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Biopharmaceutical Analysis by HPLC: Practices and Challenges

Golshan Mahdi[1], Ashkan Alamdary[2], Hooman Kaghazian[2], Nayebali Ahmadi[3], Rajab Mardani[4,*], and Alireza Gholami[2,*]

[1]Pharmaceutical Sciences Branch, Islamic Azad University, [2]Department of Research and Development, Production and Research Complex, Pasteur Institute of Iran, [3]Department of Medical Lab Technology, Proteomics Research Center, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, [4]Department of Viral Vaccine, Research and Production Complex, Pasteur Institute of Iran, Tehran, Iran

High-Performance Liquid Chromatography (HPLC) is an essential analytical technique in the biopharmaceutical industry, crucial for the separation, identification, and quantification of complex biological molecules such as monoclonal antibodies and recombinant proteins. It plays a vital role in assessing the purity, potency, and stability of biopharmaceutical products, which are critical for regulatory approval. HPLC offers high resolution and sensitivity, allowing for the detection of small quantities of compounds in complex samples. Its versatility is evident in various modes, including reversed-phase, ion-exchange, size-exclusion, and affinity chromatography. However, challenges remain, such as selecting the appropriate stationary phase, addressing peak overlapping and matrix interference, and optimizing operational parameters like flow rate and mobile phase composition. Standardization and method validation are essential for ensuring reproducibility, accuracy, and regulatory compliance in HPLC analyses. The need for reliable reference materials and calibration methods is also a significant challenge. Recent advancements in HPLC technology, including ultra-high-performance liquid chromatography (UHPLC) and hybrid systems that integrate HPLC with mass spectrometry, are helping to overcome these challenges by enhancing sensitivity, resolution, and analysis speed. In summary, as biopharmaceutical products grow more complex, HPLC's role will continue to evolve, highlighting the need for ongoing research and development to refine this critical analytical tool.

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Corresponding Author:
Alireza Gholami
Department of Research and Development, Production and Research Complex, Pasteur Institute of Iran, Tehran 3159915111, Iran
Tel: +98-9123486080
Fax: +98-2634906110
E-mail: a4gholami@yahoo.co.uk

Rajab Mardani
Department of Viral Vaccine, Research and Production Complex, Pasteur Institute of Iran, Tehran 3159915111, Iran
Tel: +98-9123381958
Fax: +98-2634916110
E-mail: rajabmardani@yahoo.com

INTRODUCTION

High-Performance Liquid Chromatography (HPLC) is essential in the analysis of protein drugs, playing a critical role in ensuring their quality, safety, and efficacy. As a fundamental analytical tool, HPLC facilitates precise characterization and quality control of protein-based pharmaceuticals. Through careful method development and validation, optimization of analytical conditions, and compliance with regulatory standards, HPLC guarantees accurate and reliable analysis, which is crucial for the successful development and commercialization of these products. Given that proteins are complex molecules with varying properties, optimizing chromatographic conditions is vital for effective analysis. Gradient elution is frequently utilized to improve resolution and minimize analysis time. Key parameters such as flow rate, gradient profile, and column temperature must be meticulously optimized to achieve the desired separation and ideal peak shape.[1]

The selection of stationary phase, mobile phase, and detection method is also crucial, with certain options being more favorable than others. For biopharmaceutical applications, reversed-phase chromatography (RPC) is com-

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monly employed due to its efficiency in separating proteins, peptides, and other biomolecules. The choice of stationary phase, such as C18 columns, is based on the specific characteristics of the biopharmaceutical being analyzed.[2] The mobile phase typically consists of a mixture of water and organic solvents, often supplemented with additives like trifluoroacetic acid (TFA) or phosphoric acid to enhance separation. In summary, HPLC is a vital technique for the analysis of protein drugs, ensuring their quality and effectiveness through meticulous method development and optimization. This overview highlights the critical aspects of HPLC application in the biopharmaceutical industry, focusing on the importance of selecting appropriate chromatographic conditions to achieve optimal results. Table 1 outlines the various chromatographic methods used in biopharmaceutical analysis, highlighting their specific purposes, key features, advantages, and associated challenges to aid in the selection of appropriate techniques for protein and glycan characterization.

CONCEPTS OF THE AFFINITY CHROMATOGRAPHY

Affinity chromatography is a technique in which, the columns are composed of a solid support matrix embedded with immobilized ligands with a polymeric bead such as dextran, acrylamide or agarose. There are a variety of ligands including specific antibodies, protein A, G and L, as well as protein tags and other specific groups of biomolecules which specifically bind to the target protein.[3] Protein A specifically targets the Fc region of antibodies, situated between the CH2 and CH3 domains, which makes it a widely utilized platform for antibody purification. When attached to polymeric beads, protein G and protein A form highly effective and user-friendly chromatography media for routine antibody purifications. Typically, the resins used in affinity columns possess hydrophobic characteristics, reducing nonspecific protein interactions and enhancing operational precision. In practice, various biological fluids, from cell culture media to other biological samples, can be applied to the column using protein A. Therefore, it is essential for affinity columns to be able to endure a wide range of operational conditions that are required for different types of samples.[4]

Using a Protein A affinity column, samples are introduced to a column equilibrated with mobile phase buffers with an approximate pH of 7.5. To elute the target protein, a low pH mobile phase containing a 0.02 M phosphate buffer (approximately pH 3-4) is employed, typically resulting in a single peak in the chromatogram. A Neutralizing buffer containing 1 M Tris-HCl, pH 9.0 should be employed for sample collection to avoid protein conformation change due to low pH. For titer determination, a standard curve could be generated from standards injected onto the column, and the titer of the sample would be calculated based on this curve. To ensure calibration accuracy, preparation of the standards from the same protein type as the sample would be essential. It is noteworthy to practice this experiment particularly for quantifying low titer samples such as those obtained from the early-phase cell culture during bioprocess development.[5]

1. Affinity chromatography method and applications

For laboratory-scale protein purification, affinity chromatography can be conducted using gravity in hand-crafted columns or, to achieve faster flow rates, with the aid of bench-top centrifuges. Various protein purification kits featuring different affinity columns are commercially available from multiple sources. Specifically, in relation to HPLC system applications for titration studies, Protein A

TABLE 1. Comparative analysis of chromatographic methods in biopharmaceuticals

Method	Target	Key features	Advantages	Challenges
Affinity chromatography	Purification of proteins (e.g., mAbs)	Utilizes specific interactions (e.g., protein A affinity)	Single step purification; scalable	Low purity/yield; complex tag removal
Hydrophilic interaction liquid chromatography (HILIC)	Glycan analysis	Uses porous packing materials; labels glycans	Retains hydrophilic compounds; versatile	Limited to hydrophilic compounds
Reversed-phase liquid chromatography (RPLC)	Peptide/protein separation	Based on hydrophobicity; uses organic solvents	High resolution; compatible with ESI-MS	Issues with larger proteins; peak broadening
Hydrophobic interaction chromatography (HIC)	Analyzing proteins under native conditions	Focuses on hydrophobic properties	Preserves protein activity; useful for ADCs	High salt concentrations complicate coupling to MS
Ion exchange chromatography (IEC)	Separation based on charge	Utilizes charged media; separates based on pH	Effective for a variety of biomolecules	Limited resolution for some protein variants
Size exclusion chromatography (SEC)	Separation based on size	Larger molecules elute first	Non-destructive; good for complex mixtures	Limited to size differences; low resolution for similar-sized molecules

affinity columns designed for monoclonal antibodies (mAbs) are widely accessible.

Typically, these columns are operated at ambient temperatures up to 35℃ and are equilibrated with a mobile phase buffer at a pH of approximately 7.5. Analysis times can be reduced to as little as two minutes when utilizing relatively high flow rates, typically between 3 to 5 mL/min. To elute the target protein from the Protein A affinity column, a low pH mobile phase (around pH 3-4) is generally employed. To prevent protein degradation caused by exposure to low pH conditions, it is crucial to add a neutralization buffer to the elution fractions immediately following protein separation. Alternatively, a neutralizing solution can be pre-added to the collection containers or sample vials to ensure that samples are neutralized promptly upon collection. This practice is vital for maintaining the integrity and functionality of the purified protein.[6] When we want to examine low-abundance proteins, it is often necessary to first remove high-abundance proteins before analysis. This allows low abundance proteins to be effectively enriched and also allows more of them to be identified and quantified. Previous research has shown that removing 7 or 14 proteins with higher abundance leads to a 25% increase in better identification of the investigated proteins.[7] Furthermore, affinity chromatography is now widely used in several "omics" studies (e.g., proteomics, metabolomics, and genomics) and is used among other methods, to develop high-throughput screening methods for potential drugs.

A successful affinity purification starts with precise selection of chromatography media, format, equipment as well as purification methods. Various factors affecting those choices include the purpose of the purification, the required purity, the purification scale, and yield. Antibodies can be adsorbed and separated directly using protein A or G, due to the specific interaction of antibodies with these proteins. Once the antibodies are immobilized on the basis of protein A or G, they can be easily replaced by using a strong detergent and the protein A or G is reconstituted for reuse and purification of new antibodies. Generally, this method is used when a high capacity/high activity resin is required. If more permanent immobilization would be desired, the antibodies could be attached to matrices using carbodiimide or dimethylpimlimidate.[8] Purification of proteins can be simpler and more effortless, if the target protein is labeled using a known peptide sequence, i.e. a protein tag. This tag can be of various sizes, from a short sequence of amino acids to the whole domains or even entire proteins. Tags can serve both as markers to show protein expression and can help facilitate protein purification. In general, the most commonly utilized tags for this purpose are histidine fusion (His or polyHis tag), glutathione-S-transferase (GST), and protein A fusion tags. Other types of fusion tags are also available, including binding protein to maltose,[9] the GB1 domain for G protein, thioredoxin, NusA, and others.[10] The decision to practice any of these labeling methods depends largely on the researcher's opinion. Protein A affinity chromatography is widely used as the initial step for the absorption of monoclonal antibodies (mAbs) in most regulatory submissions. This method efficiently removes most of the host cell proteins (HCPs) present in the mammalian cell cultures supernatant.[11] Analytical-scale affinity chromatography is widely used to measure protein quantity (titer) in cell culture fluid harvest (CCFH), which determines the performance and proper amount of CCFH to load onto an affinity column of purification-scale during production. Consequently, HPLC is commonly operated for titer analysis in both quality control (QC) test sites and development laboratories. Affinity chromatography functions as a sample clean-up step, in high-throughput laboratories, before the subsequent chromatographic analysis.

Affinity chromatography utilizing protein tags is a highly effective technique that enables the attainment of significant purity in a single purification step. However, several challenges can arise during the affinity purification of tagged proteins, including low protein purity, reduced protein yield, and complex procedures for tag removal.[12] It is generally expected that affinity purification should achieve at least 95% purity. Nevertheless, certain tagging methods, such as His-tag, may fail to meet this purity threshold, leaving some nonspecifically bound proteins present post-purification. This often necessitates an additional purification step to eliminate these unwanted contaminants. In scenarios where the protein is utilized for simple screening, retaining the tag may be acceptable. Similarly, if the protein will undergo analysis using techniques such as surface plasmon resonance (SPR) after purification, the tag may be retained. However, if the tag adversely affects protein conformation or interferes with crystallization, its removal becomes essential. This is especially true in drug discovery, where the elimination of the tag is critical to minimizing the risks of altering the protein's functional properties.

The choice of tag can significantly influence the final yield of the protein. For example, while Strep-tag™ II may enhance protein purity, it does not provide yields comparable to those achieved with tags like MBP (maltose-binding protein) or GST. Additionally, for histidine-tagged proteins, factors such as imidazole concentration and buffer conditions can also impact the purification yield. The process of tag removal often involves multiple additional steps, such as employing proteases and conducting further affinity purifications. However, each of these steps may lead to a decrease in yield, and the action of proteases during tag removal can be time-consuming, potentially affecting sensitive proteins and resulting in varying degrees of protein degradation. Therefore, careful consideration must be given to tag removal strategies, particularly in the context of drug discovery, to ensure the proper functionality of the purified protein.[12-14]

HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY (HILIC)

Glycosylation that occurs in many biopharmaceutical proteins has a significant impact on their biological functions. Since glycan properties are of great importance for therapeutic proteins and affect protein stability, pharmacokinetics, in vivo half-life and molecular affinity, glycan profiles are carefully investigated. Different methods for glycan analysis include intact glycoprotein analysis by mass spectrometry, glycopeptide analysis after enzymatic digestion, and analysis of released glycans by capillary electrophoresis (CE) or high performance liquid chromatography (HPLC). For glycan analysis by HPLC, glycans are released through chemical or enzymatic methods, labeled with UV-activated or fluorescence reagents, and then analyzed by HILIC.[15,16]

1. HILIC fundamental and method conditions

The process of glycosylation is the enzymatic addition of polysaccharides to proteins, which is one of the common post-translational modifications in therapeutic antibodies and protein productions.

In Hydrophilic Interaction Liquid Chromatography (HILIC), various packing materials are utilized to enhance separation efficiency and retention of hydrophilic compounds. Superficially Porous Particles feature a solid core with a porous outer layer, allowing for high surface area and improved mass transfer. They facilitate faster analysis times and better resolution due to reduced diffusion path lengths. Amphion-Bonded Silica is chemically modified to introduce both hydrophilic and hydrophobic characteristics, enabling it to interact effectively with a wide range of analytes. It provides flexibility in optimizing separation conditions for various applications. The traditional unbonded silica offers a high degree of hydrophilicity, making it suitable for separating polar compounds. However, it may lead to less reproducible results due to variability in the surface properties. These packing materials are crucial for achieving optimal retention and separation of glycan structures, which can vary significantly in composition and functionality.[15,17] By selecting the appropriate packing material, analysts can enhance the efficiency and effectiveness of glycan analysis in biopharmaceutical applications.

The glycosylation site on the protein can be linked to the oxygen of the alcohol group of serine and threonine amino acids (O-linked) or linked to the nitrogen of asparagine residues (N-linked). In the case of monoclonal antibodies (mAbs), this modification mainly occurs in the Fc region. Glycan structures are different in terms of the galactose number and sialic acid molecules and in the presence or absence of N-acetylglucosamine or fucose. Each of these changes can affect the mechanisms of action of products such as therapeutic antibodies or proteins.[18] Due to the ability of mammalian cell lines to produce non-immunogenic near-human antibodies, these cells are preferred for antibody production. It should be kept in mind that cell culture conditions can have a significant effect on the glycemic profile of therapeutic IgG antibodies. This issue creates the need to identify and control essential process factors for proper and stable glycosylation during the production process.[19] Peptide: N-glycosidase F, often referred to as PNGase F, is often used to release N-linked sugars, which are subsequently labeled with fluorescence or UV-activated reagents and subjected to HPLC analysis or ultra-high efficiency liquid chromatography (UHPLC) separation. UHPLC systems are equipped with fluorescence detection for optimal performance in the analysis of glycan molecules, and UHPLC columns are also designed for this purpose, and are commercially available. The aforementioned columns separate glycans released from biomedicines as 2-aminobenzamide derivatives, among these compounds are high mannose, complex, hybrid and sialylated glycans. Many manufacturers provide guidelines that guide laboratory technicians in planning and performing glycan experiments, including glycan release, labeling procedures, and chromatographic methods. HILIC methods that use UHPLC typically provide shorter assay times and curves with improved resolution compared to traditional HPLC methods.[20,21] Specialized commercial columns designed for glycoprotein profiling and glycopeptide mapping are now accessible, facilitating the analysis of entire glycoproteins, glycopeptides, and free glycans. Both hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RPLC) methods are applicable for glycan analysis, providing valuable insights, particularly when paired with mass spectrometry (MS). Data obtained from MS can illuminate various separation patterns in glycoproteins and glycopeptides. It is important to highlight that sialylated glycoforms exhibit a change in net charge due to sialic acid occupancy, which enhances their separation when utilizing ion exchange chromatography. This improved resolution allows for a more detailed analysis of the different glycoforms present in a sample.

2. HILIC applications

HILIC is a complementary technique that can be used in combination with other chromatography techniques in order to increase the purification efficiency. In HILIC, in contrast with the reverse-phase chromatography, elution occurs in the order of most hydrophilic to least hydrophilic. Therefore, HILIC effectively retains hydrophilic compounds which are poorly retained in reversed-phase chromatography. HILIC is very well compatible with mass spectrometry (MS), especially with electrospray ionization (ESI), particularly due to the high polar organic mobile phase which increases the efficiency of the ionization process. As such, HILIC–MS can surpass other analytical techniques such as HPLC, providing a quantitative and highly sensitive analysis of many complex solutions containing polar components. When RPLC–MS provides poor separation of hydrophilic compounds, HILIC–MS becomes a complementary analytical method.[22,23] Hydrophilic compounds that are poorly retained in RPLC can be better ana-

lyzed using combination of the two techniques. HILIC has a broad range of biomedical applications including Biochemistry, Chemical engineering, Agricultural, Pharmacology, and Food Industry. Correspondingly, the technique is used principally to separate and analyze polar peptides/drugs as well as other polar components such as antibodies, carbohydrates and biogenic amines. Additionally, for lot release quality control of product glycosylation pattern and batch-to-batch consistency, assessment of the glycans are employed.[24]

REVERSED-PHASE CHROMATOGRAPHY (RPLC)

Reversed-Phase Chromatography (RPLC) is a pivotal technique for the separation of proteins and peptides based on hydrophobicity under denaturing conditions. This method delivers high-resolution separation, particularly beneficial for peptide mapping and protein variant characterization.[25] For small proteins like growth hormone and insulin, RPLC can resolve variants differing by a single amino acid if the difference is located within the protein's hydrophobic interaction region with the stationary phase.[26,27] This chromatography is based on hydrophobic interaction between hydrophobic ligands attached to a column support and hydrophobic patches on the protein. Many proteins unfold upon contact with the hydrophobic ligands and by being dissolved in an organic solvent of low pH. Therefore, the total number of hydrophobic groups dominates the elution process during RP-HPLC.[28] Thus, large integral membrane proteins, containing a relatively high number of hydrophobic groups will require high concentrations of organic solvent for elution. Reversed-phase liquid chromatography (RPLC) is a highly effective and widely utilized technique for the separation of peptides and proteins, functioning primarily based on the hydrophobicity of the molecules involved. This method proves particularly advantageous for peptide mapping, as it can distinguish between molecules differing by as little as one amino acid, making it suitable for the analysis of small proteins such as insulin and growth hormone. In RPLC, the separation of peptides and proteins is achieved through hydrophobic interactions with stationary phase ligands, which typically consist of carbon chains ranging from C4 to C18, or diphenyl ligands.[29] The effectiveness of RPLC is often enhanced at elevated temperatures, and the incorporation of organic solvents aids in denaturing proteins, thereby exposing their hydrophobic cores and facilitating improved resolution during separation.[30] Shallow gradients are generally used, and a variety of proteins often emerge in a narrow part of the gradient. The pore size of the support material has an important effect on the operation. For example, antibodies and smaller proteins are analyzed by 300 and 120 Angstrom pores, respectively. RPLC generally provides higher resolution and more accurate analysis than size exclusion chromatography (SEC) and ion exchange techniques.[31] The RPLC method can more effectively analyze a variety of proteins caused by sequence changes, oxidation, misassembly (such as bispecific antibodies and homodimer types), antibody-drug conjugates, cysteine bond changes, and other post-translational modifications.

1. RPLC method and applications

The established RPLC methods used for peptide separation usually use C8 or C18 columns and use either trifluoroacetic acid (TFA) or formic acid in a water/acetonitrile mixture as mobile phase. During the analysis of antibodies and larger proteins, C4, C8, or diphenyl columns are generally preferred along with mobile phases of water/acetonitrile or isopropyl alcohol (IPA) and about 0.1% TFA as a modifier for more hydrophobic proteins.[32] In order to minimize sample carryover, elevated column temperatures, up to 110℃, are employed. Although for peptides and smaller proteins lower temperatures are preferred.[33] When improved protein recovery is desired, particularly for antibodies, IPA and n-butanol may be included. Choice of 3,3,3-trifluoropropionic acid and 2,2-difluoroacetic volatile acid as alternatives for TFA can mitigate ionization suppression in LC/MS while maintaining peak integrity.[34]

Superficially porous particles (SPPs) can significantly enhance RPLC efficiency. They contain a porous outer layer around a solid core, reducing diffusion pathways and increasing column efficiency by 20-40%, compared to fully porous particle columns. SPP columns benefit from lower backpressure, making certain small-particle SPPs (<3 μm) popular in particular.[35,36] RPLC is vastly employed to resolve protein variants associated with post-translational modifications, oxidation, and sequence variations. RPLC can particularly separate disulfide variants of IgG2. Considering the tendency of proteins to denature with RPLC application, differentiation between wild-type free thiol-containing antibodies are feasible.[37]

Enhanced resolution of free thiol antibody variants using RP-UHPLC has also been demonstrated. RP-UHPLC has also been used as a middle-down method often in tandem with mass spectrometry (MS), for peptide mapping analysis and analysis of Fc region oxidation following pepsin digestion at hinge region.[38] Peptide mapping has higher specificity over other methods such as CZE and hence is widely used in QC testing for identity confirmation. Peptide mapping is also crucial for comparing new drugs to biosimilars and for finding sequence variants that could arise during manufacturing. RPLC coupled with MS allows the acquisition of molecular weight data on desired peaks, providing valuable information for bottom-up proteomics as well as the characterization of protein therapeutics.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

The hydrophobic interaction chromatography (HIC) method serves as both a reference and an alternative to reversed-phase liquid chromatography (RPLC), as the retention of proteins in HIC is directly correlated to their hydrophobicity. In this technique, protein samples are ap-

plied to a stationary phase with moderate hydrophobicity under "salting out" conditions, which promote the aggregation of proteins based on their hydrophobic characteristics.[39] While higher temperatures typically enhance hydrophobic interactions, it can also lead to reduced recovery and unsatisfactory peak shapes for certain proteins. Therefore, careful temperature control during HIC is essential. Suitable stationary phases for the application of therapeutic proteins include silica gel or polymer-based materials with short-chained alkyl, alkylamide, alkylamine, or ether functional groups, which are effective at facilitating the purification of these biomolecules.[40] Phases with higher hydrophobicity may promote denaturation as well as incomplete protein elution. Certain achievements for moderate to satisfying protein recoveries from HIC phases have been reported.[41,42] The main parameters affecting the improvement are salt type and concentration, pH, ligand type and density, retention time and protein quality.[43] By using some weakly lyotropic salts such as ammonium acetate, setting the pH of the mobile phase somewhat lower than the pI of the protein, higher recoveries can be obtained.[44] Besides, the addition of 5-20% organic solvent and/or 0.5-1 M guanidine or arginine into desorption detergent can increase its potency and thus its recovery.[45]

1. Hydrophobic interaction chromatography theory, method and applications

Hydrophobic Interaction Chromatography (HIC) is a fundamental technique for analyzing proteins under native (non-denaturing) conditions based on their hydrophobic properties. HIC has exceptional selectivity as well as the ability to preserve protein biological activity. HIC is widely utilized in the analysis of Antibody-Drug Conjugates (ADCs) with variable drug-to-antibody ratios (DARs), allowing the calculation of average DAR and permitting the fractionation of ADC species with various DARs for additional characterization. The high salt concentration in HIC approach required in the mobile phase causes challenges for direct coupling of HIC-MS, even though specific conditions have also been developed to resolve this problem.[46,47]

Hydrophobic interaction chromatography (HIC) effectively preserves the native structures of proteins while exploiting their hydrophobic properties, making it particularly advantageous for subsequent structure-function analyses. HIC exhibits superior selectivity compared to ion exchange chromatography (IEC); proteins are initially partitioned to the hydrophobic stationary phase due to the high salt concentration in the mobile phase. As the salt concentration decreases, proteins migrate back into the mobile phase, facilitating separation. HIC is adept at separating a wide variety of monoclonal antibodies (mAbs). The method typically employs a gradient of high to low salt concentration while operating at ambient temperature to maintain protein integrity. Stationary phases used in HIC feature alkyl-based C3 or C4 ligands, albeit at lower densities than those used in RPLC. Mobile phase additives, such as ammonium acetate and ammonium sulfate, are evaluated to optimize separation based on different ligands and specific protein characteristics. One of the primary applications of HIC in biopharmaceuticals is the determination of antibody-drug conjugate (ADC) drug-to-antibody ratio (DAR). Additionally, HIC is utilized to identify protein variants, including isoaspartic isomers resulting from aspartic acid residues in antibodies, free thiol mAb and Fab variants, as well as oxidation products within the Complementarity-Determining Regions (CDRs) of antibodies. As an efficient separation technique, HIC allows for the enrichment of these species, facilitating the study of their functional activities while preserving their biological functions for further analysis.[48,49]

PROTEIN BIOPHARMACEUTICALS

Amino acids are named such, as they are composed of an amino group and a carboxylic acid group. They also carry an R group which is extremely important for the determining 20 different amino acids which comprise building blocks of life. Based on that, they are classified in four groups of acidic, basic, neutral hydrophobic, and neutral hydrophilic. These amino acids form huge quantity of proteins in living organisms with huge varieties. Short combination of 2 or more amino acids by peptide bond forms peptides. Typically, polypeptides are formed of 20 to 50 amino acid unit long unbranched chains of amino acids. Finally, proteins are made up of typically multiple polypetides bound together usually containing over 50 amino acid units. In this respect, simple amino acid sequence is called the primary structure, forming the secondary structure when forming a helix turn or a pleated sheet, depending on the amino acid compositions. Spatial arrangements of secondary structure forms the tertiary structure depending on composition and environmental conditions. Eventually, the assembly of protein subunits create the quaternary protein structure. Why proteins are important in life is due to the huge and major biological functions that they support. They are involved in biochemical functions (e.g. enzymes), act as hormones, form structural components (collagen), used in transportation of different molecules (hemoglobin), support mechanical functions (actin in muscle), and form antibodies which play defense roles and are basis of monoclonal antibodies which are a major class of biopharmaceuticals.[50]

Traditional pharmaceuticals are generally synthesized chemically with precisely defined entity.[51] They have a relatively stable structure with 1-5 reactive moieties and between 100-1,500 Daltons of molecular weight. Biopharmaceuticals are genetically engineered from living cells which are between 2-2,000 kDa which, as a function of their size, can possess several thousands of reactive groups. They are relatively to highly labile with complex structure and heterogeneity often containing a mixture of closely related variants which paves the ground to work on other biosimilars and particularly biobetters. Comparing pharma-

ceuticals with biopharmaceuticals gives clear idea of the higher complexity of the latter group and related greater challenges from analytical perspective.[52] The first biopharmaceutical product introduced to the market was the therapeutic protein insulin in 1982.[53] The market is rapidly growing since then, holding more products majorly manufactured against life threatening diseases such as cancer and autoimmune diseases. The biopharmaceutical drugs account for 20% of the global sales and are expected to cover more than 50% of the new approved drugs. The global biopharmaceuticals market in 2022, was estimated at some 263 billion U.S. dollars. However, according to this estimate, it is expected to increase to around 571.84 billion U.S. dollars by 2023 and is predictable to grow from USD 616.94 billion in 2024 to USD 1,183.72 billion by 2032.[54] This statistic demonstrates the growing expected size of the biopharmaceuticals market worldwide from 2022 to 2032.

BIOPHARMACEUTICAL PROTEIN AND PEPTIDE ANALYSIS

During the development of biopharmaceuticals, full characterization of the product is required. Due to their inherent size, complexity and heterogeneity, their analytical procedures are typically more complex. Therefore, a significant number of analyses in parallel would be necessary and one single technique generally would not be sufficient. A range of liquid chromatography techniques are commonly required for the analysis of these molecules. Some of these chromatographic approaches may need to be supported by mass spectrometric detections.[53] Reversed-phase liquid chromatography (RPLC) is a widely utilized technique in biopharmaceutical analysis due to its higher resolving power, better plate count, and narrower peak widths compared to ion exchange chromatography (IEC) or size exclusion chromatography (SEC). This makes RPLC particularly effective for analyzing a variety of biomolecules, including peptides and small proteins.

However, RPLC has its limitations when applied to the analysis of larger proteins. One significant challenge stems from the presence of a higher number of positive charges on large proteins, which can lead to slower ionic interactions with the stationary phase. These interactions may result in peak tailing, a phenomenon where the peak shape elongates and broadens, complicating the interpretation of results and reducing the overall sensitivity of the analysis. On the other hand, hydrophilic interaction liquid chromatography (HILIC) serves a crucial role in the characterization of hydrophilic species that are not effectively analyzed by RPLC. This includes compounds such as glycans and underivatized amino acids, which require different separation mechanisms due to their polar nature. In summary, while RPLC remains a powerful tool in biopharmaceutical analysis, it is essential to consider its constraints, especially when dealing with large proteins. This consideration, combined with the application of alternative techniques such as HILIC, IEC, and SEC, can provide a more comprehensive characterization of complex biomolecules.[25] To address this issue, there are modern stationary phases that can be employed. In addition, TFA can be added to the mobile phase in order to do ion pair with the stationary phase, as well as temperature increase up to 90 degrees which can enhance the ionic interaction and decrease peak tailing. Due to the big size and low diffusion coefficient of the protein, peak broadening often occur during RPLC. Longitudinal diffusion, due to void volume within the column and tubing, slow flow rate, and excessive retention in the column can cause peak broadening. Efficient stationary phases can be used to decrease peak broadening. Columns can be packed with sub-2 microns porous or core-shell particles as a strategy to overcome that issue.[55] For example, the resolving power can be drastically enhanced when using column packed with sub-2 micron fully porous particles compared with regular columns packed with 5 micron particles. When working with relatively larger proteins, it is very important to use wide pore stationary phase which possess a pore size of at least 300 angstrom instead of the regular 100-150 angstrom. Using this pore size would be much more appropriate for large proteins and will avoid peak broadening. Adsorption of protein to the stationary phase can also occur. To limit this phenomenon, it is possible to work with less hydrophobic stationary phase, such as C4 instead of C18, and it is also possible to use bio-inert materials which are available from several providers.[56]

MONOCLONAL ANTIBODY ANALYSIS

Among the biopharmaceuticals, monoclonal antibodies, known as mAb, are the most promising class of therapeutic molecules.[57] To simplify the analysis of mAbs, because it is complex to analyze the intact Ab, it is preferable to use middle up analytical strategy. The size of protein and complexity decreased by partially digesting with Papain, resulting in 50 kD fragments called Fab and Fc instead of the initial mAb with 150 kD size.[58] Alternatively, the mAb can be reduce by DTT to generate Lc and Hc of 25 and 50 kD fragments respectively. The heterogeneity of the resulting fragments is much easier to analyze than the original Ab and the small fragments are much more compatible with chromatographic conditions. In the chromatogram of the original mAb, in the case of Herceptin for instance, there is additional tail peaks which correspond to other forms of this mAb that co-exist in the commercial solution due to the high heterogeneity of such large biomolecule in reality.[59] Those small peaks could be due to charge, size or glycosylation variants of the molecule. Trastuzumab digested with papain gives Fc and Fab fragments, possessing 50 kD size.[60] Generally Fc is eluted first while Fab is higher in height and more heterogeneous and is eluted later. This pattern gives much more peaks and consequently much more information regarding the protein. The mAb reduced with DTT, two peaks will be observed, namely the light chain and the heavy chain which is more retardant than

the previous one and obviously more heterogeneous, resulting in several more additional peaks after the Hc peak.[61] Here, the mass spectrometry will be very useful to resolve the nature of those additional peaks. The cumulative data obtained from the various chromatographic analyses provide valuable insights into the characterization of monoclonal antibodies (mAbs), which are inherently complex molecules. Conducting these analyses at elevated temperatures, such as 80 degrees Celsius, can present challenges, particularly regarding protein stability. While high temperatures can be destructive, they also offer several advantages in chromatographic separation.

One of the key benefits of using elevated temperatures in reversed-phase liquid chromatography (RPLC) is the significant reduction in adsorption of proteins to the stationary phase. This can lead to improved peak shapes and resolution. Additionally, elevated temperatures can alter the selectivity for charged variants of the mAb, reduce peak widths, and modify the retention times of the analytes, which can enhance the overall separation efficiency.[33] However, it is crucial to balance these advantages with the risk of on-column degradation of the protein. Prolonged exposure to high temperatures can lead to denaturation or degradation, resulting in the formation of ghost peaks—artifacts that appear on the chromatogram due to the breakdown products of the protein. To mitigate this risk, it is advisable to limit the runtime of the analysis to around 15-20 minutes. This timeframe helps to prevent the appearance of additional peaks that could complicate the interpretation of the chromatograms while still allowing for effective separation of the mAb variants. In summary, while elevated temperatures can enhance chromatographic performance by reducing adsorption and improving resolution, careful consideration must be given to the potential for protein degradation. Optimizing the analysis conditions, including runtime and temperature, is essential to ensure accurate and reliable characterization of mAbs in biopharmaceutical applications.[62]

It is very important to characterize the glycosylation of proteins. Sequence, composition, branching, linkage of the different glycans. Glycosylation remains one of the major source of the mAb variability and it represents 3% of the mAb weight. Glycans are composed of sugar units including mannose, fructose, galactose and several others. Glycosylation can be revealed both at protein and peptide level and the content analysis is only possible when they are removed from the peptide backbone.[59] Because native glycans are very hydrophilic it cannot be sufficiently retained at RPLC condition and hence, the HILIC is preferentially employed for their analysis. Stationary phase with amide group is generally a good choice for such analysis. Glycans cannot be detected with spectrophotometry detectors and a labeling procedure is generally applied using 2-aminobenzamide (2-AB) chromophore agent which will result is successful chromatograms. The glycans, either o-linked or n-linked, are enzymatically cleaved from the peptides and then labeling is performed using 2-AB which improves separation and the detection. For o-linked glycans, chemical cleavage methods are also widely used. Therefore, alkaline conditions is used to hydrolyze the O-glycosidic linkage between the reducing glycan sugar and serine/threonine residues.[63] Derivatives such as Girard's reagent T, along with 2-AB, are commonly used for glycan labeling.[64] The analysis of the glycans is performed using HILIC method combined with fluorescent or UV or MS or any other appropriate detection method.[65]

PROTEIN HETEROGENEITY AND PEPTIDE ANALYSIS

Another way to characterize the molecule is to digest the protein with enzyme and assess the peptide map.[66] Trypsin digestion gives small peptides range between 500 to 2,000 Daltons which are therefore much easier to analyze. However, the number of peptides may be considerable and be between 50-100 samples. As a results it is important to have a powerful technique being able to discriminate between such numbers of peptides.[67,68] Normally, it is advised to use long (like 250 mm) column packed with core-shell material. In this condition, TFA would be mandatory to improve the peak shape and mask the positive charge on the surface of the protein. There might be a number of hydrophilic peptides that are not sufficiently be retained on RPLC. To better characterize this portion of the chromatogram and have better idea of type of peptides that are located there, it to employ HILIC condition might be a better choice to better retain those hydrophilic species. HILIC is much more employed for peptide map analysis.[69,70] With combination of chromatography with the power of mass spectrometry, other peaks corresponding to the post translational modifications could also be resolved gathering much more information regarding the analyte.[71]

For the peptide analysis, UHPLC should be preferentially selected as instrumentation in order to achieve high resolving power.[72] Ideally, the column should be packed with core-shell particles and have sufficient pore size like 120-150 Angstrom. There is no need for 300 Angstrom because we are working with relatively small peptides. On the other hand, columns with pore size 60-80 Angstrom will not be appropriate for the peptide analysis.[73] TFA should be added to the mobile phase at a reasonable concentration. Acetonitrile is an organic modifier of choice as it generates a reasonable back pressure or pressure drop compared to methanol or other organic solvents. A gradient time around 35 minutes should be applied to keep a balance between resolving power and analysis time. Mobile phase temperature should be set at 60 degrees and UV detection at 214-280 nm. Those conditions will modify if an intact protein is applied to the system. Isopropanol or 1-propanol can be used to change the selectivity and optimize recovery for proteins although it can increase viscosity and pressure drop.

In liquid chromatography, there is tendency to replace ion exchange chromatography (IEX) and size exclusion

chromatography by the reversed-phase liquid chromatography (RPLC) for the analysis of large biomolecules.[74] There are two important advantages for RPLC over SEC and IEX. All the mobile phases used in RPLC are compatible with ESI-MS analysis. Peptide mapping of mAbs with complete proteolytic digestion, followed by RPLC-MS/MS analysis through a bottom-up approach is often the preferred method for quantification and identification of mAbs chemical modifications.[75] Nevertheless, the analysis of intact mAbs through advanced RPLC stationary substances and instrumentations which requires minimal and less modification prone sample preparations provide a preferred method over the traditional peptide mapping. Moreover, in RPLC peak widths are significantly narrower compared to IEX and SEC. This in turn can increase the number of compounds or isoforms that can be resolved in one chromatogram. Nevertheless, certain shortcomings are associated with using of RPLC. For instance, isocratic condition is impractical for separation of large biomolecules and gradient mode is indispensable. In addition, peak broadening may be significant as the large molecules normally diffuse very slowly. Besides, decrease in the pore size of the stationary phase significantly slows down the analyte diffusion.[73] There are several chemical modifications on the antibody peptide chains resulting in antibody isoforms and increasing the overall heterogeneity of an IgG. Consequently, this micro-heterogeneity can be source of impurities and/or degradation products leading to serious health implications which necessitates analytical techniques improved for detailed characterization of the mAbs.[76] Various proteases such as Lys-C, pepsin and papain are often used in order to fragment and facilitate the investigation of mAb microheterogeneity. A wide range of analytical techniques including chromatography, electrophoresis and mass spectrometry techniques are used to demonstrate the batch to batch similarity and to determine detailed structure of mAbs.[77]

The recovery of intact mAbs increases by a factor of 20 and 30 at 60℃ compared to 40℃.[78] However, there is a well-known risk for on-column degradation of protein which is more significant at elevated working temperatures.[79] It has been shown that increasing the column residence time for mAb results in an evident increase of the area of the mAb pre- and post-peaks. Selecting appropriate column properties such as pore size and balancing between column residence time and temperature results in a proper recovery and avoids on-column thermal degradation.[79] Another solution is to add ancillary solvents, such as *n*-butanol and *n*-hexanol, to the mobile phase in order to decrease column adsorptions driven by strong secondary ionic interactions.[80]

BIOPHARMACEUTICAL STABILITY IN CHROMATOGRAPHIC SYSTEMS

The stability of proteins in the field of pharmaceuticals is an issue that has increasing importance because the number of these products being developed and registered is increasing. Degradation of therapeutic proteins and peptides can be divided into two main physical and chemical categories. Protein chemical instability includes damage to the primary structure, covalent changes of the protein through bond formation or polypeptide chain cleavage.[81,82] The heterogeneity of therapeutic protein products often complicates their analysis. A wide range of chromatographic methods such as SEC, IEX and RPLC have been used to investigate the identity, heterogeneity, impurity content as well as activity of therapeutic protein products. RPLC is capable of separating container-derived nonpolar impurities from protein, which can be identified by mass spectrometry such as organic compounds present in the rubber stopper.[83] Although RPLC has higher resolution and shorter separation time than SEC and IEX, due to difficult conditions such as mobile phase composition, temperature and pH used, it may damage the protein conformation. For example, increasing the temperature from 35 to 80℃, the peaks become narrower, but when the temperature increases from 100 to 120℃, not only the peaks become wider and smaller, but several new and small peaks appear. Therefore, to avoid the destruction of proteins, the temperature and time of analysis should be balanced.[84] Relatively high concentrations of organic modifiers can denature many globular proteins at high temperatures. Alcohols such as methanol and isopropanol in the mobile phase can induce helical structural compounds that can eventually lead to the appearance of multiple peaks in the chromatogram.

ADSORPTION OF PROTEINS ON SOLID SURFACES

Most of the protein molecules are amphipatic and they are intrinsically surface active. Therefore, low recoveries, tailed peaks, cross-contamination, carryover, varying peak areas and shifting retention times are commonly observed during protein chromatography. Chromatographic results such as selectivity and resolution, as well as peak shape and area,[85] protein and peptide adsorption to column packing materials and the frits of chromatography columns can significantly impact the efficiency and accuracy of biopharmaceutical analyses. Such adsorption can lead to sample loss, altered peak shapes, and inconsistent results, making it crucial to minimize these interactions during experimental procedures. Inert bio-liquid chromatography technology offers a promising solution to reduce the adsorption of analytes across various components of the chromatography system, including injectors, connection surfaces, tubing, and detectors.[86] By utilizing inert materials and specialized coatings, this technology can mitigate the nonspecific binding of proteins and peptides, resulting in enhanced recovery rates and improved reproducibility in analyses.

Additionally, inert instruments are designed to better withstand harsh eluent conditions commonly encountered

in biopharmaceutical analysis, such as high salt concentrations and strong acids. These conditions are often necessary to elute bound analytes effectively from the stationary phase, but they can cause degradation or alteration of both the chromatographic media and the instruments. Moreover, sample handling and preparation are critical steps in biopharmaceutical analysis, especially when working with low-concentration protein solutions. Adsorption of proteins and peptides to surfaces of pipette tips, vials, and sample tubes can lead to significant losses of analytes, which can adversely affect quantitative results.[87] Using inert materials for these components can help reduce adsorption and preserve the integrity of low-concentration samples. In summary, the use of inert bio-liquid chromatography technology alongside appropriate materials for sample handling can enhance the reliability and sensitivity of biopharmaceutical analyses, enabling more accurate characterization of proteins and peptides in various research and clinical applications.[88] Organic solvent supplements and adding acids/bases or surfactants to the sample might avoid nonspecific analyte adsorption to the container, although presence of surfactants in LC–MS analysis is not recommended. The harsh conditions and inherent denaturing aspects of the RPLC technique usually limit the recovery biologically active form of proteins. Generally higher recoveries with lower adsorption is observed using short chain C4 and C8 alkylated stationary phases. Albeit in certain cases full recoveries of therapeutic proteins from C18 columns have also been reported. Injection of a concentrated peptide or protein sample to saturate or inactivate unspecific column binding sites could be an easy way to surmount adsorption issues to the solid phase and frit surfaces.[89]

CONCLUSIONS

The characterization of biopharmaceuticals is inherently complex due to their size and heterogeneity, necessitating a diverse array of chromatographic techniques often supplemented by mass spectrometry. As the biopharmaceutical market continues to grow, the importance of High-Performance Liquid Chromatography (HPLC) and its methodologies in ensuring product quality and efficacy will remain paramount. HPLC is an indispensable tool in the characterization and quality control of protein-based pharmaceuticals. This analytical technique enables precise separation and analysis of complex biomolecules, which is crucial given the growing market for biopharmaceuticals. Effective HPLC analysis necessitates the optimization of chromatographic conditions, including the careful selection of stationary and mobile phases, as well as detection methods. Reversed-Phase Chromatography (RPC) is particularly favored for its efficiency in separating proteins and peptides. The application of affinity columns, enhances the analysis of various biological fluids, underscoring the need for robust operational conditions tailored to different sample types. For glycan analysis, the integration of mass spectrometry further amplifies the analytical capabilities of HILIC and RPC, providing insights into glycan patterns that can influence the therapeutic efficacy of biologics. While RPC is widely adopted, it is essential to consider its limitations when analyzing large biomolecules, as it often supplants other techniques like Ion Exchange Chromatography (IEX) and Size Exclusion Chromatography (SEC). Hydrophobic Interaction Chromatography (HIC) offers exceptional selectivity and maintains protein activity, making it suitable for analyzing Antibody-Drug Conjugates (ADCs) with varying drug-to-antibody ratios. However, the high salt concentrations required in HIC can complicate direct coupling with mass spectrometry, though specialized conditions have been developed to mitigate this challenge.

CONFLICT OF INTEREST STATEMENT

None declared.

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