar analytes and are a major cause of tailing, particularly for basic analytes. The level of residual silanols can be reduced by performing a secondary

Table 4.3 Common Reversed-Phase Ligands

Trimethyl
Butyl (C4)
Octyl (C8)
Octadecyl (C12, ODS)
Phenyl
Diphenyl
Cyano

silanization with a silane reagent carrying small alkyl groups (e.g., trimethylchlorosilane) so that the reagent has easier access to the remaining silanol groups. This process is termed *endcapping*, and the use of endcapped columns for polypeptides is recommended to minimize the potential for undesirable interactions of basic amino acid side chains with the silica. Columns prepared using low-activity type B silica and endcapping are termed *base deactivated*, and these are marketed for chromatography of basic drugs. Base deactivated columns are suitable for polypeptide applications if the pore diameters are appropriate.

The stability of monomeric reversed phases can be increased by modifying the structure of the silane to minimize its susceptibility to hydrolytic attack. One approach is to replace the methyl protective groups with more bulky and hydrophobic groups such as isopropyl functions. These serve to restrict access of water to the silica surface, protecting the siloxane bond. Another approach is the use of bifunctional silane reagents that react with the silica to create a two-point attachment of the chromatographic ligand. This "bidentate" attachment of the ligand reduces the likelihood that it will be stripped from the surface by hydrolysis.

Another approach to preparing a stable reversed phase with fewer residual silanols is the use of polyfunctional silanes of the type R_2SiX_2. These react to form a polymeric stationary phase that shields the siloxane bonds and restricts access to residual silanols. Polymer phases have higher carbon loads and are typically more retentive than monomeric phases. However, they are more difficult to synthesize reproducibly and may exhibit batch-to-batch variability in their properties. They also exhibit poorer mass transfer kinetics and so provide poorer efficiency than monomeric phases.

The common reversed-phase ligands are listed in Table 4.3. The most popular are the straight-chain alkyl groups. These exhibit increased retention and stability with increasing chain length. Trimethyl phases are the least hydrophobic and might be considered for chromatography of proteins that would be strongly retained on the longer-chain ligands. However, these phases are easily hydrolyzed under the conditions usually employed for protein separations. Octyl (C_8) and octadecyl (C_{18}) are the most popular phases for chromatography of small molecules because of their retentiveness and stability. They are generally the first choice for proteins and peptides, although for protein applications, octyl phases are often selected in preference to octadecyl because they are less likely to cause

denaturation and excessive retention of hydrophobic species. However, chromatography of peptides and proteins is generally carried out using gradient elution with organic modifier concentrations rarely exceeding 60 to 80%. Under these conditions, the alkyl chains probably exist largely in a self-associated state such that the effective hydrophobicity of the different alkyl chains is similar. In fact, the selectivities of butyl, octyl, and octadecyl phases for proteins and peptides are comparable.[13] Phenyl and cyano phases are likely to exhibit different selectivities than the straight-chain alkyl phases and therefore are often selected when C_8 or C_{18} columns fail to provide satisfactory resolution. Cyano is the most polar of the common reversed-phase ligands (CN columns are also used for normal-phase chromatography.), and it is the phase of choice for very hydrophobic species, e.g., membrane proteins and the hydrophobic polypeptides generated by cyanogen bromide cleavage.

Column Dimensions

A key property of column liquid chromatography is its scalability. An analytical method that has been developed on a standard 4.6-mm I.D. column can be scaled up in diameter for preparative isolation or down in diameter for improved sensitivity, analysis of volume-limited samples, or coupling to a mass spectrometer. If the column packing and mobile-phase composition are unchanged, the separation should be the same. However, the flow rate must be scaled in proportion to the difference in column diameter to avoid pressure changes and to achieve the same flow velocity and chromatographic efficiency. Assuming that the sample solvent has approximately the same eluting strength as the mobile phase, the injection volume should also be scaled according to column diameter. If the sample is introduced in a solvent that is weaker than the mobile phase (typically the case because protein and peptide preparations are often in an aqueous buffer or salt solution), large sample injections will be focused on the head of the column. This provides better sample throughput when scaling up to preparative isolation and better sensitivity when scaling down to microbore or capillary columns. Assuming a nominal flow rate of 1 ml/min on a 4.6-mm × 150-mm analytical column, comparable flow rates and sample capacities for larger and smaller columns are listed in Table 4.4.

When scaling a chromatographic separation, some precautions should be noted. When scaling up for preparative work, it is advisable (if possible) to use a column packed with material that has the same chromatographic ligand bonded to the same silica. Changes in the ligand bonding density and the type of silica can affect the chromatography by changing the protein–ligand interactions or secondary retention effects contributed by residual silanols. These changes can be subtle or (in the case of silica variations) dramatic.

Transferring a method from a standard 4.6-mm I.D. column to one of narrower diameter can cause loss of resolution due to extracolumn contributions to peak broadening. As column diameter and flow rate are scaled down, the peak

Table 4.4 Relationship Between Column Diameter, Flow Rate and Sample Capacity

Column Diameter (mm)	Flow Rate (µl/min)	Sample Capacity (µg)
21.0	21000	1000
8.0	3000	150
4.6	1000	50
3.0	400	20
2.0	200	10
1.0	50	2
0.5	10	0.5
0.1	0.5	0.02

volume will decrease in proportion to the square of the column diameter. For example, scaling to columns of 2, 1, 0.3, and 0.1 mm I.D. will decrease peak volumes by factors of approximately 5, 20, 200, and 2000, respectively. If the HPLC system is not modified to minimize extracolumn volume, these contributions to the total peak broadening can become significant enough to degrade resolution. When scaling to 1- to 3-mm columns, transfer lines should be prepared with small-diameter tubing (e.g., 0.007 or 0.005 in.) and short lengths. Also, compression fittings should be assembled correctly with care not to mismatch components such as ferrules and nuts. Scaling to capillary columns of 0.1 to 0.5 mm I.D. will require additions to the HPLC such as nanoscale injectors, flow splitters, or even a change to a nanoflow solvent delivery system.

The choice of HPLC as a separation technique in biopharmaceutical development may depend upon its scalability, even if its resolving power is less than other techniques. For example, HPLC may be used in preference to gel electrophoresis or capillary electrophoresis, where scaleup is difficult or impossible.

Mobile-Phase Composition

Aqueous Component

In reversed-phase chromatography, the mobile phase consists of an aqueous component (the weak solvent) and an organic modifier (the strong solvent), and analyte retention is regulated by the ratio of the two components. The hydrophobicity of a polypeptide is largely dependent on the ionization state of the amino acid termini and of the ionizable side chains of internal residues. Thus, the pH of the mobile phase can have profound effects on polypeptide retention, and it is necessary to control mobile-phase pH to obtain the desired selectivity and achieve reproducible separations. In practice, separations of proteins and peptides are most often performed under acidic pH conditions employing dilute organic acids as additives. Under these conditions, the ionization of residual silanols on the stationary phase is suppressed, reducing unfavorable interactions with basic

amino acid side chains that would cause tailing. Also, the organic acid additive can form ion pairs with the protonated basic side chains. The ion-paired groups will be more hydrophobic than their unpaired counterparts, favoring polypeptide retention. The ionization of terminal and side chain carboxyl groups is suppressed, which also favors retention. The most widely used organic acid in RPLC of peptides and proteins is trifluoroacetic acid (TFA), added to a concentration of 0.05 to 0.1% (approximately 5 to 10 m*M*). The stronger acidity of TFA enables the beneficial effects of low-pH operation to be achieved at much lower concentrations than required with unfluorinated organic acids. Use of lower additive concentrations reduces mobile-phase contributions to the background absorbance when using UV detection at low wavelengths. An added benefit of TFA is its high volatility, which facilitates solvent removal from collected sample fractions in preparative applications.

The use of TFA as a mobile-phase additive in LC-MS can be problematical when using electrospray ionization. In negative ion detection, the high concentration of TFA anion can suppress analyte ionization. In positive ion detection, TFA forms such strong ion pairs with peptides that ejection of peptide pseudomolecular ions into the gas phase is suppressed. This problem can be alleviated by postcolumn addition of a weaker, less volatile acid such as propionic acid.[14] This "TFA fix" allows TFA to be used with electrospray sources interfaced with quadrupole MS systems. A more convenient solution to the TFA problem in LC-MS is to simply replace TFA with acetic or formic acid. Several reversed-phase columns are commercially available that have sufficient phase coverage and reduced levels of active silanols such that they provide satisfactory peptide peak shapes using the weaker organic acid additives.[15]

A caution in the use of TFA is the effect of its ion-pairing activity on selectivity. This is clearly illustrated in a study performed by Guo et al.[16] using a series of synthetic peptides containing 0, 1, 2, 4, or 6 basic residues (Figure 4.4). As the TFA concentration was increased, the retention of basic peptides increased and elution order changed, with the more highly charged peptides showing the strongest effect. Analysts should be aware that changes in TFA concentration during routine operation can cause changes in selectivity. This could be a concern when using HPLC systems equipped with on-line degassing units.

When chromatographic resolution of species based on modifications located at the protein surface is desired, it may be advisable to use conditions that favor retention of native conformation.[17] Here, the standard acidic conditions described in the preceding text may be inappropriate, and mobile phases buffered near neutrality may be required. Buffers based on ammonium acetate, ammonium bicarbonate, and triethylammonium phosphate may prove more useful in resolving polypeptide variants with differing posttranslational modifications, amino acid substitutions, or oxidation and deamidation products. The addition of more hydrophobic ion-pairing agents may be needed to obtain polypeptide retention, and a variety of alkyl sulfonates and alkyl amines have been described for specific applications.[17]

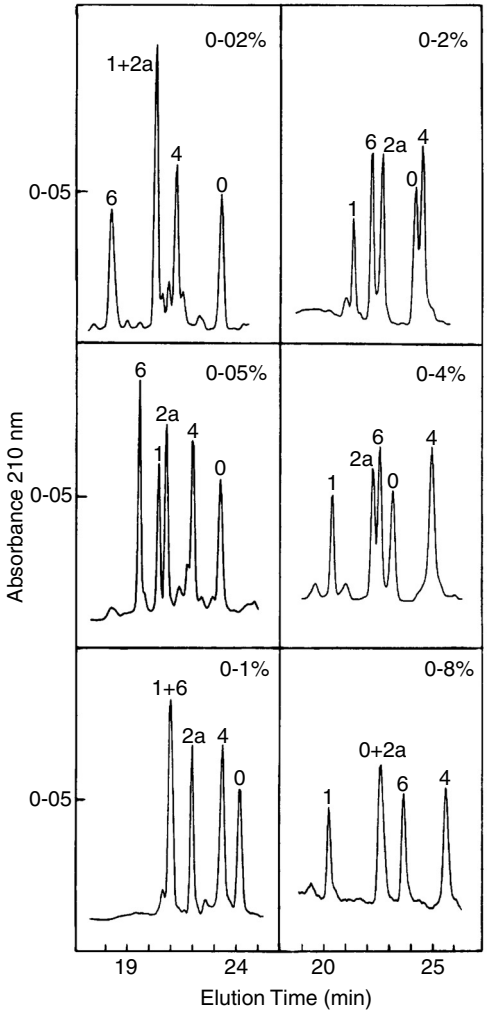

Figure 4.4 Effect of TFA concentration on peptide retention. A series of five synthetic peptides containing 0, 1, 2, 4, or 6 basic residues were separated on an octadecyl reversed-phase column using a 1%/min linear gradient from water to acetonitrile. Both solvents contained TFA at the indicated concentrations. (Reproduced from D. Guo, C.T. Mant, and R.S. Hodges, *J. Chromatogr., 386:* 205 [1987]. With permission from Elsevier Science.)

Triethylamine (TEA) is a common additive used in RPLC, which can have two beneficial effects. It can serve as an ion-pairing agent to promote retention of anionic species, and it can suppress the interaction of basic solutes with residual silanols. In fact, it is most frequently used at concentrations of 5 to 25 m*M* to

improve peaks shapes of basic drugs and metabolites. A solvent system based on triethylamine-phosphate (TEAP) and acetonitrile was one of the first mobile phases to be used successfully for RPLC of peptides.[18]

Organic Component

The criteria for selecting the organic component of the mobile phase include solvent purity and toxicity, UV absorbance, eluting strength, effects on band spacing, viscosity, and the denaturing properties of the solvent. The most popular solvents for RPLC of peptides and proteins are acetonitrile, methanol, and propanol or isopropanol. All of these are available in HPLC-grade purity, and when proper care is used in the preparation and disposal of mobile phases, these solvents do not pose severe hazards in the laboratory. Peptides and proteins are detected by the UV absorbance of the peptide bond in the 205- to 220-nm region of the spectrum. Acetonitrile has good optical transparency in this region, possessing a UV cutoff of 190 nm; it is compatible with detection at 205 nm. Methanol and propanol have a UV cutoff at 205 nm. This necessitates detection at 210 nm or above to avoid excessive baseline offsets in gradient elution. The UV response of the analytes at the longer wavelength will be somewhat less, compromising detection sensitivity. Acetonitrile–water mixtures exhibit low viscosity, which reduces resistance to mass transfer and thus improves chromatographic efficiency. The short-chain alcohols participate in hydrogen bonding interactions with water molecules, which increases the viscosity of water–organic mixtures and adversely affects mass transfer and peak shape. However, alcohols are considered to be less denaturing than acetonitrile.

The comparative behavior of these three solvents is illustrated in the gradient elution of a series of synthetic peptides (Figure 4.5). The analytes in this study were octapeptides of identical structure except for the tandem substitution of each of the protein amino acids in the internal sequence of the peptide.[6] Note that peptides with hydrophilic insertions (e.g., lysine, histidine, and arginine) were weakly retained, whereas peptides with hydrophobic insertions (e.g., isoleucine, phenylalanine, leucine, and tryptophan) were most strongly retained. The three chromatograms indicate the total analysis time increased in the order of isopropanol, acetonitrile, and methanol. This demonstrates that methanol is the weakest solvent, isopropanol is the strongest solvent, and acetonitrile is of intermediate eluting power. The best peak shapes and highest efficiencies were observed with acetonitrile, reflecting lower mobile-phase viscosity and better mass-transfer kinetics obtained with this modifer. The variations in peak positions in the three chromatograms demonstrates the different selectivities obtained with the three solvents.

Surfactants

The addition of surfactants to the mobile phase will reduce surface tension and increase eluting strength. For this reason, surfactants might be expected to be useful in the chromatography of polypeptides. This has proven not to be the case

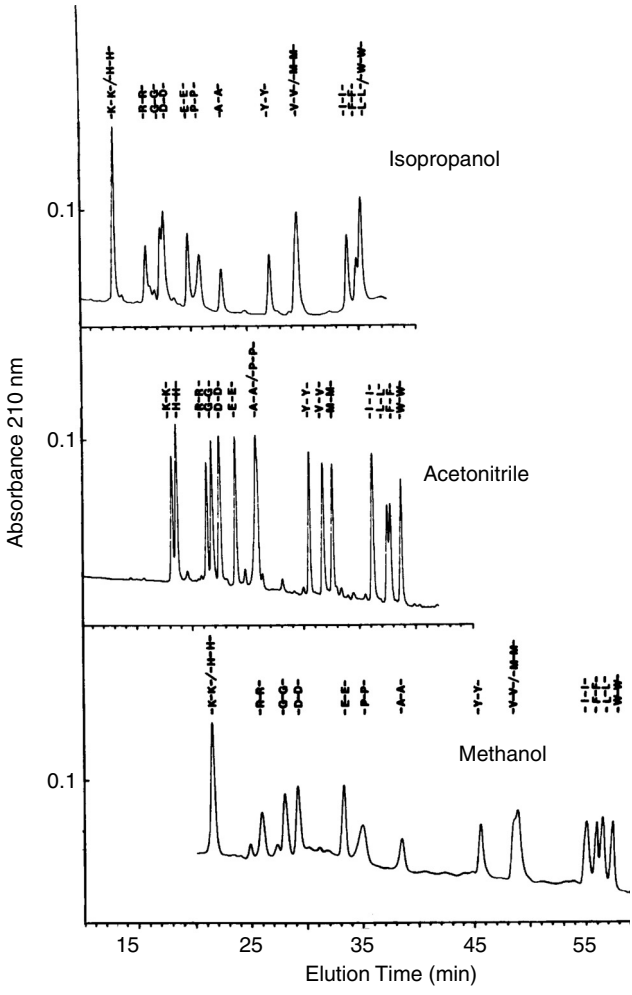

Figure 4.5 Effect of organic modifier on peptide retention. Synthetic octapeptides of the structure described in Figure 4.1 were separated by gradient elution on an octyl column using aqueous–organic systems containing a constant TFA concentration of 0.1%. (Reproduced from D. Guo, C.T. Mant, A.K. Taneja, J.M.R. Parker, and R.S. Hodges, *J. Chromatogr., 359:* 499 [1986]. With permission from Elsevier Science.)

for most separation problems. Surfactants will tend to induce protein unfolding, introducing new hydrophobic contact areas that can complicate the chromatography. Surfactants will also bind to the reversed-phase surface and can permanently change the behavior of the column. Therefore, the use of surfactants is usually reserved for applications where they are required for solubilization of the

protein and to achieve satisfactory recovery. For example, RPLC methods for integral membrane proteins often employ ionic, zwitterionic, or nonionic surfactants as additives.

Retention–Solvent Strength Relationships

The relationship between retention and solvent strength in reversed-phase chromatography is described by the linear solvent strength model:[19,20]

$$\text{Log } k = \log k_w - S\Phi \quad (1)$$

where k_w is the capacity factor (k) in water as eluent, Φ is the volume fraction of organic solvent, and S is a constant. Plots of log k vs. Φ for a small molecule (benzyl alcohol, mol wt 108), a pentapeptide (methionine enkephalin, mol wt 573), and a small protein (lysozyme, mol wt 14400) are shown in Figure 4.6.[21] The slope of the plot (S) is equivalent to the number of solvent molecules required to desorb the analyte from the stationary phase and is related to the molecular weight (M) of the solute:[19]

$$S = 0.48M^{0.44} \quad (2)$$

For small molecules, the value of S is approximately 3, and this predicts that a 10% change in organic modifier concentration will produce about a threefold change in retention as measured by k. However, for large molecules such as polypeptides, the dependency of retention on solvent strength is much greater, with S values of approximately 10 for small peptides and 25 to 100 for proteins of 10 to 100 kDa in size. This predicts that very small changes in organic modifier concentration can produce dramatic changes in polypeptide retention. For example, a 3% change in acetonitrile concentration produces a 10-fold change in lysozyme retention (Figure 4.6). This strong dependency of retention vs. solvent strength reflects the increasing hydrophobic molecular contact surface and the potential for multisite binding. The practical consequence of this behavior is that isocratic elution conditions can rarely be used for polypeptides, and gradient elution is almost universally required for multicomponent mixtures of peptides and proteins. Note that, because S is related to molecular size, the S value for unfolded states will be larger than for native conformations. Also, proteins that occupy hydrophobic environments *in vivo* (e.g., membrane proteins) should be conformationally more stable under reversed-phase conditions and display lower S values. This has been confirmed experimentally.[22]

The plots presented in Figure 4.6 display polypeptide retention data only for organic modifier concentrations of less than 50%. When this type of study is extended to higher organic concentrations, there is a reversal of retention behavior such that polypeptides exhibit enhanced retention (Figure 4.7). This behavior is observed for both peptides and proteins and is interpreted as a transition from hydrophobicity-driven retention at low organic modifier concentration to hydrophilicity-driven retention at high organic concentrations (Figure 4.8). For silica-based

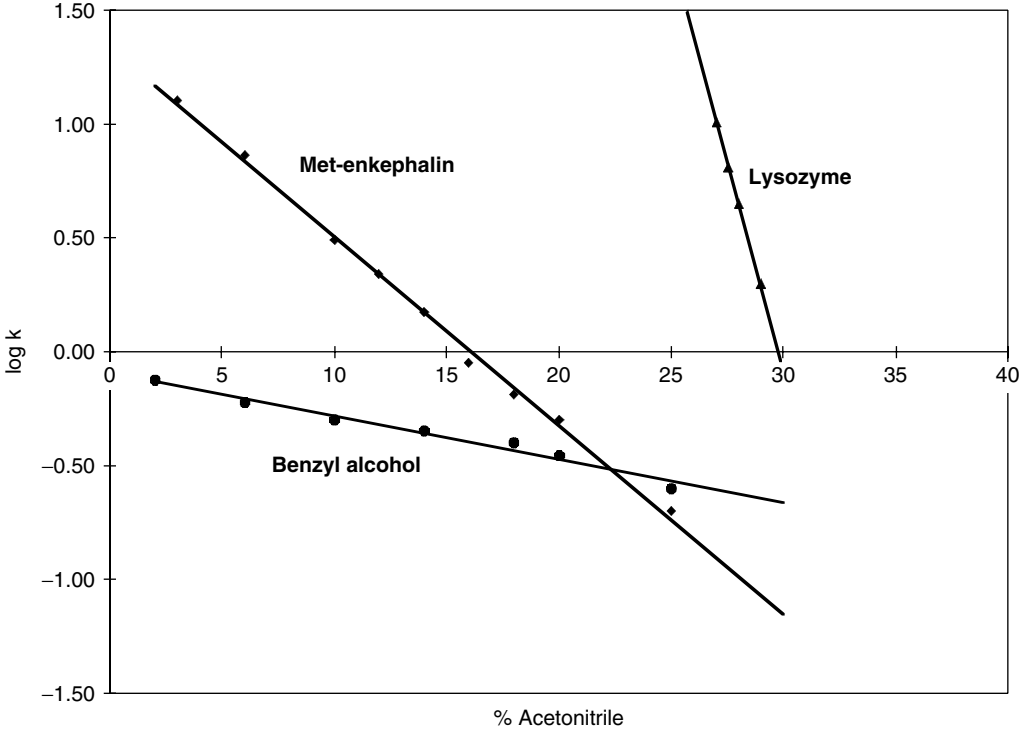

Figure 4.6 Effect of organic concentration on retention. (Reproduced from M.W. Dong, J.R. Gant, and B.R. Larsen, *BioChromatography, 4:* 19 [1989]. With permission from Eaton Publishing.)

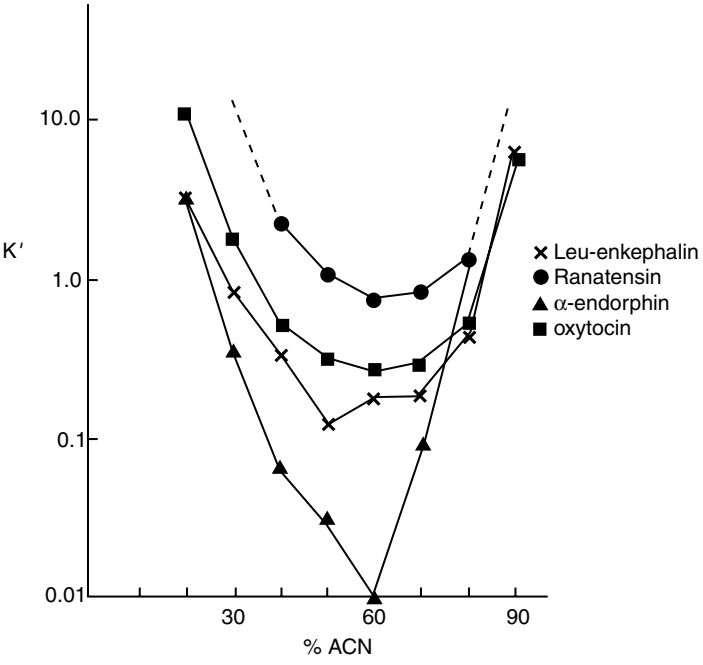

Figure 4.7 Effect of high organic modifier concentration on peptide retention. Data obtained using an octadecyl column and 20 m*M* ammonium acetate:acetonitrile mobile phases. (Reproduced from C.T. Wehr and L. Correia, *LC at Work LC-121,* Varian. With permission from Varian Associates.)

reversed-phase columns, increasing retention at $\Phi > 0.5$ occurs by silanophilic interactions with the support matrix, with the roles of the aqueous and organic components of the mobile phase reversed to yield normal-phase chromatographic behavior. This phenomenon has two practical consequences for the analyst. First, the extension of a gradient to organic modifier concentrations above 60 to 70% will be unproductive for polypeptide elution from reversed-phase columns. Second, attempts to strip contaminating proteins bound to a reversed-phase column using nonpolar solvents are likely to be fruitless. This will be true whether the analyte is proteinaceous or is a small molecule analyzed in a protein-containing matrix.

Gradient Elution

Because of the profound dependency of polypeptide retention on solvent strength, isocratic elution of proteins and peptides is generally impractical. Instead, solvent strength is increased continuously during the analysis, and each analyte elutes from the column at the point when solvent strength minimizes its binding to the stationary phase.

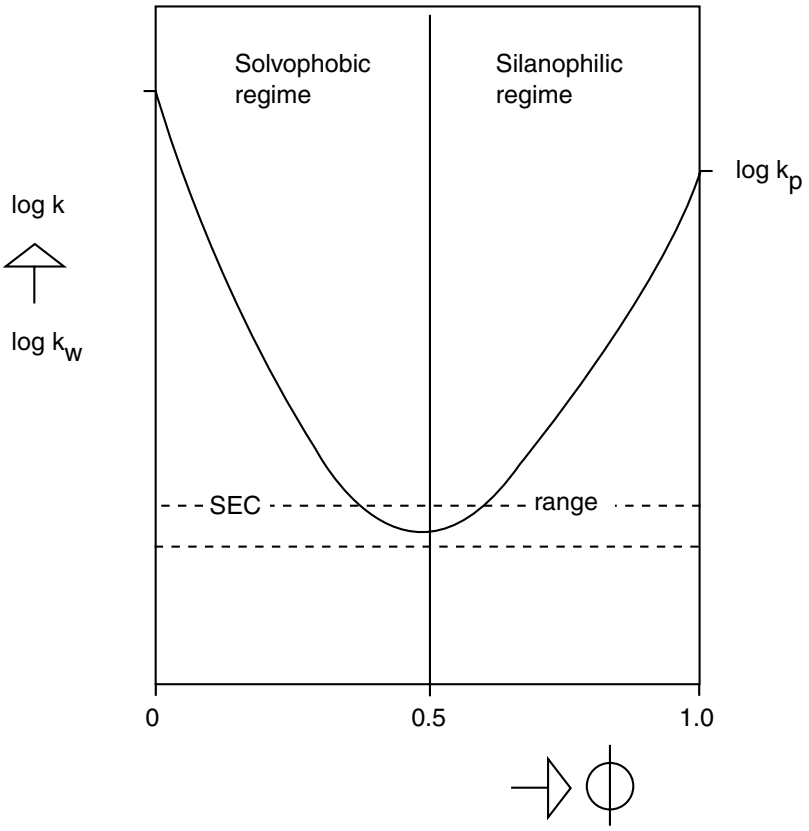

Figure 4.8 Regions of reversed-phase chromatographic mechanisms. (Reproduced from K.K. Unger, R. Janzen, and G. Jilge, *Chromatographia, 24:* 144 [1987]. With permission of Friedr. Vieweg & Sohn.)

In isocratic elution, retention is characterized by the capacity factor, k:

$$k = (t_r - t_0)/t_r \tag{3}$$

where t_r is the retention time of the analyte and t_0 is the elution time of an unretained species. The mobile-phase elution strength is adjusted to provide values of k between 1 and 10. The resolution R of two components is described by:

$$R = \tfrac{1}{4}(N)^{1/2}\,(\alpha - 1)(k/k + 1) \tag{4}$$

where N is the column efficiency in plates and is the selectivity (calculated as the ratio of the k values of two components). It should be noted that changes in k can sometimes change selectivity.

In gradient elution, the mobile-phase composition in the region of the analyte at any point in time determines an isocratic value of k, which defines the

instantaneous velocity of the peak down the column.[20] The average value of k during elution is defined as k^*, and k^* represents the value of k at the midpoint of migration down the column. The steepness of the gradient (b) is inversely related to k^*,

$$k \sim 1/b \tag{5}$$

with shallow gradients providing large k^* values and increased resolution, and steep gradients providing small values of k^* with less resolution. The dependence of k^* on gradient conditions is given by

$$k^* = t_G F/\Delta\Phi S V_m \tag{6}$$

where t_G is the gradient time, F is the flow rate, $\Delta\Phi$ is the change in the volume fraction of the organic solvent across the gradient, and V_m is the column dead volume (t_0F). Because k^* is analogous to k in isocratic separations, k^* values between 1 and 10 should yield adequate separations. Once an appropriate value of k^* has been selected, gradient conditions can be manipulated to achieve the desired resolution. As with changes in k in isocratic elution, changes in k^* in gradient elution can affect selectivity (α). Therefore, a single change in any of the gradient conditions (gradient time or range, flow rate, or column volume) may change peak spacing or elution order. The analyst must be cognizant of this characteristic of gradient elution in developing, modifying, and transferring gradient methods. For example, in the gradient elution of myoglobin tryptic peptides (Figure 4.9), changing the flow rate from 0.5 ml/min to 1.5 ml/min (while maintaining the same gradient time) results in significant changes in selectivity.[25] Inspection of Equation 6 indicates that a change in one gradient condition (e.g., an increase in flow rate) must be accompanied by a compensatory change in another variable (e.g., a collateral decrease in gradient time) to maintain a constant value of k^* and unaltered peak spacing.

Consideration of Equation 6 also suggests strategies for optimizing gradients for proteins and peptides, which have large S values. Reasonable k^* values can be achieved by increasing the gradient volume (t_GF), by reducing V_m, or by using a narrower gradient range ($\Delta\Phi$). Reduction in column volume can be accomplished by using a smaller column diameter (d) or a shorter column length (L). With the first approach, it should be noted that the flow rate must be scaled in proportion to the change in d^2 to maintain the same flow velocity to avoid loss in column efficiency. Reducing V_m by decreasing L would be expected to reduce efficiency and thereby compromise resolution. In practice, protein resolution does not seem to be strongly dependent on column length. This is because the negative effect of decreasing N is offset by the positive effect of increasing k^*.[20]

Using the preceding guidelines, a systematic approach to gradient optimization can be followed:[20]

1. Estimate the mean S values for the analytes from Equation 2.
2. Select an appropriate value for k^*, e.g., $k^* = 5$.

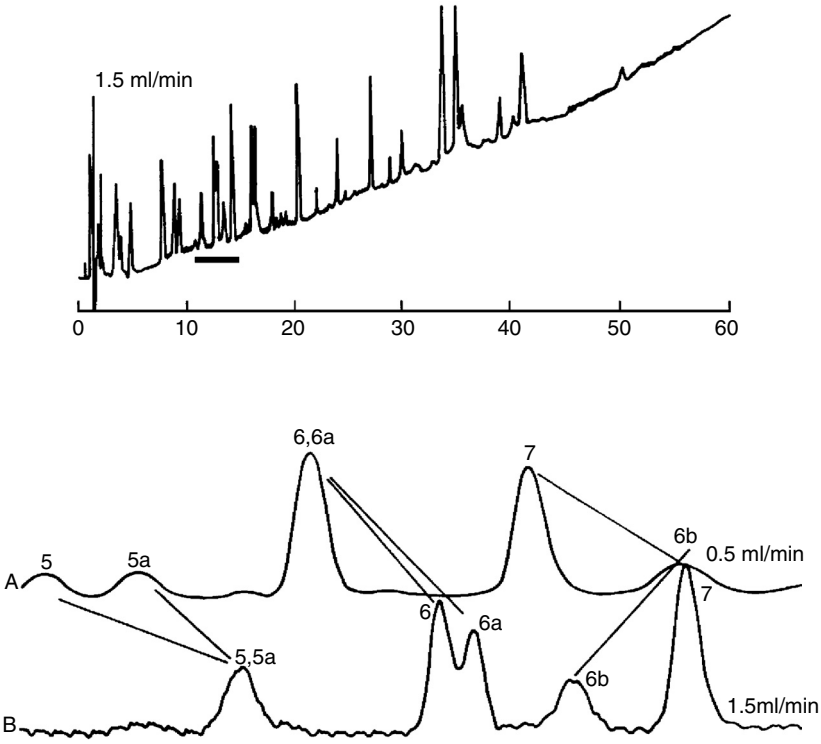

Figure 4.9 Changes in selectivity resulting from a change in flow rate from 0.5 to 1.5 ml/min for the reversed-phase gradient separation of a myoglobin tryptic digest. (A) entire chromatogram obtained at 1.5 ml/min, (B) expanded portion of the peaks eluting in the indicated segment of the chromatogram for both flow rates. (Reproduced from J.L. Glajch, M.A. Quarry, J.F. Vasta, and L.R. Snyder, *Anal. Chem., 58:* 280 [1986]. With permission from the American Chemical Society.)

3. Select a solvent range ($\Delta\Phi$) for a scouting gradient.
4. Using the values of S, k^*, and $\Delta\Phi$ of in combination with V_m and F, estimate t_G from Equation 6. For example, for a mean molecular weight of M and a k^* of 5,

$$t_G = 3\Delta\Phi\ V_m\ FM^{\,0.44}.$$

5. Run the scouting gradient, then narrow the gradient range so that the earliest peak elutes after t_0 + dwell time (t_D) and the last peak elutes near the final solvent concentration. The gradient steepness should be maintained in the narrowed gradient.

6. Fine tune t_G, F, and L for best resolution while keeping k^* constant. However, note that high values of t_G or low values of F will increase analysis time, may reduce recovery, and can induce conformational changes.
7. For complex samples, segmented gradients with varying steepness may provide the best resolution across the separation. However, multisegment gradients often have problems with precision and transferability.

Once optimal gradient conditions have been found, further manipulations of peak spacing are accomplished using the same strategies employed in isocratic separations. The mobile-phase pH, organic modifer, additive type and concentration, column temperature, and the type of stationary-phase ligand can be changed. However, as noted above, changes in these parameters can introduce changes in protein folding states that may affect peak shape.

The large S values observed for polypeptides have two beneficial consequences in gradient elution. The narrow elution range of proteins reduces peak volumes, particularly for larger proteins that tend to be more strongly retained. Gradient compression is also enhanced by large S values. Gradient compression arises from the gradient in solvent strength across the peak such that the peak tail migrates at a higher velocity than the center of the peak, creating a peak sharpening effect.

Practical considerations in performing gradient elution include baseline artifacts and solvent proportioning precision. Baseline artifacts can arise from mismatch in the UV absorbance of the mobile-phase solvents and from contaminants in the mobile-phase components. The detection wavelengths used to monitor protein and peptide separations are close to the UV cutoff of methanol and propanol, and these solvents generate continuously rising baselines across the gradient. In addition, the absorbance of TFA increases with increasing organic solvent concentration due to shifts in the absorbance spectrum of the additive in nonpolar environments. To compensate for such solvent mismatches, acetonitrile is often used in preference to alcohols, the TFA concentration in the strong solvent can be reduced, and detection can be performed at 214 to 216 nm instead of 210 nm.

Mobile-phase contaminants often elute as discrete peaks, usually late in the gradient. Contaminants may be present in the buffers and additives or (more often) in the water used to prepare the weak solvent. If these are hydrophobic in nature, they will accumulate on the column in the early part of the gradient and elute as peaks as solvent strength increases. To confirm that artifact peaks arise from mobile-phase contaminants, the weak solvent may be pumped isocratically for a period prior to initiating the gradient. An increase in the height of the artifact peaks is diagnostic of contaminants in the weak solvent. Elimination of artifact peaks may require the use of purer reagents and water, or off-line cleanup of the weak solvent with a reversed-phase stripper column (an expired analytical column is useful for this task).

The proportioning accuracy and precision of the solvent delivery system is more important in the chromatography of proteins and peptides than in the

analysis of small molecules. Small deviations in solvent composition can produce significant variances in analyte elution due to the strong dependency of polypeptide retention on solvent strength (e.g., large S values). The use of premixed mobile phases can minimize this problem. For example, a separation requiring a gradient range from 20 to 60% acetonitrile should be performed by proportioning between premixed 80:20 water:acetonitrile (solvent A) and premixed 40:60 water:acetonitrile (solvent B) and executing a gradient from 0 to 100% B.

Temperature

It might be expected that column temperature would affect retention and selectivity in the chromatography of proteins and peptides because changes in temperature can shift the ionization state of ionizable amino acid side chains and can induce conformational changes. In fact, studies with tryptic digests confirm that temperature can be an important variable in controlling peak spacing of peptides in gradient elution.[26] Moreover, selectivity effects due to temperature change are independent of effects due to changes in gradient steepness. This argues that simultaneous variation of temperature and gradient steepness should be useful in optimizing polypeptide separations. This strategy may be more convenient than changing mobile-phase composition or column chemistry because gradient and temperature changes are easily implemented by changing settings in the HPLC system controller. Chromatography simulation software that allows the analyst to find optimal separation conditions for polypeptides with input of a few experimentally established temperature and steepness data is commercially available.[26] A reason for caution in the use of temperature as a chromatographic variable is the instability of many HPLC columns under conditions of elevated temperature and low pH. Fortunately, there are several reversed-phase columns now available with enhanced stability at extremes of pH and temperature.[27,28]

ANOMALOUS BEHAVIOR OF PROTEINS IN RPLC

Many globular proteins in the 10- to 100-kDa range behave as predicted[20] in terms of peak width and shape. However, deviations from expected behavior are often observed with proteins in RPLC, particularly with those larger than 25 kDa. These anomalies include excessive bandwidth, poor recovery, the observation of multiple peaks for a single species, and "ghosting." Ghosting refers to the appearance of protein peaks in a blank gradient following a chromatographic run. Anomalous behavior has been shown to arise from interconversion of the protein between different conformational states.[29,30] If the rate of interconversion during elution is fast, a single narrow band is observed. If the rate of interconversion is slow, the band may be broad and asymmetric. Very slow interconversion rates may generate multiple peaks for the different conformers. Protein unfolding within the pores may lead to entrapment, and the phenomenon of ghosting is thought to represent refolding and subsequent elution of entrapped species. Such anomalous or "nonideal" behavior of proteins in RPLC can be minimized by

employing conditions that stabilize a particular conformer, e.g., operation at elevated temperature or extremes of pH. The use of sample pretreatment with denaturing conditions and separation at elevated temperature (60°C) has been used successfully to obtain acceptable peak shapes for "non-well-behaved" proteins.[31,32]

DETECTION TECHNIQUES

Absorbance detection in the low-UV region is the most popular method for polypeptides. Although the peptide bond exhibits strongest absorbance below 200 nm, the use of longer wavelengths (210 to 220) provides an acceptable tradeoff between obtaining satisfactory signal for analytes and minimizing background absorbance from mobile-phase components. Photodiode array (PDA) detectors enable multiwavelength detection (e.g. 215, 260, and 280 nm) and on-line acquisition of absorbance spectra. This information can be useful for confirming peak purity or the presence of aromatic side chains in the peptide primary sequence. For proteins, spectral information can provide clues about the solvent environment of aromatic residues, e.g., whether tryptophan and tyrosine residues are buried in the protein interior or exposed on the protein surface.

Fluorescence is not widely used as a general detection technique for polypeptides because only tyrosine and tryptophan residues possess native fluorescence. However, fluorescence can be used to detect the presence of these residues in peptides and to obtain information on their location in proteins. Fluorescence detectors are occasionally used in combination with postcolumn reaction systems to increase detection sensitivity for polypeptides. Fluorescamine, *o*-phthalaldehyde, and napthalenedialdehyde all react with primary amine groups to produce highly fluorescent derivatives.[33,34] These reagents can be delivered by a secondary HPLC pump and mixed with the column effluent using a low-volume tee. The derivatization reaction is carried out in a packed bed or open-tube reactor.

Multiangle light-scattering detectors are increasingly used to obtain on-line information on protein molecular weight. However, they must be used in combination with refractive index detectors, and so this technique is not compatible with reversed-phase gradient elution.

Mass spectrometry is the fastest-growing detection technique for HPLC in general and for proteins and peptides in particular. Electrospray ionization permits mobile-phase streams to be introduced directly into the MS system at the flow rates used with analytical HPLC columns. Among the chromatographic modes employed for polypeptide separations, reversed phase is ideally suited for ESI-MS detection because the common mobile-phase components are volatile. Virtually every type of mass analyzer (quadrupole, ion trap, time-of-flight, FT-MS, magnetic sector, and hybrid mass spectrometers) have been successfully coupled to RPLC-ESI systems. Electrospray is a soft-ionization technique that provides mostly pseudomolecular ions with little loss to fragmentation. Applications of LC-MS include (1) high-sensitivity detection of particular peptides in complex

mixtures using selected ion monitoring; (2) determination of peptide and protein mass using scanning detection; (3) determination of peptide sequence using tandem MS; and (4) investigation of protein conformation and protein–protein interaction.

Although RPLC-MS is a very powerful analytical tool, not all RPLC conditions are compatible with mass spectrometric detection. Nonvolatile buffers, salts, and additives (e.g., phosphates, and nonvolatile ion-pairing agents) are not compatible with electrospray sources. Electrospray systems with orthogonal sprayers can tolerate low concentrations of such mobile-phase components for brief periods, but volatile components are preferred. For low pH operation (pH 2 to 3), phosphate should be replaced with dilute solutions of volatile organic acid (e.g., 0.1 to 1% acetic or formic acid). As discussed in the preceding text, the use of TFA causes ion suppression, and it is usually replaced with acetic or formic acid. For operation at higher pH, volatile ammonia-based buffers can be used: ammonium formate (pH 2.7 to 4.7), ammonium acetate (pH 3.7 to 5.7), or ammonium bicarbonate (pH 5.4 to 7.4). These buffers should be used at concentrations below 50 m*M* to avoid ion suppression. Volatile ion-pairing agents such as heptafluorobutyric acid (HFBA) can be used for basic analytes. Alkyl amine additives such as TEA, which are often used to suppress tailing of basic analytes in RPLC, will cause strong ion suppression in positive-mode electrospray. The use of surfactants as mobile-phase additives is not recommended as they rapidly contaminate the ionization source.

Although modern electrospray sources can accommodate mobile-phase flow rates of up to 1 ml/min, lower flow rates are preferred for best ionization efficiency. This can be achieved by installing a flow splitter between the column and the ionization source. Because electrospray ionization is concentration sensitive, this has little effect on signal strength. Alternatively, small-diameter columns can be used. Conventional electrospray sources are usually coupled to a 2- to 3-mm I.D. column operated at 200 to 500 µl/min. When limited amounts of sample are available, capillary columns may be preferred to increase sensitivity and minimize on-column loss of sample components. Capillary LC-MS is the preferred technique for the separation of very complex peptide mixtures generated in proteomic studies.[35] These may be proteolytic digests of protein spots obtained from 2-D gels, or digests of proteins extracted directly from cells or tissues. In the latter case, the extraordinary complexity of the samples requires coupling of multiple chromatographic modes. Multidimensional chromatographic techniques generally employ ion exchange as the first dimension, and reversed-phase LC-MS as the second dimension. Multidimensional techniques include off-line first-dimension separations, on-line column switching, or direct coupling of a mixed-bed column to the mass spectrometer.[36] In the last approach,[37] the capillary is packed with a reversed-phase material in the outlet segment and an ion-exchange material at the inlet. Samples are loaded onto the ion-exchange bed and eluted with a series of salt solutions of increasing ionic strength. Each ion-exchange eluate is resolved on the second-dimension bed with a reversed-phase gradient.

APPLICATIONS

The primary analytical applications of RPLC in the development of biopharmaceuticals are the determination of protein purity and protein identity. Purity is established by analysis of the intact protein, and RPLC is useful in detecting the presence of protein variants, degradation products, and contaminants. Protein identity is most often established by cleavage of the protein with a site-specific protease followed by resolution of the cleavage products by RPLC. This technique, termed peptide mapping, should yield a unique pattern of product peptides for a protein that is homogeneous with respect to primary sequence.

Proteins

Reversed-phase chromatography is a powerful tool for detecting protein structural changes that are introduced during biosynthesis, isolation, or processing. These may include amino acid substitutions, side-chain oxidation and deamidation, and N- or C-terminal cleavage. Protein variants that differ in the number and type of posttranslational modifications such as gycosylation, phosphorylation, acetylation, and sulfaction may also be identified by RPLC. If structural changes are located at the protein surface, the variant will more likely be distinguished using chromatographic conditions that favor retention of native conformation.[17,38] The typically harsh RPLC conditions using acid water:acetonitrile:TFA mobile phases and hydrophobic octadecyl or octyl ligands may not be appropriate. Instead, alcohol modifiers (methanol, propanol) and neutral pH buffers may be used with short-chain (C4) or polar (cyano) stationary phases. Confidence in the purity of a protein separation is increased by the appearance of a single peak under two different sets of RPLC conditions, i.e., different pH values, temperatures, or stationary phases.[38]

Proper protein folding is a concern in the production of a bioactive protein therapeutic, and it is of interest to verify that the conformation of a recombinant protein is the same as the wild-type molecule. Because retention in RPLC depends on the surface hydrophobic contact area, comparable chromatographic behavior of a recombinant protein with that of the wild-type molecule provides evidence of similar 3-D structure.[17,38]

The literature is rich in publications describing the use of RPLC for protein purification and characterization, and a review is provided in Reference 17. Two examples are given here as illustration.

Interleukin 2 (IL-2) is a hydrophobic immune modulator protein with a mol wt of about 15,500 Da. Recombinant IL-2 expressed in *E. coli* forms insoluble aggregates located in inclusion bodies. These can be easily isolated from host cell extracts but must be solubilized under denaturing conditions and refolded to yield active protein. Reversed-phase chromatography has been used as the principle purification step for IL-2.[39,40] RPLC has also been used[41] to determine the purity of IL-2 isolated by gel permeation chromatography and to monitor the refolding of denatured IL-2 to the native state under different renaturing conditions (Figure 4.10).

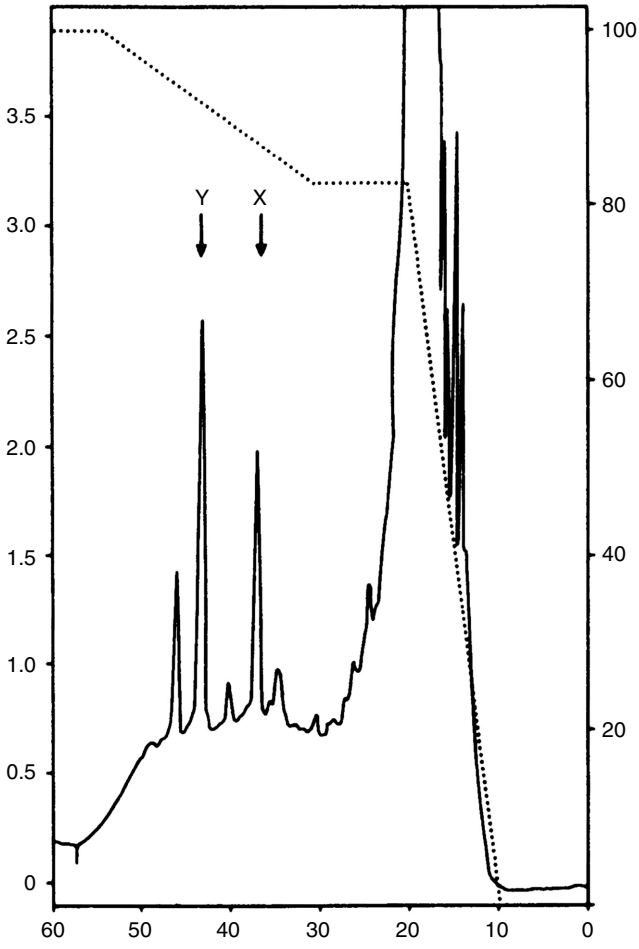

Figure 4.10 Reversed-phase chromatography of refolded IL-2. Peak X is oxidized IL-2 and peak Y is reduced IL-2. (Reproduced from M.P. Weir and J. Sparks, *Biochem. J., 245:* 85 [1987]. With permission from Portland Press.)

Amino acid variants of IL-2 have been used to investigate the relationship between retention and protein structure in gradient RPLC.[22] The protein contains three cysteine residues in its primary sequence at positions 58, 105, and 125. The two located at positions 58 and 105 are linked in a disulfide bridge in the native molecule. A series of variants in which the three cysteinyl residues were replaced with serines were compared. Substitution with serine at positions 58 or 105 forces the molecule to form an unnatural disulfide between positions 125 and 58 or 105. A methionine residue located at position 104 can also be oxidized to the sulfoxide

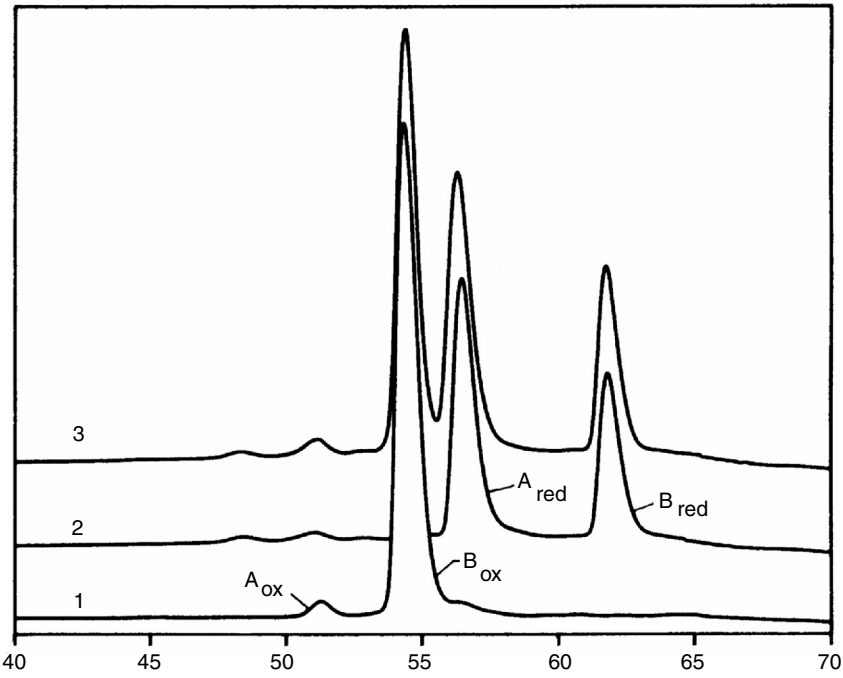

Figure 4.11 Chromatograms of: (1) desAla^1(S^{58}–S^{105})Ser^{125} IL-2, (2) desAla^1 (SH^{58}, SH^{105})Ser^{125} IL-2, and (3) mixture of all components. Peaks labeled A have methionine sulfoxide at position 104, and peaks labeled B have unoxidized methionine at position 104. Separations were performed using a gradient of 41–60% acetonitrile-water (0.1% TFA) in 60 min at 0.5 ml/min. (Reproduced from M.G. Kunitani, D.J. Johnson, and L.R. Snyder, *J. Chromatogr., 371:* 313 [1986]. With permission from Elsevier Science.)

form. All of these variants have similar molecular weight and hydrophobicity and should be expected to exhibit similar S values in gradient elution. This was not the case; variants that possessed the unnatural disulfide bridge exhibited higher-than-expected S values. This indicated conformational instability in these variants that exposed greater hydrophobic contact areas during gradient elution. Plots of k^* vs. Φ for the oxidized vs. reduced and methionyl vs. methionyl sulfoxide forms obtained by computer modeling studies predicted that these four species could be resolved by manipulation of low rate F and gradient range ($\Delta\Phi$), and this was confirmed experimentally (Figure 4.11).

Although gradient elution is generally required for RPLC separations of proteins, isocratic elution can be successful in some instances. For example, isocratic elution has been used for the determination of purity of production batches of biosynthetic human growth hormone (HGH).[42] The method was used to

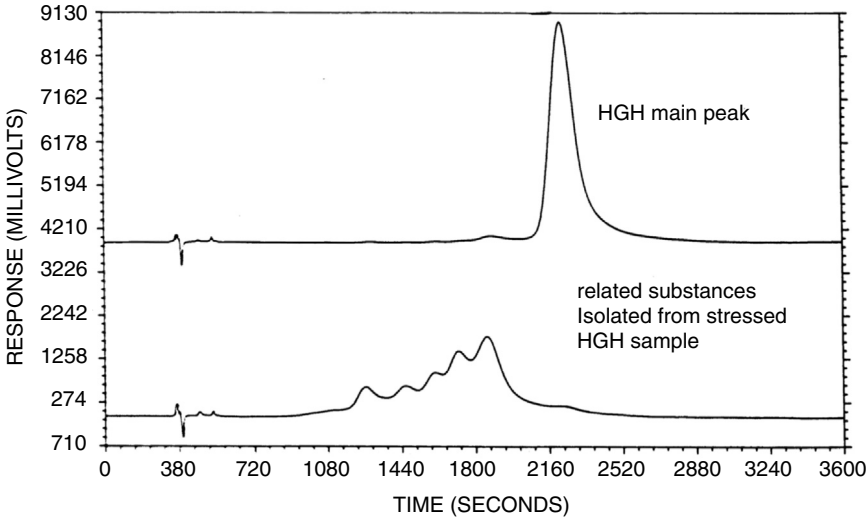

Figure 4.12 Analysis of biosynthetic human growth hormone by isocratic reversed-phase chromatography. To generate all possible degradation products, a production lot of HGH was exposed to 40°C. The profile of the unstressed HGH is shown in the upper trace. (Reproduced from R.M. Riggin, G.K. Dorulla, and D.J. Miner, *Anal. Biochem., 167:* 199 [1987]. With permission from Elsevier Science.)

resolve deamidated species and sulfoxide derivatives from native growth hormone (Figure 4.12), which could not be adequately resolved using reversed-phase gradient elution or anion-exchange chromatography. It is interesting to note that best resolution of degradation products from the parent protein required conditions which promoted the retention of native conformation (C4 stationary phase, 71:29 50 m*M* Tris buffer, pH 7.5:*n*-propanol). The use of acidic conditions (sodium phosphate, pH 2) produced poor peak shapes and inadequate resolution.

Peptides

The extraordinary ability of reversed-phase chromatography to resolve peptides with very subtle differences in sequence or structure makes it the method of choice for peptide mapping. It is not uncommon for two peptides differing in a single residue or the presence of a single side-chain modification to be fully resolved. The high efficiency of monomeric, microparticulate reversed-phase columns generates peak capacities in excess of 250, enabling separation of the complex peptide mixtures often encountered in peptide-mapping experiments.

A variety of proteases are used for peptide mapping, but trypsin is the most popular because it is stable and available in high purity with little contamination

Table 4.5 Conditions for Reversed-Phase HPLC Separations of Polypeptides

Column	4.6 × 150 mm packed with C8-bonded spherical type B silica, endcapped, 5 μm particle diameter, 30 nm pore diameter
Mobile Phase A	water + 0.1% TFA
Mobile Phase B	40:60 water:acetonitrile + 0.1% TFA
Gradient	0-100% in 60 min
Flow Rate	1.0 ml/min
Column Temperature	30°C
Detection	absorbance at 210 nm

from secondary cleavage activities. It can also be covalently modified to minimize autodigestion. Trypsin cleaves on the C-terminal side of lysine and arginine residues. These amino acids occur at frequencies of 5% and 6% (respectively) in the proteome, so tryptic peptides are usually in the range of 2 to 20 residues in length. This has several beneficial consequences for tryptic mapping experiments. First, peptides of this size possess little or no secondary structure, so that conformational effects do not cause aberrant peak morphology. Second, molecular diffusion rates are relatively high, so that mass-transfer kinetics are favorable. Third, tryptic peptides are usually within the mass range of quadrupole and ion-trap mass spectrometers. Thus, peptide mass and sequence information is easily obtained with these robust and relatively inexpensive detectors. Fourth, because every tryptic peptide has a C-terminal lysine or arginine in addition to the N-terminal amine group, they produce doubly charged ions in electrospray ionization. This facilitates fragmentation in tandem MS experiments and aids in the interpretation of MS-MS spectra.

The standard RPLC conditions used for polypeptides (Table 4.5) are usually satisfactory for tryptic peptides. The resolution of the 21 tryptic peptides of recombinant HGH[26] was obtained using these mobile-phase and column conditions with elevated temperature (Figure 4.13). Modification of side-chain residues is likely to change the chromatographic properties of a peptide such that the magnitude of that peak is diminished and a new peak appears with the retention time characteristic of the modified peptide. For example, phosphorylation of serine or threonine residues, or oxidation of methionine residues, will shift the retention of a peptide to earlier times. Similarly, deamidation of glutamine or asparagine residues will increase peptide retention.[38] Disulfide bridges can be located by observing peak shifts in digests prepared with and without reducing agents (e.g., dithiothreitol, β-mercaptoethanol). Glycosylated peptides can be recognized by a shift in peptide retention following enzymatic treatment to remove carbohydrate groups, for example, incubation with the enzyme PNGase to remove N-linked carbohydrate moieties.

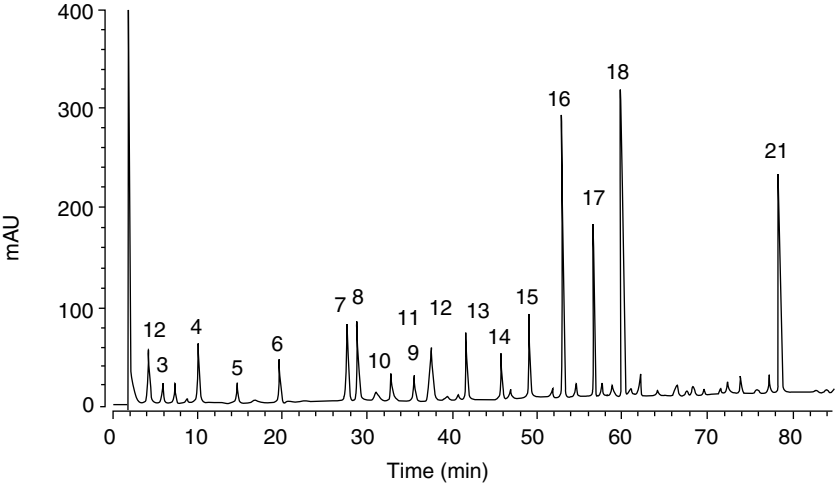

Figure 4.13 Separation of the tryptic peptides from recombinant HGH using a 120-min linear gradient at 60°C from water + 0.1% TFA to 40:60 water:acetonitrile + 0.1% TFA. Column: Zorbax SB-C8, 4.6 × 150 mm, 30-nm pore, 5-μm particle size. (Reprinted from W.S. Hancock, R.C. Chloupek, J.J. Kirkland, and L.R. Snyder, *J. Chromatogr. A, 686:* 31 [1994]. With permission from Elsevier Science.)

COMPARISON WITH ALTERNATIVE TECHNIQUES

Reversed-phase HPLC can be compared in terms of its utility with other modes of chromatography and with other separation techniques such as gel electrophoresis, capillary electrophoresis (CE), and capillary electrochromatography.

Other Chromatographic Modes

Other modes of chromatography that are used for protein and peptide separations include size exclusion chromatography (SEC), ion-exchange chromatography (IEC), HIC, immobilized metal affinity chromatography (IMAC), affinity chromatography, and hydrophilic interaction chromatography (HILIC). All of these tend to be less denaturing than RPLC and will be preferred for preparative isolation of proteins with retention of native conformation and biological activity. Reversed phase can be used as a preparative tool and is most successful for small proteins (<25 kDa), those that retain native conformation under reversed-phase conditions, or proteins that easily refold to the native state following chromatography. Preparative isolation of a protein from a complex sample often requires sequential purification steps with two or more chromatographic modes. Where possible, reversed phase may be included in a purification scheme because it offers a unique chromatographic selectivity. For analytical applications, reversed-phase chromatography is usually the technique of choice because of its high

efficiency and selectivity, the stability of the column, the compatibility of the solvents with UV and MS detection, and the relative ease of method development. However, RPLC will not be the analytical tool of choice when conformational effects compromise resolution and confound issues of product purity or microheterogeneity. For peptides, RPLC is the premier technique for purification, for purity determination of synthetic peptides, and for peptide mapping.

Other chromatographic modes may be used in preference to reversed phase to address specific analytical questions. SEC, when calibrated with appropriate protein standards, can provide estimates of protein molecular weight or aggregation state. More accurate estimates of protein mass or aggregation state can be obtained by coupling SEC with tandem multiangle light-scattering and refractive index detectors. Both of these detectors require isocratic elution conditions and are quite compatible with SEC. Because SEC is a gentle technique that does not perturb protein structure, it can be used for protein–ligand binding studies.[43] For protein isolation, SEC provides a size-based fractionation step in a protein purification scheme. In spite of its limited resolving power and low sample capacity, the excellent recovery of protein mass and activity from SEC columns favors the inclusion of SEC steps in purification protocols.

IEC is usually carried out under physiological conditions, e.g., at neutral pH with salt solutions. It is therefore widely used for purification of proteins in their native state with high recovery of mass and activity. From an analytical perspective, ion exchange may be used in preference to reversed phase to determine purity or charge heterogeneity in cases where the denaturing conditions of RPLC would yield confusing results. In particular, IEC can provide information about protein variants with differences in surface charge.

HIC, like IEC, is performed under conditions that preserve protein shape and activity. It is used in preparative applications to obtain a selectivity complimentary to IEC and akin to RPLC but without the denaturing properties of the latter technique. Although HIC and RPLC share a mechanism based on hydrophobic partitioning, the actual peak spacing and elution order of the two techniques can be different. This arises from the different hydrophobic contact points presented by the protein under native (HIC) and denaturing (RPLC) conditions. Although not widely used for analytical separations, HIC can be used to answer questions about accessible hydrophobic surface area that cannot be addressed by RPLC.[44]

Affinity chromatography and related techniques (e.g., thiol chromatography and IMAC) are widely used for preparative isolation because they enable a single protein or class of proteins to be selectively purified from very complex mixtures. They may be occasionally used as analytical tools. For example, protein A affinity chromatography has been used for quantitative analysis of immunoglobulins in ascites fluid.[45] Information about surface-accessible histidine and phosphate groups may be obtained using IMAC.

HILIC is a variant of normal-phase chromatography that employs polar stationary phases and RPLC-type mobile phases. Because HILIC separations occur by a normal-phase mechanism, the organic component of the mobile phase

(typically acetonitrile) is the weak solvent and the aqueous component (an acidic or neutral pH buffer) is the strong solvent. Elution is generally accomplished with a gradient from low to high percentages of the aqueous component. Polar polymeric stationary phases are used, such as polyhydroxyethyl aspartamide.[46] HILIC offers an alternative to RPLC for very polar species that are poorly retained on reversed-phase columns, such as phosphorylated peptides[47] and hydroxyproline-rich peptides.[48] HILIC elution solvents are also compatible with electrospray–MS. In contrast to RPLC, less-retained analytes from a HILIC column will elute at high organic solvent concentrations that are more easily desolvated in the ionization process.

Gel Electrophoresis

For decades, polyacrylamide gel electrophoresis (PAGE) has been the primary tool of the protein biochemist for characterizing protein mixtures, estimating protein molecular weight and isoelectric point, monitoring protein purification, and determining microheterogeneity. Gel electrophoretic techniques include native PAGE, SDS-PAGE, and isoelectric focusing (IEF). Two-dimensional PAGE couples the latter two techniques in a two-step process. Native and SDS-PAGE offer several advantages compared to column chromatographic techniques. When separations are performed on slab gels, several samples can be run in parallel lanes on the gel, and individual separations can be directly compared with each other and with standards and reference materials run on the same gel. If the PAGE experiment is terminated at the appropriate time, all sample components will be confined between the origin and the tracking dye. This is in contrast to chromatography in which sample components that are strongly retained on the stationary phase will not be detected. The disadvantages of PAGE include the requirement for staining to detect protein bands, the labor involved in gel preparation and processing, and gel-to-gel reproducibility. Stain intensity is either nonlinear with protein mass or linear over a narrow range, and staining intensity varies from protein to protein. Gel electrophoresis is therefore a nonquantitative or, at best, semiquantitative technique. Casting, running, staining, and destaining gels are time consuming, and reproducibility of the results is dependent on the expertise of the analyst. These limitations are minimized by the use of commercial precast gels and robotic systems for processing gels. Also, because a dozen or more lanes can be run on a single gel, the analysis time on a per-sample basis can compare favorably with serial techniques such as chromatography. For all these reasons, PAGE is often the preferred technique for capturing a "snapshot" of the progress of a protein purification process.

For analytical applications, chromatography will be preferred over PAGE where quantitative information is necessary and where automation is desired. As an instrumental technique, all components of a chromatographic analysis including injection, separation, detection, and data analysis are fully automated and under single-point control by the HPLC workstation. The limitation of serial

analysis is offset by the ability to analyze a hundred or more samples with no further operator intervention after loading samples in the autosampler. Analytes are detected by their native absorbance with linear dynamic ranges of up to five orders of magnitude, and peak-area precision is typically better than 2% RSD. Reversed-phase separations are highly reproducible, with retention time precision typically 1% RSD or better. When regulatory considerations require precise quantitation of product and impurity levels, chromatography will be the method most likely to return reliable information. Reversed-phase chromatography is almost always the first choice for the analysis of peptides. Peptides do not stain well with the standard PAGE staining techniques, and they can easily be lost by diffusion at the end of the electrophoretic separation.

For accurate determination of protein molecular weight, mass spectrometry and LC-MS have largely displaced SDS-PAGE. However, SDS-PAGE will still be used where estimates of molecular weight suffice or where MS instrument time is limited.

Capillary Electrophoresis and Capillary Electrochromatography

Because it shares the separation chemistries of gel electrophoresis and the instrumental advantages of HPLC, CE provides an alternative when either of these two competing techniques fail to deliver the desired performance.[49] Like gel electrophoresis, CE separations are based on differential migration in an electric field, and CE analogs for each gel electrophoresis mode are available: capillary zone electrophoresis (CZE [native PAGE]), capillary IEF, and SDS-entangled polymer sieving (SDS-PAGE). As an instrumental technique, CE is fully automated and employs on-line optical detection with similar benefits to LC-UV detection. The potential for replacing gel electrophoresis with precise and quantitative methods has spurred the growth of CE in the biotechnology and biopharmaceutical industries.

CE is strictly an analytical technique. The dimensions of the fused silica capillaries limit sample loads to picogram amounts, which precludes its use as a preparative tool. However, this requirement for limited sample amounts is an advantage when using CE as a complement to RPLC. CZE has been remarkably successful for the separation of peptides.[50] Because the separation of peptides in CZE is dependent on differences in mass/charge ratio, the separation selectivity of CZE is distinct from that of RPLC (in which separation is based on differences in hydrophobicity). This orthogonality in separation selectivity predicts that peptides that coelute in RPLC might be resolvable by CZE. This in fact is often the case.[51] Consequently, CZE is used as a companion technique to RPLC to confirm protein identity by peptide mapping. Also, it can be used for rapid assessment of the purity of peaks collected from an RPLC peptide-mapping experiment and to guide the analyst in further characterization of the fraction by mass spectrometric or chemical sequence analysis.

Micellar electrokinetic chromatography (MEKC) and capillary electrokinetic chromatography (CEC) are, as their names imply, chromatographic techniques

performed with CE instrumentation. Both are based on hydrophobic partitioning into a nonpolar phase and use electroosmotic flow (EOF) as a means of transporting the mobile phase through the column. In MEKC, the capillary is filled with an aqueous solution of a surfactant, typically SDS. The nonpolar phase is created by the formation of SDS micelles, which are in rapid equilibrium with surfactant monomers. Micelles and monomers move electrophoretically toward the anode, while EOF carries the bulk solution toward the cathode. Separation is accomplished by differential partitioning of analytes into the micellar phase in combination with electrophoretic contributions dictated by the analyte mass and charge state. MEKC has been occasionally used for peptide separations,[52] where it can offer the benefits of nanoscale separations with a selectivity similar to RPLC. MEKC-like conditions have also been used for protein separations,[53] although the mechanism probably includes contributions from micellar partitioning and protein–surfactant complexation.

CEC employs capillaries packed with conventional microparticulate materials. Most CEC separations have been achieved with reversed-phase packings and aqueous–organic mobile phases. EOF provides the driving force for transporting the mobile phase. In contrast to pressure-driven flow, laminar flow band broadening is eliminated and column back pressure is absent. Thus, CEC separations are characterized by high efficiencies compared to pressure-driven HPLC. Peptide separations have been performed by CEC,[54] but the difficulty in performing gradient elution on commercial systems limits the usefulness of this technique in practical applications.

All three of these capillary techniques (CE, MEKC, and CEC) share two limitations that have hindered their adoption as general replacements for conventional RPLC. First, the nanoliter volumes of sample and mobile phase require the use of on-tube detection. The reduction of detector light path from millimeters in HPLC to micrometers in CE compromises detection sensitivity. Second, changes in the state of the wall in CE and MEKC or of the particle surface in CEC will alter the magnitude of EOF, causing poor reproducibility of migration/retention times and peak areas. Sample adsorption to active sites on the wall or packing is the most frequent cause of drifting EOF. Much work has been done to minimize this problem, including the use of coated capillaries, capillary regeneration techniques, and electrolyte additives. To date, CZE separation of peptides has met with the greatest success because the low-pH electrolytes used for peptide separations suppress silanol ionization and peptide adsorption.

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