"Validation and assessment of the stability indicatingcapability of aCEX-HPLC for charge variant analysis anda SEC-HPLC method forsize heterogeneity analysis of a monoclonal antibody; Sunmab"

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MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

BY

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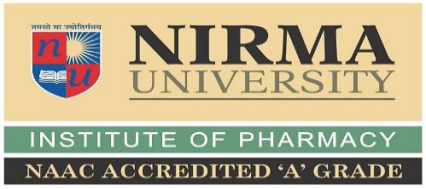

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May 2021

CERTIFICATE

This is to certify that the dissertation work entitled "Validation and assessment of the stability indicating capability of a CEX-HPLC for charge variant analysis and a SEC-HPLC method for size heterogeneity analysis of a monoclonal antibody; Sunmab" submitted by Mr. Ashutosh Saxena with Regn. No. (19MPH302) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under my/our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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CERTIFICATE OF ORIGINALITY OF WORK

This is to undertake that the dissertation work entitled "Validation and assessment of the stability indicating capability of a CEX-HPLC for charge variant analysis and a SEC-HPLC method for size heterogeneity analysis of a monoclonal antibody; Sunmab" Submitted by Ashutosh Saxena (19MPH302) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by me at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under the guidance of Dr. Priti J. Mehta and at SUN Pharmaceuticals Industries Limited under the guidance of Dr. Ravishankara M.N. I am aware about the rules and regulations of Plagiarism policy of Nirma University, Ahmedabad. According to that, this work is original and not reported anywhere as per best of my knowledge.

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DECLARATION

I hereby declare that the dissertation entitled "Validation and assessment of the stability indicating capability of a CEX-HPLC for charge variant analysis and a SEC-HPLC method for size heterogeneity analysis of a monoclonal antibody; Sunmab", is based on the original work carried out by me under the guidance of Dr. Priti J. Mehta, Head of Department, under the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University and Dr. Ravishankara M.N., Senior General Manager, Sun Pharmaceuticals Industries Limited. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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LIST OF ABBREVIATIONS

Sl no.	Abbreviation	Full Form
1.	CEX	Cation Exchange Chromatography
2.	SEC	Size Exclusion Chromatography
3.	pI	Isoelectric point
4.	µg	Microgram
5.	µl	Microliter
6.	ml	Milliliter
7.	mg	Milligram
8.	g	Gram
9.	mAbs	Monoclonal Antibodies
10.	LOD	Limit Of Detection
11.	LOQ	Limit Of Quantitation
12.	HMW	High Molecular Weights
13.	LMW	Low Molecular Weights
14.	PTM	Post Translational Modifications
15.	cIEF	Capillary Isoelectric Focusing
16.	API	Active Pharmaceutical Ingredient
17.	GMP	Good Manufacturing Practices
18.	GLP	Good Laboratory Practices
19.	ICH	International Conference on Harmonization
20.	VH	Variable Heavy chain
21.	CH	Constant Heavy chain
22.	HC	Heavy chain
23.	LC	Light Chain
24.	CQA	Critical Quality Attributes
25.	Pro	Proline
26.	Gly	Glycine
27.	Lys	Lysine
28.	Asp	Aspartate
29.	Ser	Serine
30.	His	Histidine
31.	R.S.	Reference Standard
32.	%age	Percentage
33.	% RSD	Percent Relative Standard Deviation
34.	rpm	Rotations per minute

ABSTRACT

ABSTRACT

Monoclonal antibodies (mAb) are prone to different kinds of Post Translational Modifications (PTMs) that may lead to different kinds of heterogeneities which result in the formation of mAb variants. These heterogeneities may arise at different stages of the life cycle of mAb. Two of the most common heterogeneities that are encountered are charge and size heterogeneity. On the hand, charge heterogeneity leads to the generation of charge variants in the form of acidic and basic species. The analysis of such variants can be analyzed with the help of Ion Exchange chromatography. On the other hand, size heterogeneity leads to the generation of size variants in the form of HMWs and LMWs species. The analysis of such variants can be easily done with the help of Size Exclusion Chromatography method. In reference to the current prospects, the CEX and SEC methods were developed for Sunmab which is a therapeutic monoclonal antibody developed and marketed by SUN Pharmaceuticals Industries Limited. The methods were validated for different validation parameters according to the ICH guidelines. The methods were also evaluated for their stability indicating capabilities with the help of a forced degradation study based on different stress parameters. Upon validation of the CEX method, the given analytical was found to be precise and accurate. The method was linear over a concentration range of 0.5-375% of the reference standard concentration with a correlation co-efficient of 0.9997. The method was able to quantify (LOQ) the main species up to a concentration of 0.4 mg/ml and detect (LOD) the same up to concentration of 0.08 mg/ml. Similarly, the SEC method was also precise and accurate. The range over which the method was linear was 0.5-125% of the reference standard concentration with a co-efficient of 0.9909. The LOD and LOQ of the method was found to be 0.5 ug/ml and 1.25 ug/ml respectively. Both the methods showed significant prowess in their stability indicating capabilities upon forced degradation of the sample via different stress parameters.

CHAPTER 1:
INTRODUCTION

1.1 Introduction to Monoclonal Antibodies

Monoclonal antibodies are glycoproteins of high molecular weight which primarily resemble the gamma-immunoglobulin (IgG). IgG is a glycoprotein consisting of 2 identical heavy chains (HC) and 2 identicallight chains (LC)(Abeer et al, 2017). There are one variable (VH) and three constant domains (CH1, CH2 and CH3) in each heavy chain and one variable (V1) and one constant domain (C1) in each light chain. The heavy and light chains are connected by disulfide bonds and 2 heavy chains are linked together by multiple disulfide bonds in the hinge region. The 3 loops in VH and V1 domains contain the antigen-binding sites and are termed as the complementarity determining regions (CDRs). The antigen-binding Fragment or Fab represents the VH and CH1 domains along with the disulfide linked light chain. The crystallizable fragment or Fc represents the disulfide bonded CH2 and CH3 domains in the hinge region. The CH2 domain also consists of a single N-linked antennary oligosaccharide situated on a conserved asparaginase residue. IgGs are further classified into 4 groups, namely, IgG1, IgG2, IgG3 and IgG4 based on the heavy chain sequences and interchange disulfide bond patterns. The groups IgG1, IgG2 and IgG4 are widely used as therapeutic monoclonal antibodies, whereas, IgG3 is rarely used due to their unusually short serum half-life(Xiaobin et al, 2016).

Monoclonal antibodies when compared to small molecule drugs show various desirable characteristics such as target selectivity, potency, low incidence of side-effects as well as long serum half-life (Puttrevu et al,2019). Unlike small molecu1es, they have fairly high molecular weights, have a complex structure and are formulated at fairly high concentration levels (Abeer et al, 2017). A1so, stability of monoclonal antibodies is a major concern.

Therapeutic monoclonal antibodies are primarily used for targeted therapy and their success is mainly owed to their long serum half-life along with their target selectivity and specificity abilities. The diminished risk of unwanted immunogenicity due to their similarity in the sequences of chimeric or humanized monoclonal antibodies with human monoclonal antibodies makes them an ideal choice of therapeutic proteins (Nguyen, 2018).

1.2 Heterogeneity in Monoclonal Antibodies

The monoclonal antibodies of therapeutic importance are drug molecules with a high degree of heterogeneity including charge variants, aggregates, fragments which arise mainly due to post-translational modifications (PTMs) and physicochemical transformations that can arise during the life span of the product.

PTMs can be defined as enzyme-catalyzed processing of the protein polypeptide chain following translation that are responsible for functional regulation, structural/conformational rearrangements, cellular regulation and signal transduction.(Li et al., 2015)

Common PTMs 1ike glycosylation, deamidation, oxidation, glycation, etc can be introduced over the life span of monoclonal antibodies during production, storage and in-vivo circulation by chemical or enzymatic modifications. These modifications are influenced by several chemical and physical factors such as temperature of storage, oxidation, photosensitivity, ionic strength and shear stress. Heterogeneity gives rise to two main categories of products i.e., product related substances and product related impurities.

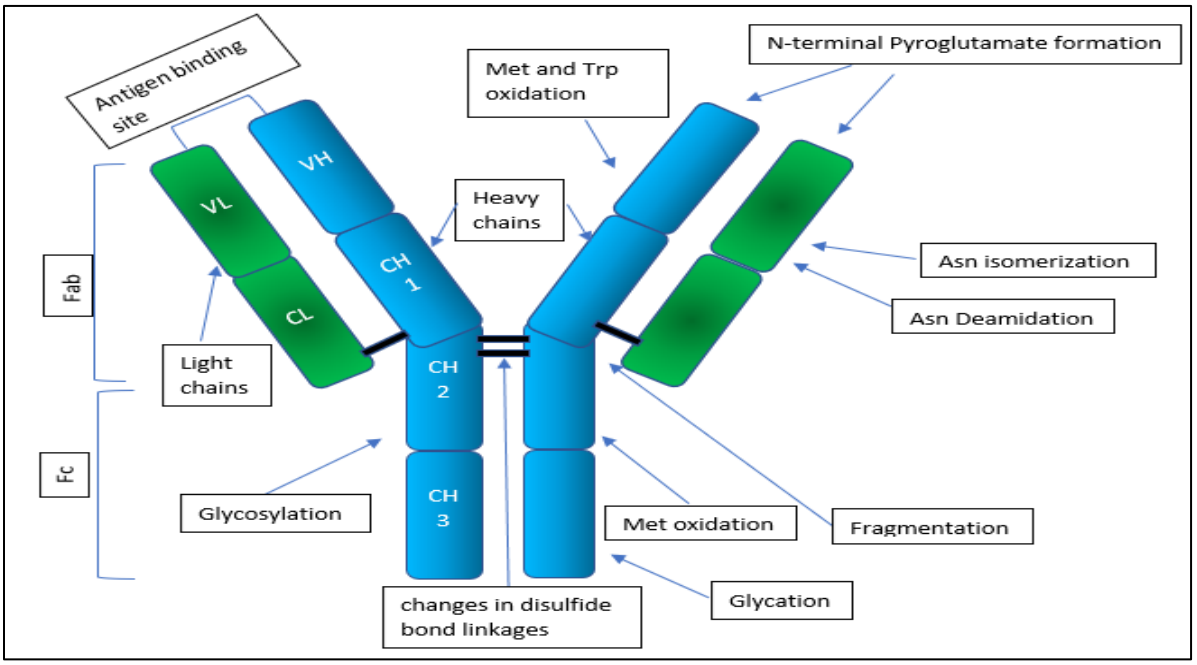

Fig 1.1: Typical forms of heterogeneity associated with monoclonal antibodies

Product related substances can be described as molecular variants that are generated during manufacture and/or storage of the desired therapeutic product that are considered to be active with no deleterious impact on the safety and efficacy of the product. Hence, these

products are not regarded as impurities. Product related impurities on the other hand, are variants that

have properties different from that of the desired product and hence are required to be completely characterized in terms of their chemical nature and impact on stability, activity, efficacy and safety.(Beck, 2019)

1.3 Charge and Size Heterogeneity

The most common types of heterogeneities encountered when talking about mAbs are charge and size heterogeneity. Charge heterogeneity arises due to multiple PTMs that bring about a considerable change in the charge distribution on the surface of the protein. Such changes could be a result of a direct alteration in the charge state an indirect change in the surface charge owing mainly to a conformational change. Charge variants can be categorized mainly as acidic and basic species. Hence, charge variant analysis is a widely used tool for characterization of mAbs as it ensures consistency of the therapeutic product with no unwanted changes to the protein.(Baek et al., 2020)

Size heterogeneity is mainly concerned with heterogeneity in molecular size and weight that are presented in the form of aggregates referred to as High Molecular Weight species (HMWs) and fragments referred to as Low Molecular Weight species (LMWs). Size variants mainly arise due to varying hydrophobic interactions that induce structural variations in the drug product that leads to denaturation of the native state of the protein. Hence, size variant analysis becomes imperative as these variants are known to cause a potent impact on safety of the product.

1.4 Impact of PTMs

PTMs have the potential to impact the safety and/or efficacy of monoclonal antibodies. They can also bring about changes in the safety profi1e of monoclonal antibodies by enhancing immunogenicity or off-target bindings or may affect the interaction of therapeutic monoc1ona1 antibodies to its target antigen, there by affecting the efficacy of the same(Xiaobin Xu, 2016).

Therefore, it becomes essential that these undesirable products need to be characterized and controlled in order to successfully develop a drug product intended for therapeutic action. These heterogeneities are often considered as Critical Quality Attributes (CQA) and hence appropriate analytical tools must be used for their analysis and characterization. A thorough understanding of the chemical and physical instabilities that act as pathways for the

generation of PTMs becomes necessary for development of a robust formulation(Krause & Sahin, 2019).

Analysis of the product variants are also a prerequisite for assessment of the potential novel mAb structure and formats(Beyer et al., 2018). Their analysis has also gained significant attention post the commanding success of biosimilars. In order for a successful biosimilar product, a complete characterization of its originator molecule is necessary. Additionally, regulatory authorities have laid down stringent rules that require for close monitoring and comparison of the heterogeneityprofiles between batches with a set of appropriate methods that is able to detect as well as characterize the products.

The analysis of different variants can be easily achieved with the help of universal methods like chromatographic or electrophoretic methods which achieve separation based on the different physicochemical properties of the mAb isoforms. These methods provide with affirmative information about the presence and relative abundance and these results can be associated with relevant PTMs. For example, CEX and cIEF can be used for charge heterogeneity analysis of mAbs which separates the acidic and basic variants and interpretation of this obtained data can be related to the mechanism or PTM responsible for the particular heterogeneity.

Similarly, techniques such as SEC and Light Scattering techniques are well established and can be used for the analysis of size variants. Nevertheless, these techniques possess certain limitations which makes it efficient to perform analysis with an array of orthogonal techniques suited for the purpose.

1.5 Stability Indicating Method and Forced Degradation Study

Forced degradation can be considered as a process that involves the degradation of the therapeutic drug product at stress conditions that are more severe than accelerated conditions. This leads to the generation of degradation products which are then studied to elucidate the degradation pathway and hence determine the stability of the molecule (Blessy et al., 2014).

The choice of the stress conditions used to achieve products of forced degradation must be carefully considered. The conditions selected must be such that they bring about decomposition of the drug product. Stress conditions must be selected keeping in mind the structural and physicochemical properties of the product under consideration (Tamizi & Jouyban, 2016).

Other than the proposed conditions would lead to any deleterious effect on the drug product. They also aid in the identification of a stable and robust formulation in a short period of time.

It must be noted that forced degradation studies go alongside analytical characterization as the outcomes of these degradation studies can only be substantiated and validated provided there are stability indicating analytical methods available to identify the degradation products.

A stability indicating method is an analytical procedure used for quantification of the reduction in the amount of the API in the drug product. It shows capability in specifically and accurately measuring significant level of change in the concentration of the active ingredients in the drug product under consideration. The samples obtained from the forced degradation studies are used in order to develop and assess the stability indicating capability of the given analytical method.

It must be noted that the analytical method used to determine stability must be sensitive enough to detect impurities at extremely low levels i.e., 0.05% of analyte of interest or lower. Also, the peak responses should fall within the range of detector's linearity. The method under consideration should be able to detect all impurities formed due to the different stress condition (Psimadas et al, 2012).

1.6 Analytical Method Validation

In the most general sense, validation is considered as a confirmation by examination and provision of objective evidence in relation to the fact that certain necessary requirements to be fulfilled in order for an analytical method to be used for an intended purpose (Araujo, 2009).

Analytical method development may be defined as a process by which a given analytical method for a specific drug product needs to be developed for the entire life cycle of the product. Later, a mini validation procedure has to be carried out prior to commencement of analyses of routine samples of the drug product under consideration (Izydor Apostol, 2014).

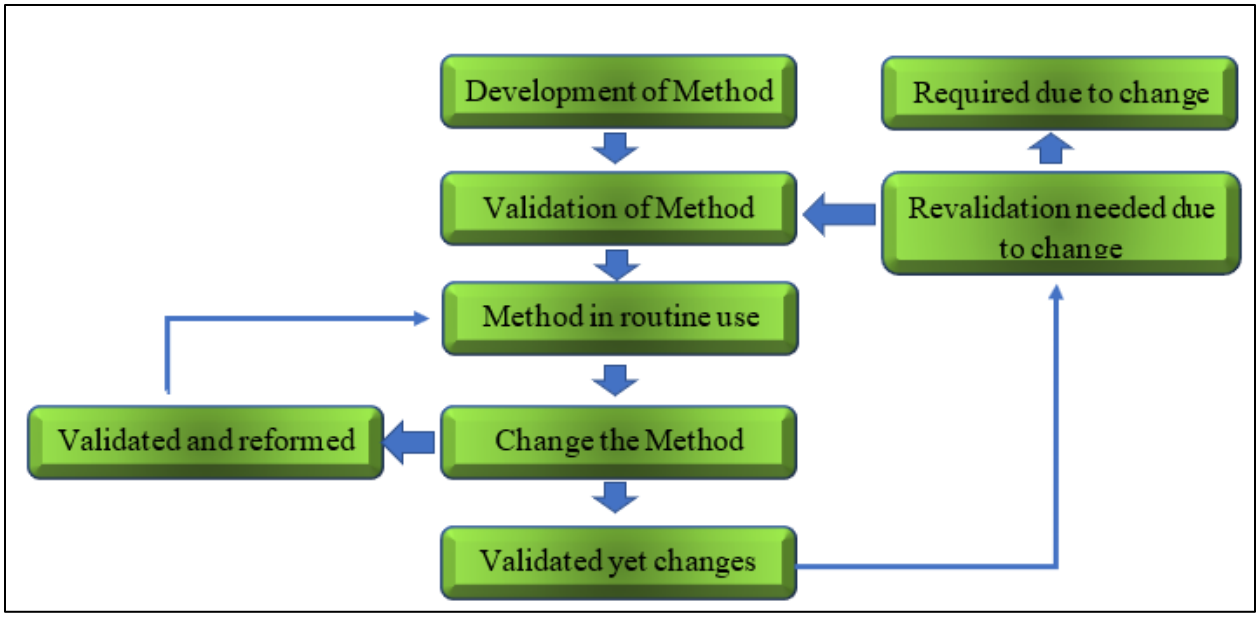

Fig 1.2: Life Cycle of an Analytical Method

The development of an Analytical Method must be performed adhering to the protocols and specific acceptance criteria with reference to ICH guidelines Q2(R1). It must be kept in mind that the given analytical method in intended for use in GLP and GMP environments only (Bharti Mittu & Chauhan, 2015). It happens to be extremely crucial as its success ultimately culminates into a marketing approval (Izydor Apostol, 2014).

The different steps involved in analytical method development are:

- Standardization of the working standard from the reference standard.
- Optimization of the analytical method parameters critical to the method's intended application.
- Analytical method verification prior to method transfer for routine samples (Izydor Apostol, 2014).

It is well known that no analytical method can be truly universal and the specifications of the method cater to only a single or group of drug products. Hence, it is inevitable that the method must be modified and subsequently verified, for which it requires different levels of validation.

Hence, validation although not prescribed by regulatory requirements is often considered as an integral part of cGMP practice (Geetha et al., 2012)

A method validation in broad terms, is the process of establishing documented evidence that provides a high degree of assurance that the drug product considered for the particular method will meet the necessary requirements for the intended analytical applications (Geetha et al., 2012).

Validation of a given analytical method justifies scientific soundness of the analytical measurement. It helps the analyst in understanding the responsiveness of the method and assists in establishment of performance limits for the method (Bharti Mittu & Chauhan, 2015). Validation also becomes necessary in order to fulfill the quality control requirements (Geetha et al., 2012). The Code of Federal Regulations (CFR) 311.165c explicitly states that "the accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented.

The different validation parameters that need to be evaluated and confirmed for an analytical method are:

Sl no.	**Validation Parameter**	**Description**	**Remarks**	**References**
1.	Accuracy	An expression of the closeness of agreement between theaccepted reference value and the value found.	Accuracy should be established across the specified range of the analytical procedure. It should be reported as percent recovery by the assay of known added amount of analyte in the sample.	(Geetha et al., 2012).

Sl no.	Validation Parameter	Description	Remarks	References
2.	Precision	An expression of the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling. of the same homogeneous sample under the prescribed conditions.	The sampling must be done from the same homogenous sample under the prescribed conditions. Three types namely, reproducibility, repeatability and	(Geetha et al., 2012).
3.	Range	It isthe interval between the upper and lowerconcentration (amounts) of analyte in the sample(including these concentrations) for which it hasbeen demonstrated that the analytical procedurehas a suitable level of precision, accuracy andlinearity	Range is calculated from the linearity study.	(Geetha et al., 2012).
4.	Limit of Detection	It is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value	Can be calculated based on: Visual Evaluation Signal-to-Noise or Standard Deviation of the Response (S.D) and the Slope (m) using the formula	(Geetha et al., 2012).

Sl no.	Validation Parameter	Description	Remarks	References
5.	Limit of quantitation	The quantitationlimit of an individual analytical procedure is thelowest amount of analyte in a sample which canbe quantitatively determined with suitableprecision and accuracy.	Can be calculated based on: Visual Evaluation Signal-to-Noise or Standard Deviation of the Response and the Slope using the formula 10 (S.D/m)	(Geetha et al., 2012).
6.	Robustness	It is a measure of its capacity to remainunaffected by small, but deliberate variations inmethod parameters.	Provides an indication ofits reliability during normal usage.	(Geetha et al., 2012).
7.	Ruggedness	It is a measure of reproducibility in the test results.	The conditions are changes in laboratory and/or analyst which are indeed expected to occur.	(Geetha et al., 2012).
8.	System suitability testing	It involves routine tests to check if all the components constituting a system are operating as expected	It forms an integral part of any analytical procedure. The components comprise of equipment, electronics, analytical operations and samples to be analyzed	(Geetha et al., 2012).

Sl no.	Validation Parameter	Description	Remarks	References
9.	Specificity	It is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present It refers to the extent to which it can determine particular analyte(s) in a complex mixture without interference from other components.	The method must have the ability to separate each known impurity and degradation product at the quantitation level	(Geetha et al., 2012). (Ravichandran et al., 2010) (Izydor Apostol, 2014).
10.	Linearity	The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.	Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. Following which, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares.	(Geetha et al., 2012) (Izydor Apostol, 2014).

Table 1.1: Different validation parameters evaluated in Analytical Method Validation

CHAPTER 2:

SIZE HETEROGENEITY (AGGREGATION) IN MONOCLONAL ANTIBODIES

2.1 Introduction

In order to function effectively, therapeutic proteins require a 3D folded structure. Such a structure is possible due to certain fundamental forces between amino acids such as Van der Waals and hydrophobic interactions, hydrogen bonding, etc. these kinds of interactions are also critical in maintaining structural integrity(Khodabandehloo, 2017).

However, there are certain intrinsic and extrinsic factors that can cause chemical and/or physical instabilities. Such instabilities, mainly physical instabilities lead to the generation of six variants in the form of High molecular weight (HMW) and/or Low molecular weight (LMW) species.

Protein aggregates which represent HMWs are composed of multimers of natively conformed or denatured monomers(Rosenberg, 2006). On the other hand, protein fragments which represent LMWs are fragmented portions of proteins that are of lower molecular weight than the protein itself.

These size variants are formed by different processes, namely denaturation, aggregation, precipitation and adsorption of the protein. Failure of a protein to remain in its tertiary and secondary structure represents a denatured protein. The transformation of a protein from its native stable state to a denatured state can be due to a direct unfolding process or a series of intermediate unfolded states thereby leading to exposure of hydrophobic residues to the aqueous environment.

Further, the intermolecular interactions of the exposed hydrophobic regions of two denatured protein molecules leads to the formation of aggregates. Failure to prevent such interactions leads to formation of soluble particles. This happens due to interactions of clusters of aggregated proteins with other denatured proteins. Ultimately, the soluble particles form insoluble, macroscopic aggregates which are termed as precipitates. The formation of precipitates from soluble aggregates is usually smooth. Protein association is a reversible process that involves the intermolecular interactions of native protein molecules which can be re-dissolved to obtain native proteins. Aggregation by adsorption of denatured proteins onto hydrophobic surfaces and air-water interfaces is another form of aggregation(Frie et al., 2005).

Hence, aggregation represents a physical instability reaction that leads to formation of particles ranging from few nanometer sized dimers to micron sized sub-visible particles that eventually lead to millimeter sized precipitates (Bansal et al., 2019).

2.2 Mechanism of Aggregation

There is no single pathway or mechanism that can possibly describe protein aggregation conclusively. However, in recent years, there have been different mechanisms of aggregation that have been proposed by different researchers such as reversible association of native monomers, aggregation of conformationally altered monomer, aggregation of chemically modified monomer, nucleation-controlled aggregation and surface-induced aggregation (Singla et al., 2016).

The pathway that leads to aggregation may depend on a variety of factors such as the protein itself, the initial state of the protein that is prone to aggregation and different environmental conditions (HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012).

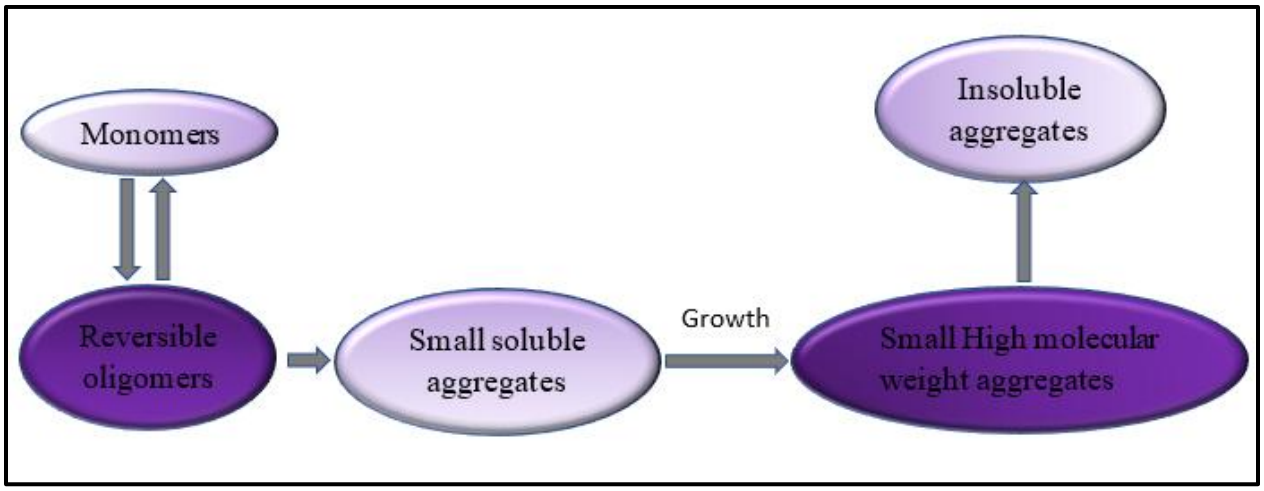

Fig 2.1: Schematic representation of the stepwise mechanism of Protein aggregation

In a recent study, aggregation was owed to protein conformational changes that lead to exposure of stretches of hydrophobic amino acids, thereby leading to protein-protein interactions which results in an irreversible contact between proteins via formation of inter-protein beta sheets (Ghosh et al., 2016).

2.3 Types of Protein Aggregates

Protein aggregates can be classified into the following categories;

a) **By type of bond:** non-covalent aggregates that are formed solely via weak forces namely, Van der Waals, hydrogen bonding, hydrophobic and electrostatic interactions whereas, covalent aggregates are formed via disulphide bond linkages through free thiol groups or by non-disulphide cross-linking pathways such as di-tyrosine formation (HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012).

b) **By reversibility**: reversible aggregates which form by self-assembly of protein molecules due to changes in pH or ionic strength of protein solution and irreversible aggregates

c) **By size**: soluble aggregates in the form of dimers, tetramers, oligomers (Rosenberg, 2006). Insoluble aggregates, on the other hand, lie in the size range of 1-25 mm that occur in the form of amorphous or fibrillar material which in turn depends on proteins and its environment (HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012).

2.4 FACTORS AFFECTING PROTEIN AGGREGATION

Broadly, heterogeneities in mAbs can arise due to degradation via different instability reactions at different stages of the product life cycle from drug product manufacturing to delivery of the formulation to the patient. A wide variety of factors are responsible for the generation of protein aggregation that can be classified as internal as well as external factors.

Internal factors are mainly concerned with changes in the primary as well as secondary structure of protein. It has been previously established that the tendency of a protein to aggregate is inherently a function of its sequence. Any change in the protein sequence caused by a mutation or chemical alteration can lead to a change in its hydrophobicity and/or surface charge distribution which can cause aggregation (Singla et al., 2016).

On the other hand, external factors impact the aggregation propensity of the protein. They include different environmental factors such as pH, temperature, salt concentration, buffer type, protein concentration, ionic strength, mechanical stress, presence of metal ions, freeze drying, freeze thawing and reconstitution. Each of these factors lead to different patterns in

the size and degree pf aggregation of the protein. Hence, in order to study their impact, the Lumry-Eyring model is commonly used. This model suggests that aggregation follows a

simple, two step, non-native mechanism which incorporates a rate limiting reversible conformational transition of the protein. Subsequently, irreversible conglomeration of the protein into aggregates takes place (Singla et al., 2016).

The different factors and how they impact the propensity of aggregation are described as under:

a) **Temperature**: an increase in temperature acts a catalyst to oxidation and deamidation reactions that can lead to significant aggregation. High temperatures are also associated with conformational changes in the quaternary, tertiary and secondary structures of protein that can induce unfolding and subsequent aggregation (HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012).

b) **Freezing and Thawing**: the creation of new ice/solution interfaces, adsorption to container surfaces, cryo-concentration of protein along with pH changes due to crystallization of buffer components could be possible mechanism leading to aggregation in mAbs.

c) **Agitation**: Mechanical stress in the form of agitation is considered as a potent factor that leads to aggregation. Agitation leads to cavitation i.e., the rapid formation of bubbles and voids within the liquid. When thee collapse, they produce shock waves which result in the generation of hydroxyl and hydrogen radicals that in turn lead to aggregates(HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012).

d) **Protein concentration**: since the aggregation of protein sis considered to be due to biomolecular interactions of proteins, it is said to be concentration dependent. At high protein concentrations, macro-molecular crowding occurs which leads to self-assembly of proteins into aggregates according to excluded volume theory. At low protein concentrations, aggregates formed by weak reversible interactions can dissociate(HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012).

e) **Solvent and surface effects**: solvent effects in the form of pH changes strongly influences aggregation rate as it alters the charge distribution on the surface of proteins which in turn affects the electrostatic interactions. Also, under acidic conditions, protein cleavage is predominant whereas, deamidation and oxidation are prevalent under neutral

to alkaline conditions. The solution components also impact aggregation behavior as there are certain polysorbates and surfactants that quench aggregation.

An increase in salt concentration in the buffer may enhance hydrophobic interactions coupled with weakening of electrostatic interactions. That lead to a destabilizing effect on the protein structure aggravating a protein's susceptibility to aggregation (Singla et al., 2016).

Different buffers may have varying impacts on mAb aggregation due to different types of complex molecular interactions that may take place between the buffer species and the Fc domain of the antibody molecule.

f) **Chemical modification of protein**: modifications of the proteins by chemical reactions like deamidation, isomerization, hydrolysis, etc can modify the amino acid side chains thereby distorting the conformation of proteins leading to protein aggregation. These chemical reactions can in turn be aggravated by different environmental factors such as pH, temperature, light exposure, etc(HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012).

The impact that different factors have on the aggregation rates have been elaborately described by Osho et al. According to this study, aggregation was first induced by exposure of the mAb to a slightly acidic pH followed by an increase in ionic strength with the addition of NaCl

The concept of aggregation index was employed to the impact of aggregation. Aggregation index is a concentration independent measure of the aggregate content of the protein solution. It is obtained by normalizing the turbidity signal A_{340} with ($A_{280} - A_{340}$). The aggregation index (AI) was found to be maximal at low pH and high ionic strength suggesting that these conditions had a negative impact on the conformational and colloidal stability(Bickel et al., 2016).

2.5 Impact of Size Heterogeneity

Protein aggregates cause potential adverse effects ranging from immunogenicity to loss of bioactivity (HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012). HMWs are mainly concerned with a potential increase in immunogenicity by eliciting an immune response independent of T-cell help whereas, LMWs mainly lead to decreased activity, reduced serum half-life owing to missing Fab and/or Fc fragments (Renee et al., 2015),(Rosenberg, 2006).

Immunogenicity causes neutralization of the endogenous protein leading to life-threatening situation for the patient (HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA

GRAUSCHOPF, 2012). Protein aggregates directly affect the quality of the final product in terms of efficacy and safety (Hernández-jiménez et al., 2018).

The size and complex structure of therapeutic proteins lead to different chemical and/or physical instabilities which in turn make development of stable aqueous formulations a challenging task (Frie et al., 2005).

2.6 Need for analysis of size variants

Aggregation is often considered as a critical quality attribute (CQA) during biopharmaceutical development as it represents a process-related impurity and/or a degradation product that must be characterized as well as controlled to lowest levels of concentration (Fekete et al., 2014).

Protein aggregates cover a dynamic range of types and sizes which may lead to different immunogenicity reactions along with loss of safety and efficacy of the final therapeutic product (Philo, 2014),(Rosenberg, 2006). Hence, the formation of aggregates under different conditions should be thoroughly investigated to not only ensure the safety and stability of the protein formulation but also becomes critical for maintaining the quality of the therapeutic product (Singla et al., 2016).

The dynamic properties of aggregates lead to a significant analytical challenge and hence requires an array of orthogonal approaches to successfully detect as well as characterize the entire spectrum of aggregate species. The technological and scientific advancement has led to the development ofnovel and emerging tools that possess the potential scope for aiding the prediction and profiling of aggregates. In addition to these, the in-vitro and in-vivo screening of protein aggregation can significantly advance the understanding of which molecular mechanisms cause the aggregation in the therapeutic protein species (Khodabandehloo, 2017).

CHAPTER 3:

SIZE EXCLUSION CHROMATOGRAPHY FOR SIZE VARIANT ANALYSIS

3.1 Introduction

Ideally, in analysis of mAb formulations for the analysis of size variants, one would like to acquire precise information about the amount and size of every species that is present upto levels of 0.1% or less. But, due to the huge variety of different sizes and amounts of size variants, this is not always possible. However, with the currently available analytical techniques, the molecular mass of these variants can be easily determined which in turn gives us information about the stoichiometry of the aggregates(Philo, 2014).

One such analytical methodology commonly adopted is the Size Exclusion Chromatography (SEC) method. It is a high-throughput analytical method that allows the determination and quantification of the level of aggregates and fragments of purified antibodies through isocratic elution. In SEC, separation of different size variants is achieved based on the differential steric exclusion from the pores of the packing material contained in a bed of porous particles as the stationary phase. The larger components i.e., protein aggregates penetrate the matrix particles to a lesser extent and are therefore eluted from the column early.

Hence, they elute ahead of the comparatively smaller components i.e., protein monomers and fragments. The fragments being the smallest components penetrate the matrix readily and are eluted at the last. The monomer being intermediate in size elutes after the aggregate species and before the fragment species (Sec-hplc et al., n.d.).

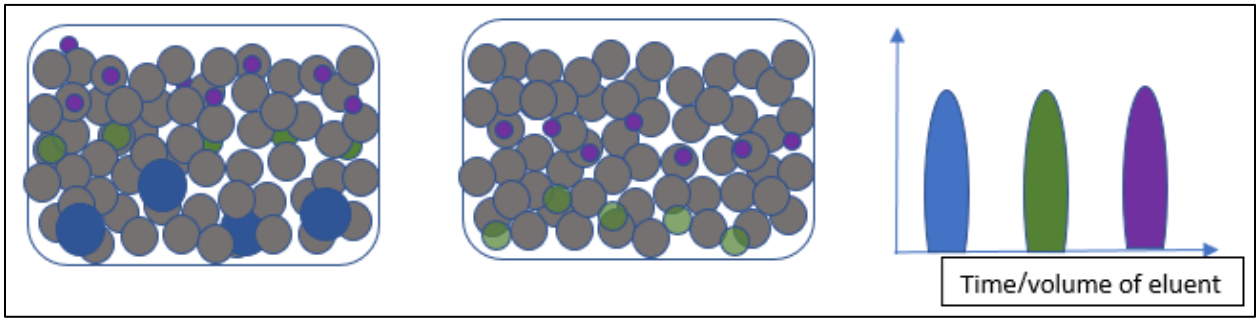

Fig 3.1: Representation of separation of components on a SEC stationary phase resin

SEC is also called gel filtration chromatography when the mobile phase is aqueous and Gel Permeation chromatography when the mobile phase is an organic solvent(Fekete et al., 2014).

3.2 Principle

SEC separates biomolecules according to the hydrodynamic diameter of the species. In this type of separation, the analyte species elute based on their differences in their molecular sizes without any retention on the stationary phase(Arakawa et al., 2010).

3.3 Stationary Phases in SEC

The surface of proteins is known to be highly heterogenous consisting of many functional groups which are essential for their therapeutic actions ad they confer proteins with the ability to bind. Such heterogenous groups provide additional binding specificity and affinity for molecules with similar heterogeneities. Hence, while selecting a stationary phase for size exclusion chromatography, the ideal choice would be one that has the affinity for any one of the functional groups inherently present on the surface of proteins (Arakawa et al., 2010). The stationary phase therefore consists of a packing material of choice packed along with spherical porous particles with a controlled pore size and pore size distribution. The packing materials used in SEC are mostly silica, which may or may not contain surface modifications; cross-linked polymeric packings that are accompanied by a hydrophobic, hydrophilic or ionic character (Fekete et al., 2014).

Having said that, the initial binding of protein to the stationary phase resin brings about significant conformational changes in the protein structure. This leads to an increase in the number of contacts between the protein and the surface and may lead to irreversible protein adsorption that may significantly impact protein recovery.

Also, proteins tend to interact with the charged surface sites of the stationary phase. The type of interactions is mainly dominated by electrostatic and hydrophobic interactions. Electrostatic interactions in which the protein and stationary phase are similarly charged can lead to elution time shifting, band tailing and/or asymmetrical band elution due to ion-exclusion. On the other hand, cases in which the protein and stationary phase are oppositely charged can lead to increased elution time due to protein adsorption (Fekete et al., 2014). Increased elution time is also observed in cases where hydrophobic interactions are dominant. Most commonly, a diol-bonded silica stationary phase remains prevalent mainly owing to its properties of high chemical stability as well as hydrophilic character (Goyon et al., 2018).

3.4 Mobile Phases in SEC

The apt selection of a suitable mobile phase for analysis by SEC is essential as it is known to critically impact the retention and recovery of proteins. As mentioned earlier, both proteins and stationary phases consist of different functional groups which goes to show that proteins bind to the stationary phase by mixed-mode interactions. The mobile phase components may also significantly affect the extent of size heterogeneity and can therefore hinder the true results of analysis. Hence, the composition of the mobile phase should be carefully done (Arakawa et al., 2010).

An ideal mobile phase would be one that reduces interactions forces between the protein and resin surfaces and at the same time remains in equilibrium with the stationary phase.

Organic solvents have shown significant impact on reduction of unnecessary hydrophobic interactions. But they have also shown the ability to aggravate the electrostatic interactions as well as denature proteins. Nevertheless, organic solvents like alcohol and acetonitrile are often used pro the suppression of protein adsorption (Arakawa et al., 2010).

The use of salts like NaCl or KH_2PO_4 in the form of buffers may aid in the suppression of undesirable electrostatic interactions between the protein and the stationary phase resin, but simultaneously may also increase the hydrophobic interactions. It has also been observed that at very high or very low phosphate concentrations, both reversible and irreversible binding occurs which leads to delayed elution and recovery losses.

Similarly, co-solvents such as arginine may stabilize the native conformation thereby inhibiting undesirable protein adsorption to the column.

It must be duly noted that although a combination of different mobile phase components do help in enhancement of the chromatographic performance, they should not alter the aggregation state of the protein (Arakawa et al., 2010).

3.5 Method Development considerations

While developing an ideal and robust SEC method for size heterogeneity analysis, it is important to consider that the method gives reproducible results despite factors that may interfere with the same. Hence, it must be noted that those factors need to be given due consideration in order to develop a size based chromatographic set up for the size heterogeneity analysis of mAbs.

It is well established that the chromatographic column is one of the most crucial components in liquid chromatographic analysis of a sample. Earlier, it was assumed that the separation power of a SEC column is directly proportional to the square root of the column length and hence long columns were desired for separation and subsequent analysis of complex sample (Fekete et al., 2014). The application of a small column volume would lead to a low peak volume and also the peaks would elute before the column dead volume itself. Therefore, larger columns with low flow rates and low pressures were used (Goyon et al., 2018). Recently, it has been studied that small column packed with sub 3-um particles offer faster separation of protein size variants than the conventionally used longer columns. This advancement was accessory to the fact that pore size of the column packing is essential as it determines the molecular weight range of the proteins that can be successfully analyzed in the particular column.

Additionally, to improve the efficiency of size-exclusion chromatographic analysis which is known to rely almost entirely on the intra-particle pores, increase in the total porosity of the columns is beneficial (Goyon et al., 2018).

It is often noticed that in a sequence of multiple injections of a protein sample on a column, the binding of the protein is higher for the initial injections. This factor must be addressed as it is essential that the recovery of protein sample in SEC analysis should be completely independent of the amount of sample injected (Arakawa et al., 2010).

The binding of the protein to the column stationary phase is another very prominent interference that needs to be monitored and controlled. A new column has a natural tendency to bind to proteins and hence less adsorptive columns must be selected as well as preconditioning protocols should e employed before the utilization of the column for SEC analysis (Arakawa et al., 2010).

Electrostatic, hydrophobic and hydrogen binding interactions are the main interactions that are said to affect the elution as well as the separation of proteins (Goyon et al., 2018). Electrostatic interactions can be categorized as ion-exchange (if the protein and stationary phase resin are oppositely charged) and ion-exclusion (if the protein and stationary phase are similarly charged). In order to reduce electrostatic interactions, the ionic strength of the mobile phase can be increased by increasing the salt concentration (Hong et al.,

n.d.),(Fekete et al., 2014). This ultimately leads to improvement in peak symmetry and shape, retention

time and precise quantitation. The recovery of the aggregates can also be substantially increased by the addition of salts (Fekete et al., 2014).

However, it is also seen that changes in the ionic strength may causer either dissociation of reversible aggregates or could also lead to induction of new aggregates (Arakawa et al., 2010).

Also, the choice of buffer plays a crucial role. For example, potassium-based salts are better than sodium-based salts to limit electrostatic interactions.

Organic solvents are another crucial mobile phase modifier that impact the efficiency of SEC separations. These organic solvents may enhance resolution and recovery by reducing the hydrophobic interactions as they have a higher eluent strength compared to that of water.The pH of mobile phase can also affect the type and extent of interactions between the protein ad stationary phase. Ideally, the pH should be close to the pI of the protein which leads to reduction of secondary interactions. However, there are certain consequences such as ion-exclusion effects which are seen when pH is lower than pI and ion-exchange effects when the pH of the mobile phase is higher than the pI of the protein (Fekete et al., 2014).

Mobile phase additives such as arginine could aid in reduction of possible secondary interactions which would thus lead to an improvement in protein aggregates quantitation as well as peak shape.

It is important to note that the modification of the mobile phases buy incorporating different sets of changes can lead to improvement in resolution, peak symmetry and/or recovery. But it may unnecessarily lead to the development of an inaccurate method as it alters the size distribution of the species to be analyzed (Arakawa et al., 2010). Hence, the different factors must be considered that would lead to the development of a robust analytical method which can be utilized to its complete capability.

3.6 Advantages and disadvantages of SEC

SEC has significant prowess in terms of detection of size variants in the range of 1-25 nm. It is the most common analytical tool used for size variant analysis as well as characterization. It shows good sensitivity, is relatively inexpensive as compared to other techniques used for the same purpose, easy to implement and shows significantly good throughput mainly due to auto sampling capabilities. Also, there are wide variety of HPLC equipment and columns to choose from (Bansal et al., 2019).

There are some inherent disadvantages in SEC when it is considered for the size variant analysis of mAbs. One of the most common problems encountered are that the SEC column can act as a filter which can lead to the removal of aggregates either due to binding interactions with column matrix. There are chances that aggregates may break up due to hydrodynamic shear forces (Bansal et al., 2019). In the analysis of larger aggregates, it maybe possible that they may elute in the void volume of the column due to quick exclusion from the column used. SEC analysis inevitably includes the dilution of the sample for better results and this may potentially lead to dissociation of the reversible aggregated species. Another inherent disadvantage of SEC is that it cannot reliably measure the true molecular mass based on the elution position relative to standard proteins.

3.7 Other Techniques for Size heterogeneity analysis

SEC is one of the most powerful and widely used techniques for size heterogeneity analysis of proteins. However, one of the most crucial disadvantages of this method is its limited sensitivity for a specific size range. Therefore, it becomes essential for the analysis of the protein sample using a set of different orthogonal techniques as no single technique is fully able to analyze and characterize the entire spectrum of size variants. Orthogonal techniques are described as the use of a combination of a variety of different analytical techniques wherein each technique utilizes its own measuring principle(HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, 2012).

The different techniques commonly used alongside SEC are sedimentation velocity, light scattering techniques and SEC-MS. Each of these techniques have their own strengths in size heterogeneity analysis of mAbs and can thus either supplement or complement SEC as an analytical tool for characterization and analysis of size variants(Philo, 2014).

AUC as an analytical method comprises of a simple centrifuge along with an optical system, rotor and centrifuge cells that allow for the estimation of the distribution of the material inside each of the cell while the rotor spins. This method can be subdivided into 2 types namely, sedimentation velocity and sedimentation equilibrium.

In sedimentation velocity, the rotor spins at a pace of 30,000-60,000 rpm such that the entire sample pellets over a period of 2-6 hours. The optical system measures the rate at which the different components of the sample sediment. Hence, it utilizes the principle of separation based on the sedimentation co-efficient which varies with molecular mass and shape.

Sedimentation equilibrium, on the other hand, uses a low rotor speed such that the applied centrifugal force pushes the macromolecules towards the outside of the rotor. This motion is opposed by diffusion forces within the cell. This state of opposite forces finally reaches a steady state wherein the flow due to sedimentation is balanced by the flow due to diffusion. Therefore, in sedimentation equilibrium, the final equilibrium distribution depends only on the solution molecular mass (Philo, 2014).

Analytical Method	Aggregate size (µm)	Advantages	Disadvantages
Dynamic light scattering	0.001-5	Sensitive; non destructive; capable of analysis of broad range of analyte concentration/ size range	Semi-quantitative; unsuitable for complex samples
Size exclusion chromatography	0.001-0.05	Highly sensitive; reproducible; small sample volume; combination with online detectors; robust; high resolution	Requires filtration; sample dilution required; limited particle size range
Analytical ultracentrifugation	0.001-0.1	High resolution; absolute; broad analyte concentrations; size/ shape measurement	Low sample throughput; sample dilution; complex data analysis; expert operator needed

Table 3.1: Different methods available for analysis of spectrum of aggregates

Light scattering techniques are another analytical tool for size heterogeneity analysis. The two main principles that are covered in this methodology are static and dynamic light scattering. Static light scattering involves the measurement of the total intensity of the scattered light observed from one or more angles that is relative to the incident light beam.

Multi-angle light scattering involves measurement from multiple angles at the same time. This makes it possible to determine the angular dependence of the scattering light intensity to an angle where intensity is independent of the size and shape of the molecule(Philo, 2014).

Dynamic light scattering measures fluctuations of the scattering intensity over time. These fluctuations are related to the Brownian motion of the scattering molecules (Philo, 2014). The rate of this Brownian motion in turn depends on the diffusion rate of the particles which is a function of particle size, viscosity of the solution and temperature (Khodabandehloo, 2017).

The hyphenation of light scattering measurement technique as a detection mode to Size based separation methods like SEC offers numerous advantages in terms of higher sensitivity, faster

data acquisition, etc(Khodabandehloo, 2017). Additionally, it becomes possible to study the properties of the individual separated components(Philo, 2014).

SEC coupled with static light scattering principle allows for distinction between samples of different process steps and provides information about their composition (Ahrer et al., 2003).

To conclude, different analytical techniques can be used to cover the entire range of size variants generated at different stages during the life span of the protein. But all these techniques up to some extent suffer from complexity of the methodology as well as the need for highly skilled and trainedanalysts. The best analytical tool should therefore be selected based on the nature of the product and its particular pathway that leads to size variants (Philo, 2014).

CHAPTER 4:

CHARGE HETEROGENEITY IN MONOCLONAL ANTIBODIES

4.1 Introduction

The production process of biopharmaceuticals such as mAbs involves arrange of steps that include expression of the protein, subsequent filtration, protein purification, buffer exchange and other formulation steps which ultimately provides us with a finished biopharmaceutical product intended for therapeutic use. This product is then stored under frozen conditions until it has to be administered (Singla et al., 2016).

The development of a successful drug product highly relies on proving the safety and efficacy of the product throughout its shelf life. Hence, meeting the requirements concerned with CQAs at various stages during the life span of the biopharmaceutical product ensures successful manufacturing as well as overall safety and efficacy of the product. The development of a robust formulation of proteinaceous origin hence requires thorough understanding of the chemical and physical instabilities that can possibly emerge throughout the time frame from product manufacturing until its complete shelf-life (Singla et al., 2016).

An in-depth understanding of the structure of mAbs suggests that their surfaces are mainly comprised of charged and polar amino acids especially in an aqueous environment. Hence, molecular interactions of these surface charge components with the solution components may lead to multiple chemical and/or enzymatic modifications that mainly result in differences in their electrostatic surfaces (Rosenberg, 2006). These modifications can be incorporated either as a change in the local charge distribution or may bring about am alteration in the overall surface charge distribution of the antibody (Beck, 2019).

This charge heterogeneity may lead to a variety of protein variants as a consequence of multiple PTMs (Goyon et al., 2018). Most of the modifications are induced by degradation pathways that either directly affect the charge distribution or bring about a conformational change in the protein thereby leading to a heterogeneous mixture of charge variants (Beck, 2019).

4.2 Different PTMs generating charge heterogeneity

The different Post translational Modifications and their effect on the charge variant profile of mAbs has been discussed in detail.

4.2.1. GLYCOSYLATION:

4.2.1.1 N-Glycosylation

Mabs are glycosylated mostly at the N-terminal by oligosaccharides comprising mainly of galactosylation, fucosylation and sialylation. This glycosylation can occur throughout the antibody(Beck, 2019). The N-terminal glycan residue is influenced mainly by the glucose feed as a cell culture condition, dissolved oxygen and pH. However, the cell line and the glycoprotein are the major factors affecting the glycosylation rate. Additionally, the conserved, canonical N-glycosylation site in the Fc region influences the conformation of the antibody (Beyer et al., 2018).

Glycosylation shows marked inhibitory effect on complement activation, antibody-dependent cell cytotoxicity, etc. It can also be held accountable for effects on protein folding, conformation, localization and activity(Li et al., 2015).

Galactosylation brings about subtle conformational changes around the glycosylation site with little or no impact on mAb stability(Beck, 2019). However, mAbs with depleted galactose showed significant reduction in binding affinity. Also, the presence of fructose also exhibits decreased binding affinity. Mabs glycosylated with mannose exhibit faster serum clearance than usual whereas, the pharmacodynamics seem unaffected(Beyer et al., 2018).

The presence of sialic acid also leads to local conformational changes that exerts no negative impact on the potency and activity of the mAb(Beck, 2019).

The change in charge distribution of a mAb brought about by glycosylation primarily depends on the oligosaccharide incorporated for example, the presence of sialic acid makes the antibody more acidic and appears as acidic species in the ion exchange chromatography.

4.2.1.2 Glycation

Glycation is a classic example of Maillard reaction which can be considered as the non-enzymatic reaction of a monosaccharide with an amino acid residue via formationof an unstable Schiff base which rearranges to form a keto-amine derivative known as an Amadori product(Li et al., 2015).

Glycation occurs mainly due to the presence of reducing sugars such as glucose at different stages in the development of the product. The reducing sugars in the cell culture media seem to be the major reason for glycation. Glycation is also possible during administration or in the patient's serum(Beck, 2019).

The most abundant end product of glycation is Nε fructosyl-Lys, which arises from glycation of a Lys residue. The glycation products can also undergo further transformation to form advanced glycation products (Li et al., 2015).

It must be noted that the presence of carboxylic acids in the vicinity of the glycation site can act as a catalyst.

Glycation in the complementarity determining region (CDR) could lead to complete loss of antigen binding affinity(Beck, 2019)

Since glycation results in the loss of a positive charge due to blocking of a lysine residue it appears as an acidic species upon Ion-Exchange chromatography(Li et al., 2015).

4.2.2. Terminal Modifications

4.2.2.1 C-terminal Lysine Clipping

The C-terminal of Mabs usually terminate with a Pro-Gly-Lys sequence(Beyer et al., 2018). The terminating Lys residue is removed during manufacturing by proteolysis with carboxypeptidases. This leads to a loss of a positive charge that leads to an acidic variant due to a shift in the antibody's isoelectric point(Li et al., 2015).

However, incomplete removal of these terminal lysine residues is often encountered and this results in mAbs with additional unclipped lysine residues (Beck, 2019). The major reason for this is considered to be due to the presence of trace levels of metals such as copper or zinc(Li et al., 2015).

The incomplete C-terminal Lysine clipping is known to reduce the cell-dependent toxicity activity while it does not have any considerable impact on the antibody function(Beyer et al., 2018).

4.2.2.2 C-terminal Amidation

The amidation of the amino acid residues at the Carboxy terminal are considered to be catalyzed by peptidyl glycine alpha-amidating monooxygenase (PAM). The copperconcentration in the cell culture media is known to impact the extent of amidation. This type of reaction generates basic variants of the antibody and these variants do not have any significant impact on the structure, stability and biological activity of the antibody(Beyer et al., 2018).

4.2.2.3 N-terminal Pyroglutamate Formation

Glutamine and glutamic acid appear to be the most common amino acid residues situated at the N-terminal of heavy as well as light chains of the antibody (Beyer et al., 2018). This makes them susceptible to modifications that ultimately results in the formation of pyroglutamate by a non-enzymatic/enzymatic reaction (Beck, 2019). It must be noted that the cyclization rate of glutamine is faster than glutamic acid (Li et al., 2015).

This conversion occurs primarily during the production process under cell culture conditions, but it may also occur during the processing and storage of the antibody (Beyer et al., 2018). Hence, the rate and extent of this reaction depends on the buffer composition, pH and temperature (Beck, 2019).

The enzymatic reaction proceeds by the action of glutaminyl cyclase (Beyer et al., 2018).

Since, the conversion of glutamine to pyroglutamate is associated with a loss of positive charge, it creates variants that are more acidic than the main variant(Li et al., 2015).

The pyroglutamate formation does not have any major impacts on the biological activity of the mAb owing to the fact that this modification occurs far from the functionally relevant regions in the molecule (Beyer et al., 2018).

4.2.2.4 Incomplete Signal Peptide Cleavage

N-terminal signal peptides are essential during the initial phases of product development as they initiate translocation of the molecule. Subsequently, they are enzymatically removed to obtain the mature form of the protein. This removal ideally should occur in a site-specific manner. However, sometimes there may be possibilities of unspecific cleavage which leaves certain signal peptides still attached to the final product. This results in mAb variants with truncated signal peptides of different sizes. These variants, mainly elute as basic species but there are cases where they may be present as acidic species.These variants are known to affect the target binding affinity of the mAb mainly due to the fact that these modifications share close proximity with the antigen binding region of the mAb (Beck, 2019),(Beyer et al., 2018).

4.2.3. AMINO ACID SIDE CHAIN MODIFICATIONS

4.2.3.1 Asparagine and Glutamine Deamidation

4.2.3.1.1 Deamidation

Deamidation appears to be the most common non-enzymatic protein modification leading to the generation of charge variants. The most susceptible residues to deamidation are asparagine and glutamine. The Asn residues in the CDR are most susceptible to deamidation owing to their high flexibility and easy exposure to solvents. It has been observed that the most common deamidation site appears to be the crystallizable fragment of the mAb (Beck, 2019).

Deamidation is observed as acidic species as it leads to an addition of a negative charge to the molecule.

The deamidation rates of susceptible amino acid residues is found to majorly depend on the buffer composition, pH and temperature.

The effect of deamidation on the potency of the antibody depends on the reaction site. Deamidation in the CDR region results in decreased target affinity and potency (Beyer et al., 2018).

4.2.3.1.2 Aspartic Acid Isomerization

The deamidation of Asn residues to aspartate is followed by isomerization to iso-aspartate in cases where aspartate is followed by glycine residues (Beyer et al., 2018). It has been noted that the isomerization is mainly seen in the CDR region due to the flexibility and increased exposure of the residues (Beck, 2019).

The presence of iso-aspartate generates basic charge variants which results in a later elution from the column in cation exchange chromatography (Beyer et al., 2018).

In the three-dimensional fold of IgG molecule, the placement of potential isomerization sites is of decisive importance for the impact of this

The impact of isomerization on the potency and activity of the antibody Is found to be majorly dependent on the reaction site. For example, isomerization in the CDR results in substantial loss of antigen binding affinity thereby decreasing potency(Beck, 2019).

Depending on the isomerization site, acidic or basic charge variants can be generated.

Isomerization of aspartate to iso-aspartate often occurs as a follow-up of a deamidation reaction, especially on aspartate residues that are directly followed by a glycine in thesequence.

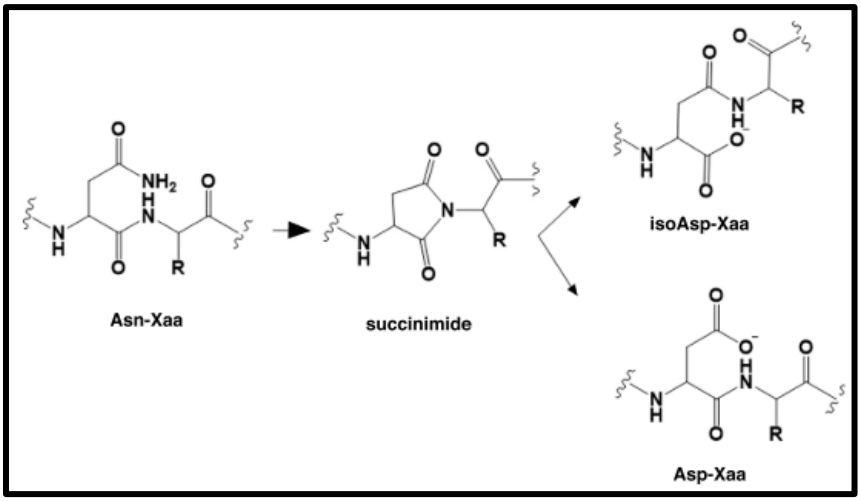

Fig 4.1: Mechanism of deamidation reaction

The sequences most sensitive to isomerization include Asp-Gly, Asp-Ser, and His-Asp. Native Asp first converts to a cyclic imide intermediate, and then either hydrolyses back to Asp or isomerizes to iso-Asp. Aspartate (Asp) isomerization has been commonly observed in mAbs in CDRs due to higher levels of flexibility and exposure. From a biophysical point of view iso-aspartate would be slightly more acidic than the aspartate precursor. However, the isomerization reaction also introduces an additional methyl group, which is likely to cause a change in the three-dimensional structure of the molecule.

antibody variants containing iso-aspartate instead of aspartate have been reported to elute later in cation-exchange chromatography (Beyer et al., 2018).

The location of potential isomerization sites in the three-dimensional fold of the IgG molecule is of decisive importance for the impact of this modification on the biologicalactivity of the antibody. Isomerization of Asp in CDRs has been shown to cause a decrease in antigen binding affinity. Which in turn affects the potency.

Since there is no charge difference between Asp and iso-Asp, the observed decrease in potency is probably caused by conformational changes due to the introduction of a methyl group into the peptide backbone. depending on the specific location, isomerization can either generate acidic or basic species (Beck, 2019).

4.2.3.1.3 Succinimide

Succinimide is known to be the common intermediate of both Asn deamidation as well as aspartate isomerization(Beck, 2019). Deamidation can be acid or base catalyzed. Under acidic pH, Asn undergoes direct hydrolysis to produce Asp. On the other hand, at neutral to basic pH, Asn deamidation happens via formation of a cyclic imide intermediate known as succinimide which is a result of the nucleophilic attack. Subsequently, succinimide hydrolyzes to form aspartate and iso-aspartate in a 3:1 ratio.

The sequence of the reaction site as well as the tertiary structure determines the

rate of succinimide formation(Li et al., 2015). The presence of succinimide intermediate generates acidic charge variants and these variants are known to cause a decrease in potency (Beck, 2019).

4.2.3.2 Oxidation

Attack by free radicals in the presence of oxygen can lead to potential oxidation of the amino acid side chain groups. The oxidants that may be responsible for oxidation can be radical or non-radical. The amino acids that are the most susceptible to oxidation are the Sulphur-containing residues i.e., Cysteine and Methionine. However, the rate and extent of oxidation widely depends on the properties of the affected amino acid as well as the sequence associated with it, the source of oxidation and environmental parameters such as temperature and pH (Li et al., 2015).

Oxidation of a particular susceptible amino acid leads to the generation of charge variants that are mainly acidic but, in some cases basic variants have also been observed.

Oxidation of methionine residues generally results in a multitude of negative impacts including decreased thermal stability, increased aggregation, diminished complementdependent cytotoxicity, decreased binding affinityshorter in-vivo half-life(Beck, 2019).

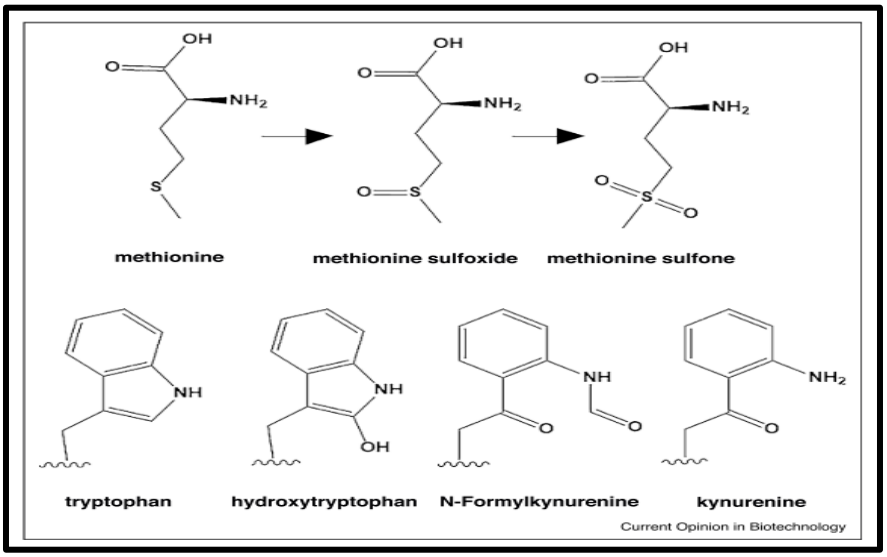

Fig 4.2: Different Oxidation Products for Mabs

4.2.3.3 Disulphide Bond Variants

The most well-known structure of an antibody normally consists of 12 intrachain disulphide bridges, one interchain bond between the Fab heavy and light chain as well as two interchain linkages in the hinge region. These disulphide bridges are majorly responsible for the proper folding and structural stability of the antibody molecule as they are the only covalent attachment between the different parts of the molecule. Hence, variations associated with these disulphide bridges are a source of heterogeneity that is given due consideration(Beyer et al., 2018).

There are several variants associated with incorrect disulphide linkages that have been observed including the presence of free cysteine residues, scrambling of disulphide bridges, trisulfide bonding, formation of thioether and cysteine racemization (Beck, 2019).

Firstly, non-classical linkages envelope those variants that consist of disulphide links at the wrong amino acid residues. Free sulfhydryl groups represent another repercussion of incomplete formation of disulphide bonds mainly due to influence by external factors. The highly reactive nature of free thiol groups might promote the formation of dimers that in turn affects the safety of the therapeutic antibody (Beyer et al., 2018).

The decomposition of disulphide bridges to initial cysteine residues occurs by a Beta-elimination reaction that includes dehydroalanine and persulfate as intermediates. The subsequent cross-linking of dehydroalanine and cysteine results in the formation of a thioether bond. Another common modification related to disulphide bonds are the formation of trisulfide bonds. This type of modification generally occurs during the fermentation stage due to the presence of hydrogen sulphide which interacts with the newly formed disulphide bond.

The presence of trisulfide bonds has no significant impact on the antigen binding as well as the biological activity of the antibody molecule. The thioether linkage leads to the change in bond length and this impacts the orientation of the molecule and hence influences the stability of the molecule.

Since, disulphide bond variants induce changes in the structure of the antibody, the development of charge variants varies depending on the site of heterogeneity(Beyer

et al., 2018). However, it has been observed that the absence of disulphide linkages leads to species that are rich in acidic species (Beck, 2019).

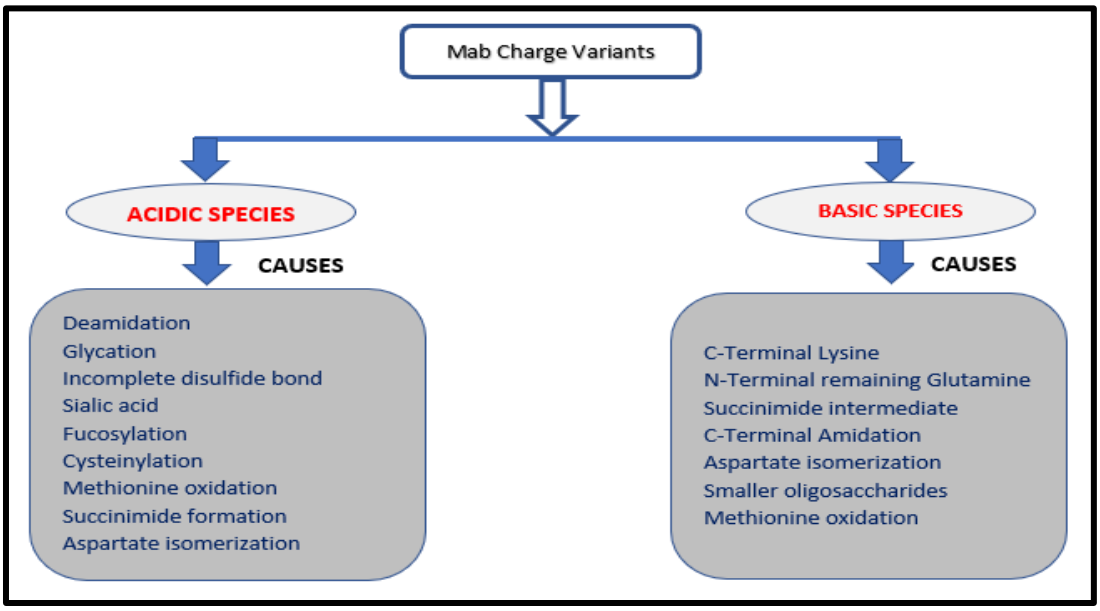

Fig 4.3: Summary of PTMs leading to acidic and basic charge variants in mAbs

Hence, it can be affirmatively said that there are wide variety of factors that lead to the generation of charge variants via a multitude of mechanisms as discussed above. The use of analytical techniques that specifically cater to the apt analysis and characterization of these variants becomes essential for the successful development of quality biopharmaceutical products.

CHAPTER 5:

CATION EXCHANGE CHROMATOGRAPHY FOR CHARGE VARIANT ANALYSI

5.1 Introduction

A high throughput analysis of these charge variants becomes necessary as it inherently affects drug properties such as serum half-life, effector functions, solubility and stability. Charge heterogeneity is considered as a major CQA as even minor alterations in optimum conditions can induce heterogeneity in charge distribution leading to significant change in isoform distribution (Lingg et al., 2013).

Hence, the determination, optimization and monitoring of charge variant profile of mAb is essential at all stages of the product's life cycle (Trappe et al., 2018). The analysis of the charge variant profile ensures stability of not only the product but also the production process employed (Bai et al., 2000). This analysis ensures product consistency and inhibits any undesirable changes to the protein (Ponniah et al., 2015).

There is an array of analytical methodologies that can be successfully employed for the charge variant analysis of mAbs. These include Ion-Exchange Chromatography (IEC), Iso-Electric Focusing (IEF), capillary IEF, Capillary Zone Electrophoresis (CZE), etc.

IEF was traditionally used for the charge variant analysis of mAbs. The separation principle of this technique relies on differences in the isoelectric point (pI) of the antibody charge variants. It uses a mixture of ampholytes that help set up a pH gradient within a gel. With the help of an externally applied electric field, the antibody variants migrate to a zone where pI of the charge variant equals the pH of that zone (Vlasak & Ionescu, 2008). This technique was semi-quantitative, labor intensive and relied entirely on dye staining for detection. It also showed low throughput, lacked automation and exhibited poor reproducibility (Xu et al., 2019).

cIEF was then introduced to curb the inherent limitations of traditional IEF technique. This utilized the development of the pH gradient inside a capillary. The separated variants then mobilized towards an on-column detector located at one end of the capillary (Talebi et al., 2013). cIEF offered prominent advantages such as high sensitivity, automation capability as well as less sample consumption. Another technique named Capillary Zone Electrophoresis (CZE) was also employed which separated mAb charge variants based on both charge and hydrodynamic radii.

This method was easy to implement and provided the analyst with a relatively higher throughput than cIEF (Xu et al., 2019).

It must be kept in mind that only analysis and separation of charge variants is not sufficient. Characterization of the analyzed variants is extremely vital as it helps in determining the factors that must be controlled in order to minimize the heterogeneities. Although cIEF is considered to be one of the most powerful techniques for separation of charge variants, the inherent disadvantage of not being to collect fractions of the separated variants makes this method confined to only monitoring of variants (Talebi et al., 2013).

Hence, IEC was introduced which is considered as the gold standard for the charge heterogeneity analysis of mAbs. It is applicable for not only monitoring of charge variants but also for the preparative isolation and characterization of the generated variants.

Ion Exchange- High Performance Liquid Chromatography (IEX-HPLC) or Ion Exchange Chromatography (IEC) is a non-denaturing high throughput technique that is employed for the qualitative and quantitative evaluation of charge heterogeneity of therapeutic proteins (Fekete et al., 2015),(Iex-hplc et al., 2014).

IEC separates charge variant isoforms of a particular mAb based on differential interactions on a charged support (Fekete et al., 2015). The stationary phase usually consists of charged groups chemically bound to a hydrophilic surface and the mobile phase is usually a buffer solution along with am eluting salt.

IEC can be sub-divided into two main types namely, Cation Exchange Chromatography (CEX) and Anion Exchange Chromatography (AEX). Practically, for the analysis of charge heterogeneity in mAbs, CEX is more widely used than AEX as the pI of a majority of mAbs lies in the neutral to basic region I.e., 6.8-9.4. also, the mobile phase used in CEX are more compatible with mAb as they are prepared at mild pH conditions (Wang et al., 2020).

Hence, CEX appears to be a robust method for the purpose of charge variant analysis; that utilizes the small binding differences of the charge variants with the stationary phase which can be modified using a suitable elution mechanism (Lingg et al., 2013).

IEX in general, allows for direct fraction collection of the variants that helps in the in-depth characterization of the concerned variants. This in turn helps in setting-up stability specifications for mAb formulations. Also, the separation of mAb charge variants are not only

dependent on the overall charge, but also on the local surface charge distribution. This helps us elucidate the site as well as the mechanism of modifications (Wang et al., 2020).

5.2 Principle

CEX is based on the electrostatic interactions between the negatively charged groups on the stationary phase and positively charged groups of the analyte. In this form of chromatography, the mAb sample is loaded on top of the column. At this point, the net charge of the protein is opposite to the charged surface of the stationary phase which assures binding. Subsequently, the mobile phase is introduced, whose composition depends on the elution mechanism to be followed. The charge variants of a mAb are eluted by either a salt gradient, pH gradient or a mixed gradient(Beck, 2019). In salt gradient, the elution takes place solely due to differences in the ionic strengths between the salt incorporated in the mobile phase and the mAb. pH gradient based elution employs an externally or internally generated pH gradient which brings about a change in the charge of the variant at a pH equal to its pI.

As mentioned before, CEX is more widely used as it is more compatible with the mAbs. In CEX, variants which elute earlier than the main peak are considered as acidic species as they are less positively charged as a result, are less firmly bound to the column. Similarly, variants that elute after the main peak are considered as basic variants as they are relatively more positively charged compared to the main species. It must be duly noted that CEX involves separation based on the net-surface charge, charge distribution as well as on the overall geometry of the mAb molecule (Vlasak & Ionescu, 2008).

5.3 Instrumentation

5.3.1 Stationary Phases

The stationary phase in CEX consists of as hydrophilic support with negatively charged ionic groups. These groups provide sites for electrostatic binding with positively chargedanalytes. There are two main aspects that need to be considered while selecting an appropriate stationary phase for the purpose of charge variants analysis, namely, the strength of interaction and associated retention i.e., strong/weak exchangers and the achievable efficiency. Strong cation exchangers such as, sulphonyl, are comprised of strong acids whereas, weak cation exchangers such as, carboxymethyl, consist of weak acids. As the name suggests, a strong exchanger will be able to retain its charge over a

wide range of pH whereas, a weak cation exchanger will eventually lose its charge as the pH of the mobile phase decreases.

Commercially, the CEX columns are packed with silica or polymer packing materials. They are available as porous and non-porous particles, but non-porous particles are generally not preferred for mAbs(Fekete et al., 2015).

mAbs exhibit high molecular weights as well as large stokes radii which imparts them poor mass transfer properties. This inadvertently leads to unnecessary band broadening effects (Weitzhandler et al., 2001). Therefore, it is advised to use non-porous particles or make use of polymeric particles with a fluid impervious core which helps eradicate any intra-particle mass transfer. Also, the usage of pellicular supports minimizes the unwanted hydrophobic interactions between the analyte and the stationary phase (Weitzhandler et al., 2001). Hence, highly cross linked non-porous polymeric particles are most frequently utilized for charge variant analysis (Fekete et al., 2015).

5.3.2 Mobile Phase and Elution Mechanisms

CEX separates the charge variants based on differential magnitude in surface charge which influences their interaction with the stationary phase (Fekete et al., 2015). The mobile phase composition largely varies with the type of elution mechanism that is to be used.

Traditionally, upon binding of the positively charged analytes to the negatively charged groups on the stationary phase, its retention was modulated by a changing salt concentration in the mobile phase. The mobile phase generally consists of a suitable buffer to maintain a stable pH along with a variable concentration of a suitable salt that acts as a counter-ion to control the retention of the analyte ions. In this case, the charge on the counter-ion is same as that of the sample ions which allows it to competitively bind to the charged sites on the CEX stationary phase (Fekete et al., 2015). By gradually elevating the salt concentration, the competition for the charged sites on the stationary phase increases between the analyte and the salt. At an adequate salt concentration, the retention of the analyte diminishes due to attenuation of the electrostatic interaction between the stationary phased and the charged analyte (Ståhlberg et al., 1991).

Hence, in salt gradient elution mechanism, charge variants are eluted in the order of increasing magnitude if binding charge, where in charge variants that are more positively charged are eluted later than variants that are comparatively less positively charged (Fekete et al., 2015). The proteins are pushed down the column from one exchange site to another by displacement from the salt ions. As a result, a focusing effect occurs upon an increase in the salt concentration (Ponniah et al., 2015).

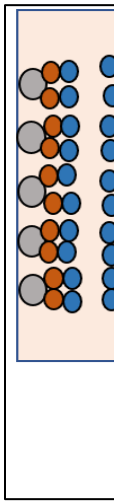

Fig 5.1: Workflow for a typical salt gradient elution for CEX of mAb depicting:-
1. Equilibration
2. Sample loading and washing
3. Introduction to elution buffer
4. Elution of less positively charged component by increasing salt concentration
5. Elution of high positively charged mAb species by a relatively higher salt concentration

h a CEX technique with a salt gradient elution pattern is widely used, it suffers from certain prominent limitations that diminishes its applicability in the current scenario. Salt gradient elution possesses low stability and robustness. Minor fluctuations in eluent composition and/or pH brings about a drastic change in the retention time, resolution as well as peak content (Rozhkova, 2009). It must also be noted that the adsorption affinity of the charge variants is highest at low salt concentrations but this is certainly not possible as high salt concentrations are essential to achieve elution (Kang & Frey, 2003). Another limitation of this elution mode is that the retention of variants increases as the

column temperature increases. This may be due to a possible increase in the diffusion co-efficient

of the mAbs or because of a decrease in the mobile phase conductivity (Trappe et al., 2018). Owing to these complexities, a more mAb friendly approach utilizing a pH gradient was adopted in routine practice.

In light of this demand, pH gradient CEX also known as chromato-focusing was developed which acts as a useful alternative to classic salt mediated elution of mAb charge variants. It utilizes differences in the pI of the charged isoforms to achieve separation (Kang & Frey, 2003). It employs a pH gradient that is formed at a low ionic strength of mobile phase in order to elute variants at a pH that corresponds to the apparent isoelectric point of the mAb which is often close to the true pI of the mAb(Fekete et al., 2015).

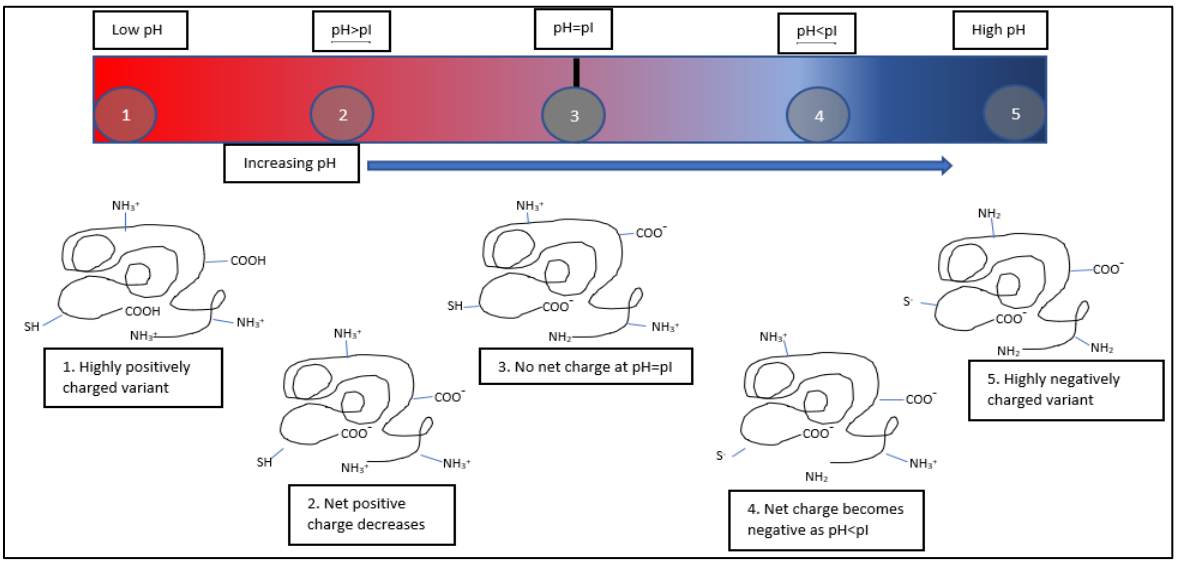

Fig 5.2: Isoelectric point of a protein and how it impacts elution in pH gradient

In this form of elution, net charge of mAb variants is modified by the pH gradient. This happens due to the protonation- deprotonation of the functional groups associated with the charge isoforms (Fekete et al., 2015).

This form of elution mechanism was first explored by Sluyterman and his co-workers in 1920s. A protein sample was first applied to an ion-exchange column that had already been equilibrated with a starting buffer. The pH and composition of the start buffer was selected such that it promoted complete binding of all the components that were intended to be separated.

The subsequent elution of the bound analytes was then achieved with the help of a stepwise change in the pH of the elution buffer. The composition and pH of the elution buffer was

carefully composed such that none of the bound analytes were bound on the column. This was done with the help of carefully calculated mixtures of ampholytes. As the elution buffer descends down the column, the buffer components bind differentially to the column thereby generating a pH gradient. This ultimately leads to detachment of the bound analyte in a sequential manner at a pH close to its respective pI which are eluted from the chromatographic column (Rozhkova, 2009).

It is understood that a major portion of the mAb surface gets to participate in binding with the column packing. This is because the proteins are mildly absorbed during their transit time through the column.

Hence, pH gradient CEX combines the resolving power of isoelectric focusing and the flexibility and simplicity of chromatography. This elution mechanism has been shown to be useful for separation of proteins owing to its high resolution as well as the ability to retain the native state of the mAb as well as its biological activity (Kang & Frey, 2003).

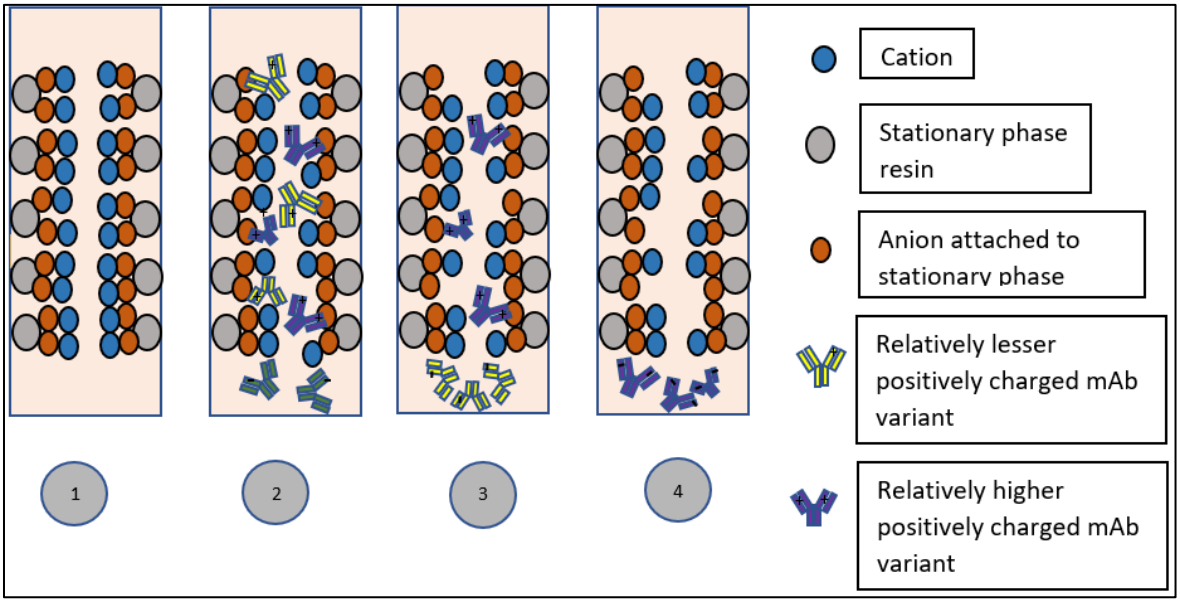

Fig 5.3: Workflow for a typical pH gradient elution for CEX of mAb depicting:-
1. Equilibration 2. Sample loading and washing
3. Introduction to elution buffer which increases pH inside the column
4. The positively charged species become negatively charged when pH>pI

This conventional form of pH CEX though seems to be a reliable alternative, it does possess certain limitations. One of the most critical limitations is that in order for it to serve its

purpose, polymeric ampholyte buffers must be used. These buffers are expensive and showpoor chromatographic reproducibility as they showcase large variability in their physical and chemical properties. Also, a low concentration of buffers is preferred in order to develop a pH gradient that is not too steep which in turn would provide reasonable resolution. A high buffer concentration would generate a very steep pH gradient as the mobile phase buffering capacity surpasses the column's buffering capacity. Another potential drawback of the conventional pH CEX technique is its inability to control the slope of the pH gradient (Shan & Anderson, 2002).

In order to combat these critical drawbacks, pH gradient CEX methodology that employs an externally developed gradient has recently been developed. This technique makes use of low molecular mass buffering species, the proportion of which is altered gradually with the help of a gradient pump. The development of an external pH gradient overcomes all the drawbacks of the conventional pH gradient technique which involves an internal development of the desired pH gradient. Additionally, this technique offers an advantage of being capable of focusing the eluting bands when compared to the salt gradient technique (Shan & Anderson, 2002). Also, employing this method allows the measurement of the effective change of the analyte under consideration (Publishers et al., 1986).

Overall, pH gradient CEX is a far better choice for the charge variant analysis of mAbs as it shows robustness against critical experimental parameters like changes in composition of the eluent. It also provides high reproducibility along with sharper peaks as a result of its superior focusing effect (Publishers et al., 1986).

A rather recent development in the conventional techniques is the salt mediated pH-CEX method that can be optimized for each mAb. Firstly, it would require the pH gradient to be adjusted in order to achieve faster and efficient separation. Secondly, by utilizing a short pH range of approximately 2 pH units, the separation can be achieved at a faster time. Also, the ionic strength of the elution buffer can be adjusted relevant to each mAb to achieve optimal resolution (Zhang et al., 2013). This can be done by controlling the buffer concentration or modulating the salt concentration.

5.4 Method Optimization

Under the current circumstances, particle packed columns appear to be the most commonly used stationary phases for the charge variant analysis of mAbs by CEX. In spite of their widespread applicability, there always seems to be discrimination between small molecules and large molecules such as mAbs, in terms of speed and efficiency in separation. The main reasons for this are slow rate of mass transfer and the inherent large void volume present between the packed particles (Talebi et al., 2013).

In order to overcome these challenges and in the search of a more efficient stationary phase column packing, monolithic columns have shown to be a promising advancement. A monolithic column can be defined as a continuous solid porous matrix which incorporates inter-connected flow paths (Fekete et al., 2015).

The large flow through channels and non-porous support allow a rapid mass transfer of the analytes by convection rather than diffusion, which leads to high resolution and helps achieve faster separation. Additionally, they also offer high permeability which allows for the use of higher flow rates (Fekete et al., 2015).

Recently, it has been understood that biocompatibility of the employed stationary phase is essential during analysis of mAbs. A biocompatible stationary phase is one which is capable of resisting non-specific adsorption of biomolecules in general, and is able to preserve the bioactivity of the biomolecules (Talebi et al., 2013). Monolithic columns are one such category that are considered as biocompatible for application of biopharmaceuticals.

Monolithic columns can be further categorized as organic and silica-based monoliths. As far as the analysis of mAbs are concerned, organic monoliths like poly(meth)acrylate and polyacrylamide are widely used (Talebi et al., 2013),(Fekete et al., 2015).

Additionally, monolithic columns give enhanced separations even with a shallow pH gradient generated by simple component buffer systems. Also, monolithic columns make it possible to achieve high throughput analysis along with faster re-equilibration time even in dilute buffer systems (Talebi et al., 2013).

It is well known that CEX deals with the analysis of charge variants and hence resolution between the separated species seems to be of utmost importance. The resolution as well as optimization of the method varies with the type of elution pattern utilized as they differ in their separation principle and composition.

In salt gradient elution, one of the most important parameters is the choice of a suitable pH and to maintain that pH with the help of a suitable buffer. An optimum pH will allow desirable resolution of the charge variants (Ponniah et al., 2015)

Upon selection of an appropriate pH to carry out analysis, the choice of a suitable buffer that would maintain that pH is essential. It is known that the surface charge of proteins changes rapidly as the pH varies which could lead to a shift in the retention time of the analyte. The pH also changes upon introduction of the salt.

Besides, the column's inherent buffering capacity must also be given due consideration. The buffering capacity of a column ultimately depends on the column capacity and the nature of the charged groups bound to the stationary phase resin. Having said that, it must be noted that weak cation exchangers are much more susceptible to this form of pH change than strong cation exchangers.

Also, there shouldn't be any drastic change in pH as it would although lead to improve in peak shape, it could also cause co-elution of certain previously eluted species. It may also result in variable and non-reproducible results between buffer preparations, new columns and/or different HPLC systems.

As mentioned earlier, only the charged amino acids at the surface of mAbs in their native state can interact with the ion-exchange sites on the column. Hence, the ionization of certain specific individual groups on the surface can impact the overall elution. Also, any conformational change due to a pH change can induce additional complexity and must be given due consideration (Ponniah et al., 2015).

In pH gradient form of elution, a mixture of buffers is used to cover a wide pH range. This makes it essential that the gradient should be able to control the pH in a predictable and reproducible fashion. Resolution in this type of elution depends on the concentration of buffer, the outlet pH gradient profile and the column pH gradient. Usually, optimum resolution is achieved at high buffer concentration. This is owed mainly to the effect of high buffer concentration on peak separation by an increase in the electrophoretic pI of variants which enhances resolution (Shan & Anderson, 2002), (Talebi et al., 2013).

The outlet pH gradient profile mainly affects peak width and it has been concluded that lower outlet pH gradient slopes lead to narrow peak widths and hence impart superior resolution. Theoretically speaking, an increased column pH gradient leads to a focusing effect of the peaks

thereby improving resolution (Shan & Anderson, 2002). The use of a mixture of buffer components allows each component to buffer at its own ability which leads to disruption on the charged sites of the column during the generated gradient and equilibration. Hence, the type and capacity of the column used should be carefully considered. For example, a weak ion exchanger will dissociate more easily than a strong ion exchanger. This leads to a stronger buffering effect on the column. This results in a delay in equilibration as well as a shift in the retention time (Ponniah et al., 2015). Hence, it can be concluded that recent advances in ion-exchange chromatography, especially Cation Exchange Chromatography provides widespread applications in the characterization as well as analysis of mAb charge variant isoforms.

AIM & OBJECTIVE OF WORK

AIM

- To validate and assess the stability indicating capability of a CEX-HPLC method for the charge heterogeneity analysis of a monoclonal antibody; Sunmab
- To validate and assess the stability indicating capability of a SEC-HPLC method for the size heterogeneity analysis of a monoclonal antibody; Sunmab

OBJECTIVE

- To conduct method trials for the given Sunmab sample in order to qualify the authenticity of the sample and the given analytical methods.
- To perform validation of the Cation exchange chromatography (CEX) and Size Exclusion Chromatography (SEC) analytical methods with reference to SPIL guidelines based on ICH guidelines.
- To assess the stability indicating capability of the two methods by evaluation of a forced degradation study on the samples with relevant stress conditions.
- To analyse and compile the data obtained from the above studies and reach a satisfactory conclusion with regards to the stability indicating capabilities of the two methods.

CHAPTER 6:

EXPERIMENTAL WORK

6.1 CHEMICALS AND MATERIALS

MES hydrate, MES sodium salt, Sodium Dihydrogen Phosphate monohydrate, Di-Sodium Hydrogen Phosphate dihydrate, Sodium Hydroxide and Sodium Chloride were produced from MERCK Laboratories Ltd.

Milli-Q water was obtained from the in-house institutional facility. HPLC grade Acetonitrile was procured from Rankem Laboratories Ltd.

6.2 INSTRUMENTS AND EQUIPMENT

All instruments and equipment that were utilized during the entire course of the project were calibrated at defined intervals as per the given standard operating procedure of Department of Analytical Development, Sun Pharma Industries Limited.

Sl no.	Name of Instrument	Make	Model
1.	HPLC	Waters	e2695
2.	UV-detector	Waters	e2690
3.	pH meter	Thermo scientific	Orion 4 star
4.	Weighing balance	Sartorius	BT 224 S
5.	Vortex shaker	Spinwin tarson	MC02

Table 6.1: List of instruments used

6.3Cation Exchange Chromatography for Charge Variant Analysis of Sunmab

6.3.1 Trial of the Method

6.3.1.1 Sample Preparation of Reference standard

160 ul of the Sunmab drug substance was taken with the help of a micropipette in an Eppendorf tube and diluted to 2 ml.

6.3.1.2 Preparation of Mobile Phase

Mobile Phase A: 2.58 g of MES hydrate, 2.54 g of MES sodium salt were accurately weighed and mixed in 900 mL of Milli-Q water. The volume was made up to1000 ml.

40 mL of Acetonitrile was added and mixed on a magnetic stirrer. The mobile phase was filtered through 0.45 µ filter prior to use.

Mobile Phase B: 0.193 g of Sodium Dihydrogen Phosphate monohydrate, 3.29 g of Di-Sodium Hydrogen Phosphate Dihydrate and 5.55 g of Sodium Chloride was accurately weighed and added to 900 ml of Milli-Q water. The volume was made up to 1000 ml. 40 ml of Acetonitrile was added and mixed on a magnetic stirrer. The mobile phase was filtered through 0.45 µ filter prior to use.

6.3.1.3 Analytical Method Parameters

A WATERS e2695 HPLC system with a DionexProPac WCX-10 (4 x 250 mm) column was used for the charge variant analysis of Sunmab. 10 µl of Sunmab at its reference standard concentration was injected into the system. Mobile Phase A (24mM MES, pH 6) and Mobile Phase B (20 mM phosphate, 95 mM NaCl, pH 8) were used at a flow rate of 0.5ml/min with a gradient of 66% A to 25% A in 44 minutes, 25% A to 0% A in 8 minutes and then back to 66% A in the next 12 minutes. The eluted components were analyzed using a WATERS e2690 UV-detector at a detection wavelength of 280 nm.

6.3.2 METHOD VALIDATION

The validation of the given analytical method was carried out as per the SPIL guidelines that were based on the ICH guidelines. The different validation parameters that were evaluated include precision, accuracy, linearity, range and determination of LOD and LOQ.

6.3.2.1 Precision

There were two types of precision namely, method and instrument precision that were evaluated.

6.3.2.1.1 Method Precision

6 preparations of the reference standard were made and 1 injection from each preparation were injected into the HPLC system. The % RSD of the results was used to evaluate the precision of the analytical method.

6.3.2.1.2 Instrument Precision

1 preparation of the reference standard was made and 6 injections of the preparation were injected into the HPLC system. The % RSD of the results was used to evaluate the precision of the employed HPLC instrument.

6.3.2.2 Specificity

One injection each of Mobile Phase A, Mobile Phase B and diluent were injected into the HPLC system. The resulting chromatograms obtained were compared to the reference standard chromatogram in order to confirm no matrix interferences.

6.3.2.3 Linearity

Triplicate preparations of linearity test solutions of 5-375% of 20% of reference standard concentration were prepared from the stock solution i.e., the reference standard solution. Duplicate injections of each preparation were introduced into the HPLC system. The concentration vs mean area graph was plotted to determine the regression equation as well as the correlation coefficient.

Conc. %	Conc. (mg/ml)	Vol. of Stock (ml)	Vol. of Blank (ml)	Total Volume (ml)
5	0.08	0.01	0.99	1
25	0.4	0.05	0.95	1
50	0.8	0.1	0.9	1
75	1.2	0.15	0.85	1
100	1.6	0.2	0.8	1
125	2	0.25	0.75	1
250	4	0.5	0.5	1
375	6	0.75	0.25	1

Table 6.1: Preparation of Linearity test solutions

NOTE: The concentration at 100% was chosen to be 1.6 mg/ml as the permitted levels of charge variants for Sunmab was 20% of the reference standard concentration.

6.3.2.4 Range

The range of the given analytical method was calculated from the linearity studies by evaluation of the % RSD of the obtained results

6.3.2.5 Determination of LOD

The LOD concentration was determined by evaluating the signal to noise ratio and the %RSD of the individual concentrations. The concentration at which %RSD was >2% and the signal to noise ratio was >3 was considered as the LOD concentration.

6.3.2.6 Determination of LOQ

The LOQ concentration was determined by evaluating the signal to noise ratio and the %RSD of the individual concentrations. The concentration at which the signal to noise ratio was >10 was considered as the LOQ concentration.

6.3.2.7 Accuracy

The accuracy of the method was determined by recovery studies. 1 preparation each of 0.4 mg/ml, 0.8 mg/ml and 1.2 mg/ml were prepared by dilution from the reference standard solution. Triplicate injections of each preparation were injected into the HPLC system. The % recovery was calculated using the regression equation from the Linearity studies.

6.3.3 FORCED DEGRADATION STUDY

The forced degradation study was performed in order to elucidate the different stress factors that were responsible for the generation of charge variant species in Sunmab. However, this study is limited to identification of stress factors that lead to the generation of different acidic and basic species. The stress factors were applied on the test sample solution of Sunmab that resembles the reference standard solution. The control sample for this study was the test sample solution of concentration 8mg/ml.

6.3.3.1 Oxidation

The test sample solution was mixed with 5% H_2O_2. Blank and control sample were injected into the system. The prepared stress sample was injected in duplicate into the system. The area %age change in acidic, basic and main species was observed by comparison with the reference standard chromatogram.

6.3.3.2 Reduction

The test sample solution was mixed with 5µl of β-mercaptoethanol. Blank andcontrol sample were injected into the system. The prepared sample was

injected in duplicate into the system. The area %age change in acidic, basic and main species was observed by comparison with the reference standard chromatogram.

6.3.3.3 pH shifts in Formulation Buffer

6.3.3.3.1 Acidic shift in formulation buffer

300 µl of test sample was taken in an Eppendorf tube and 0.5M HCl was added dropwise to obtain a pH of 3. Blank and control sample were injected into the system. The prepared sample was injected in duplicate into the system. The area %age change in acidic, basic and main species was observed by comparison with the reference standard chromatogram.

6.3.3.3.2 Basic shift in formulation buffer

300 µl of test sample was taken in an Eppendorf tube and 0.5 M NaOH was added to obtain a pH of 11. Blank and control sample were injected into the system. The prepared sample was injected in duplicate into the system. The area %age change in acidic, basic and main species was observed by comparison with the reference standard chromatogram.

6.3.3.4 Vortex

300 µl of test sample was taken in an Eppendorf tube. The solution was vortexed for 20 minutes at 100 rpm. Blank and control sample were injected into the system. The prepared sample was injected in duplicate into the system. The area %age change in acidic, basic and main species was observed by comparison with the reference standard chromatogram.

6.3.3.5 Temperature Induced Stress

500 µl of sample was taken in an Eppendorf tube. The sample as incubated at 60°C for 24 hours. Blank and control sample were injected into the system. The prepared sample was injected in duplicate into the system. The area %agechange in acidic, basic and main species was observed by comparison with the reference standard chromatogram.

6.4 Size Exclusion Chromatography for Size Heterogeneity Analysis of Sunmab

6.4.1 Trial of the Method

6.4.1.1 Sample Preparation of Reference standard

20 ul of the Sunmab drug substance was taken with the help of a micropipette in anEppendorf tube and diluted to 2 ml.

6.4.1.2 Preparation of Mobile Phase

2.7 g of Sodium Dihydrogen Phosphate monohydrate, 5.4 g of Di-Sodium Hydrogen Phosphate Dihydrate and 11.7 g of Sodium Chloride was accurately weighed and added to 900 ml of Milli-Q water. The volume was made up to 1000 ml. The pH was adjusted to 7 with 50% NaOH. 40 ml of Acetonitrile was added and mixed on a magnetic stirrer. The mobile phase was filtered through 0.45 µ filter prior to use.

6.4.1.3 Analytical Method Parameters

A WATERS e2695 HPLC system with a YMC Pack Diol-2000 (8 x 300 mm, 5µm) column was used for the size variant analysis of Sunmab. 10 µl of Sunmab at its reference standard concentration was injected into the system. Mobile Phase (50 mM phosphate, 250 mM NaCl, pH 7) was used at a flow rate of 0.5ml/min in isocratic mode. The eluted components were analyzed using a WATERS e2690 UV-detector at a detection wavelength of 214 nm.

6.4.2 METHOD VALIDATION

The validation of the given analytical method was carried out as per the SPIL guidelines that were based on the ICH guidelines. The different validation parameters that were evaluated include precision, accuracy, linearity, range and determination of LOD and LOQ.

6.4.2.1 Precision

There were two types of precision namely, method and instrument precision that were evaluated

6.4.2.1.1 Method Precision

6 preparations of the reference standard were made and 1 injection from each preparation were injected into the HPLC system. The % RSD of the results was used to evaluate the precision of the analytical method.

6.4.2.1.2 Instrument Precision

1 preparation of the reference standard was made and 6 injections of the preparation were injected into the HPLC system. The % RSD of the results was used to evaluate the precision of the employed HPLC instrument.

6.4.2.2 Specificity

One injection each of Mobile Phase and diluent were injected into the HPLC system. The resulting chromatograms obtained were compared to the reference standard chromatogram in order to confirm no matrix interferences.

6.4.2.3 Linearity

Triplicate preparations of linearity test solutions of 5-125% of 0.5 % of reference standard concentration were prepared from the stock solution. The stock solution was prepared by taking 0.1 ml of reference standard solution and diluting up to 10 ml. Duplicate injections of each preparation were introduced into the HPLC system. The concentration vs mean area graph was plotted to determine the regression equation as well as the correlation coefficient.

Conc. %	Conc. (µg/ml)	Vol. of Stock (µl)	Vol. of Blank (µl)	Total Volume (ml)
5	0.25	25	975	1
10	0.5	50	950	1
25	1.25	125	875	1
50	2.5	250	750	1
75	3.75	375	625	1
100	5	500	500	1
125	6.25	625	375	1

Table 6.2: Preparation of Linearity Test Solution

NOTE: The concentration at 100% was chosen to be 5 ug/ml as the permitted levels of size variants for Sunmab was 0.5% of the reference standard concentration.

6.4.2.4 Range

The range of the given analytical method was calculated from the linearity studies by evaluation of the % RSD of the obtained results.

6.4.2.5 Determination of LOD

The LOD concentration was determined by evaluating the signal to noise ratio and the %RSD of the individual concentrations. The concentration at which %RSD was >2% and the signal to noise ratio was >3 was considered as the LOD concentration.

6.4.2.6 Determination of LOQ

The LOQ concentration was determined by evaluating the signal to noise ratio and the %RSD of the individual concentrations. The concentration at which the signal to noise ratio was >10 was considered as the LOQ concentration.

6.4.2.7 Accuracy

The accuracy of the method was determined by recovery studies. 1 preparation each of 1.25 µg/ml, 5 µg/ml and 6.25 µg/ml were prepared by dilution from the reference standard solution. Triplicate injections of each preparation were injected into the HPLC system. The % recovery was calculated using the regression equation from the Linearity studies.

6.4.3 FORCED DEGRADATION STUDY

The forced degradation study was performed in order to elucidate the different stress factors that were responsible for the generation of size variant species in Sunmab. However, this study is limited to identification of stress factors that lead to the generation of different HMW and LMW species. The stress factors were applied on thetest sample solution that resembles the reference standard solution prepared. Also, the control sample is the as such sample with the same concentration as the reference standard.

6.4.3.1 Vortex

300 µl of the test sample solution was transferred to each of the four Eppendorf tubes. One of each tube were vortexed for 10,20,30 and 60 minutes at 100 rpm. Blank and control were injected into the system. The prepared sample was injected into the system. The area % species were compared with reference chromatogram.

6.4.3.2 Temperature Induced Stress

500 µl of the test sample was transferred to an Eppendorf tube. The sample was incubated at 70°C for 24 hours. Blank and control sample were injected into the system. The prepared sample was injected into the system. The area % species were compared with reference chromatogram.

6.4.3.3 Heat coupled Vortex stress

500 µl of the test sample was transferred to an Eppendorf tube. The sample was incubated at 70°C for 6 hours. The incubated sample was then vortexed at 100 rpm for 30 minutes. Blank and control sample were injected into the system. The prepared sample was injected into the system. The area % species were compared with reference chromatogram.

6.4.3.4 Alkaline shift in formulation buffer

500 µl of the test sample was transferred to an Eppendorf tube. 0.5M NaOH was added dropwise till the pH of the sample was 11.0. Blank and control sample were injected into the system. The prepared sample was injected into the system. The area % species were compared with reference chromatogram.

6.4.3.5 Vortex in Alkaline stress

500 µl of the test sample was transferred to an Eppendorf tube. 0.5M NaOH was added dropwise till the pH of the sample was 11.0. The sample was vortexed for 30 minutes. Blank and control sample were injected into the system. The prepared sample was injected into the system. The area % species were compared with reference chromatogram.

CHAPTER 7:

RESULTS AND DISCUSSIONS

7.1 Cation Exchange Chromatography for Charge Variant Analysis of Sunmab

7.1.1 Trial of the Method

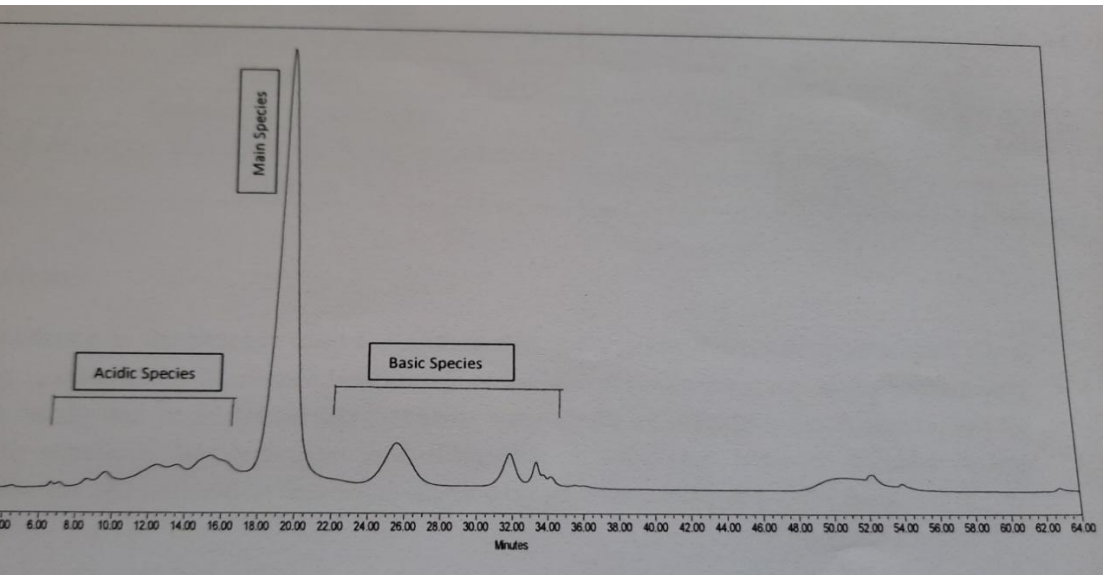

Fig 7.1: Trial chromatogram of Sunmab Reference Standard

Upon integration of the different species, it was found that the reference standard consisted 18.6% of acidic species, 16.58% of basic species and the remaining 63.23%was constituted by the main species.

7.1.2 METHOD VALIDATION

7.1.2.1 Precision

7.1.2.1.1 Method Precision

The given analytical method was found to be precise as the %RSD of the observations was <2%.

	Acidic Species		Main Peak		Basic Species	
Injection	**Area**	**%Area**	**Area**	**%Area**	**Area**	**%Area**
1	2559123	18.32	8846955	63.32	2396320	17.15
2	2506161	18.12	8781798	63.49	2360619	17.07
3	2528629	18.15	8837623	63.44	2385388	17.12
4	2535883	18.33	8731865	63.12	2379502	17.2
5	2554734	18.37	8816133	63.38	2374589	12.07
6	2551731	18.20	8854704	63.15	2446798	17.45
Mean	2539377	18.24833	8811513	63.31667	2390536	17.1767
Std dev	20046.91	0.104960	47013.74155	0.15214	29988.17119	0.14278
%RSD	0.79	0.58	0.53	0.24	1.25	0.83

Table 7.1: Observation Table for Method Precision

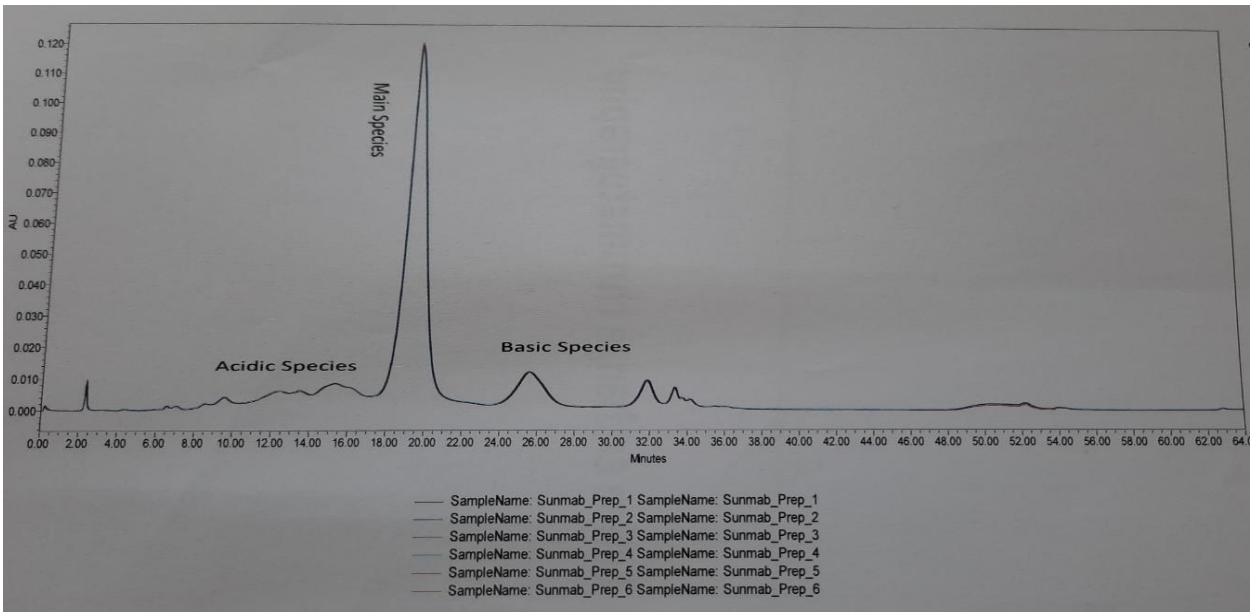

Fig. 7.2: Overlay Chromatogram for Method Precision of Sunmab

7.1.2.1.2 Instrument Precision

The given analytical instrument was found to be precise as the %RSD of the observations was <2%.

	Acidic Species		Main Peak		Basic Species	
Injection	**Area**	**%Area**	**Area**	**%Area**	**Area**	**%Area**
1	2698532	18.26	9365709	63.38	2521640	17.06
2	2657783	18.2	9266198	63.46	2490388	17.06
3	2692474	18.23	9358951	63.38	2534121	17.16
4	2665170	18.13	9321576	63.42	2519111	17.14
5	2686266	18.25	9324914	63.35	2533588	17.21
6	2686081	18.23	9326923	63.3	253345	17.2
Mean	2681051.00000	18.21667	9327378.50000	63.38167	2522065.50000	17.13833
Std dev	16013.57799	0.04719	35352.66274	0.05529	16865.68120	0.06585
%RSD	0.6	0.26	0.38	0.09	0.67	0.38

Table 7.2: Observation Table for Instrument Precision

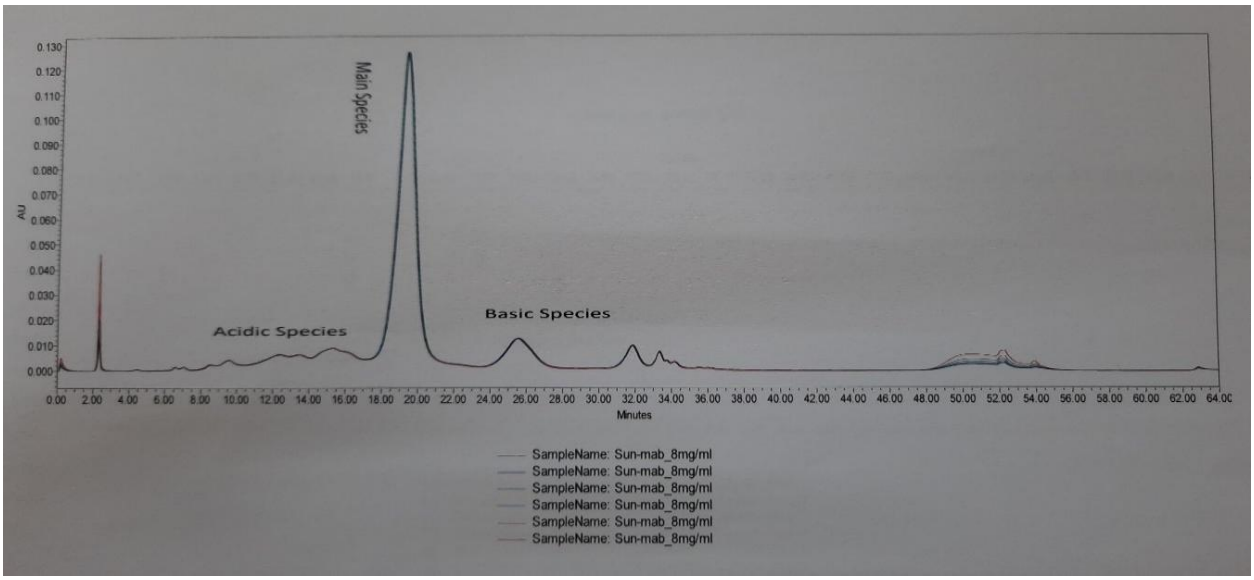

Fig. 7.3: Overlay Chromatogram for Instrument Precision of Sunmab

7.1.2.2 Specificity

There were no matrix interferences observed due to the mobile phase and diluent as there were no contributing peaks in the respective overlay chromatograms with the chromatogram of the reference standard.

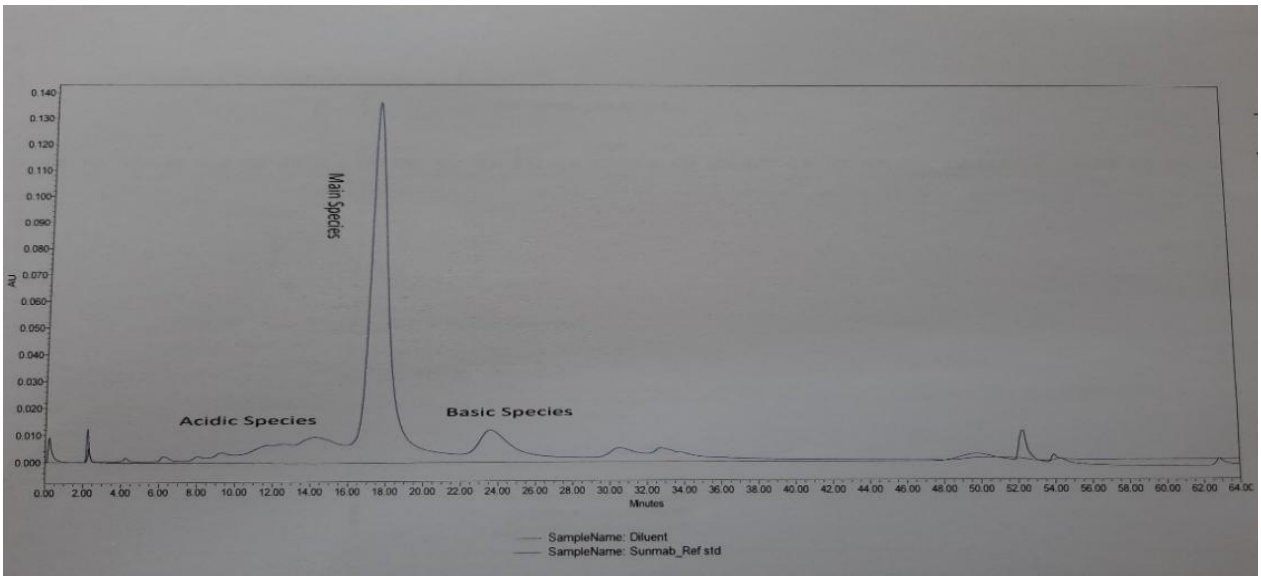

Fig. 7.4: Overlay Chromatogram for Mobile Phase A and Reference standard of Sunmab

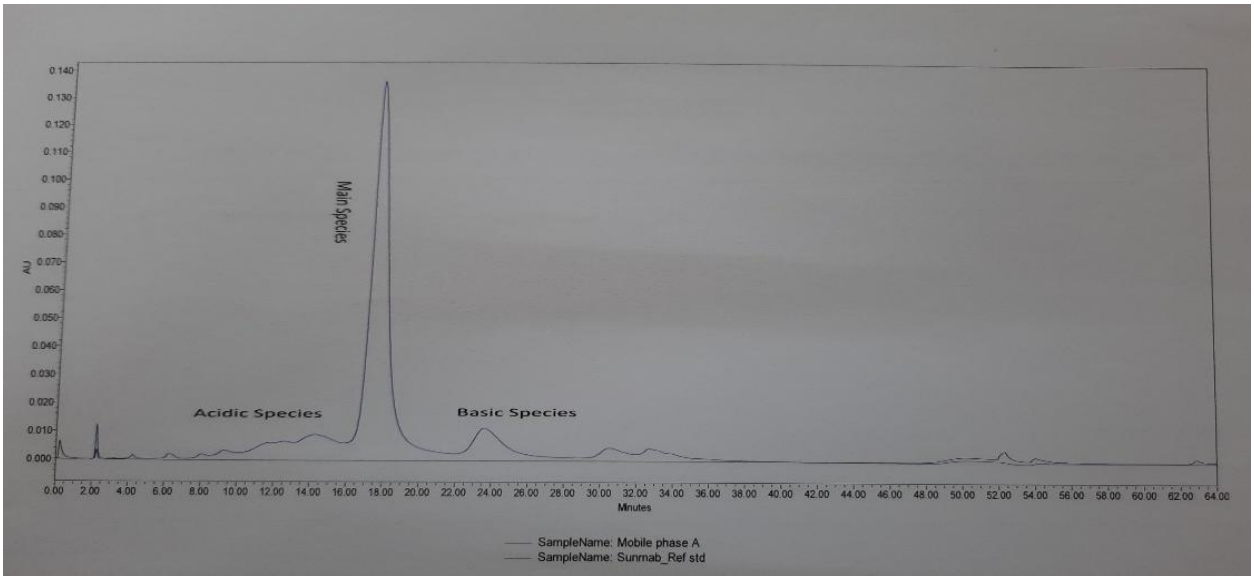

Fig. 7.5: Overlay Chromatogram for Mobile Phase B and Reference standard of Sunmab

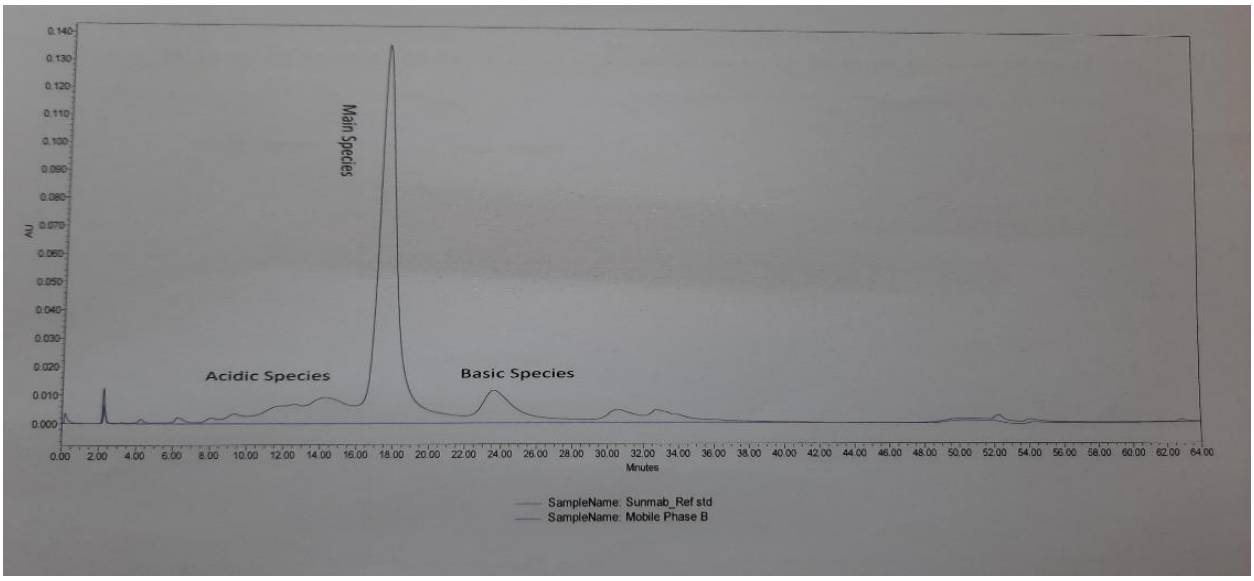

Fig. 7.6: Overlay Chromatogram for Diluent and Reference standard of Sunmab

7.1.2.3 Linearity

The given method was linear over a concentration of 10% - 375% and the correlation coefficient obtained from the calibration curve was found to be 0.9996. The equation of the calibration curve was y = 989308x + 39604.

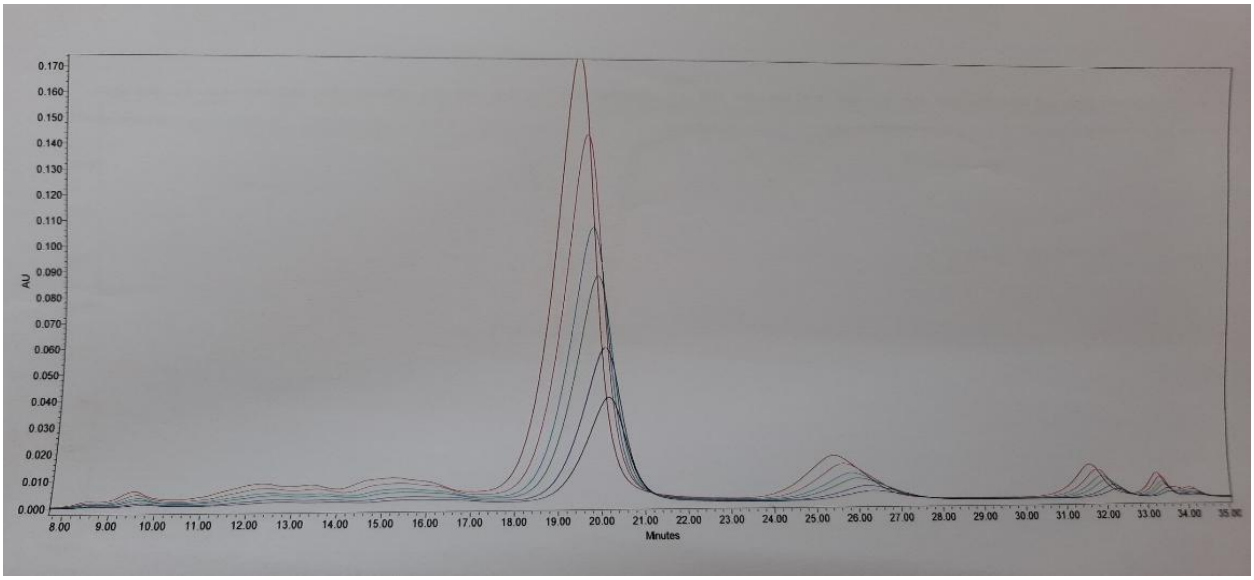

Fig 7.7: Overlay chromatogram of linearity test solutions

Conc%	Conc. mg/ml	Mean area	Std dev	%RSD
5	0.08	94774.2	18658.5	19.7
25	0.4	430246.3	3396.6	0.8
50	0.8	863134.8	15428.7	1.8
75	1.2	1242293.2	14906.1	1.2
100	1.6	1638760.0	31524.2	1.9
125	2	1954627.8	86533.5	1.4
250	4	4052775.0	31745.6	0.8
375	6	5948300.0	17133.9	0.3

Table 7.3: Observation Table for Linearity Study

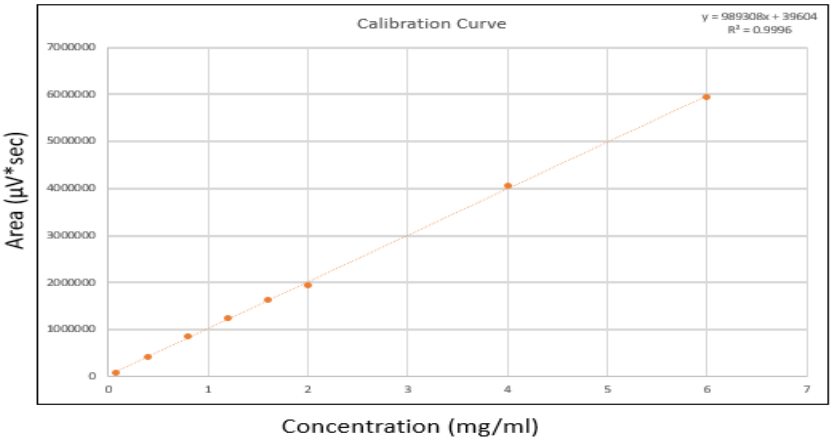

Fig 7.8: Calibration Curve for Sunmab

7.1.2.4 Range

The range of the analytical method for the charge variant analysis was 10% - 375% corresponding to 0.4 mg/ml to 6 mg/ml.

7.1.2.5 Determination of LOD

The Limit of Detection was found to be 0.08 mg/ml as the S/N ratio at this concentration was >3 and the % RSD was >2%. This implies that the analytical method was able to detect the analyte up to 0.08 mg/ml.

7.1.2.6 Determination of LOQ

The Limit of Quantification was found to be 0.4 mg/ml as the S/N ratio at this concentration was >10 and the % RSD was >2%. This implies that the analytical method was able to quantify the analyte up to 0.4 mg/ml.

7.1.2.7 Accuracy

The accuracy of the method was calculated by determining the % recovery. The % recovery of the test solutions was calculated from the regression equation in the linearity study. The % recovery turned out to be in the range of 99-105 %.

Conc.%	Conc(mg/ml)	Area	Back cal conc. (mg/ml)	%Recovery
25	0.4	429251	0.4	103.9
		427114	0.4	103.4
		434375	0.4	105.2
75	1.2	1253614	1.2	103.6
		1240857	1.2	102.6
		1232409	1.2	101.9
125	1.6	1651175	1.6	102.7
		1665406	1.7	103.6
		1599700	1.6	99.4

Table 7.4: Observation Table for % recovery study for accuracy

7.1.3 FORCED DEGRADATION STUDY

7.1.3.1 Oxidation

There is substantial increase in basic species from 16.58% in control sample to 44.49% in the stress sample. A reduction in the acidic species was seen from 18.6% in the control sample to 13.88%in stress sample.

This suggests that oxidation of the mAb sample generates basic charge variants.

7.1.3.2 Reduction

Upon reduction of the sample, a significant increase in the acidic species was observed as the % acidic species elevated from 18.6% (control) to 27.13% (stress). Additionally, a slight increase in %age basic species to 17.34% (stress) from 16.58% (control) was observed.

This study can thus lead us to the conclusion that reduction of the sample generates acidic variants with little or no effect on the basic charge variant profile.

7.1.3.3 pH shifts in the formulation buffer

7.1.3.3.1 Acidic shift in formulation buffer

An acidic shift in formulation buffer lead to decrease in the acidic charge species from 18.6% (control) to 14.41 % in the stress sample. Whereas, the basic charge variants were found to increase under identical stress conditions to 27.12 % from 16.58% in the control sample.

This lays accessory to the fact that an acidic shift in the buffer generates basic charge variants while leads to a significant decrease in the acidic charge species.

7.1.3.3.2 Basic shift in formulation buffer

A basic shift in formulation buffer lead to increase in the acidic charge species from 18.6% (control) to 20.76 % in the stress sample. Whereas, the basic charge variants were found to decrease insignificantly under identical stress conditions to 15.52 % from 16.58% in the control sample.

This suggests that a basic shift in the buffer generates acidic charge variants while leads to a significant decrease in the basic charge species.

7.1.3.4 Vortex

There was only a slight variation in the % charge variants post vortexing of the sample. The % acidic charge variants decreased by a small percentage from 18.6% to 17.92% whereas, %basic species increased to 17.02% in the stress sample from 16.58% in the control sample.

The impact of mechanical stress in the form of vortex is insignificant in terms of a change in the % charge variants of Sunmab.

7.1.3.5 Temperature Induced Stress

Upon incubation of the sample at 60°C, it was seen that there is a considerable increase in the %age of both acidic and basic charge variants from 18.6 % (control) to 21.21% (stress) and from 16.58 % (control) to 17.50 % (stress) respectively. Hence, it can be said that upon incubation of the sample at elevated temperatures impacts the charge variant profile by leading to an increase in both acidic and basic charge species.

7.1.4 Summary of Results

VALIDATION PARAMETER		RESULT
Precision	Method Precision	% RSD < 2%
	Instrument Precision	% RSD < 2%
Accuracy (% recovery)		99-105%
Linearity	Range	5% - 375 %
	r^2	0.9996
	Y-intercept (c)	39604
	Slope (m)	989308
LOD		0.08 mg/ml
LOQ		0.4 mg/ml

Table 7.5: Summary of Method Validation Study

SAMPLE	% ACIDIC SPECIES	% BASIC SPECIES	INFERENCE
Control	18.6	16.58	Significant % charge variants
Oxidation Stress	13.88	44.79	Increases Basic species Decreases acidic species
Reduction Stress	27.13	17.34	Decreases Basic species Increases acidic species
Acidic Shift in buffer	14.41	27.12	Increases Basic species Decreases acidic species
Basic Shift in buffer	20.76	15.52	Decreases Basic species Increases acidic species
Vortex	17.92	17.02	Increases Basic species Decreases acidic species
Heat stress	21.21	17.50	Increases Basic species Increases acidic species

Table 7.6: Summary of Forced Degradation Study

7.2 Size Exclusion Chromatography for Size Variant Analysis of Sunmab

7.2.1 Trial of the Method

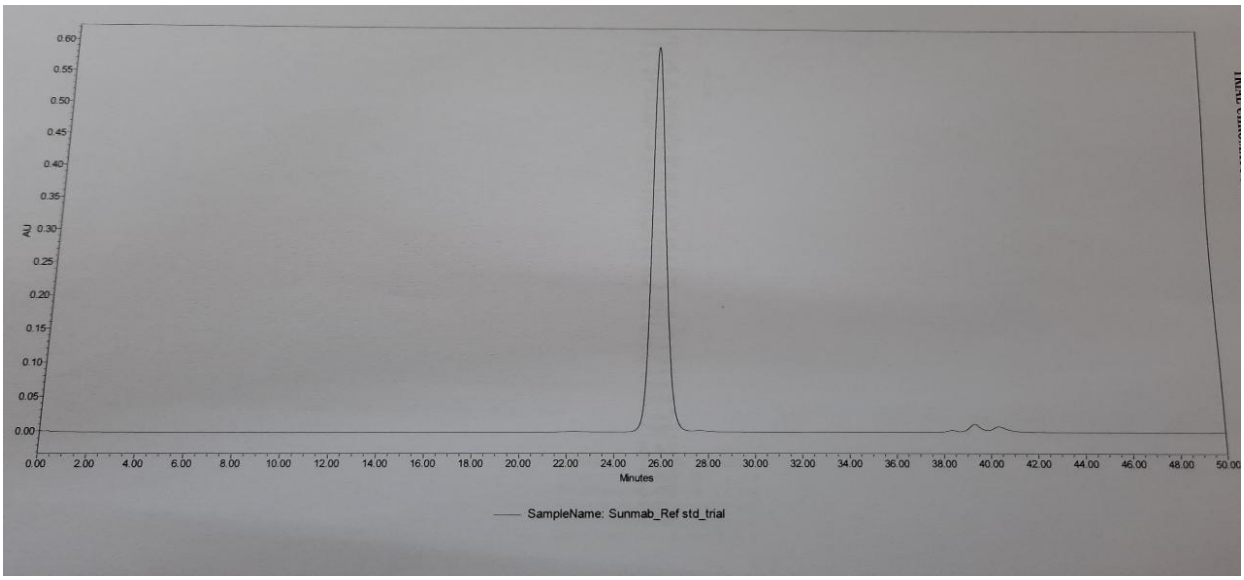

Fig 7.9: Trial chromatogram of Sunmab Reference Standard

Upon integration of the different species, it was found that the reference standard consisted 0.19% of HMW species, 0.32% of basic species and the remaining 99.49% was constituted by the monomer species.

7.2.2 METHOD VALIDATION

7.2.2.1 Precision

7.2.2.1.1 Method Precision

The given analytical method was found to be precise as the %RSD of the observations was <2%.

Injection	HMW		MONOMER		LMW	
	Area	%Area	Area	%Area	Area	%Area
1	43464	0.18	24455268	99.52	73649	0.3
2	43777	0.18	24488959	99.52	73858	0.3
3	43632	0.18	24453680	99.52	73415	0.3
4	43796	0.18	24731948	99.53	73784	0.3
5	44020	0.186	24622692	99.53	72703	0.29
6	43579	0.18	2468038	99.53	73949	0.3
Mean	43711.33	0.181	24572142	99.525	73559.67	0.298
Std dev	195.82	0.002	121988	0.005	459.063	0.004
%RSD	0.45	1.353	0.5	0.006	0.624	1.368

Table 7.7: Observation Table for Method Precision

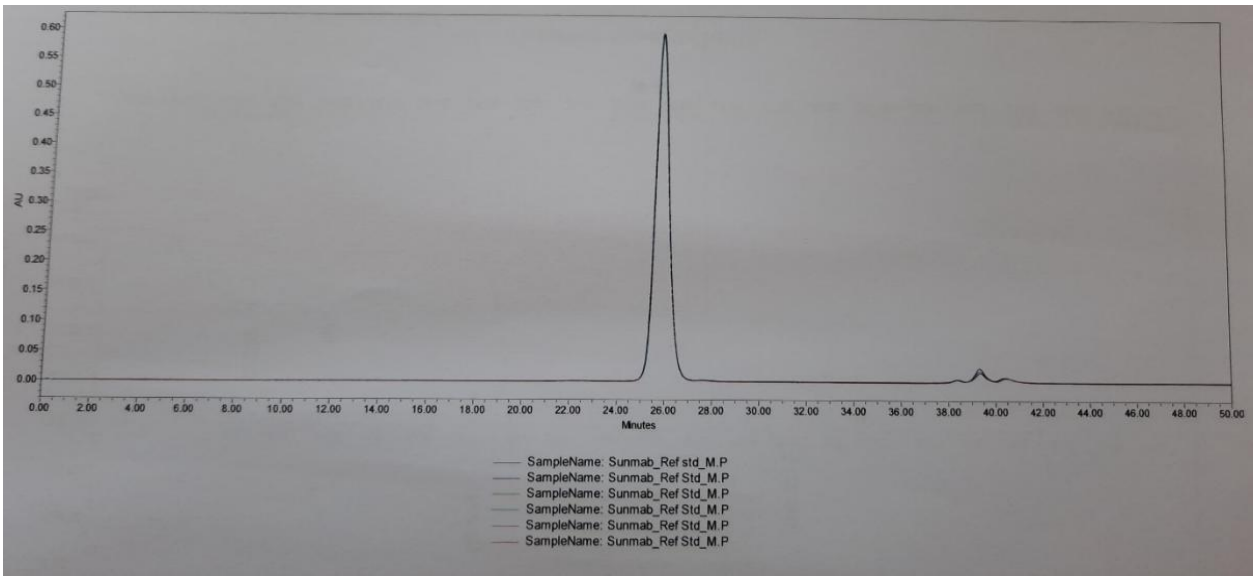

Fig. 7.10: Overlay Chromatogram for Method Precision of Sunmab

7.2.2.1.2 Instrument Precision

The given analytical instrument was found to be precise as the %RSD of the observations was <2%.

Preparation	HMW		MONOMER		LMW	
	Area	%Area	Area	%Area	Area	%Area
1	43338	0.19	23002128	99.49	73802	0.32
2	43996	0.19	23072291	99.5	72832	0.31
3	43517	0.19	22973148	99.49	73373	0.32
4	43621	0.19	23219411	99.5	73679	0.32
5	43188	0.19	23063902	99.5	73696	0.32
6	42904	0.19	23081702	99.5	73096	0.32
Mean	43427.33	0.19	23068763	99.497	73413	0.318
Std dev	376.32	0	85417.5	0.005	385.179	0.004385
%RSD	0.87	0.125	0.4	0.005	0.525	1.282

Table 7.8: Observation Table for Instrument Precision

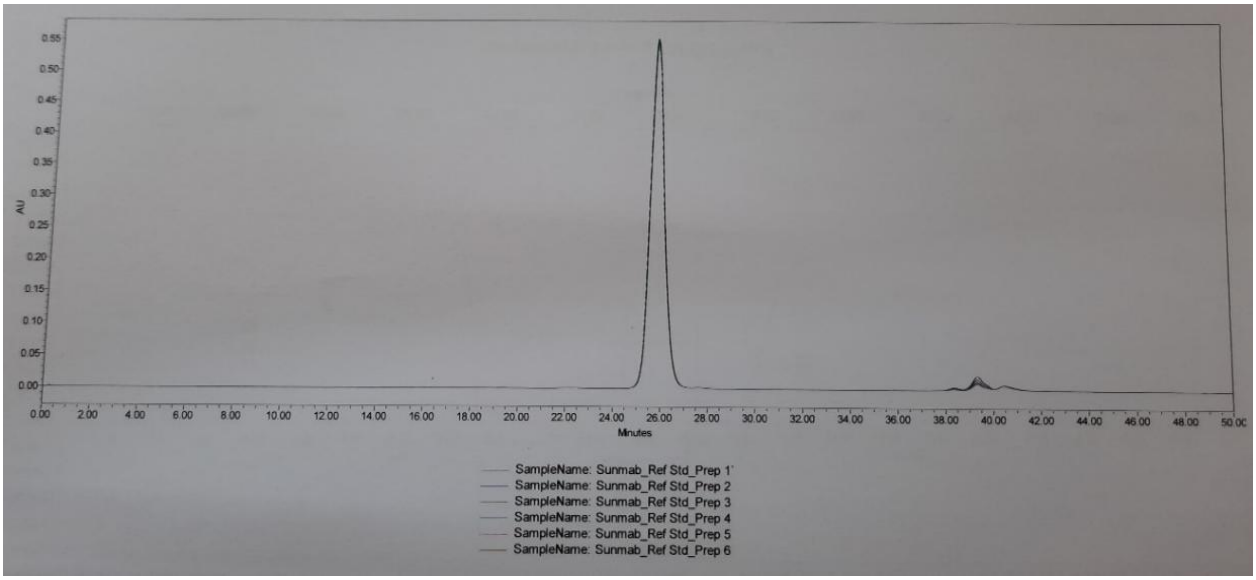

Fig. 7.11: Overlay Chromatogram for Instrument Precision of Sunmab

7.2.2.2 Specificity

There were no matrix interferences observed due to the mobile phase and diluent as there were no contributing peaks in the respective overlay chromatograms with the chromatogram of the reference standard.

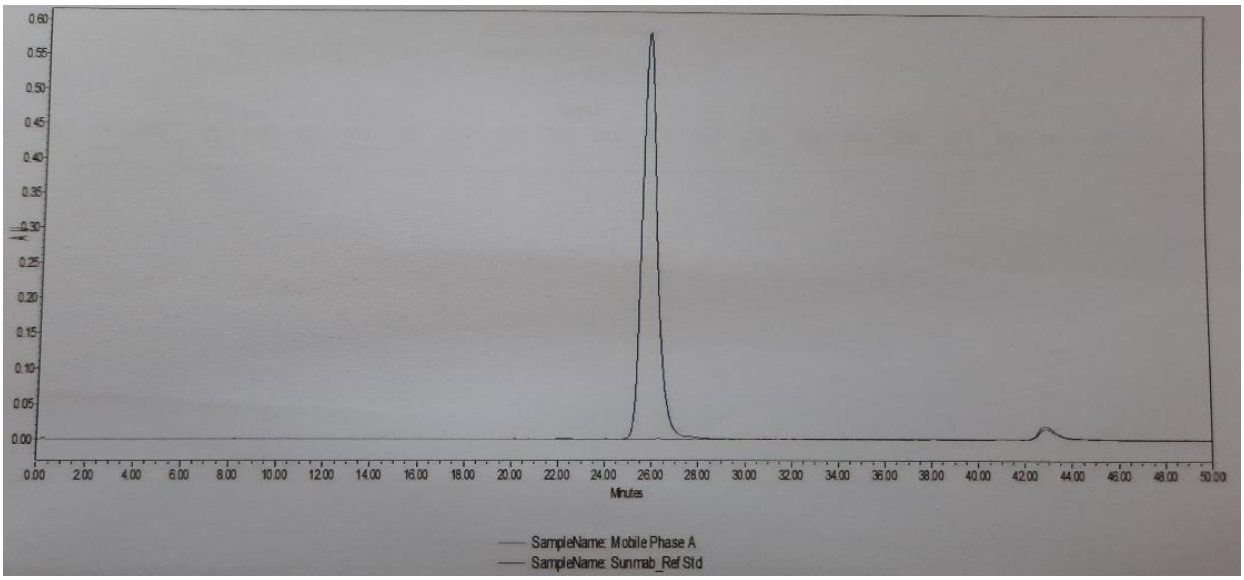

Fig. 7.12: Overlay Chromatogram for Mobile Phase and Reference standard of Sunma

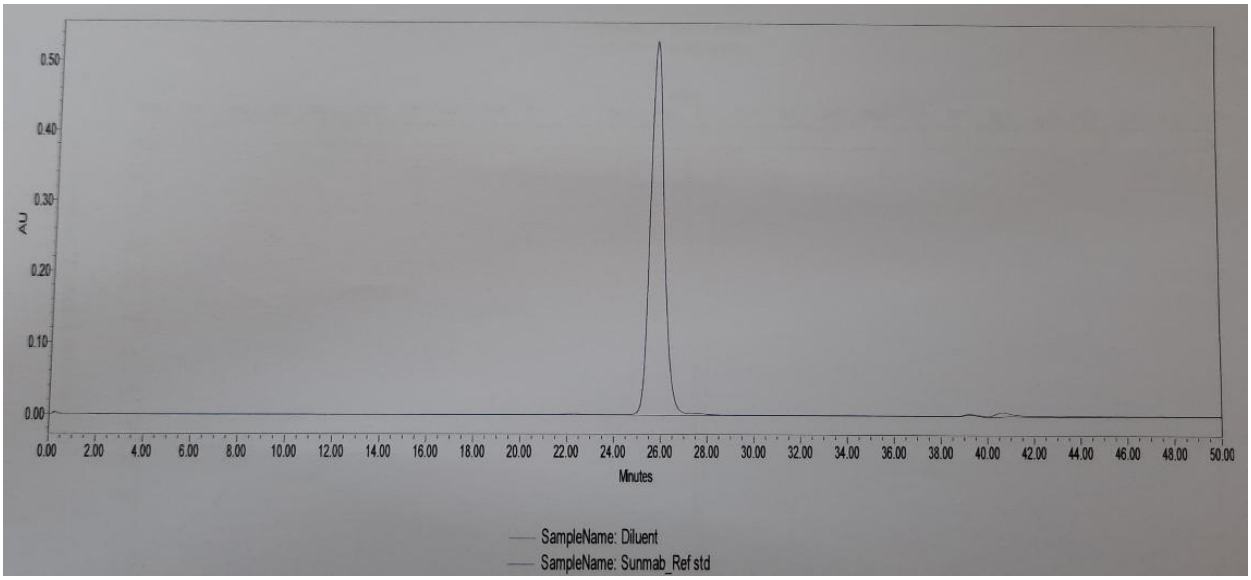

Fig. 7.13: Overlay Chromatogram for Mobile Phase B and Reference standard of Sunmab

7.2.2.3 Linearity

The given method was linear over a concentration of 10% - 125% and the correlation coefficient obtained from the calibration curve was found to be 0.9909.

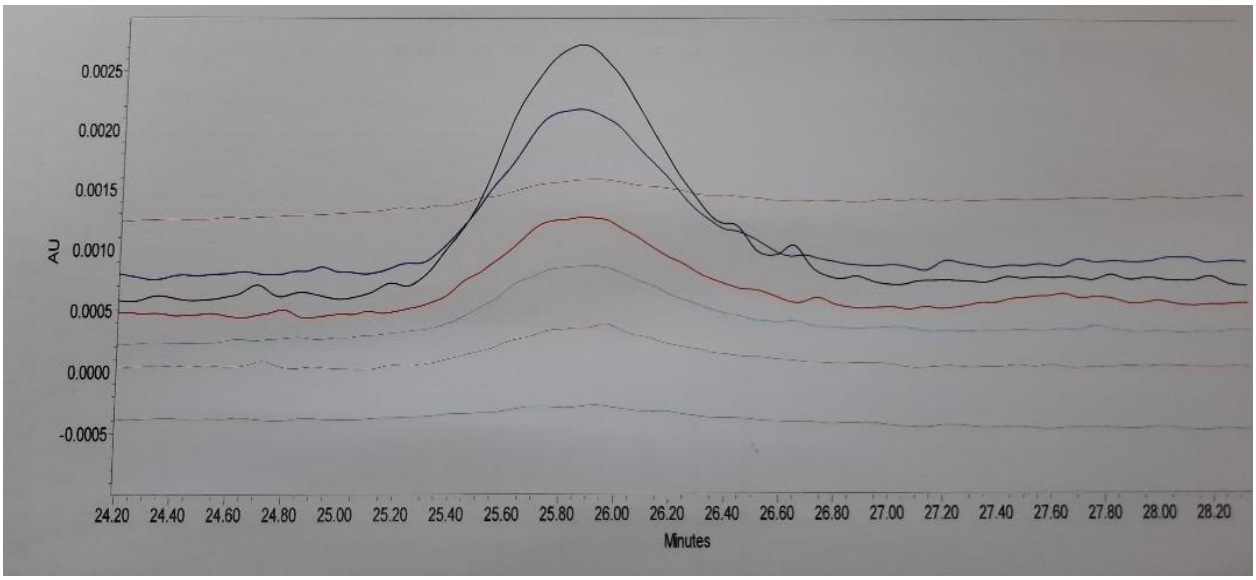

Fig 7.14: Overlay chromatogram of linearity test soluti

Conc%	Conc ug/ml	Mean	Std dev	%RSD
5	0.25	2887	699.1	24.2
10	0.5	7723	30.2	3.9
25	1.25	13143	168.8	1.3
50	2.5	25273.67	1853.9	7.3
75	3.75	39676	2575.6	6.5
100	5	56855.33	2562.2	4.5
125	6.25	75486	1498.8	1.99

Table 7.9: Observation Table for Linearity Study

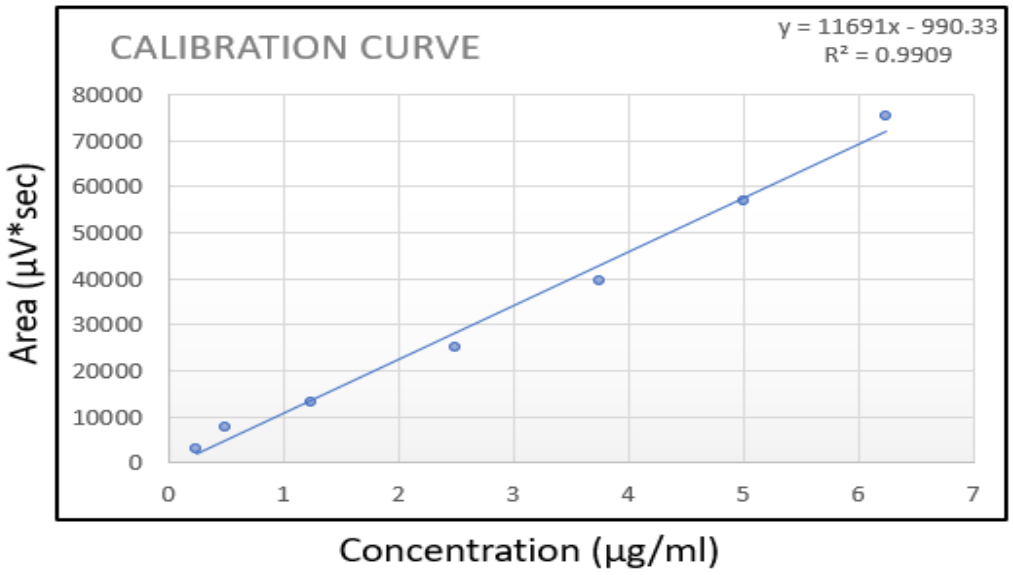

Fig 7.15: Calibration Curve for Sunmab

7.2.2.4 Range

The range of the analytical method for the charge variant analysis was 10% - 125% corresponding to 0.5 µg/ml to 6.25 µg/ml.

7.2.2.5 Determination of LOD

The Limit of Detection was found to be 0.5 µg/ml as the S/N ratio at this concentration was >3 and the % RSD was >2%. This implies that the analytical method was able to detect the analyte up to 0.5 µg/ml.

7.2.2.6 Determination of LOQ

The Limit of Quantification was found to be 1.25 µg/ml as the S/N ratio at this concentration was >10 and the % RSD was >2%. This implies that the analytical method was able to quantify the analyte up to 1.25 µg/ml.

7.2.2.7 Accuracy

The accuracy of the method was calculated by determining the % recovery. The % recovery of the test solutions was calculated from the regression equation in the linearity study. The % recovery turned out to be in the range of 95-105 %.

Conc.%	Conc(ug/ml)	Area	Back cal conc. (ug/ml)	%Recovery
25	1.25	13334	1.2	98
		13013	1.2	95.8
		13083	1.2	96.3
100	5	56048	4.9	97.6
		59724	5.2	103.9
		54794	4.8	95.4
125	6.25	77212	6.7	107
		74516	6.5	103.3
		74729	6.5	103.6

Table 7.10: Observation Table for % Recovery study for accuracy

7.2.3 FORCED DEGRADATION STUDY

7.2.3.1 Heat Stress

There is substantial decrease in HMW species from 0.19% in control sample to 0.01% in the stress sample. An elevation in the LMW species was seen from 0.32% in the control sample to 4.14 %in stress sample.

This suggests that upon incubation of the mAb sample at 70°C generates LMW size variants and almost completely eliminates the HMW species.

7.2.3.2 Vortex

Upon vortex of the sample, a minor increase in both HMW and LMW species was observed as the % HMW species elevated from 0.19 % (control) to 0.28 % (stress) and % LMW species to 0.45% (stress) from 0.32 % (control) was observed.

This study can thus lead us to the conclusion that mechanical stress of the sample in the form of vortex generates both types of size variants.

7.1.3.3 Basic shift in formulation buffer

A basic shift in formulation buffer lead to increase in the LMW size variant species from 0.32 % (control) to 4.85 % in the stress sample. Whereas, the HMW size variants were found to decrease insignificantly under identical stress conditions to 0.12 % from 0.19% in the control sample.

This suggests that a basic shift in the buffer generates LMW size variants while leads to a significant decrease in the HMW species.

7.1.3.4 Vortex coupled with Heat stress

There was a significant increase in the % HMW species from 0.19 % to 4.9 % in the stress sample. On the contrary, the %LMW species were found to decrease from 0.32 % to 0.17 % in the stress sample.

The impact of mechanical stress in the form of vortex coupled with elevated temperature is substantial in terms of the size variant profile of Sunmab.

7.1.3.5 Basic Shift in formulation buffer coupled with Vortex

Upon basic shift in pH of the formulation buffer and subsequent vortex of the sample, the % HMW species reduced to 0.08 % in the stress sample from 0.19 % in the control sample. On the other hand, a large increase was observed in the% LMW species from 0.32 % (control) to 6.33 % (stress).

Hence, it can be said that upon basic shift in pH followed by vortex, impacts the size variant profile by leading to an increase in LMW species and a minor decree se in the HMW species.

7.1.4 Summary of Results

VALIDATION PARAMETER		RESULT
Precision	Method Precision	% RSD < 2%
	Instrument Precision	% RSD < 2%
Accuracy (% recovery)		95-105%
Linearity	Range	10% - 125 %
	r^2	0.9909
	Y-intercept (c)	-990.3
	Slope (m)	11691
LOD		0.5 µg/ml
LOQ		1.25 µg/ml

Table 7.11: Summary of Method Validation Study

SAMPLE	% HMW SPECIES	% LMW SPECIES	INFERENCE
Control	0.19	16.58	
Heat Stress	0.01	4.14	Increases LMW species Decreases HMW species
Vortex Stress	0.28	0.45	Decreases LMW species Increases HMW species
Basic Shift in buffer	0.12	4.85	Decreases HMW species Increases LMW species
Vortex + basic shift in buffer	0.08	6.33	Increases HMW species Decreases LMW species
Heat stress + vortex	4.9	0.7	Increases HMW species Decreases LMW species

Table 7.12: Summary of Forced Degradation Study

CHAPTER 8:

CONCLUSION

Monoclonal antibodies are being developed in high propensity mainly due to their wide range of therapeutic capabilities. In order to successfully utilize this capability, the production of high-quality products becomes necessary. However, it must be noted that heterogeneities in monoclonal antibodies are inevitable and must be controlled for obtaining quality products. Two of the most common heterogeneities are charge and size heterogeneity.

Charge heterogeneity arises in the form of acidic and basic species whereas, size heterogeneity arises in the form of high molecular and low molecular weight species. These heterogeneities can arise as a result of various Post Translational Modifications (PTMs) that could be incorporated during the entire life span of the product; from development of the biopharmaceutical product to the time of administration of the drug by the patient.

Hence, it becomes imperative to characterize and control these impurities as they can directly affect the safety and efficacy of the product for which sensitive analytical methods need to be developed and then validated.

Here, a Cation Exchange chromatographic method for charge variant analysis and a Size Exclusion chromatographic method for size variant analysis have been studied. The CEX and SEC methods were validated according to SPIL guidelines which have been built in the lines of the ICH Q2R1 guidelines. The validation was carried out for different parameters relevant to the analytical methods, namely, precision, accuracy, specificity, linearity, range, LOD and LOQ determination.

One important aspect of producing high quality biopharmaceutical products is the characterization of the different degradation products and elucidation of the underlying mechanisms for such products. In this context, forced degradation studies provide an opportunity to gain an in-depth understanding of the biochemical and biophysical properties of the molecules. Forced degradation study (or stress testing) isan umbrella term covering all forms of applying stress to drug substance or drug product exceeding the conditions used for stability testing.

In order to analyze the effects of forced degradation study, a stability- indicating analytical method is absolutely essential. Also, the stress conditions that must be selected for the forced degradation study should cater to the analytical method and its underlying principle for analysis.

In order to assess the stability indicating capability of the CEX method for charge variant analysis of Sunmab; oxidation, reduction, pH shifts in formulation buffer, vortex and elevated

temperatures were used as the stress conditions. Upon, completion of the study it was found that the % acidic charge variants increased upon reduction, basic shift in formulation buffer and at elevated temperatures. On the other hand, % basic species were found to decrease upon basic shift in formulation buffer while all other stress conditions led to its increase in comparison with the control sample.

Similarly, a forced degradation study of Sunmab by SEC for the effect of stress conditions such as, elevated temperatures, vortex, basic shift in pH of formulation buffer, basic shift coupled with vortex and elevated temperature incubation of vortexed sample on the size variant profile was performed. The results of this study revealed that all the stress conditions lead to an increase in the % LMW species while the % HMW species were found to increase only on incubation of the vortexed sample at elevated temperature.

It can affirmatively be concluded that the CEX and SEC analytical methods show appreciable prowess in their stability-indicating capabilities for the charge and size heterogeneity analysis of Sunmab.

CHAPTER: 9

REFERENCES

1.Ishikawa, T., Ito, T., Endo, R., Nakagawa, K., Sawa, E., & Wakamatsu, K. (2010). "Influence of pH on heat-induced aggregation and degradation of therapeutic monoclonal antibodies".Biological and Pharmaceutical Bulletin, 33(8), 1413–1417. https://doi.org/10.1248/bpb.33.1413

2. Bansal, R., Gupta, S., & Rathore, A. S. (2019). Analytical Platform for Monitoring Aggregation of Monoclonal Antibody Therapeutics. Pharmaceutical Research, 36:152. 1–11. https://doi.org/10.1007/s11095-019-2690-8

3. Philo, J. S. (2014). A Critical Review of Methods for Size Characterization of Non-Particulate Protein Aggregates. Current Pharmaceutical Biotechnology, 10, 359-372. https://doi.org/10.2174/138920109788488815

4. Sec-hplc, C., Schrag, D., Corbier, M., & Raimondi, S. (n.d.). Chapter 31. 1131, 507–512. https://doi.org/10.1007/978-1-62703-992-5

5. HANNS-CHRISTIAN MAHLER WOLFGANG FRIESS, ULLA GRAUSCHOPF, S. K. (2012). Protein aggregation: pathways, induction factors and analysis. Journal of Pharmaceutical Sciences, 101(7), 2271–2280. https://doi.org/10.1002/jps

6. Ahrer, K., Buchacher, A., Iberer, G., Josic, D., &Jungbauer, A. (2003). Analysis of aggregates of human immunoglobulin G using size- exclusion chromatography, static and dynamic light scattering. Journal of Chromatography A, 1009, 89–96.https://doi.org/10.1016/S0021-9673(03)00433-3

7. Renee, A., Yun, Y., Bing, T., Xuemei, Z., Alice, L., Yonghua, L., & Zhang, T. (2015). High resolution separation of recombinant monoclonal antibodies by size exclusion ultra-high-performance liquid chromatography (SE-UHPLC). Journal of Pharmaceutical and Biomedical Analysis. https://doi.org/10.1016/j.jpba.2015.02.032

8. Hernández-jiménez, J., Martínez-ortega, A., Cabeza, J., Prados, J. C., &Ortíz, R. (2018). Study of aggregation in therapeutic monoclonal antibodies subjected to stress and long-term stability tests by analyzing size exclusion liquid chromatographic profiles. International Journal of Biological Macromolecules. https://doi.org/10.1016/j.ijbiomac.2018.06.105

9. Frie, W., Mahler, H., Mu, R., Delille, A., & Matheus, S. (2005). Induction and analysis of aggregates in a liquid IgG1-antibody formulation.European Journal of Pharmaceutics and Biopharmaceutics,59, 407–417. https://doi.org/10.1016/j.ejpb.2004.12.004

10. Bickel, F., Herold, E. M., Marrahi, A. S., Romeijn, S., & Kiefer, H. (2016). Reversible NaCl-Induced Aggregation of a Monoclonal Antibody at low pH: Characterization of Aggregates and Factors Affecting Aggregation. European Journal of Pharmaceutics and Biopharmaceutics. https://doi.org/10.1016/j.ejpb.2016.07.020

11. Arakawa, T., Ejima, D., Li, T., & Philo, J. S. (2010). The Critical Role of Mobile Phase Composition in Size Exclusion Chromatography of Protein Pharmaceuticals. 99(4), 1674–1692. https://doi.org/10.1002/jps

12. Goyon, A., Fekete, S., Beck, A., Veuthey, J., &Guillarme, D. (2018). Unraveling the mysteries of modern size exclusion chromatography - the way to achieve con fi dent characterization of therapeutic proteins. Journal of Chromatography B, 1092(June), 368–378. https://doi.org/10.1016/j.jchromb.2018.06.029

13. Hamrang, Z., Rattray, N. J. W., &Pluen, A. (2013). Proteins behaving badly: emerging technologies in profiling biopharmaceutical aggregation. Trends in Biotechnology, 31(8), 448–458. https://doi.org/10.1016/j.tibtech.2013.05.004

14. Ghosh, R., Calero-rubio, C., Saluja, A., & Roberts, C. J. (2016). Relating Protein - Protein Interactions and Aggregation Rates from Low to High Concentrations. Journal of Pharmaceutical Sciences, 105(3), 1086–1096. https://doi.org/10.1016/j.xphs.2016.01.004

15. Singla, A., Bansal, R., Joshi, V., & Rathore, A. S. (2016). Aggregation kinetics for IgG1-based monoclonal antibody therapeutics. AAPS Journal, 18(3), 689–702. https://doi.org/10.1208/s12248-016-9887-0

16. Rosenberg, A. S. (2006). Effects of protein aggregates: An Immunologic perspective. AAPS Journal, 8(3), 501–507. https://doi.org/10.1208/aapsj080359

17. Hong, P., Koza, S., Bouvier, E. S. P., & Corporation, W. (n.d.). Journal of Liquid Chromatography & A REVIEW SIZE-EXCLUSION CHROMATOGRAPHY FOR THE ANALYSIS OF PROTEIN BIOTHERAPEUTICS AND THEIR AGGREGATES. May 2015, 37–41. https://doi.org/10.1080/10826076.2012.743724

18. Fekete, S., Beck, A., Veuthey, J., &Guillarme, D. (2014). Journal of Pharmaceutical and Biomedical Analysis Theory and practice of size exclusion chromatography for the analysis of protein aggregates. Journal of Pharmaceutical and Biomedical Analysis, 101, 161–173. https://doi.org/10.1016/j.jpba.2014.04.011

19. Khodabandehloo, A. (2017). Particle sizing methods for the detection of protein aggregates in biopharmaceuticals.

20. Beck, A. (2019). Macro- and Micro-Heterogeneity of Natural and Recombinant IgG Antibodies. 1–22. https://doi.org/10.3390/antib8010018

21. Baek, J., Schwahn, A. B., Lin, S., Pohl, C. A., Pra, M. De, Tremintin, S. M., & Cook, K. (2020). New Insights into the Chromatography Mechanisms of Ion- Exchange Charge Variant Analysis: Dispelling Myths and Providing Guidance for Robust Method Optimization. https://doi.org/10.1021/acs.analchem.0c02775

22. Beyer, B., Schuster, M., Jungbauer, A., &Lingg, N. (2018). Microheterogeneity of Recombinant Antibodies: Analytics and Functional Impact. 1700476, 1–11. https://doi.org/10.1002/biot.201700476

23. Li, W., Kerwin, J. L., Schiel, J., Formolo, T., Davis, D., Mahan, A., Benchaar, S. A., Oaks, T., & States, U. (2015). Structural Elucidation of Post-Translational Modifications in Monoclonal Antibodies.

24. Krause, M. E., &Sahin, E. (2019). ScienceDirect Chemical and physical instabilities in manufacturing and storage of therapeutic proteins. Current Opinion in Biotechnology, 60, 159–167. https://doi.org/10.1016/j.copbio.2019.01.014

25. Xiaobin Xu (2016), In vivo characterization of therapeutic monoclonal antibodies JOURNAL OF APPLIED BIOANALYSIS, Vol. 2, No. 1; 10-15. http://dx.doi.org/10.17145/jab.16.003 (ISSN 2405-710X)

26. Hoda E. Mohamed, Abeer A. Mohamed, Medhat A. Al-Ghobashy, Faten A. Fathalla, Samah S. Abbas (2017), Stability assessment of antibody-drug conjugate Trastuzumab emtansine in comparison to parent monoclonal antibody using orthogonal testing protocol; Journal of Pharmaceutical and Biomedical Analysis; DOI: https://doi.org/10.1016/j.jpba.2017.12.022

27. Blessy, M., Patel, R. D., Prajapati, P. N., & Agrawal, Y. K. (2014). Development of forced degradation and stability indicating studies of drugs - A review. Journal of Pharmaceutical Analysis, 4(3), 159–165. https://doi.org/10.1016/j.jpha.2013.09.003

28. Psimadas, D., Georgoulias, P., Valotassiou, V., &Loudos, G. (2012). Molecular Nanomedicine Towards Cancer: Journal of Pharmaceutical Sciences, 101(7), 2271–2280. https://doi.org/10.1002/jps

29. Tamizi, E., &Jouyban, A. (2016). Forced degradation studies of biopharmaceuticals: Selection of stress conditions. *European Journal of Pharmaceutics and Biopharmaceutics*, *98*(November), 26–46. https://doi.org/10.1016/j.ejpb.2015.10.016

30. Shan, L., & Anderson, D. J. (2002). Gradient chromate-focusing. Versatile pH gradient separation of proteins in ion-exchange HPLC: Characterization studies. *Analytical Chemistry*, *74*(21), 5641–5649. https://doi.org/10.1021/ac020169q

31. Shen, H., & Frey, D. D. (2004). Charge regulation in protein ion-exchange chromatography: Development and experimental evaluation of a theory based on hydrogen ion Donnan equilibrium. *Journal of Chromatography A*, *1034*(1–2), 55–68. https://doi.org/10.1016/j.chroma.2004.01.039

32. L. A. Sluyterman and A. Elgersma. (1978). Chromato-focusing: Iso-electric focusing on Ion-Exchange. Journal of Chromatography, 150 (1978) 17-30.

33. R.W. Stout, S.I. Sivakoff and R.D Ricker. (1986). Separation od proteins by gradient elution fromion exchange column-optimizing experimental conditions. Journal of Chromatography, 353 (1986) 439-463.

34. Ståhlberg, J., Jönsson, B., &Horváth, C. (1991). Theory for Electrostatic Interaction Chromatography of Proteins. *Analytical Chemistry*, *63*(17), 1867–1874. https://doi.org/10.1021/ac00017a036

35. Talebi, M., Nordborg, A., Gaspar, A., Lacher, N. A., Wang, Q., He, X. Z., Haddad, P. R., &Hilder, E. F. (2013). Charge heterogeneity profiling of monoclonal antibodies using low ionic strength ion-exchange chromatography and well-controlled pH gradients on monolithic columns. *Journal of Chromatography A*, *1317*, 148–154. https://doi.org/10.1016/j.chroma.2013.08.061

36. Weitzhandler, M., Farnan, D., Rohrer, J. S., &Avdalovic, N. (2001). Protein variant separations using cation exchange chromatography on grafted, polymeric stationary phases. *Proteomics*, *1*(2), 179–185. https://doi.org/10.1002/1615-9861(200102)1:2<179::AID-PROT179>3.0.CO;2-8

37. Kang, X., & Frey, D. D. (2003). High-performance cation-exchange chromate-focusing of proteins. *Journal of Chromatography A*, *991*(1), 117–128. https://doi.org/10.1016/S0021-9673(03)00210-3

38. Rozhkova, A. (2009). Quantitative analysis of monoclonal antibodies by cation-exchange chromatofocusing. *Journal of Chromatography A*, *1216*(32), 5989–5994. https://doi.org/10.1016/j.chroma.2009.06.045

39. Bai, L., Burman, S., & Gledhill, L. (2000). Development of ion exchange chromatography methods for monoclonal antibodies. *Journal of Pharmaceutical and Biomedical Analysis*, *22*(3), 605–611. https://doi.org/10.1016/S0731-7085(00)00236-3

40. Ståhlberg, J., &Jönsson, B. (1996). Influence off charge regulation in electrostatic interaction chromatography of proteins. *Analytical Chemistry*, *68*(9), 1536–1544. https://doi.org/10.1021/ac9509972

41. Fekete, S., Beck, A., Veuthey, J., &Guillarme, D. (2015). Journal of Pharmaceutical and Biomedical Analysis Ion-exchange chromatography for the characterization of biopharmaceuticals. *Journal of Pharmaceutical and Biomedical Analysis*, *113*, 43–55. https://doi.org/10.1016/j.jpba.2015.02.037

42. Zhang, L., Patapoff, T., Farnan, D., & Zhang, B. (2013). Improving pH gradient cation-exchange chromatography of monoclonal antibodies by controlling ionic strength. *Journal of Chromatography A*, *1272*, 56–64.https://doi.org/10.1016/j.chroma.2012.11.060

43. Xu, Y., Wang, D., Mason, B., Rossomando, T., Li, N., Liu, D., Cheung, J. K., Xu, W., Raghava, S., Katiyar, A., Nowak, C., Xiang, T., Dong, D. D., Sun, J., Beck, A., Xu, Y., Wang, D., Mason, B., Rossomando, T., … Structure, H. L. (2019). Structure , heterogeneity and developability assessment of therapeutic antibodies. *MAbs*, *11*(2), 239–264. https://doi.org/10.1080/19420862.2018.1553476

44. Wang, D., Nowak, C., Mason, B., Katiyar, A., & Liu, H. (2020). Journal of Pharmaceutical and Biomedical Analysis Analytical artifacts in characterization of recombinant monoclonal antibody therapeutics. *Journal of Pharmaceutical and Biomedical Analysis*, *183*, 113131. https://doi.org/10.1016/j.jpba.2020.113131

45. Trappe, A., Füssl, F., Carillo, S., Zaborowska, I., &Meleady, P. (2018). Rapid charge variant analysis of monoclonal antibodies to support lead candidate biopharmaceutical development. *Journal of Chromatography B*, *1095*(July), 166–176. https://doi.org/10.1016/j.jchromb.2018.07.037

46. Vlasak, J., & Ionescu, R. (2008). Heterogeneity of Monoclonal Antibodies Revealed by Charge-Sensitive Methods. Current Pharmaceutical Biotechnology, 2008, 9, 468–481.

47. Ponniah, G., Kita, A., Nowak, C., Neill, A., Kori, Y., Rajendran, S., & Liu, H. (2015). *Characterization of the acidic species of a monoclonal antibody using weak cation exchange chromatography and LC-MS*. https://doi.org/10.1021/acs.analchem.5b02385

48. Lingg, N., Tan, E., Hintersteiner, B., Bardor, M., &Jungbauer, A. (2013). Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range. *Journal of Chromatography A*, *1319*, 65–71. https://doi.org/10.1016/j.chroma.2013.10.028

49. Leblanc, Y., Ramon, C., Bihoreau, N., &Chevreux, G. (2017). Charge variants characterization of a monoclonal antibody by ion exchange chromatography coupled on-line to native mass spectrometry: Case study after a long-term storage at + 5 ◦ C. *Journal of Chromatography B*, *1048*, 130–139. https://doi.org/10.1016/j.jchromb.2017.02.017

50. Iex-hplc, C., Corbier, M., Schrag, D., & Raimondi, S. (2014). *Chapter 30*. *1131*, 501–506. https://doi.org/10.1007/978-1-62703-992-5

51. G. Lavanya, M. Sunil, M.M. Eswarudu*, M. ChinnaEswaraiah, K. Harisudha and B. Naga Spandana, "ANALYTICAL METHOD VALIDATION: AN UPDATED REVIEW", IJPSR, 2013; Vol. 4(4): 1280-12

52. Geetha, G., Naga, K., Raju, G., Kumar, B. V., & Raja, M. G. (2012). Analytical Method Validation: An Updated Review of analytical method and validation. *International Journal of Pharmaceutical Sciences and Research*, *1*(1), 64–71. www.ijpsr.com

53. Araujo, P. (2009). Key aspects of analytical method validation and linearity evaluation. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *877*(23), 2224–2234. https://doi.org/10.1016/j.jchromb.2008.09.030

54. Bharti Mittu, A. C., & Chauhan, P. (2015). Analytical Method Development and Validation: A Concise Review. *Journal of Analytical & Bioanalytical Techniques*, *06*(01), 1–5. https://doi.org/10.4172/2155-9872.1000233

55. Ravichandran, V., Shalini, S., Sundram, K. M., &Rajak, H. (2010). Validation of analytical methods - Strategies & importance. *International Journal of Pharmacy and Pharmaceutical Sciences*, *2*(SUPPL. 3), 18–22.

56. Puttrevu, S. K., Laxman, T. S., Tripathi, A. K., Yadav, A. K., Verma, S. K., Mishra, A., Pradhan, R., Verma, N. K., Ghosh, J. K., & Bhatta, R. S. (2019). Liquid chromatography–tandem mass spectrometry-based method development and validation of S016-1271 (LR8P), a novel cationic antimicrobial peptide for its application to pharmacokinetic studies. Journal of Pharmaceutical and Biomedical Analysis, 169, 116–126. https://doi.org/10.1016/j.jpba.2019.01.046

57. Nguyen, T. T. T. N., Mistarz, U. H., Costa, N., Herbet, A., Boquet, D., Becher, F., & Rand, K. D. (2018). Investigating the utility of minimized sample preparation and high-resolution mass spectrometry for quantification of monoclonal antibody drugs. Journal of Pharmaceutical and Biomedical Analysis, 159, 384–392. https://doi.org/10.1016/j.jpba.2018.07.012

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