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Review Article

HPLC METHOD DEVELOPMENTFOR PROTEINSAND POLYPEPTIDES

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ABSTRACT

In the pharmaceutical field, there is considerable interest in the use of peptides and proteins for therapeutic purposes. High performance liquid chromatography (HPLC) and its methods of complex peptide or protein mixtures remains a general method of choice because of the resolution it provides. Unlike small organic molecules whose chromatographic behavior is described by a finite partitioning equilibrium between the stationary phase and the mobile phase, proteins and peptides typically do not exhibit such an effect. Instead, they exhibit an adsorption phenomenon in which the polypeptide is adsorbed onto the stationary phase and elutes only when the solvent strength of the mobile phase is sufficient to compete with the hydrophobic forces keeping it there. For this reason, elution of peptides or proteins from reversed-phase supports is by gradients of increasing solvent strength. There are other differences that one needs to be aware of in order to develop HPLC methods for separations of proteins and peptides as efficiently as possible.

Keywords: Proteins, Peptides, HPLC.

INTRODUCTION

The novel trend in drug development is the design and development of biomolecule drugs. Nowadays the pharmaceutical industry has focused its attention on manufacturing protein and polypeptide drugs. However, in order to use these molecules as active pharmaceutical ingredients (API), the purified compound must be available and also a method of analysis must be developed for accurate quantification of the component. The developed method thereafter must be validated following the International Conference on Harmonization (ICH) Q2A and Q2B guidelines[1].

High-performance liquid chromatography (HPLC) is now firmly established as the premier technique for the analysis and purification of a wide range of molecules. In particular, HPLC in its various modes has become the central technique in the characterization of peptides and proteins due to its ease of use, wide range of selectivity, High recoveries and excellent resolution[2]. Exactly these features made HPLC technique the first choice when dealing with biomolecules.

Protein molecules are characterized with large molecular weight and presence of multiple functional groups, which make their HPLC analysis quite different than the analysis of small molecules. However, today's versatility of HPLC modes, columns, detectors and solutions has simplified the process of method development.

Protein Basics[3]:

Protein molecules are generally large molecules with a molecular weight greater than 5000 Da. When developing a **HPLC** method for proteins, one must know the primary amino acid sequence and tertiary structure of the target protein. The primary amino acid sequence gives us information on which functional groups are present in the molecule and in which quantity. Peptides and proteins interact with the chromatographic surface in an orientation specific manner, in which their retention time is determined by the molecular composition of specific contact regions. For larger polypeptides and proteins that adopt a significant degree of secondary and tertiary structure, the chromatographic contact region comprises a small proportion of the total molecular surface. Hence, the unique orientation of a peptide or protein at a particular stationary phase surface forms the basis of the exquisite selectivity that can be achieved with HPLC techniques[2].

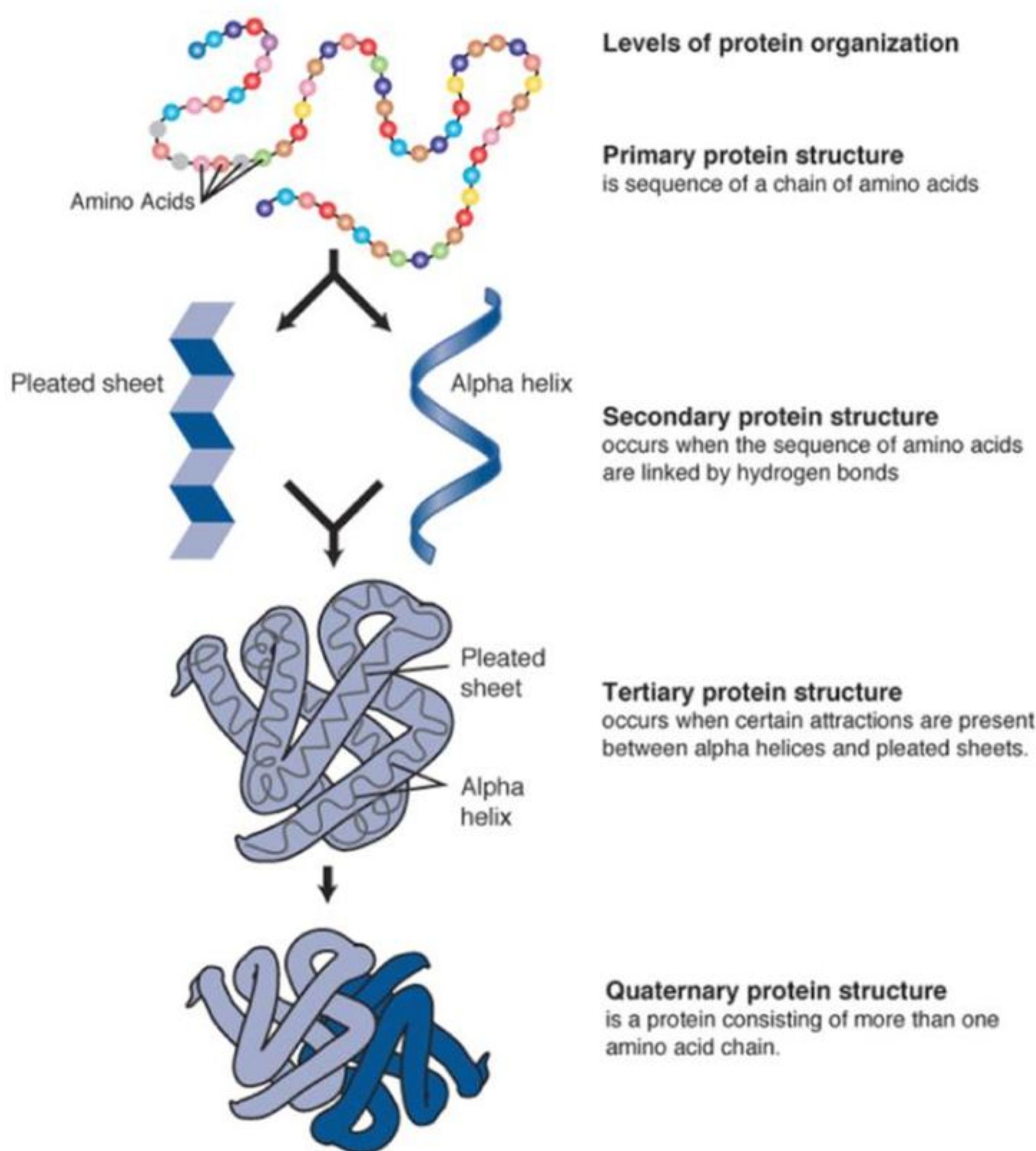

Figure 1: Four degrees of Protein structure[4]

When the protein molecule unfolds, interior hydrophobic amino acids side chains will be exposed, leading to increase in retention time and decrease in separation efficiency. There are numerous factors that contribute protein unfolding[5], such as: stationary phase hydrophobicity, mobile phase polarity, mobile phase pH, ion-pairing agents, detergents and even oven temperature[6].

HPLC MODES FOR PROTEIN ANALYSIS

RP-HPLC Mode[7]:

The Reverse Phase Liquid Chromatography[8] (RP-HPLC) is the most popular modes of separation or purification of proteins. The main problem when dealing with protein molecules with molecular weight larger than 20,000 Da is the fact that these molecules then to unfold more than the smaller ones. In that case the molecule's interior portion with its high concentration of hydrophobic[9,10] amino acid residues will lead to interaction to the non-polar ligates on the column. In many cases not all of the protein undergoes unfolding and the same protein will elute in two or more bands.

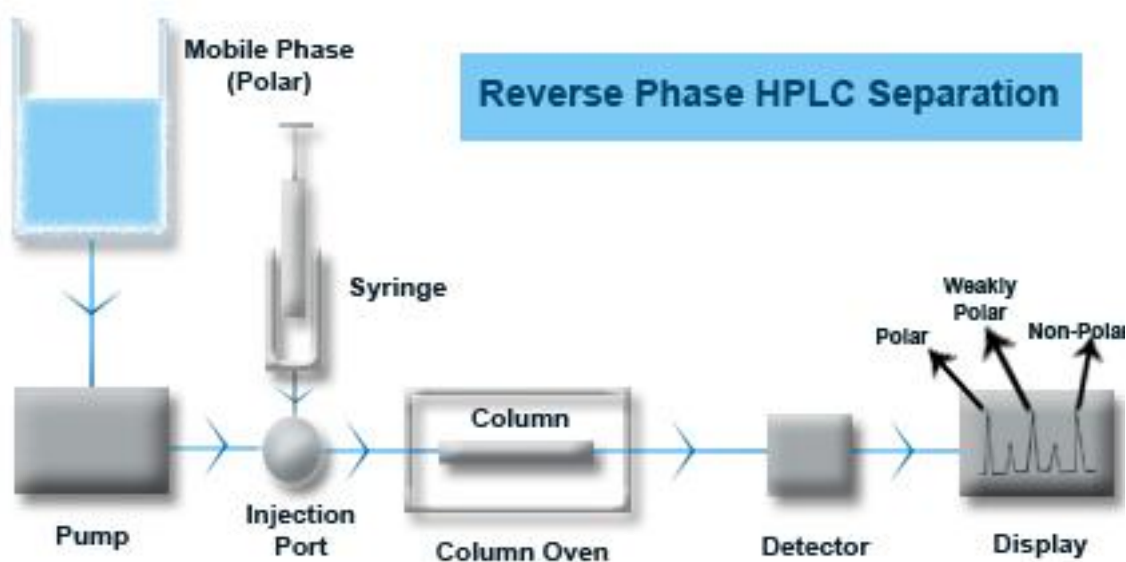

Figure 2: Reversed-Phase Chromatography[11]

There are several factors that need to be taken into consideration when developing a RPLC[12] method for analysis of protein molecules. Those are stationary phase support, ligates[13], surface tension, mobile phase polarity, temperature, pH and mobile phase additives. The aspects of the column that are particularly important to protein analysis are protein size and particle size. The RPLC column columns range from 1.5-25μm.

The minimum pore diameter that is acceptable for protein-ligate interaction is four times the protein's diameter. The most suitable combination of mobile phase is water-organic eluent. The use of aqueous-based mobile phases is preferable because non-aqueous phases contribute to protein denaturation.

The use of mobile phases with low pH is quite favorable because it stabilizes the ionizable side-chains.

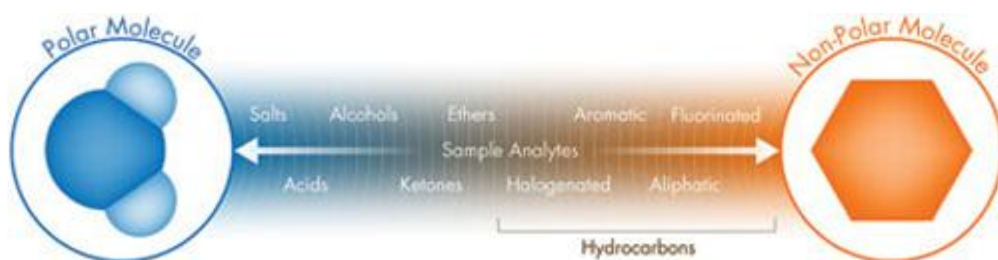

Figure 3: Sample polarity[4]

Hydrophilic Interaction Chromatography (HILIC)[14]:

The mode of chromatography utilizing hydrophilic interactions to separate solutes is referred to as normal-phase chromatography. It is characterized by its use of polar stationary phases including bare silica in combination with a polar eluents. Under typical HILIC conditions, retention of solutes increases with increasing solute hydrophobicity and decreasing mobile phase polarity and specific interactions of a hydrophilic solute with a hydrophilic stationary phase which are responsible for this particular chromatographic behavior.

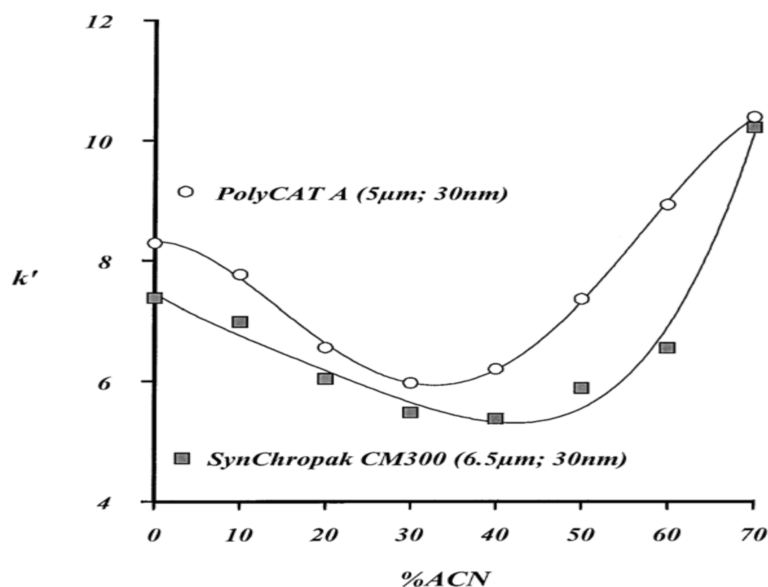

Figure 4: Effect of organic solvent concentration on protein retention[14].

Above graph shows the retention of a basic peptide (expressed as k′) as a function of acetonitrile content, whereby an increase in the acetonitrile concentration corresponds to a decrease in solvent polarity.

The polar packing material is a commonly used weak cation exchanger having carboxymethyl groups, and the solute is eluted by an increasing linear salt gradient.

Stationary Phase packing materials having average particle diameters of 5–10 μm and a pore size between 30 and 150 nm with successful employed in the pH region between2.0 and 8.0. Elution of peptides and proteins in HIC is accomplished by increasing the polarity of the mobile phase. This can usually be done in two ways, namely by increasing the amount of water in the eluent, i.e., using a decreasing organic solvent gradient, or by running an increasing salt gradient. Solvents commonly used are acetonitrile, methanol, and 2-propanol at concentrations of up to 85%.

Ion Exchange Chromatography:

Ion exchange chromatography (IEC) is a common technique for separation of charged molecules. Protein molecules usually have multiple charged functional groups; therefore this is one of the most suitable **HPLC** modes for analysis. The retention exists as a result of the electrostatic interaction between analyte and the ligand. In singly charged molecules, the elution is linear in response to pH change. Whereas when multiple interactions occur the elution becomes nonlinear with pH.

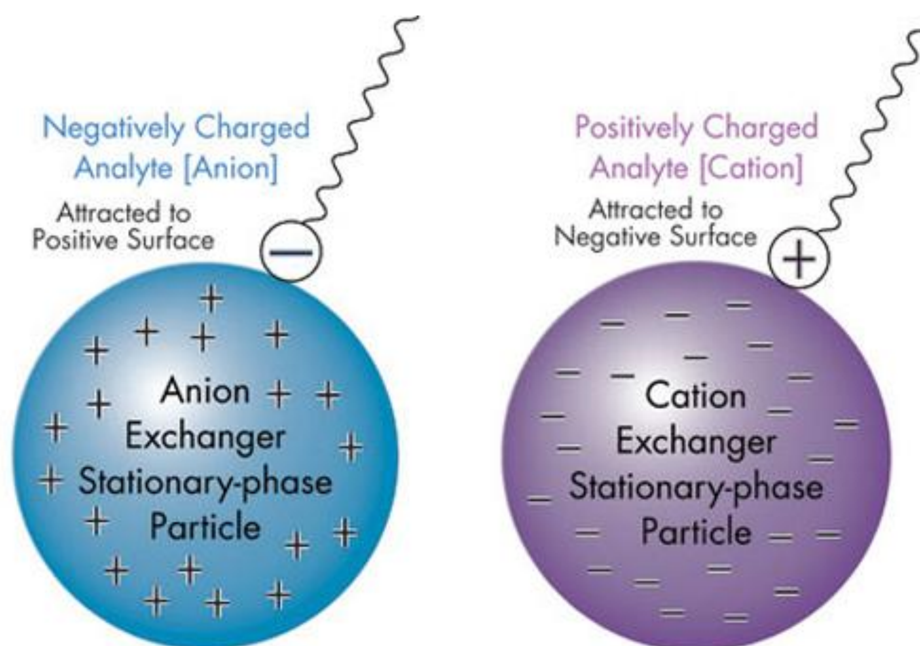

Figure 4: Principle of Ion Exchange Chromatography[4]

Acidic proteins are analyzed on an anion exchange column, and basic proteins are analyzed on a cation exchange column. Native proteins are negatively charged, thus should be analyzed on a cationexchange

column[15,16].

The column consists of the stationary phase resin with immobilized strong or weak cation or anion exchangers. The exchangers are divided into strong or weak. Strong anion or cation exchangers' ability to affect an exchange does not change with the change in pH. Therefore, separation of proteins with the use of strong exchangers is more straightforward. On the other hand the exchange capacity of weak exchangers is variable, and changes as mobile phase pH approaches the pKa of the exchanger's functional group.

Analyte Type	Weak ACID e.g., $pK_a \sim 5$		Strong ACID	Weak BASE e.g., $pK_a \sim 10$		Strong BASE
Charge State vs. pH*	No charge at pH < 3	− [anion] at pH > 7	− [anion] Always Charged	+ [cation] at pH < 8	No Charge at pH > 12	+ [cation] Always Charged

Stationary Phase Particle	Strong Anion Exchanger	Weak Anion Exchanger e.g., $pK_a \sim 10$		Strong Cation Exchanger	Weak Cation Exchanger e.g., $pK_a \sim 5$	
Charge State vs. pH*	+ Always Charged	+ at pH < 8	No Charge at pH > 12	− Always Charged	No Charge at pH < 3	− at pH > 7
Mobile Phase pH Range						
to Retain analyte [capture]	pH > 7	pH < 8		pH < 8	pH > 7	
to Release analyte [elute]	pH < 3	pH > 12		pH > 12	pH < 3	

Figure 5: Ion-Exchange Guidelines[4]

Gradient of increasing salt concentration is the minimum requirement for an IEC mobile phase. The gradient should increase the salt concentration very gradually, because small change in ionic strength may have a large effect on retention since many groups on a protein may be affected at one time.

The salt type and pH of the mobile phase have major effect on selectivity. Such adjustments may be useful for increasing or decreasing retention time. Sometimes, small amounts of organic modifier may be added in order to minimize hydrophobic interactions with the stationary phase.

Size Exclusion Chromatography:

The Size Exclusion Chromatography (SEC) has very limited use when discussing the analysis of protein molecules[17]. Its low resolution makes it unsuitable for the purpose of identification or quantification of protein molecules. Thus this **HPLC** mode is used only when dealing with protein aggregates or protein molecules with very high molecular weight.

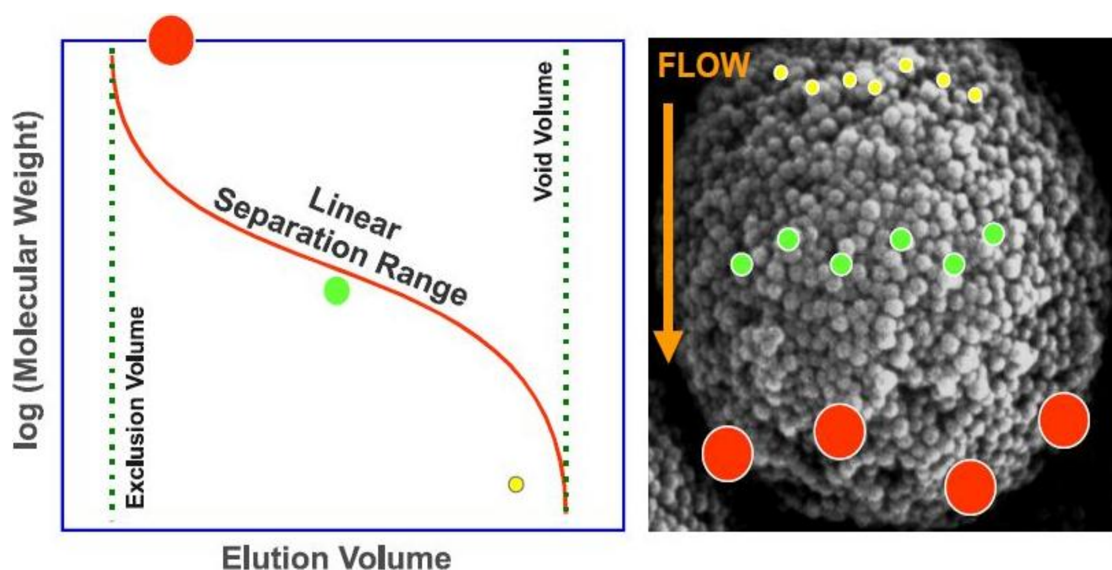

Figure 6: Mechanism of SEC

The SEC technique gained its name because it separates based on molecular size. However, in the case of protein molecules we do not discuss the molecular size but yet another parameter the hydrodynamic volume. Due to the aqueous nature of the SEC mobile phase, polar interactions between the water molecules and protein molecules may result in creating water-protein associates. The association gives the molecule an apparently larger molecular size. However, the degree of binding water molecules is not linear with the increase of the protein molecular weight, because proteins have different degrees of hydration[18]. Therefore, predicting the elution order based solely on molecular weight becomes quite difficult.

Molecular weight range	Pore size
100 - 1000	50 A
250 - 2500	100 A
1,000 - 18,000	500 A
5,000 - 40,000	10^3 A
10,000 - 200,000	10^4 A
50,000 - 1,000,000	10^5 A
200,000 - > 5,000,000	10^6 A
500,000 - ~20,000,000	10^7 A
~1,000 - 10,000,000	Mixed Bed - High
~100 - 100,000	Mixed Bed - Low

Figure 6: Column pore size selection based on molecular weight of the protein

Finally, there are several method development parameters that need to be taken into consideration for SEC: column type, buffer choice, salt concentration, organic modifier and flow rate. The SEC columns come with inorganic or organic packing. Columns with inorganic oxide packing are suitable for use in an acidic environment (P_H8).

Detector selection:

There are several detectors[19] that can be used for the **HPLC** analysis of protein molecules, UV, fluorescent, electrochemical and evaporative light scattering detector (ELSD). The UV detector is the most popular detector for the protein analysis. Usually proteins are detected at 210-220nm due to the absorbance by the peptide bond. The protein molecule is expected to have larger peak at 210nm and a smaller one at 280nm due to the aromatic amino acid side chains that absorb at 280nm. When choosing an UV detector for protein analysis is advisable to use a photodiode array (PDA) detector. This detector offers increased sensitivity and multiple wavelength analysis suitable for determination of peak purity[20].

The fluorescent detector is suitable for proteins that have native fluorescence or that can be easily made to fluoresce through derivatization. The positive side of the fluorescent detector is that it can be as much as 100 times more sensitive than a UV detector.

If the protein molecule is present in its oxidized or reduces form, than the electrochemical detector

(EC) is the one to choose. The EC detector is one of the most sensitive and selective **HPLC** detectors available. However, the EC detector requires the use of electrically conductive mobile phases.

The ELSD detector works on the principle of evaporation (nebulization) of the mobile phase followed by measurement of the light scattered by the resulting particles. Unlike the UV detector where chromophores are required, the ELSD response is related to the absolute quantity of the analyte present and it depends only of its capability to be nebulized without causing damage to the protein molecule. Another commonly used detector is the laser light scattering detector (LLSD) that makes measurements in solution as opposed to particles suspended in a gas. The scattered light is measured at multiple angles and using the proper mathematical transformations, the mass of an analyte can be determined without the use of reference standards. [5]

CONCLUSION

There are several **HPLC** modes that one analyst can use in order to analyze the needed protein molecules, those are: RPLC, IEC and SEC. These various modes have many variables that should be taken into consideration when developing a method for analysis of protein molecules. These different **HPLC** modes come with different detectors. Detectors can be selected depending on the purpose of the method and depending on various protein characteristics.

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