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Analytical Techniques Required to Ensure a Well-Characterized Biological Reference Material

Abstract:

Due to their complexity, characterization of biological reference materials is a significantly more difficult task than for typical small molecules. Further, regulatory agencies require orthogonal techniques when available to better understand the structure and stability of biological reference standards. The poster will outline some of the techniques required to fully characterize a protein reference standard and why each is important.

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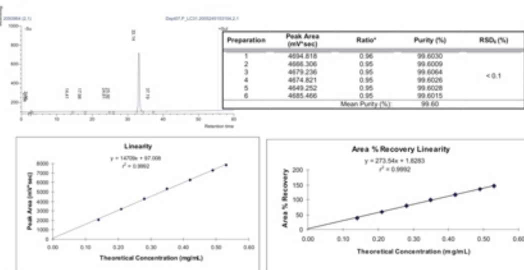
Introduction:

Many of the drug candidates in the discovery stage are biologics including proteins, peptides, monoclonal antibodies, etc. A tremendous amount of analytical testing is required to support a biopharmaceutical product - from discovery, through development, clinical trials, and manufacturing and eventually to the market. This all begins with the ability to obtain a well-characterized reference material. Due to the complexity of these large molecules, vast arrays of methods are utilized to attempt to fully characterize these complex molecules - significantly more than for a typical small synthetic molecule. Since biopharmaceuticals are expressed (produced) by living systems, i.e. E. coli, yeast, or mammalian cells, there may be nonhomogeneity and post translational modifications requiring advanced analytical techniques to delineate. Further, many agencies are expecting orthogonal techniques when available to better understand the structure and stability of a biopharmaceutical. The poster will outline techniques including biochromatography, mass spectrometry, spectroscopy, gel and capillary electrophoresis; and demonstrate what information each approach can yield.

Purity

Reversed phase is one of the most common forms of HPLC. Biomolecules are retained on the stationary phase in highly aqueous mobile phase and then are eluted with an organic mobile phase. HPLC is an extremely precise and accurate way to determine purity.



Preparation	Peak Area (mV*sec)	Ratio*	Purity (%)	RSD (%)
1	4694.818	0.96	99.6030	
2	4666.306	0.95	99.6009	
3	4679.236	0.95	99.6064	< 0.1
4	4674.821	0.95	99.6026	
5	4649.252	0.95	99.6028	
6	4685.466	0.95	99.6015	
		Mean Purity (%)	99.60	

Biochromatography

The field of biochromatography employs separation techniques to characterize biomolecules, such as proteins, peptides, nucleic acids and carbohydrates. As an accurate, sensitive, and reproducible alternative to electrophoretic methods, biochromatography has the ability to observe proteins while retaining their biological activity (non-denaturation). Despite the diverse activity, shape, size, solubility, and charge differences of these molecules, characterization is accomplished utilizing various methods of high performance liquid chromatography (HPLC). Reverse-phase, ion exchange and size-exclusion (SEC) chromatography are three of the most common approaches. These instruments are coupled with photodiode array, ultraviolet (UV), fluorescence, and mass spectrometric (MS) detection to yield a powerful analytical tool. Newer instruments employ higher pressures and smaller particle size stationary phases. This technique is known as ultra or UHPLC.

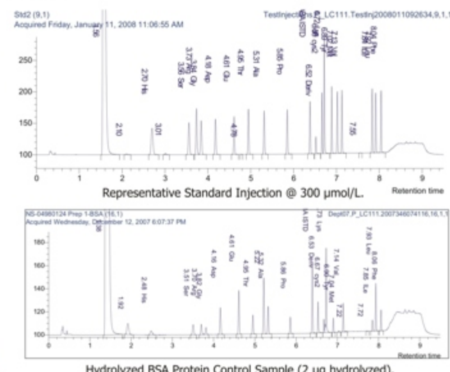
Reversed phase is one of the most common forms of HPLC. Biomolecules are retained on the stationary phase in highly aqueous mobile phase and then are eluted with an organic mobile phase. Therefore, compounds are separated based on their hydrophobic character. This technique can be used for assay, impurity, and peptide mapping.

In ion exchange chromatography, the solid phase of the column contains a charge. As a mixture of proteins elutes through the column, the target protein is retained since the charge of the beads is selected to have the opposite charge of the target protein. After the contaminant proteins are separated from the target protein, a high salt buffer is used to pull the target protein from the column. This technique can be used for assay and impurity analysis.

Size-exclusion Chromatography (SEC) is based on the different size and shape of proteins. Proteins of different size penetrate into the internal pores of the beads to different degrees. The column retards small protein molecules while large molecules pass through, more rapidly. Since the migration distance is correlated to the size of the molecule this technique can also be used for the determination of the molecular weight of a protein. This can be useful in studying aggregates and oligomers.

Amino Acid Analysis (AAA)

Amino acids are the primary building blocks of proteins. Accurate determination of amino acid concentrations is critical to a well-characterized protein. Protein samples undergo vapor phase acid hydrolysis using the Eldex Hydrolysis/Derivatization Workstation followed by AccQ-Tag derivatization. The amino acids are then determined by Ultra Performance Liquid Chromatography (UPLC) with UV detection. Quantitation of the amino acids is carried out with standards curves using reference materials. Bovine serum albumin (BSA) can be used as a control protein. Of the twenty amino acids typically found in a protein, eighteen of the twenty are readily hydrolyzed and evaluated. The remaining two amino acids, cysteine and tryptophan, are destroyed by acid hydrolysis and are typically not represented in protein characterizations. Additionally, glutamine and asparagine are converted to glutamic acid and aspartic acid during this hydrolysis procedure. Hence glutamine and asparagine content are included in the glutamic and aspartic acid values reported.

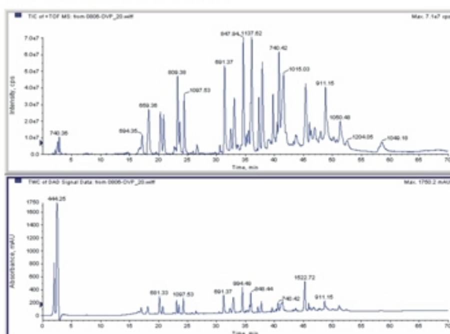


Representative Standard Injection @ 300 µmol/L.

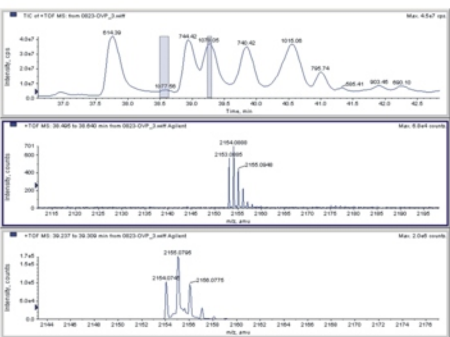
Hydrolyzed BSA Protein Control Sample (2 µg hydrolyzed).

Peptide Mapping and Post-Translational Modifications

Proteins are constructed from peptides, which are sequences of amino acid chains. Another common approach to help characterize a protein is called peptide mapping. Proteins are digested with enzymes, which cleave at specific locations along the chain. The digest can then be analyzed by HPLC. The resulting chromatogram is essentially a "fingerprint" (shown below) of the protein, based on the peptide fragments.



When coupled with a mass spec detector, a wealth of additional information can be determined. In the example below, a post-translational modification (deamidation) site can be identified.



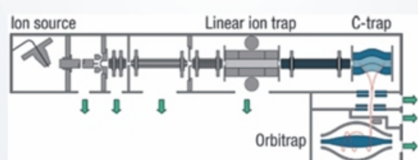
Mass Spectrometry

Mass spectrometry (mass spec or MS) is an extremely powerful analytical technique. The basic theory is as follows. A molecule is introduced into the mass spectrometer where it is transformed into an ionized state. The ions are then subsequently filtered or separated corresponding to their mass-to-charge (m/z) ratio and finally detected. The resulting output is a mass spectrum plot of the (relative) abundance of the ions as a function of the m/z ratio.

Mass spectrometers are typically coupled with a chromatographic (separation) technique such as HPLC or GC. Mass spec equipment can also come in various configurations from a single and triple quadrupole, ion trap, and time-of-flight instruments. The Orbitrap (shown below) is one of the most sophisticated instruments on the market for characterization of large molecules. Each of the different mass spec configurations and techniques has distinct applications, advantages, and disadvantages.

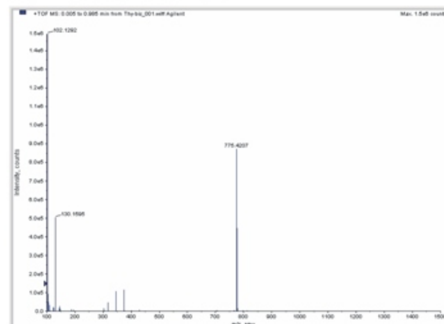
Mass spectrometry allows one to determine molecular weight and other structural information not easily extracted from other techniques. Several mass spec experiments are described here including accurate mass determination and determination of post-translational modifications (PTM).

ThermoFisher LTQ-Orbitrap (LC/MS/MS)



Accurate Mass

Mass spectrometers such as a time-of-flight and Orbitrap are capable of providing accurate mass measurements, a very precise molecular weight. This can be used to confirm structure of reference materials.



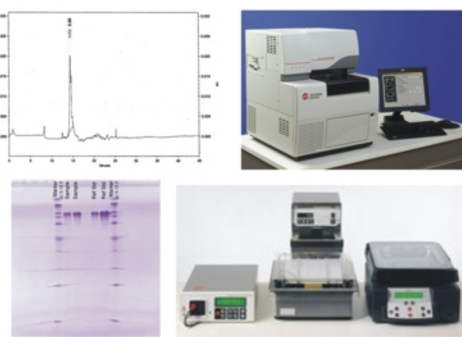
Deoxyribonucleoside Phosphodiamidite Material

Compound	Theoretical Exact Mass	trial	Measured $(M+H)^+$	M	Mass Error ppm
$C_{43}H_{59}N_4O_7P$	774.4121	1	775.4207	774.4134	1.7
		2	775.4183	774.4110	-1.4
		3	775.4188	774.4115	-0.8
		4	775.4199	774.4126	0.6
		5	775.4200	774.4127	0.8
			ave	**774.4122**	**0.2**
			std dev	**9.711E-04**	

Isolelectric Focusing

An important technique used to verify the identity of a reference standard is isoelectric Focusing (IEF). In general, IEF is a method of separating proteins based on their relative content of acidic and basic residues. This content is represented by a value known as an isoelectric point, or pI. Proteins are introduced into a gel (composed of polyacrylamide, starch, agarose, etc.), which has an established pH gradient or is capable of establishing such a gradient after applying an electrical current. This gradient is established by subjecting a mixture of polyampholytes, small polymers that have different pI values, to electrophoresis before the application of proteins. Proteins that are introduced into the gel migrate until they reach a place in the gel where the pH is equal to the isoelectric point of the protein. Isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01.

Below are examples of capillary electrophoresis and gel electrophoresis.



Spectroscopy

Spectroscopy is the branch of analytical chemistry that deals with the interaction between matter and radiation as a function of wavelength or frequency. Techniques range through the electromagnetic spectrum from radio waves and nuclear magnetic resonance (NMR) to infrared (IR) to visible-ultraviolet (Vis-UV) to x-rays. Some of these techniques can be used to study higher order structure of proteins, while others can be used to calculate concentration of the protein in solution.

UV

The ultraviolet absorbance can be measured. If the extinction coefficient is known then an accurate concentration can be calculated.

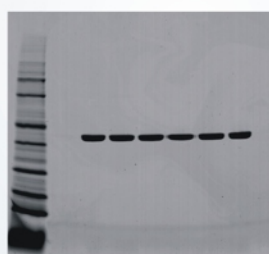
IR and CD

The folding of peptide chains into -helices, -sheets, and random coils in a protein defines its secondary structure. Fourier-transform infrared spectroscopy (FTIR) can be used to obtain secondary structure information by analyzing the amide bands. This can be used in conjunction with circular dichroism spectroscopy (CD).

Electrophoresis

Electrophoretic methods are based upon the migration of a biomolecules through a gelatinous media as a function of its size, charge and conformation. Several of the most common techniques are polyacrylamide and agarose-based gels, capillary electrophoresis, and isoelectric focusing.

SDS-PAGE (shown below) is one of the most widely used techniques. The column on the left is the result of a mixture of molecular weight markers. The bands across the middle of the gel are multiple runs of a protein reference material and can be used to estimate the molecular weight and demonstrate purity.



Conclusion

The successful and efficient development of a biopharmaceutical product depends on the quality of the reference materials. Biologic materials require a vast array of orthogonal techniques in order to be considered well characterized. Various approaches have been described here including biochromatography, mass spectrometry, spectroscopy, and electrophoresis. Advances in analytical chemistry have provided more sophisticated methods for characterization of these complex molecules.