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Chromatographic Techniques for the Characterization of Proteins

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1. INTRODUCTION

The improvements in the chromatographic analysis of proteins and peptides during the last decade contributed significantly to the development of (recombinant) pharmaceutical proteins. The availability of these analytical methods for the characterization and purity determination of proteins enabled the improvement of the overall manufacturing process of pharmaceutical proteins.

As with all other pharmaceutical preparations, (recombinant) pharmaceutical protein products must be thoroughly characterized. Protein drugs must meet the same standards of safety, purity, and potency as conventional drugs. The characterization of the structure of a protein is complex because of the presence of a primary, secondary, tertiary, and quarternary structure. Small structural changes in a protein can influence the physicochemical properties as well as the activity and potency of the protein. Different purification steps are used during the manufacturing that may change the structural characteristics of the protein. Such changes may range from minor modifications (e.g., deamidation of an Asp) (Patel and Borchardt, 1990) to unfolding of the protein chain yielding biologically

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inactive molecules. Unfolding may result in the decomposition (such as oxydation and fragmentation) of the protein due to the fact that certain parts of the molecule, normally part of the inner side of the tertiary structure, are exposed to chemicals, water, and so forth.

For the determination of the purity of proteins the detection of impurities is of importance. The impurities are process, host, or product related (Garnick *et al.*, 1988). Product-related impurities can be the result of posttranslational changes or variability but are in most cases decomposition products that are formed during the purification process or upon storage. Table I presents an overview of possible processes that can change the structure of a protein (Geigert, 1989). Recently an extended overview was published on the inactivation of proteins during chromatographic analysis (Sadana, 1992).

The protein can also be changed intentionally. With the introduction of large amounts of recombinant proteins, more and more time is spent on the development of chemical modifications of the proteins in order to change their disposition *in vivo*. Covalent attachment of, e.g., polyethylene glycol (PEG) chains shows promise for increasing the stability and protein solubility. The resulting product contains a mixture of species characterized by a distribution in both the number and position of attachment of the PEG molecules. The situation is even more complicated by the fact that PEG is inherently polydispersal. Characterization of such a pegylated protein is important in order to be able to assure product consistency.

The qualitative and quantitative analysis of a pharmaceutical protein and its related compounds relies on the use of sophisticated analytical methods for demonstrating the structural identity and homogeneity of the proteins to be separated. The application of multiple methods is imperative to ensure that a protein is thoroughly identified and characterized and its purity accurately assessed. For the analysis of pharmaceutical proteins the method must be able to

Table I. Possible Modifications of Proteins

Degradation pathways	Mutation	Posttranslational process	Chemical conjugation
Fragmentation	Site-directed mutagenesis	Glycosylation	Pegylation
Deamidation		Formylation	
Oxidation		Acylation	
Disulfide scrambling		Phosphorylation	
Oligomerization		Sulfatation	
Aggregation			
Cross-linking			
Proteolysis			
Denaturation			

separate compounds that may only differ by one or a few amino acids or functional groups. Considering the low diffusion coefficients of large biopolymers, it is a real challenge to develop analytical chromatographic methods that enable the required separation.

Proteins are ampholytes and therefore the pH and the ionic strength of the mobile phase play an important role in their chromatographic behavior. In addition, temperature, protein concentration, and adsorbent surface characteristics affect the analysis in all modes of interactive chromatography as well as in noninteractive (size-exclusion) chromatography. These parameters are important because the three-dimensional structure of the protein may change when the protein is in a nonphysiological environment and may be sensitive to slight changes in this environment; these parameters also can be used to manipulate the protein structure in order to achieve separation.

This chapter presents an overview of the analytical chromatographic methods used for the characterization of proteins with an emphasis on pharmaceutical proteins. This chapter does not pretend to give a detailed and complete overview on the chromatography of proteins and peptides, but rather presents an overview of approaches used to characterize proteins, including the significant problems that can arise and relevant examples. General information on the chromatography of peptides and proteins, including the column materials used, can be found elsewhere (Mant and Hodges, 1991).

2. REVERSED-PHASE CHROMATOGRAPHY

2.1. General

Reversed-phase chromatography (RPC) is the most frequently applied chromatographic technique for the analysis (especially for the determination of the purity) of peptides and intact proteins as well as for the analysis of peptide fragments obtained after enzymatic or chemical cleavage of the protein (see also section 2.4.3). In RPC the protein generally loses its native structure and denatures (Lau *et al.*, 1984). Denaturation occurs when the hydrophobic and coulombic interactions with the stationary and/or mobile phase are stronger than those that are maintaining the protein native structure. The level of denaturation often depends on the time the sample resides on the column. The elution of a protein is determined by the presence of hydrophobic amino acids as well as by the exposure of hydrophobic regions to the column surface, determined by a sequence of hydrophobic amino acids. The retention of the intact protein is based on the number of hydrophobic residues that interact with the hydrophobic column support and therefore is dependent on whether these hydrophobic areas are present on

the outside of the protein when dissolved in the mobile phase. The less polar residues can be shielded by conformational effects, which results in an exposition of the more polar residues at the protein's surface during the analysis (Nugent *et al.*, 1988). For peptides up to 15 amino acids there is a correlation between the hydrophobicity and retention on a C_4, C_8, and C_{18} bonded phase. With larger compounds also the polypeptide chain length must be taken into account because the length of the chain determines the spatial structure. Using the overall hydrophobicity of proteins (based on retention parameters derived from small peptides), a correlation was found with retention behavior, under denaturing conditions, for proteins ranging in molecular weight from 3.5 to 32 kDa (Mant *et al.*, 1989). The conformation of the protein determines the exposure of hydrophobic regions to the stationary phase, and thus regulates the retention of the protein. Resolution of the denatured protein in RPC is achieved on the basis of the overall hydrophobicity of the sample and is mainly dominated by solvent effects.

Conformational changes on reversed-phase and hydrophobic supports can lead to multiple states of the protein, which can result in multiple or distorted peaks. The control of the peak shape and the number of peaks in protein chromatography require the understanding of equilibria and the kinetic processes involved. One can distinguish three kinetic processes where protein structural changes can affect behavior. In the first case, the changes are slow relative to the chromatographic migration time and the process is apparently irreversible. In such cases several chromatographic peaks, representing native and (partially) denatured compounds, may be observed. In the second case, retention and half-lives are comparable and distorted peaks can be observed. Finally, rapid changes occur and the system may act as though it were behaving ideally, while at the same time significant changes have taken place on the chromatographic surface. In principle one would like to prevent such changes in order to enhance the possibility of analyzing the native biologically active protein (Karger and Blanco, 1989); but in RPC this is almost an impossible task. Therefore, in order to prevent conformational changes during the analysis, it is important to choose the circumstances in such a way that the analyte is present in one single conformation, where in most cases the denaturation of the protein is complete. The unfolding of proteins in a chromatographic system can be controlled by the hydrophobicity of the packing material. Sometimes it is possible to chromatograph proteins under reversed-phase conditions in the folded conformation by carefully choosing the column material (Hanson *et al.*, 1992).

Changes in the quaternary structure often result in a significant change in the molecular weight or hydrophobic properties of the molecule because of dissociation or aggregation of the molecule. Oligomers are high-molecular-weight compounds and in most cases more hydrophylic when compared with the monomeric proteins. Oligomers are therefore easily separated from the corresponding monomers/subunits by RPC.

Peptide mapping is often used in the quality control of pharmaceutical proteins and is then used to detect lot-to-lot variations. Isolation of peptides following the chromatographic analysis and the determination of the amino acid sequence of these peptides are tools that are used to determine the full sequence of a protein. Peptide mapping of proteins is used to generate information on the primary structure of proteins. Peptides produced from proteins by two or more different chain cleavage methods and analyzed by RPC give information on the identity of the protein. Peptide mapping consists of an enzymatic (e.g., trypsine) or chemical [e.g., cyanobromide (CNBr)] digestion often preceded by reduction and carboxymethylation of free sulfhydryl groups with iodoacetic acid. With peptide mapping it is possible to detect small changes in the protein, e.g., replacement of a single amino acid. The replacement of one amino acid in a peptide has in general a dramatic influence on the retention behavior on RPC. In general, RPC gives a high-resolution separation of tryptic digests. A typical run takes between 30 and 60 min in order to obtain optimum separation of the peptide mixture. The use of short reversed-phase columns packed with 2-μm pellicular particles can reduce the run time of a peptide to 15 min or less at elevated temperatures, as was shown by Kalghatgi and Horváth (1988) for recombinant tissue plasminogen activator and recombinant human growth hormone (rhGH). For the determination of impurities the method is less suitable. In general, it is difficult to identify protein impurities below 10% by peptide mapping using RPC with UV detection, as was shown by Clogston *et al.* (1992) for recombinant human granulocyte colony stimulating factor.

2.2. Stationary Phase

The stationary phase used in RPC contains a hydrophobic surface and often consists of coated silica, polymeric bonded silica, and cross-linked polymers (Mant and Hodges, 1991; Kennedy *et al.*, 1989). The matrices are in most cases coated with hydrophobic ligands such as (in increasing order of hydrophobicity), C_2, C_4, C_8, C_{18}, phenyl, and cyanopropyl groups. In general, the retention time increases with increasing hydrophobicity of the ligands. Decreasing particle size results in an increasing surface area, and thus in a higher resolution.

The most frequently used column materials are silica based with a pore size of 100 to 300 Å. These phases are only stable at pH 2 to 8. The phases consisting of a polymer coating on silica are more inert to chemical degradation when compared with the conventional silica bound packings. These stationary phases are stable in the pH region of 2 to 10.

Replacement of the silica as base of the reversed-phase material by a polymer increases the stability further. An example is the C_4, C_8, and C_{18} anchored to the

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