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Mini-review on Chromatography of Proteomics

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Abstract

This literature mini-review focuses on chromatography and its application for separation of proteomics, Factors affecting RP-HPLC of proteins and their peptide mapping. Only two drugs are cited in the mini-review, which are erythropoietin hormone and carbetocin where mode of action and analytical techniques used for their determination were described.

Keywords: Mini-Review; Chromatography; Proteomics; Erythropoietin Hormone; Carbetocin.

Overview on Chromatography

HPLC has become the most significant analytical tool in the past fifty years. Its significance revolutionized to cover larger areas of analytical chemistry of proteins. HPLC became awell-established technique for isolation, analysis, and structural elucidation of peptides and proteins. Development of new stationary phase supports increased the capability of HPLC to separate closely related structural proteins and peptides without losing the biological activity of such proteins. Among the various types of stationary phases (including size exclusion chromatography, ion-exchange chromatography), reversed phase liquid chromatography (RPLC) is the most powerful tool for the analysis of proteins and peptides due to several factors [1]. First of all, their excellent separation of close structurally related molecules can be achieved with good resolution. Their selectivity can be adjusted easily via changing the mobile phase characteristics. RPLC has a high speed of analysis without loss of efficiency. And finally they have high reproducibility and recovery results. RPLC is used for analytical as well as preparative applications for isolation of proteins and peptides obtained from different synthetic, biotechnology or biological sources. However, RPLC can cause denaturation of proteins and hence can reduce the ability to recover the protein molecule in a biologically active state. That's why RPLC is employed less for the isolation of proteins when recovery of the protein in a biologically active form is required (e.g. protein purification).

Mechanism of separation of proteomics on RPLC

Separation of peptides or proteins on RP-column is considered either by adsorption of the molecules on the hydrophobic stationary phase or by partitioning between mobile and stationary phases [2]. Hydrocarbon groups attached on silica surface render it hydrophobic. Since peptides and proteins are large molecules, so only a portion of protein is adsorbed on the hydrophobic surface until the concentration of the organic solvent reaches a level enough to cause desorption. By increasing solvent strength of the mobile phase, attractive forces are weakened and the solute is eluted. This adsorption/desorption mechanism gives rise to difference in behavior of proteomics than smaller molecules on RP-columns. While smaller molecules change retention slowly as a function of organic solvent concentration, protein molecules retention changes abruptly once the required organic solvent concentration is reached.

This fact has two main results [3] the first is gaining sharper peaks for proteins and peptides. The second is that the application of gradient solvent programming is generally

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superior to isocratic elution because separation is achieved in shorter time frame and avoiding the peak broadening of later-eluting peaks and thus sensitivity increased.

The second assumed mechanism is the partitioning of the molecules between the stationary and organic phases. The selectivity will then depend on the partition coefficient of the solute. Both retention principles have their supporting factors; however the effective process is largely dependent on the bonded RP packing and the size and conformation of the solute.

Factors Affecting RP-HPLC of Proteins

Separation of proteins and peptides on RP-LC is affected mainly by two systems; the mobile phase and the stationary phase. Factors related to each phase will be discussed briefly.

Mobile phase: Proteins are desorbed from the hydrophobic bonding sites of the stationary phase when the organic solvent reaches the precise concentration required for desorption of specific molecule. The mobile phase used for RP-LC is composed of aqueous part and organic solvent part. Among the organic solvents, acetonitrile (ACN) is the most widely used organic solvent in peptides RPLC because of several factors including volatility, low viscosity, low UV-cut, low backpressure and wide applications[4]. However; ethanol (EtOH) is used for large scale protein purification due to its lower toxicity [5].The aqueous art of the mobile phase is usually a buffer of low concentration of strong acid or salt. Additives in the aqueous phase affect elution time, order (selectivity) and also improve peak shape. Trifluoroacetic acid (TFA) is the most widely used ion-pairing reagent in proteomics separation. At concentration about 0.1%, it can enhance peak shapes of separated proteins. Phosphoric acid and phosphates can be used also as additives, but they are not volatile making them very difficult to remove from the elates [3].

The technique used also affects proteins chromatographic separation. Polypeptides are almost eluted using a solvent gradient. The slower the rate of organic solvent percentage change, the better the resolution between different molecules of the mixture. The flow rate has very low effect on the separation of proteins. However, the total gradient volume must be constant so that we can maintain the resolution.

Stationary phase: the column is the core where all separations take place. Choice of column matrix is very important for optimization of chromatographic separations. Some of the problems associated with RP-LC of proteins such as poor recovery and peak shape are related to the chosen column [6]. While selecting the best column, we have to consider some variables. Bonded stationary phases (for instance C18) is the most widely used ligands, however C4 & C8 are used also for highly hydrophobic molecules. The type of n-alkyl substitution affects the selectivity of separation of proteins. It also affects recoveries. Higher recoveries were obtained on shorter alkyl chains (e.g. C8) [7]. Two dimensional LC of proteomics is also experimented using different types of stationary phases [8]. The dimensions of the chromatographic column have some other effects. Column length has little or no effect on protein separation. Short columns separate proteins as well as long columns. Moreover, longer columns can have adverse effect on recovery. The choice of internal diameter (ID) of the column is based on the sample capacity and required sensitivity. Usually, 4.6 mm ID is used. However for smaller sample amounts, smaller ID of 1-2 mm can be used to increase the sensitivity [9]. The pore size is very important in LC separation of proteins. The solute molecular diameter must be at least 1/10 the pore size to avoid restricted diffusion and also to allow interaction with the total surface of the stationary phase [9]. Wide pore silica allows proteins and larger peptides to enter the pore and fully interact with the surface, resulting in better peak shape and enhanced resolution. Usually 100-300 Å pore size is acceptable for peptides. The Stationary phase geometry has certainly several effects on separations [10]. Theory predicts that column performance should increase with decreasing particle size. On decreasing the particle size, improved peak shape is attained [9].

Peptide mapping

One of the most widely used peptide separation technique is peptide mapping. Peptide mapping is a means of characterization of proteins, especially those intended for manufacturing as therapeutic drug products. This technique involves two steps; the first is breaking the protein into pieces by breaking its backbone using protease enzymes or trypsin. The second step is analyzing such cryptic digests by HPLC to draw a map of peptides resulting from this cleavage.

Much information can be obtained by peptide mapping of protein fragments such as ensuring the quality and purity of therapeutic proteins by comparing the peptide map with a reference protein of high purity. Protein tertiary structure can be identified by verifying the disulfide bond linkages. Protein tertiary structure is important to identify its therapeutic efficacy. Peptide mapping also provides valuable information about protein stability and determination of degradation products. Protease digests can be used for identification and quantitation of proteins on studying proteomics [11]. As the number of peptides separated during peptide mapping increases, more information can be obtained.

Examples of Pharmacologically Active Proteins

Erythropoietin hormone [12,13] has a molecular formula of $C_{815}H_{1317}N_{233}O_{241}S_5$ and molecular weight of 30400 Dalton. Erythropoietin is a 165-amino acid erythropoiesis-stimulating glycoprotein manufactured by recombinant DNA technology. Erythropoietin is a glycosylated protein hormone and a hematopoietic growth factor. It is secreted primarily by the kidneys. It regulates erythropoiesis by stimulating the release of reticulocytes into the circulation and the synthesis of hemoglobin. The release of erythropoietin is promoted by

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hypoxia or anaemia. Erythropoietin is used in the treatment of symptomatic anaemia associated with chronic renal failure in adult and pediatric patients. Several papers described the peptide mapping of erythropoietin hormone [14-16]. Carbetocin (CBT) [12,13] is another polypeptide that has the molecular formula of $C_{45}H_{69}N_{11}O_{12}S$ and molecular weight of 988.17 g/mol. Its IUPAC name is(2S)-N-[(2S)-1-[(2-amino-2-oxoethyl)amino]-4-methyl-1-oxopentan-2-yl]-1-[(3R,6S,9S,12S,15S)-6-(2-amino-2-oxoethyl)-9-(3-amino-3-oxopropyl)-12-[(2S)-butan-2-yl]-15-[(4methoxyphenyl) methyl]-5,8,11,14,17-pentaoxo-1-thia-4,7,10,13,16-pentazacycloicosane-3-carbonyl]pyrrolidine-2-carboxamide (**Figure 1**).

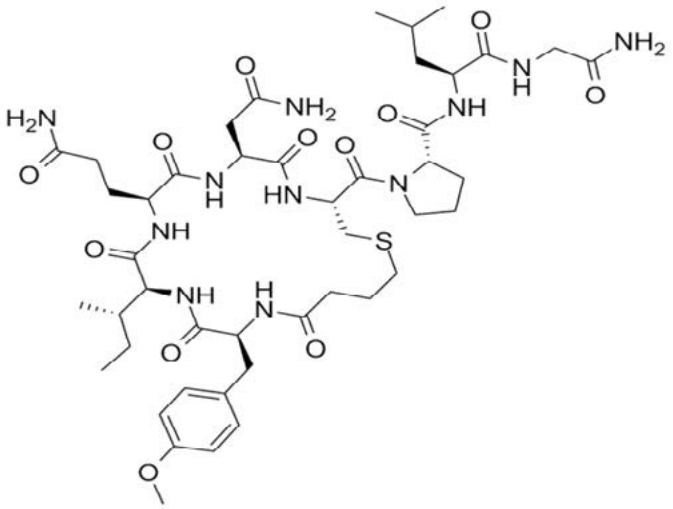

Figure 1: Carbetocin chemical structure.

CBT is a white fluffy powder. It has low water solubility of about 0.03mg/mL and pKa value of 11.4.CBT is a synthetic oligo-peptide containing a sequence of eight amino acids joined by peptide bonds. CBT is an analogue of oxytocin (OXT) with a longer duration of action. OXT is a cyclic nonapeptide (9 amino acids) having the structure of the hormone produced by the posterior lobe of the pituitary gland that stimulates contraction of the uterus and milk ejection in mammals amino acids sequences are shown in (**Figure 2**). CBT binds to oxytocin receptors that are present on the uterine smooth muscles, resulting in rhythmic uterine contractions, increased frequency of existing contractions, and increased uterine tone. The oxytocin receptor content of the uterus is low in the non-pregnant state, and increases during pregnancy, reaching a peak at the time of delivery. CBT is used for the prevention of uterine a tony and excessive bleeding after caesarean section under anesthesia. CBT dose is a single 100 µg injection that can be given by slow intravenous injection reviewing literature revealed liquid chromatographic methods for the determination of CBT in presence of its impurities using phosphate buffer and ACN as mobile phase at flow rate of 1.2 mL/min [17]. Column temperature was set at 60°C and the detection wavelength was set at 220 nm. Another method was reported using ethanol instead of ACN as greener mobile phase modifier [15].

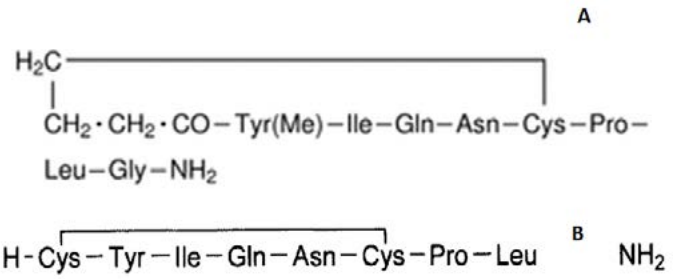

Figure 2: Carbetocin (A) and Oxytocin (B) amino acids sequence.

Conclusion

This literature mini-review is introducing brief summary about chromatography and its application for separation of proteomics, Factors affecting RP-HPLC of proteins and their peptide mapping.Only two drugs are cited in the mini-review which are erythropoietin hormone and carbetocin where mode of action and analytical techniques used for their determination were described.

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