Trends in Analytical Chemistry 63 (2014) 76–84

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Ultra-high-performance liquid chromatography for the characterization of therapeutic proteins

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ARTICLE INFO

Keywords:
Biopharmaceutical
Characterization
Hydrophilic interaction chromatography (HILIC)
Ion-exchange chromatography (IEX)
Mass spectrometry (MS)
Monoclonal antibody (mAb)
Reversed-phase liquid chromatography (RPLC)
Size-exclusion chromatography (SEC)
Therapeutic protein
Ultra-high-performance liquid chromatography (UHPLC)

ABSTRACT

The pharmaceutical market has markedly changed over the past few years, and there are today an increasing number of therapeutic drugs produced from biological sources. These biopharmaceuticals include recombinant peptides, proteins, and monoclonal antibodies (mAbs). Their detailed characterization could be difficult and time consuming, so it requires powerful chromatographic and spectroscopic methods. In this context, the use of columns packed with sub-2-µm particles at very high pressure, also known as ultra-high-performance (or pressure) liquid chromatography (UHPLC) has been reported as successful. Various modes of chromatography are compatible with UHPLC columns and conditions, including reversed-phase liquid chromatography (RPLC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEX) and hydrophilic interaction chromatography (HILIC).

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1. Introduction

The large majority of traditional pharmaceuticals are chemically-synthesized small-molecule compounds. In addition to these "chemical" substances, there are a number of substances that are produced from biological sources (i.e., biological systems or biological molecules). These "biopharmaceuticals" include recombinant peptides, proteins or glycoproteins [1,2]. The pharmaceutical potential of numerous proteins (e.g., interferons, interleukins, and growth factors) that are naturally produced in the body was originally demonstrated more than 40 years ago. These molecules have obvious advantages, including high efficacy, high specificity, wide therapeutic range, limited side effects, and exceptional chemical and biological diversity. The clinical use of therapeutic proteins has enabled the treatment of a wide range of life-threatening diseases, which were considered incurable or untreatable only a few decades ago. Dozens of new drugs for the treatment of cancer, AIDS and arthritis are on the market or are very close to regulatory approval [3]. Today, the global protein therapeutics market is worth over $100b, thereby evolving towards a total pharmaceutical market

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http://dx.doi.org/10.1016/j.trac.2014.05.012
0165-9936/

share of 20% [4,5]. We expect that, within the current decade, more than 50% of the new drug approvals will be biologics [4,6,7].

Therapeutic proteins are large, heterogeneous and subject to a variety of enzymatic and chemical modifications during expression, purification and long-term storage [5]. These changes include several possible modifications, such as oxidation, deamidation, glycosylation, aggregation, misfolding, or adsorption, leading to a potential loss of therapeutic efficacy or unwanted immune reactions.

Because the development of biopharmaceuticals and biosimilars is especially complex, regulatory agencies, such as the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA), require a demonstration of detailed characterization (e.g., verifying primary structure and appropriate post-translational modifications, secondary and tertiary structure), lot-to-lot and batch-to-batch comparisons, stability studies, impurity profiling, glycoprofiling, and determination of related proteins, excipients and protein aggregates [8]. For this purpose, a single analytical technique is generally not sufficient, and a variety of orthogonal methods are required to characterize such a complex sample fully, as summarized elsewhere [9].

The primary structure of proteins can be identified with two reference techniques, namely mass spectrometry (MS) and Edman degradation/sequencing [10]. The use of liquid chromatography (LC) with tandem MS (LC/MS/MS) in *de novo* sequencing dominates the process of sequencing proteins, and with peptide-mass fingerprinting combined with MS/MS became the preferred techniques. Numerous spectroscopic techniques are available to assess protein secondary and tertiary structure, such as X-ray crystallography, nuclear magnetic resonance (NMR), UV/Vis spectrophotometry, fluorescence, circular dichroism (CD), dynamic light scattering (DLS), static light scattering (SLS), differential scanning calorimetry (DSC) and infrared spectrophotometry (IR) [11,12].

In addition to these methodologies, electrophoresis is also a key technique for protein analysis, and different modes can be employed in slab gel or capillary format. Isoelectric focusing (IEF) is able to distinguish charge differences (isoelectric point) among proteins through the use of a pH gradient [13]. Capillary zone electrophoresis (CZE) has several well-established attractive features for the characterization of such complex samples, including its high resolving power and throughput. High-performance LC (HPLC) is another option for the detailed characterization of proteins [14].

The three most common modes of chromatography are size-exclusion chromatography (SEC), which separates proteins based on their size or hydrodynamic volume, ion-exchange chromatography (IEX), able to separate proteins based on their charge, and reversed-phase chromatography (RPLC), where separation occurs on the basis of the hydrophobicity of the proteins. This last strategy offers a higher resolving power than SEC and IEX.

Today, one of the most widely-used analytical methods for therapeutic protein characterization is LC, probably due to the impressive developments of the past few years, enabling a new level of chromatographic performance. Recent developments in LC columns, such as ultra-high-performance LC (UHPLC), packed with wide-pore superficially porous particles (SPPs) and organic monolith columns allow a dramatic increase in the efficiency and the resolution of protein separations, even with large, intact molecules.

The aim of this article is to review the current trends in UHPLC and the potential for UHPLC strategy for the characterization of therapeutic proteins. In this review, we focus solely on unidimensional separations and particle-based, stationary-phase formats for routine UHPLC applications.

2. Need for high kinetic performance in protein analysis

Higher separation efficiencies and throughput have always been of great interest in LC. The pharmaceutical industry is interested in using rapid, efficient procedures for qualitative and quantitative analyses to cope with the large number of samples and to reduce the time required to deliver results [14]. When dealing with protein analysis, selectivity is often limited so the only way to improve separation is to increase chromatographic efficiency. Moreover, high-molecular-weight compounds, such as intact proteins, may have numerous conformations, post-translational modifications, or multiple isoforms that can cause broadened peaks and shifted retention times in the chromatograms. Another reason for broadened peaks is the slow molecular diffusion of these compounds due to their large size.

Typical routine tasks are the separation of oxidized, deamidated or reduced forms of intact proteins. Because the differences in molecular structure are small, similar retention behaviors of the different forms are expected. In many cases, the selectivity cannot be improved and, as a result, enhancement of separation efficiency must be considered. The stationary-phase dimensions and morphology, and the mobile-phase temperature are the two most relevant parameters for improving the efficiency of protein separations. Thanks to the latest stationary-phase technologies, such as sub-2-μm porous particles, superficially porous particles (SPPs), or wide-pore monolithic columns, the separation power was significantly increased in recent years. In addition, it is also possible to extend the column length (e.g., coupling columns in series) to achieve the required plate count or peak capacity. The use of elevated mobile-phase temperature in the range 60–90°C further improves performance. At higher temperature, the viscosity of the mobile phase decreases and the diffusivity of large proteins increases, leading to sharp chromatographic peaks. The other benefit of elevated mobile-phase temperature is reduction in adsorption of undesired proteins at the surface of the stationary phase.

3. Reversed-phase liquid chromatography (RPLC)

In RPLC, solute retention is predominantly mediated through the hydrophobic interactions between the non-polar amino-acid residues of the proteins and the bonded n-alkyl ligands of the stationary phase. Proteins are thus eluted based on their hydrophobicity. Large molecules possess a so-called “on-off” retention mechanism. Their retention strongly depends on minor variations in the solvent strength, and a small change (<1%) in the organic modifier content can lead to a significant retention change. For this reason, isocratic conditions are impractical, and gradient elution mode is recommended. The efficiency of RPLC is superior to other chromatographic modes and its robustness makes it well suited for use in a routine analysis environment [5]. Mobile phases typically consist of water, acetonitrile or methanol and 0.1% trifluoroacetic acid or formic acid (TFA, FA). The separation mechanism is based on a combination of solvophobic and electrostatic interactions, the latter governed by the ion-pairing interaction of TFA with basic side chains of a few amino acids (i.e., arginine, lysine, histidine) and the N-terminus amino groups [14].

It was recognized very early that one of the best approaches to improving intact protein RPLC separations would be to use small-particle packing material [15]. It came into practice in the late 1990s, when very fine particles (sub-2-μm or sub-μm) could strongly improve the separation performance, but required a huge increase in system pressure. To overcome the pressure limitations of conventional HPLC with a pressure limit of 400 bar, the research groups of Jorgenson [16,17] and Lee [18] constructed prototype instrumentation and experimental columns packed with very small non-porous material and performed analyses at very high pressures (up to 7200 bar). New nomenclatures have appeared to describe this higher back-pressure requirement, including ultra-high-pressure liquid chromatography, ultra-high-performance liquid