From Development to Validation: Analytical Method LCM and its Associated Challenges

Abstract

Analytical methods are the backbone for the release of a quality pharmaceutical product. The development of an analytical method is thus based on the capability of an analytical technique to discern the critical quality attributes desired of a product, with consistent precision and accuracy. The true challenge of this seemingly simple tool development task is apparent only when automated techniques with the capability to simplify the complex molecular structure are designed to support robustness and data repository. The approach to development is usually traditional with elements of DOE evaluated during the qualification phase, which assess the method capability and robustness over myriad parameters, that instead of which are verified during validation performed in a controlled environment. The journey of a validated method is tracked through its life cycle management. While selection of a method is defined by the quality target profile, the life cycle management is defined by the analytical target profile of the method. In this article the journey of an analytical method, from development to lifecycle management will be discussed along with the associated regulatory and compliance considerations.



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Introduction

Analytical methods are intrinsic to quality. Sensitivity and robustness in a test method are critical to ensure product quality for every lot manufactured and released. The guidelines published by various regulatory authorities unequivocally uphold the ICH Q2 (R1) recommendations on analytical testing method requirements of robust analytical tests that determine identity, purity, impurities, efficacy and safety for product release[1]. The analytical techniques for each attribute are standard across the pharmaceutical industry, nuanced by the molecular structure complexity of the active pharmaceutical ingredient (API). For instance, liquid chromatography techniques are employed for impurity quantitation and a functional *in vitro* test method for assessing activity of the API. The testing approach for therapeutic proteins in industry is similar although it is considered a distinct field within the industry, owing to the challenges posed by the sheer size and multi-layered complex structure. Distinct guidelines on analytical method requirement from regulatory agencies worldwide have been introduced for biological entities owing to complex structure and susceptibility to variation during manufacturing process[2].

In this article, challenges associated with adopting requirements of some of the established techniques in routine analytics for therapeutic proteins is presented.

Analytical Method: *Selection to Validation*

Manufacturing of therapeutic protein products involves two primary steps, cellular expression and protein purification. The choice of a suitable host (prokaryotic, or eukaryotic microbes, or mammalian cell lines) is governed by the structure & post translational modification desired of the therapeutic protein, or as per precedence which is the case with biosimilars[3]. It is not unlikely for the expression construct to influence translational and post-translational events, such as titer, N-terminal/ C-terminal truncations or glycosylation pattern. Therefore, the requirement of suitable analytical method for quality and titer estimation is necessary at the very early stage of the therapeutic protein manufacturing process. An overview of analytics requirement along the product lifecycle is indicated in the schematic illustrated in Figure 1. The need for suitable analytical techniques is necessary at the clone selection stage itself, which is also usually the beginning of an analytical method development work package to support clone selection and process development.

Method Selection

A pre-requisite for the development of an analytical method is an understanding of the objective/capability desired from a test method. Proteins minimally comprise of 3 layered structures - primary, secondary and tertiary, with an additional quaternary structure for proteins composed of more than one polypeptide chain. The expectation is to develop tools that are sensitive to detect subtle changes in any of these structural levels. With protein therapeutics this becomes a challenge, as the API is highly susceptible to structural perturbations, which may be introduced during manufacturing or product storage. Thus, a protein API would invariably comprise of a population of more than one form. The purification process ensures enrichment of the targeted form but can never assure elimination of the 'unwanted'. A key challenge is to understand the impact of the 'unwanted' additional forms on efficacy. Most of the techniques employed for detecting such subtle differences at physico-chemical levels are liquid chromatography derived, which employ shallow gradients to distinguish between these subtle variants/ unwanted forms. HPLC based methods are still preferred as Table 1 lists, both because of choice of availability of columns with respect to matrix, chemistry, length, particle size, etc. aswell as the shallow gradients deployed to achieve resolution. For instance deamidation of asparagine amino acid residue which is one of the commonly encountered variation would result in pI difference of about 0.07 units with increase in mass by only 1 Da as illustrated in Figure 2 for a hypothetical sequence of 10 amino acid residues. However, an increase in polypeptide length change in pI due to deamidation may be less evident as illustrated in Figure 2 with the 100 amino acid residue hypothetical polypeptide. Traditional methods of isoelectric-focussing may thus not be suited for proteins of longer amino acid residues lengths. It has been observed that surface charge distribution is a more reliable parameter and hence ion exchange chromatography methods are usually more successful in discerning such modifications. The gradients used are salt or pH but very shallow, which have a tendency to introduce retention time drifts within a sequence. Nonetheless, reproducibility for these methods is dependable w.r.t impurity resolution and quantitation. These tests can be employed at intermediate stages of protein manufacturing. Both cation and anion exchangers, weak and strong have been demonstrated for such industrial applications. Identity for proteins is established through tests that verify primary structure. This is perfomed using peptide mapping, wherein the protein is subjected to enzymatic fragmentation and the resulting peptides are resolved by reversed phase chromatography techniques. This test is restricted to the analysis of purified protein.

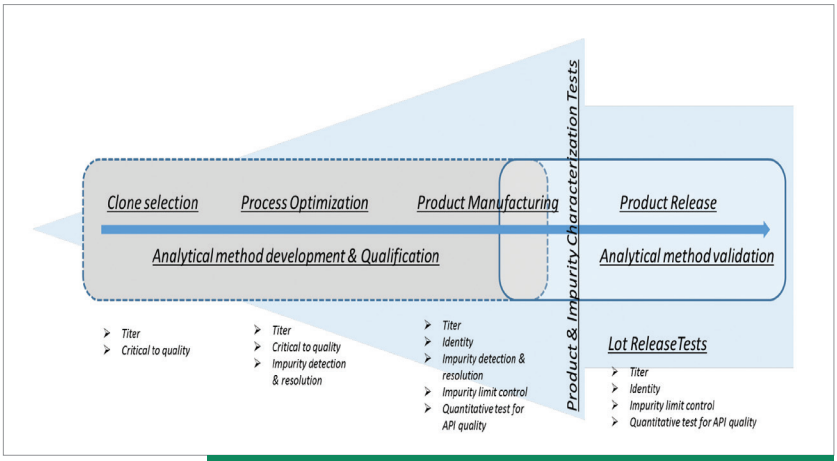
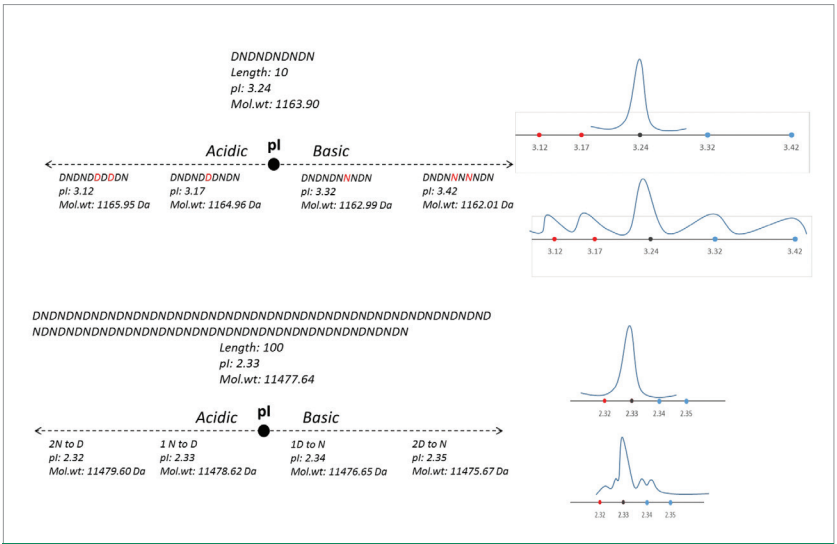


Figure 1. Schematic showing the change in gamut of analytical test & method stage across the therapeutic protein life cycle.


DNDNDNDNDN
Length: 10
pI: 3.24
Mol.wt: 1163.90
Acidic
pI
Basic
DNDNDDDDDN
pI: 3.12
Mol.wt: 1165.95 Da
DNDNDDDNDN
pI: 3.17
Mol.wt: 1164.96 Da
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pI: 3.32
Mol.wt: 1162.99 Da
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pI: 3.42
Mol.wt: 1162.01 Da
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The chromatography techniques employ primarily UV-vis detection systems, which provide an absorbance read for peptide bond or aromatic amino acid residues. Use of fluorescence detection system is restricted to profiling of labelled glycan structures or for protein characterization studies.

Selection of a titer test method would vary depending on the stage of product development. At final stage of

Technique	Purpose	Associated Challenges			Limitation
		Technique	Data analysis	Robustness	
Iso-electric focussing	To distinguish between differently charged species	Standard technique, however limited choice for ampholyte ranges	The bands observed are very close by and not distinguishable for subtle changes in charge	The test can be employed in a qualitative manner only with not absolute resolution	With increase in amino acid residue length of a sequence, the difference in pI units with change in acidic or basic residues is still subtle to be picked up. Only effect of bulk charges such as those introduced by sialic acid can be observed.
Ion exchange HPLC	To separate molecules based on surface charge	Standard technique, however, changes introduced by conformational variations can also provide input. Shallow gradients are required to resolve impurities	The data analysis is complex, as shallow gradients are highly dependent on column lots and maintenance procedures	Variabilities observed over long term usage, primarily with column lots and lack of equivalent columns. As subtle differences in matrix, packing density may impact resolution significantly	The surface charge variants of a protein entity are too complex to be resolved absolutely. This results in a profile like a continuum, which makes autointegration a challenge. Also, lack of equivalent columns available in market manufactured especially for biomolecules pose a challenge in long run.
Reverse Phase-HPLC	To separate surface polarity variants	The method fails to work effectively for large intact proteins, which tend to elute as a single entity. Design of gradients and selection of column chemistry have been complex. The technique is usually employed after fragmenting parent molecule into smaller fragments using enzyme or chemical processing	Design of gradients are shallow, as surface polarity differences between peptides of similar length are not significant. Choice of enzyme or chemical to fragment is a trade- off, resulting in too many small peptides or too little which may not truly be indicative of subtle changes.	Method is highly sensitive to subtle changes in mobile phase preparation & slight variations in ion-pairing reagents.	The primary challenge is sample preparation. Although this is one of the techniques best employed in LC-MS mode. However, leads to trade-off between ideal choice of ion pairing reagent and mass spec compatibility
Gel permeation-HPLC	To separate size based variants	Very effective for resolution between high molecular weight. However, fragmented proteins cannot be detected owing to non-covalent interactions with monomer and inability of mobile phase to resolve.	Method is robust to pick significant changes in hydrodynamic volumes between variants (primarily increase), and the outcome does not reflect the molecular weight unlike that observed with small molecules. Also, slight changes can get introduced by the mobile phase itself.	Method is robust. But not absolute in ascertaining the nature of high molecular weight species observed.	Cannot be a standalone technique. Requires characterization of variants observed by complex instrumentation such as light scattering detectors, for establishing identity of the molecule.
SDS-PAGE	To separate size based variants	The electrophoretic mobility is not necessarily a reflection of molecular weight. Post translational modifications can significantly impact the relative mobility on a gel.	Slight truncations would go undetected in this technique. Additionally, the detection using stains, may not be uniform for all variants.	Method is primarily employed in qualitative mode. Densitometric analysis is complex as certain protein diffusion is inherent due to post translational modifications.	SDS-PAGE cannot be considered an absolute way to determine the molecular weight from relative mobility for all therapeutic proteins. It remains primarily a test for purity and biased by the nature of impurity.
Mass Spectrometry	To determine primary sequence and molecular weight	This technique routinely employed as identity for small molecules poses a challenge for proteins. Determination of intact mass with precision remains challenge. Mass obtained after truncation of the protein may not always be conclusive due to variations introduced during sample preparation	With advances in technology this still remain a challenge and a specialized field. Sample preparation induced changes tend to give a population based result and hence minimize on the sensitivity	Method is employed only at characterization phase. The quantum of data generated usually is too complex and exhaustive to be analysed in routine analysis.	The technique fails to provide an absolute read. Multiple approaches within the technique need to be thus employed to support characterization package. This technique makes data interpretation and analysis specialized/expensive with limited access.
Bioassay	To determine the efficacy of the molecule	This technique is most suited when a commercial line or an in-house cell line is used to produce a sensitive read or to develop an in-house cell line. Maintenance of reagent and cell banks remain a challenge.	Data analysis is complex, especially for cell proliferation assays, which are more sensitive to subtle variations during experiment set-up. Also when it comes to choosing the best test to represent efficacy there is a trade-off between robustness desired and activity.	Inherently the methodology is complex.	Data representation and quality systems associated with bioassay techniques require a separate discussion, which is beyond the scope of this article.

Table 1. Challenges encountered with standard techniques