GMP Conform Protein Analysis

New Analytical Solutions

The success of protein based therapeutics, e.g. monoclonal antibodies, interferons, hormones etc., in the last years generated one of the fastest growing segments in the pharmaceutical industry. Furthermore, whilst some of these blockbusters drugs are already out of patent protection, an increasing number of companies enter into the field of biogenerics production. Since the withdrawal of Tysabri (Biogen Idec) from the market in 2005 and the severe side effects of Supermab recognized in a clinical phase I study in 2006, biotherapeutics and their precise analysis on the molecular level came even more into the focus of the regulatory bodies like FDA and EMEA. The need for new technologies to address the safety issues is therefore obvious. The present article will give an overview on state-of-the-art GMP compliant analysis of protein drugs and will cover the newest developments and trends.

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Analysis	Goal
1. Structural characterization	
Amino acid sequence	Amino Acid Sequence of protein as compared to theoretical sequence
Amino acid composition	Amino acid composition of unknown protein
N/C-terminal sequencing	Identify nature and homogeneity of termini
Peptide map	Confirm identity of protein after digestion
S-S-Bridge analysis	Confirm location and /or connectivity of S-groups
Glycosylation analysis	Sugar content and further analysis (structure, modification site)
2. Physicochemical properties	
Molecular weight (MW) and size	MW of protein
Isoform pattern	Relative quantification of all isoforms
Extinction coefficient (EC)	EC of known substance
Electrophoretic patterns	Show the identity, homogeneity and purity of a substance
Liquid chromatographic patterns	Show the identity, homogeneity and purity of a substance
Spectroscopic Profiles	Determine higher-order structure
3. Process- and product-related impurities	
Host cell proteins	Find contamination via host cell proteins
Truncated forms	Find truncated forms of a protein
Post-translational modifications (PTM)	Identification of PTMs
Aggregates	Find protein complexes

Fig. 1: Overview over the analytical portfolio required by ICH guideline Q6B for release testing of protein drugs. State-of-the-art technical equipment and expertise is needed to fulfill the requirements.

Drug safety and its regulation by the responsible authorities is an area of still growing importance which is governed by standards known as Good Manufacturing Practice (GMP). Primary GMP was addressed to the production of small molecules of well defined structure and composition.

But with the appearance of protein drugs new challenges were posed to the regulatory authorities and to the analytical community. Protein drugs can not, like small molecule drugs, be manufactured by standard chemical synthesis. Relatively simple protein drugs as insulin and growth hormone are produced in bacterial cell lines. More complex proteins like Erythropoietin or monoclonal antibodies have to be expressed in mammalian cell lines as their activity depends on a correct assembly and post-translational modification. At present, the most commonly used cell lines are Chinese hamster ovary cells (CHO) or baby hamster kidney cells (BHK), which e.g. guarantee human-like but not human glycosylation. All these cell lines do not produce a single product. Instead microheterogeneity is introduced, i.e. a large mixture of very similar, but not identical molecules is produced.

A further challenge for all involved in drug safety issues is the advent of biosimilars, copies of the out-of-patent blockbuster biopharmaceuticals. As it is impossible to exactly define one protein structure for the original drug, it is also impossible to conclusively show the overall identity of a biosimilar to the original marketed product.

Regulatory View of GMP Conform Analysis

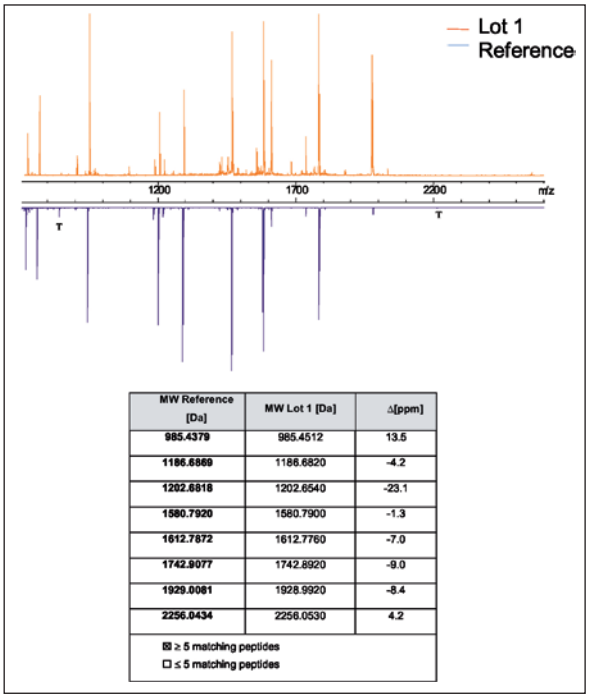
The major regulatory authorities of the industrialized nations, i.e. FDA and EMEA, have faced the challenges posed by this new class of drug substances, the innovative biological entities (IBE). The rules and guidelines for the approval of new drugs had to be altered and expanded to cover protein drugs as well.

The guidelines from the different agencies were harmonized by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). These ICH guidelines are the first point of information for anyone concerned with GMP conform analysis of drug substances. Of these, especially guidelines "Q6B: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products" (fig. 1) and "Q7: Good Manufacturing Practices for Pharmaceutical Ingredients" deal with protein drugs manufactured by biotechnological processes.

Another resource for regulatory information on analytical challenges are the Pharmacopoeias in which the specific tests for the release of a drug substance are stated as well as information about the general analytical methods.

New Developments

The analytical challenges posed by protein drugs lead to the development of new analytical methods that were capable of analysing such complex samples. According to guideline ICH Q6B the characterization of a biotechnological or



MW Reference [Da]	MW Lot 1 [Da]	Δ[ppm]
985.4379	985.4512	13.5
1186.6869	1186.6820	-4.2
1202.6818	1202.6540	-23.1
1580.7920	1580.7900	-1.3
1612.7872	1612.7760	-7.0
1742.9077	1742.8920	-9.0
1929.0081	1928.9920	-8.4
2256.0434	2256.0530	4.2

☒ ≥ 5 matching peptides
☐ ≤ 5 matching peptides

Fig. 2: Detailed characterization of a protein compared to its reference using a peptide map of the tryptic peptide digest. At the same time, the identity of each isolated peptide is confirmed by mass spectrometry. Representative reference peptides masses were used for the efficient, fast and reliable confirmation of the identity of the analysed molecule. (Dr. P. Bulau, Protagen)

Tab. 1: Regulatory Documents/References

Document	(Regulatory) Authority	Link
US Pharmacopeia (USP)	USP	www.usp.org
European Pharmacopeia	EDQM (European Directorate for the Quality of Medicines & Health Care)	www.edqm.eu
EudraLex (The rules governing medical products in the European Union) Volume 4	European Comission	Ec.europa.eu/enterprise/pharmaceuticals/eudralex
FDA (Guides): • Code of Federal Regulation (CFR) • Guidance for Industry	FDA (US Food and Drug Administration)	www.fda.gov
EMEA Guides	EMEA (European Agency for the Evaluation of Medical products)	www.emea.eu
ICH Guides	ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use)	www.ich.org

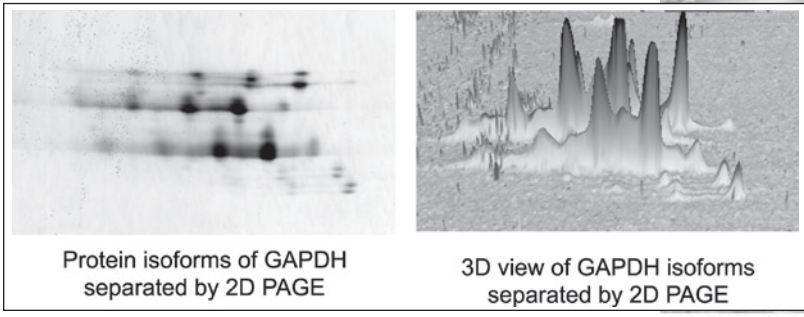


Fig. 3: Enlargement of a 2D gel region showing Glycerinaldehyde-3-phosphate- dehydrogenase (GAPDH) protein isoforms. The relative peak hights of the isoforms can be visualized in a three-dimensional view of the spot. (P. Lutter, Protagen)

biological product includes the determination of physicochemical properties, biological activity, immunochemical properties, purity and impurities.

The physicochemical characterisation of a therapeutic protein will deal with the structural characterization of the protein, especially in terms of the inherent microheterogeneity of the therapeutic product.

The structural characterisation of a protein includes the amino acid sequence as well as posttranslational modifications (e.g. phosphorylation or glycosylation). Here, glycosylation is the major post-translational modification of eukaryotic, especially human proteins and plays a major role in the activity, pharmacokinetics, immunogenicity and other crucial properties.

Impurities are divided into process-related impurities such as contaminants and product-related impurities e.g. degradation products. The impurity analysis is especially important, as proteins and DNA from the host cells could still be present at low level and can cause side effects, may influence activity or induce immune responses.

Typically, GMP compliant protein drug production involves three different phases. First of all, proteins from representative production batches are fully characterized by all available analytical methods. Typical state of the art technologies are ELISA, Liquid Chromatography (TLC, RP-HPLC, SEC, IEX), Electrophoresis (sodium dodecylsulfate polyacrylamide electrophoresis or SDS-PAGE, capillary electrophoresis or CE) or Mass Spectrometry. For the determination of correct glycosylation of protein drugs, HPAEC-PAD is presently state-of-the-art. Only by applying all these methods, a better but by far not complete picture of the protein drug begins to emerge.

In a second step, reference material batches are produced and analyzed. In a third step, the lot release testing of drug substances is performed by comparing the product with the reference material. For this, the crucial tests for the actual release of the drug substance are selected. This subset of methods is then diligently validated according to all regulatory guidelines to ensure a reproducible and correct analysis.

Examples

In a typical scenario, 1D gel electrophoresis or HPLC are used in combination with mass spectrometry in order to show the equivalence of the reference material and the production lot with regard to identity, purity and physicochemical properties.

In figure 2 the mass spectrum of a production lot of a protein is compared to a reference standard for lot release. When comparing the mass spectra visually, the identity is obvious to the trained eye. In order to obtain a validated result, the peak lists rather than the raw data are compared. For a GMP conform result, it is important to set the specifications for a successful comparison in a format that the risk of error is minimized. Here, a statistically valid set of criteria was used for lot release.

High resolution 2D gel electrophoresis gives even deeper insights into the isoform pattern or the impurities of a protein preparation (fig. 3).

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