"Method Development and Validation of Reverse-phase HPLC for purity analysis and Size-exclusion HPLC for aggregate analysis of monoclonal antibody, SUNmAb"

A Thesis Submitted to

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in Partial Fulfilment for the Award of the Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

BY

KHUSHBOO RAMNANI (19MPH308), B. PHARM.

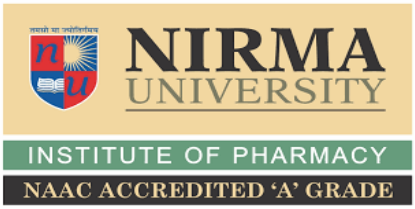
Under the guidance of

Dr. PRITI J. MEHTA

HOD, Department of Pharmaceutical Analysis

Dr. RAVISHANKARA. M.N.

Sr. General Manager, Sun Pharamceutical Industries Limited



Department of Pharmaceutical Analysis

Institute of Pharmacy

NIRMA University

Ahmedabad-382481

Gujarat, India.

May 2021

CERTIFICATE

This is to certify that the dissertation work entitled "Method Development and Validation of Reverse-phase HPLC for purity analysis and Size-exclusion HPLC for aggregate analysis of Monoclonal Antibody, SUNmAb" submitted by Ms. Khushboo Ramnani with Regn. No. (19MPH308) in partial fulfilment 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 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.

Guide

Dr. Priti J. Mehta
HOD,
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University

Prof. Manjunath Ghate
Director
Institute of Pharmacy,
Nirma University

28 May 2021

20 May 2021

Sun Pharmaceutical Industries Ltd.
Tandalja, Vadodara - 390 012, INDIA
Tel. : 91-265-6615500/6615600/6615700
Fax : 91-265-2354897
CIN : L24230GJ1993PLC019050

SUN PHARMA

22 January 2021

To whomsoever it may concern

This is to certify that Khushboo Ashokkumar Ramnani student of M.Pharm final year at the Nirma University, Ahmedabad has undergone her project training in Biotechology in R&D Baroda.

Duration of training: 6 months

- 18rd July 2020 to 22nd January 2021

Her dissertation work will be submitted after three months to the Project Guide. The brief training report is submitted to the organization.

During her training, her performance was found to be satisfactory.

We wish her all the best in her future endeavors.

Sincerely

For Sun Pharmaceutical Inds Limited

Sajam A S

Sr. GM – Human Resources.

CERTIFICATE OF ORIGINALITY OF WORK

This is to undertake that the dissertation work entitled "Method Development and Validation of Reverse-phase HPLC for purity analysis and Size-exclusion HPLC for aggregate analysis of Monoclonal Antibody, SUNmAb" submitted in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by me at the Institute of Pharmacy, Nirma University under the guidance of Dr. Priti J. Mehta and at the SUN Pharmaceuticals Industries Limited under 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.

Ms. Khushboo Ramnani,
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University

Guide
Dr. Priti .J. Mehta
HOD,
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University

28 May 2021

20 May, 2021

DECLARATION

I hereby declare that the dissertation entitled "Method Development and Validation of Reverse-phase HPLC for purity analysis and Size-exclusion HPLC for aggregate analysis of 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., Sr. General Manager, Department of Biotechnology, 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.

Ms. Khushboo Ramnani,
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University

20 May, 2021

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LIST OF ABBREVIATIONS

Sr No.	Abbreviation	Full Form
1.	RP	Reverse Phase
2.	SEC	Size Exclusion Chromatography
3.	HPLC	High Pressure Liquid 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.	°C	Degree Celsius
11.	CE-SDS	Capillary Electrophoresis-Sodium Dodecyl Sulphate
12.	FTIR	Fourier Transform Infrared Spectroscopy
13.	NMR	Nuclear Magnetic Resonance
14.	ELISA	Enzyme Linked Immunosorbent Assay
15.	LC-MS	Liquid Chromatography Mass Spectroscopy

ABSTRACT

Development of analytical method for therapeutic protein as compared to small molecules is a challenging task because of their high molecular weight, storage conditions, environmental conditions. During analytical method development of therapeutic protein, they come in contact with different solvents, parameters like pH, temperature may change which can lead to instability of protein molecule and so method development for protein requires expertise. Here, we are presenting our research work which includes the development of Reverse Phase-HPLC (RP-HPLC) method for purity analysis of therapeutic protein using Zorbax 300SB C-18 (3.5 µm 150x4.6 mm, Agilent) column with solvents like water, acetonitrile, and propanol. Different combination of mobile phases were tried and the results were observed and interpreted. A gradient method with combination of 1-propanol and acetonitrile at 220 nm was found to provide satisfactory results. The method was qualified and was found to be accurate, precise, specific and provided linear responses. Similarly, we have developed a SEC-HPLC method for aggregate analysis of therapeutic protein using TSKgel G2000 SWxL 5µm, 125 Å (300 x 7.8mm, Tosoh) and Biosep SEC s2000 5 µm, 150 Å (300 x 7.8mm, Phenoemenex) column with Sodium and Potassium based buffers as mobile phase. A method with Biosep SEC s2000 column and Potassium based buffer as a mobile phase at 215 nm was found to provide satisfactory results. The method was qualified and was found to be accurate, precise, specific and provided linear responses.

CHAPTER 1

INTRODUCTION

1.1 Therapeutic proteins

Once rarely used subset of medical treatments, the use of protein therapeutics has tremendously increased since introduction of recombinant protein therapeutic – insulin. Protein therapeutics have now become an important part in almost every field of medicine. [Leader et al., 2008] European union and USA have approved more than 100 of genuine and similar protein therapeutics from 2010. [Buchanan & Revell., 2015] Protein therapeutics have a great potential and many of them positively impact on functions and conditions of human body. Protein therapeutics are also called as degradable biopharmaceuticals or biodrugs. [Dimitrov., 2012] Protein therapeutics are basically large molecules with complex structure and function. They are made up of amino acids and folded into secondary, tertiary and quaternary structure. They are also sometimes modified by attachment of other molecular entities like glycan, polyethylene glycol naturally or by designing to extend their half-life or to reduce immunogenicity. [Fang et al., 2017] These therapeutic proteins have evolved and are engaged in various activities from signaling as ligands and receptors, acting as transporter, regulating gene expression to catalyzing reactions in the body.[Struble et al., 2016] These protein therapeutics have to play their role in order to ensure proper functioning of body. According to their activities, they can be categorized into five groups : 1) replacing deficient or abnormal protein, 2) enhance working of existing pathways, 3) providing novel function or activity, 4) interfering with a molecule or organism, 5) delivering compounds such as drugs or effector proteins.[Fang et al., 2017] Different type of protein therapeutics include antibody-based drugs, fusion proteins, blood, factors, bone proteins, engineered proteins, enzymes, growth factors, hormones, interferons, interleukins and thrombolytic. These can also be classified on basis of their mechanism of activity as : a) binding covalently to target e.g., monoclonal antibodies b) affecting covalent bonds e.g., enzymes, c) exerting activity without specific interaction e.g., serum albumin. Therefore, these compounds are highly potent and specific with very low toxicity.[Joseph et al., 2017]

1.2 Monoclonal Antibodies

Monoclonal antibodies are compounds formulated from living systems with help oftechnology. Evolution in molecular biology through recombinant DNA technology made production of monoclonal antibodies possible. [Santos at al., 2021] Monoclonal antibodies are

produced from B-cell and they are target specific compounds. Because of hybridoma technique, it has become possible to obtain pure mAbs in large amounts.[Lue et al., 2020]

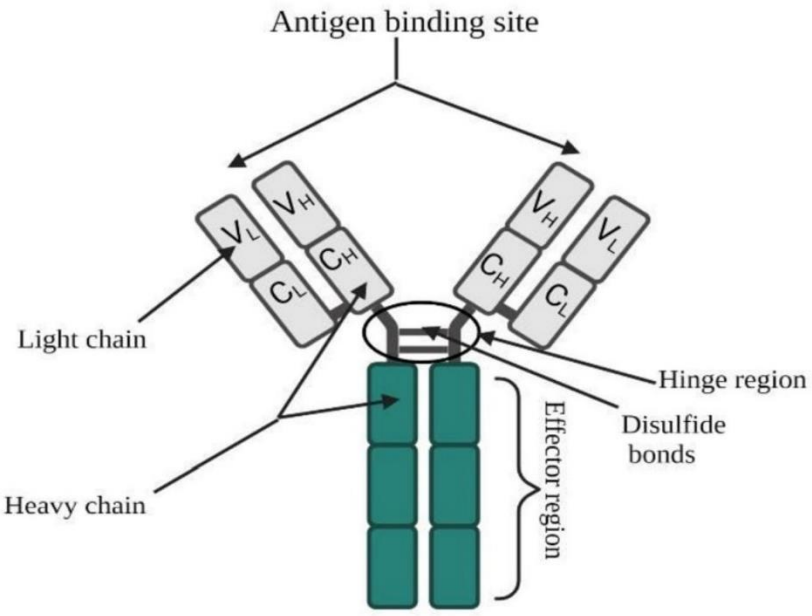

Figure 1.1 : Structure of Monoclonal Antibody

Monoclonal antibodies can also be defined as “molecules produced in laboratory with help of some technologies to serve as a substitute antibody that can restore, enhance, or mimic the immunesystem’s response to any disease”.[Bayer., 2019] This category of therapeutic protein has become very popular now and is used in many diseases like cancer, diabetes, arthritis, psoriasis, asthma etc.

Advancement in technologies and progress in antibody engineering has allowed us to yield different types of monoclonal antibodies. These monoclonal antibodies may have same principle but have different targets and applications. The purpose of their application, availability, and effectiveness are the factors to choose a method for their production. These monoclonal antibodies may be classified as :

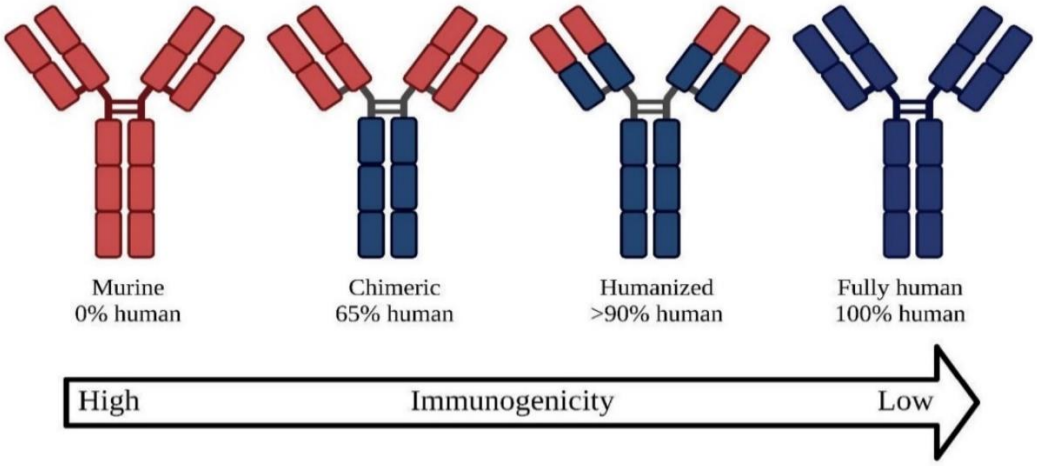

Figure1.2 : Types of Monoclonal Antibody

Murine mAbs : The first to be discovered and produced. This type of mAbs are obtained by taking B-cell from spleen of mice and then mixed with an immortal myeloma cell line lacking the hypoxanthine-guanine-phosphoribosyl-transferase (HTPR) gene. These mAbs are identified by "-omab" at the end of their name e.g., muromonab-CD3, blinatumomab, capromab. They have allergic reaction when used in humans and also, they have shorter half-life. The use of murine antibodies produced by hybridoma technology for humans is limited because of difference between human and rodent immune systems.

Chimeric mAbs : Chimeric are special type of mAbs produced by manipulation of human constant region and mouse variable region.These mAbs have variable region of a murine mAb but the light and heavy chains are of human. It was possible to achieve this with help of genetic engineering which resulted in mAbs that are approximately 65% human and 35% murine. They are identified by "-ximab" suffix e.g., Infliximab, Rituximab, Abciximab. They have extended half-life and are less immunogenic when compared to murine mAbs.

Humanize mAbs : Humanized mAbs are considered as natural drugs due to their safety for in vivo activities. These are produced by grating the murine hypervariable regions of light and heavy chains onto a human antibody framework. As a result, they are approximately 95% human. They are sometimes weaker than the parent murine monoclonal antibody in term of binding with antigen. They are identified by "-zumab" suffix e.g., Trastuzumab, Bevacizumab, Alemtuzumab.

Human mAbs : Production of human mAb by conventional hybridoma techniques is bit difficult because of stress involved to maintain immortalized cell lines and human hybridoma. However, human mAbs can be produced by expression of antibody fragment or single cell variable fragment in bacteria. These are made by utilizing animals which carry human Ig genes. Human mAbs serve as an alternative to re-engineer murine mAbs with low immunogenicity. Human mAbs are less antigenic and better tolerated as compared to other class of mAbs. Also, they are present in humanbody's circulation. These are identified by"-umab" suffix e.g., Ofatumumab, Daratumumab, Denosumab.

Depending upon how they are administered and used, they are classified as : unconjugated or naked, conjugated and bispecific.

Unconjugated mAbs : These antibodies function by themselves and most commonly used in cancer. They attach to antigens in most cases. These mAbs attract immune cells and helps in recognition of cancer by immune system and leads to increased apoptosis. Other unconjugated mAbs block antigens that help in expanding and proliferation of cancer cells.

Conjugated mAbs : These are mAbs combined with a chemotherapy agent or a radioactive particle. These mAbs circulated through patient's body and delivers the chemotherapy agent or radioactive particle to the target antigen. This helps to minimize the harm to normal cells.

Bispecific mAbs : These are combination of two mAbs and this unique combination allows mAb to attach to two different antigens at same time. One target is protein on cancer cell while other is protein found on immune cell. This combination brings cancer cell and immune cell together in hopes of an increased immune response and destruction of cancer cells. [Bayer., 2019, Mahmuda et al., 2017]

It's been three decades since first monoclonal antibody was approved by USFDA in 1986, and with time changing, antibody engineering has evolved tremendously.

Antibodies available today have fewer adverse effects as their specificity has increased and therefore monoclonal antibodies have become an important part of therapies today.

From last five years, monoclonal antibodies have become the best-selling drugs in market, and in 2018, eight of top 10 were biologics. The global market of monoclonal antibodies was valued 115.2 USD in 2018 and is expected to reach 300 USD by 2025.[Lue et al., 2020]

Now-a-days, Biosimilars are gaining popularity which can be called as generic versions of original monoclonal antibody product. Biosimilars are basically the products that are "similar" to the originator or reference product and have same efficacy at a lower cost. Once the patent of originator monoclonal antibody expires, it is open for companies and allows to formulate biosimilars. These biosimilars are released into the market after a complete establishment of similarity with the originator product. The similarity is established by set of different techniques.

With increasing popularity and being an integral part of modern medicine, it becomes important for scientists to take care that these revolutionizing molecules are produced, formulated, and characterized in order to maintain their stability, safety and efficacy they offer.

1.3 Characterization of Monoclonal Antibodies

Monoclonal Antibodies have extremely complicated and dynamic structure and so it is always a challenging task to establish well-characterized monoclonal antibody. Characterization of monoclonal antibodies include determination of physico-chemical properties, biological activity, immunochemical properties, purity and impurities by appropriate techniques. [ICH Q6B]

Property	Purpose	Methods	Techniques
Physcico-chemical property	Determination of composition, physical properties, primary structure, sometimes higher order structure	Molecular weight or size, Isoform pattern, Electrophoretic patterns, Chromatographic patterns, Spectroscopic profiles	SEC, SDS-PAGE, Iso-electric focusing, Mass spectrometry,
Structural Propertie	To determine amino acic sequence and composition, sulfhydryl and disulfide bridges, terminal amino acid sequence	Chromatographic and spectroscopic patterns	Peptide mapping by LC-MS, Circular Dichroism, FTIR, NMR
Biological activity	Potency	Potency assays	ELISA, Westernblotting
Immunochemical properties	Identity, Homogeneity	Chromatographic patterns	Gel electrophoresis, iso-electric focusing, SDS-PAGE, Western blotting
Purity, impurity and contaminants	Assessment of purity, identification and study of impurities and contaminants	Chromatographic patterns	RP-HPLC, SEC-HPLC, Ion-exchange chromatography, affinity chromatography
Quantity	Measurement of protein content	Spectroscopic profiles	Protein assays, UV absorption, ELISA, Nitrogen determination

Table 1.1 Characterization of Monoclonal Antibodies

1.4 Analytical Techniques for Characterization of Monoclonal Antibodies

With the advancement in high-throughput technologies, the discipline of analytical methodologies has seen a surge in the growth and its scope. From being low-sensitive, consuming time and high amounts of samples and reagents, analytical techniques have now evolved to become automated, having high selectivity, lower and cost-effective.

Significant developments in field of medicine have led scientist to understand monoclonal antibodies and their complexities related to them and as result, development of automated, real-time, reliable, reproducible and cost-effective advanced analytical technologies took place. With the use of such technologies, the monitoring and detection of antibodies has become accurate and more efficient. [Kaur et al., 2019]

Monoclonal antibodies-based therapeutics are playing and important part in treatment or prevention of many diseases. Monoclonal antibodies are far more complex than the small molecules because of their structures, heterogeneities, stability. The criticalattributes associated with them such as the structure, post-translational modifications, functions at biomolecular and cellular levels requires, purities, impurities and stability need to be defined and profiled in details during their development. [Wang et al., 2018]

The characterization of monoclonal antibodies is lengthy and complicated procedure which depends on the intended use of antibody. Characterization always goes in parallel with the development of monoclonal antibody.

Below are some of the important analytical techniques characterization used at different stages of monoclonal antibodies development :

Technique	Principle	Use
Reversed-phase HPLC	Separation of proteins based on affinity towards polar mobile phase	For purity, impurities identification and analysis
Size exclusion HPLC	Separation of proteins on basis of their hydrodynamic radius	Molecular weight determination, Identification and determination of aggregation
Ion-exchange chromatography	Based on reversible exchange of ions between protein and separation matrix	Study ofcharge heterogeneities
Gel Electrophoresis	Separation based on molecular weight in polyacrylamide gel	Molecular weight determination
Capillary Iso-electric focusing	Separation based on iso-electric point of protein, performed in capillary	Iso-electric point determination, Identification of protein
CE-SDS	Separation of proteins on basis of their hydrodynamic radius, performed in capillar	Molecular weight determination, Identification
FTIR	Based on vibrations inside the protein backbone	For structural determinations
Circular Dichroism	Based on the absorption of plane polarized light by protein molecule	Determination of secondary structure, information about α and β chains
NMR	Based on spinning of nuclei by magnetic radiation	For structural determinations
ELISA	Based on interaction of protein antibody with antigens	For identification of protein antibody
Western blotting	Based on interaction of protein antibody wi specific antigens, it is a step after SDS-PAGE	For confirmation of protein antibody
Surface Plasmon Resonance	Based on interaction of proteins antibody ar receptors on metal surface	To study binding of anti-body, affinity and kinetics of binding

Table 1.2 Analytical techniques for characterization of mAbs

1.5 Reverse Phase-High Pressure Liquid Chromatography

Characterization of monoclonal antibodies-based therapeutics using high quality of analytical methods is very important to ensure maximum characterization in less amount of time. Among different analytical techniques available, Reversed-phase high performance liquid chromatography is among the essential methods to characterize the intact proteins of monoclonal antibodies which allows faster analysis with high resolution.[Sousa et al., 2017] Reversed-phase liquid chromatography (RP-HPLC) is widely used technique to analyze intact, reduced, and subunits of mAb proteins.[Wang et al., 2020] Reversed-phase liquid chromatography is an important tool for intact protein analysis because of its ability to separate species on minor structural differences and when coupled with specific detectors, it can also provide quantitative information about main component,variants and cleaved compounds.[Le & Bondarenko., 2005] Separation using reversed-phase liquid chromatography can be achieved in minutes, especially with automated systems and therefore this technique is also employed for process control. [Flatman et al., 2007]

1.5.1 Principle involved in Reverse-phase Chromatography

Reversed-phase liquid chromatography involves separation based on the hydrophobicity of a protein molecules. In RP-HPLC, stationary phase is a hydrophobic silica gel or a synthetic polymer and bears the hydrophobic ligands mainly C4, C8 or C18 alkyl chains which is non-polar in nature, while the mobile phase used are water and water-miscible organic solvents such as acetonitrile, methanol, iso-propanol which are polar in nature. Sometimes acids like formic, acetic, trifluoroacetic acid are added to retain the proteins positively charged and to reduce interactions with stationary phase. [Josic & Kovac., 2010]

In reversed-phase chromatographic separation, the hydrocarbon groups are chemically attached to surface of stationary phase which makes it very hydrophobic. Monoclonal antibodies are adsorbed by the face to the surface, which is termed as “hydrophobic foot”. Monoclonal antibodies are large as compared to thickness of hydrophobic surface and so only a part of mAb gets adsorbed onto the hydrophobic surface and much of the mAb remains in contact with the mobile phase. The interaction between the surface and mAb is strong enough to keep mAb adsorbed to it until a specific concentration of organic solvent is obtained. As soon as, a specific concentration is available, which is favorable to mAb, it starts to desorbs

from the hydrophobic surface of stationary phase and separation is achieved by single adsorption/desorption process.

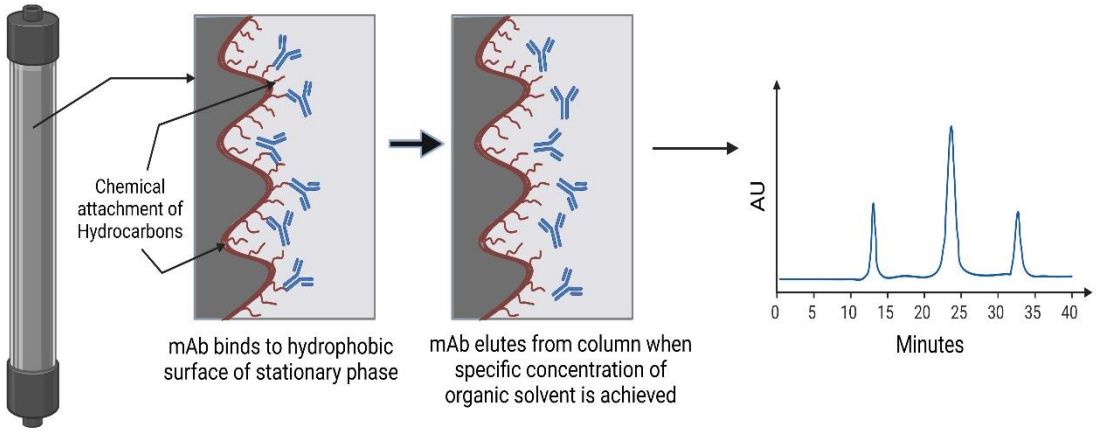

Figure 1.3 : Principle involved in RP-HPLC

The retention behavior of mAb is different than that of small molecules. Small molecules change retention slowly with change in organic solvent while the retention of mAb is abruptly affected once the required concentration of organic solvent is reached which results in rapid change of retention and mAbs have a sharp peak. This change in retention of mAbs with small changes in concentration of organic solvent shows that isocratic elution is not very useful for mAbs because peaks may become broad and small changes in concentration of organic solvent leads to changes in mAb retention.

Reversed-phase HPLC can be used a general purity test for monoclonal antibodies. The impurities will include proteins i.e., product related fragments and also non-proteins derived from manufacturing process i.e., chemical leachates and extractables.[Josic & Kovac., 2010]

1.5.2 Key consideration for method development of RP-HPLC

Monoclonal antibodies being complex and hydrophobic may have low recovery on silica based stationary phase making use of RP-HPLC limited to mAb characterization and quantification. Therefore, a proper RP-HPLC method is required to be developed and some of the important parameters are to be considered. First, a gradient mode is required because small changes in strength of solvent affects the mAb retention. Second, columns with wide pore i.e., 300Å-1000Å is should be used due to high molecular weight of mAbs. Third, the secondary interaction

between mAb and stationary phase leads peak tailing and broadening, to prevent this, ion pairing agents should be used. [Sousa et al., 2017]

Below are listed some of the key components of Reversed-phase chromatographic system :

1. Silica purity : The separation performance in RP-HPLC may be affected by purity of silica used. A lower resolution and tailing of peak may be caused by metal impurities and so ion pairing agents are used in high amount to maintain a good peak. Silica with high purity may require ion pairing agents in concentration of as low as 0.005% for a good peak.

2. Pore diameter : Small pore (100Å) silica results gives improper separations of proteins. Wide pore silica (300Å or more) gives a much better separation. Small pores do not allow protein to enter the surface, while wide pore silica allows mAb to interact with the surface which results in good separation and obtaining a good peak.

3. Separation surface : Various organic groups are used to modify the silica surface according to proteins. The attachment of linear, aliphatic eighteen carbon chain is common giving a C18 or Octa-Decyl silica column. Particularly for separation of peptides less than 2-3000 Da, C18 column is used whereas, attachment of butyl group to silica surface (C4) is suitable for protein separations.

4. Column Selection :

Column Length : More interaction between mAb molecules and hydrophobic surface of stationary phase give a better resolution. Generally, long columns give high resolution than shorter columns. mAb molecules however adsorb near top of the column and once desorbed, do not react with the surface.Fifteen- or twenty-five-centimeters columns are recommended for separation.

Column diameter : The standard diameter of HPLC columns is 4.6mm and run best at flow rates of 1ml/min. Smaller bore columns are also available and are used for some specific purpose. Narrow bore columns having internal diameter 2mm are also available and run at flow rate of 200 microliters/min and thus less solvent is used. But when using narrow bore column, the performance requirements for HPLC solvent delivering systems become more stringent as low flow rates are used.

5. Mobile Phase : The mobile phase for proteins generally contains a buffer component, an organic modifier and often an ion pairing agent for selectivity. All of them must be free from any impurities, since any contaminants may lead to unwanted extra peaks, ghost peaks and contaminate the proteins.

Organic solvent : The commonly used organic solvents for RP-HPLC are acetonitrile, propanol, methanol, ethanol and water. Two solvents with different polarity may be mixed resulting in a solvent with intermediate polarity those of original solvents. Lower the polarity of solvent mixture, higher is the eluting power in reversed-phase chromatography. The properties of some solvents are listed below :

Acetonitrile and methanol are used mostly because of their low viscosity and UV transparency. Propan-2-ol has low polarity and has higher eluting strength, however its use results in viscous mobile phase but it is excellent for cleaning of columns. Ethanol is used for large scale process purification of mAbs due its low toxicity.

pH : Reversed phase separation are usually performed at low pH values between 2-4. It results in good solubility of sample and ion suppression. Mobile phase having ammonium acetate or phosphate salts are used for pH closer to neutral. Basic proteins often tail during elution at low pH so for basic compounds, better resolution is achieved above 8 pH.

Ion pairing agent : Ion pairing agents are believed to bind to solute molecules by ionic interactions to increase their hydrophobicity and change selectivity. Ion pairing agents prevents reaction between protein molecule and stationary phase and prevent peak tailing. Trifluoroacetic acid, hepta-butyric acid, pentafluoropropionic acid, ammonium acetate, phosphoric acid, formic acid are some of the ion pairing agents used. Ion pairing agents such as trifluoroacetic acid also acts to maintain pH of the mobile phase. Ion pairing agents are used in concentration ranging from 0.01% - 0.1% or between 10 – 100 mM.

1.6 Size-Exclusion High Pressure Liquid Chromatography

Size exclusion chromatography is a historical technique used for detailed characterization of monoclonal antibodies. It is considered as a standard technique for qualitative and quantitative analysis of monoclonal antibodies. Size exclusion chromatography is also known as gel filtration chromatography and is used for separation of mAbs and other biomacromolecules. Size exclusion chromatography is widely used to monitor the level of higher molecular weight species such as aggregates and is used as a quality control technique for monoclonal antibodies. [Fekete et al., 2010]

1.6.1 Principle involved in Size Exclusion Chromatography

Unlike other chromatographic techniques, size exclusion does not include adsorption as separation mechanism. Size exclusion chromatography involves separation of protein analytes according to their hydrodynamic radius. Separation can be seen as partitioning into the liquid volumes.[Cutler., 2004] Size exclusion chromatography separates molecules on basis of their hydrodynamic radius. It includes partition of molecules between interstitial volume of particles of column and particle pore volume. Separation of particle is dependent upon ability to permeate the pore of particles. Larger hydrodynamic radii molecules elute earlier. As partitioning increases, it slows the velocity of particle to move through the column and so molecules with small hydrodynamic radius elute late. [Farnan et al., 2009]

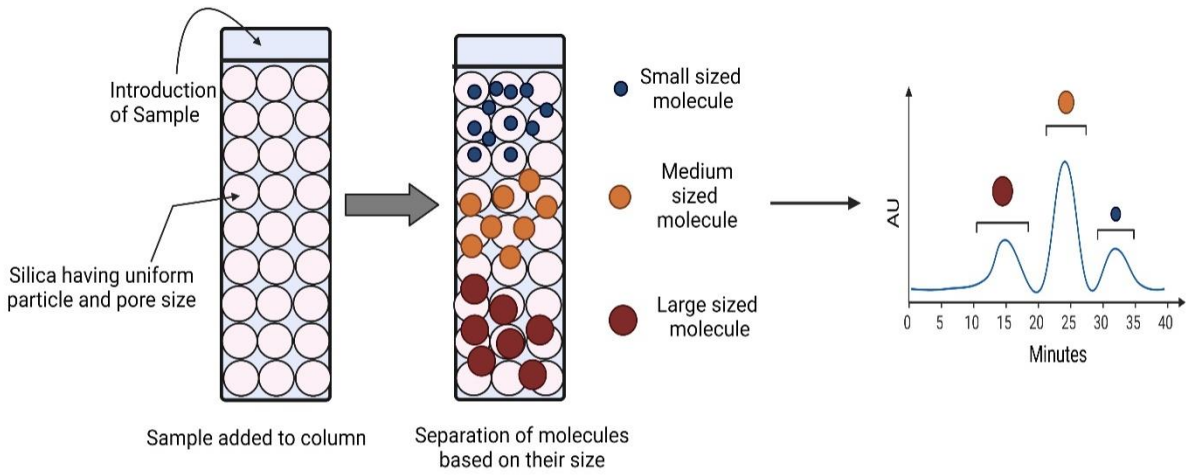

Figure 1.4 : Principle involved in SEC-HPLC

The solid phase matrix of stationary phase contains porous beads which have controlled pore size, through which proteins diffuse on basis of their molecular size. The mobile phase flowing has access to volumes both inside the pores and outside the beads. The proteins being dissolved in mobile phase can interact with the beads and separation is achieved. [Fekete et al., 2010]

1.6.2 Theory of Size exclusion chromatography

Size exclusion chromatography is an entropy-controlled process without any adsorption and thus Gibbs free-energy equation becomes

$$n\ K_D = -\Delta S_0 / R$$

Where $K_D$is thermodynamic retention factor, ΔS_0 is change in system entropy and R is gas constant. In SEC, the thermodynamic factor is fraction of intraparticle pore volume that is available to analyte.[Hong et al., 2012] An analogous retention factor k* was suggested by Engelhardt as ratio of probability of sample staying in mobile phase inside the pores and in mobile phase moving through the particle surface.[Engelhardt., 1983] This k* is limited and its maximum value is given by ratio of pore volume (V_p) and the interstitial volume (V_z) of a column or by pore porosity (ε_p) and the interstitial porosity (ε_z):

$$k^* = V_{elu} – V_z / V_z$$

$$k^*_{max} = V_p / V_z = \varepsilon_p / \varepsilon_z$$

Therefore, in SEC, retention range can be determined by ratio of pore porosity and interstitial porosity. These parameters may get alter by change in column packing or by using larger pore volumes. [Fekete et al., 2013]

1.6.3 Aggregation of Monoclonal antibodies

Monoclonal antibodies being complex in their protein structure, are generally susceptible to degradation than small molecules. Several efforts are taken to retain the stability of mAbs, but their degradation cannot be fully prevented. One such common way of degradation is aggregation of proteins of monoclonal antibodies.[Kijanka et al., 2020] Protein aggregation simply means association of protein molecules. The protein molecules come together and form a cluster is called an aggregate. [Bond et al., 2010] This formation of protein aggregate

can be induced by various factors such as light, pH, UV exposure, agitation, elevated temperatures

etc. Aggregates may vary in sizes and may affect the stability and biological function of monoclonal antibodies differently. [Kijanka et al., 2020] Major implication of aggregation could be loss of efficacy and/or appearance of effects such as immunological reactions.[Lahlou et al., 2009] Antibodies may aggregate by various interactions such as Vander Waals force, hydrophobic interactions, disulphide linkages, hydrogen bonds etc.[Ma et al., 2020]

1.6.4 Mechanism of mAb aggregation

Proteins can aggregate through different mechanism or pathway which can roughly be explained by following figure:

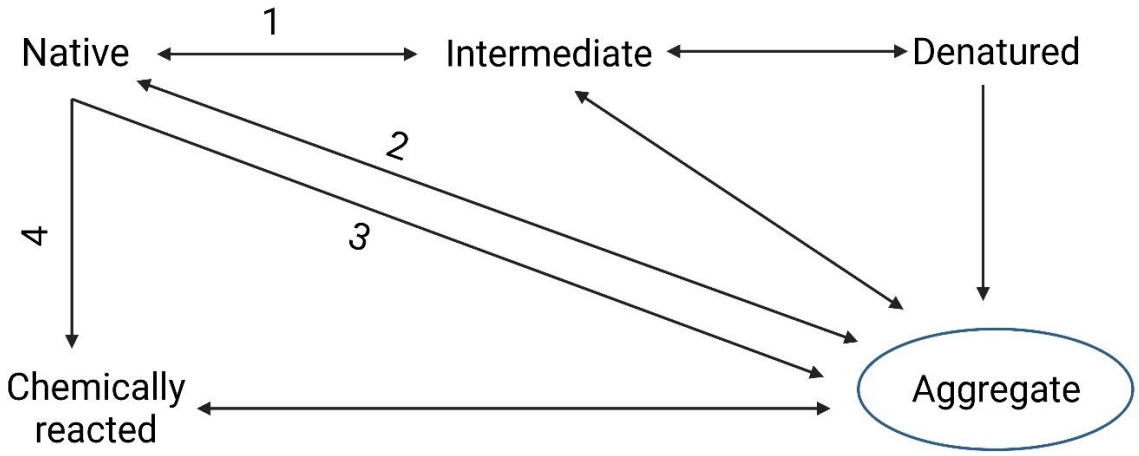

Figure 1.5 : Pathways for mAb aggregation

1.6.4.1 Aggregation through intermediates: A mAb solution under normal conditions is in equilibrium with the small amounts of unfolded intermediates which are in equilibrium with completely unfolded mAbs as in above figure. Depending on degree of unfolding, intermediate may be divided into two categories: intermediates of native protein and intermediates of unfolded protein. The folding/unfolding intermediates have more exposed hydrophobic patches and so they become the precursors of mAb aggregation. Interaction of these intermediates leads to mAb aggregation. The initial mAb aggregate may be soluble but gradually become insoluble by exceeding the limit of size and solubility.[Wang et al., 2010]

1.6.4.2 Aggregation through self-association

Monoclonal antibodies can directly associate and form aggregates without any intermediate. Such aggregates formation can be because of electrostatic or both electrostatic or hydrophobic

interactions between the mAb molecules. Other forces such as Van Der Waals forces may also influence the aggregation. Self-association of proteins may or may not lead to significant conformational changes and often causes reversible aggregation. [Wang et al., 2010, Weis et al., 2009]

The tendency of self-association of mAbs can be determined by osmotic second virial co-efficient. A positive B_{22}valueindicates repulsion whereas negative B_{22}indicates attraction between mAb molecules. The B_{22}values can significantly depend on pH and ionic strength of protein solution. It can be also possible for proteins with high and positive $B_{22}$to aggregate, if the measured B_{22}value is not true reflection of monomer form. [Alford et al., 2008]

1.6.4.3 Aggregation through direct chemical linkages

Crosslink of proteins by various chemical bonds can cause aggregation. Formation of inter-molecular disulfide bonds is most common chemical linkage and it can further influence the mAb aggregation.[Cabre et al., 2008] Other than non-disulfide, cross linking due to formaldehyde, dityrosine formation, oxidation and maillard type reactions are also reported. [Wang et al., 2010]

1.6.4.4 Aggregation through chemical degradation

The increased tendency of mAbs to aggregate by chemical degradation have been reported by many studies. These include oxidation [Rosenfeld et al., 2009], dimerization [Roostaee et al., 2009], deamidation [Takata et al., 2008], hydrolysis [Van Buren et al., 2009], and glycation [Wei et al., 2009]. Chemical degradations often affect mAbs in terms of hydrophobicity, association tendency, structures, and thermodynamic barrier to protein unfolding and therefore it is important to analyse the aggregates and determine true cause of it.[Wang et al., 2010

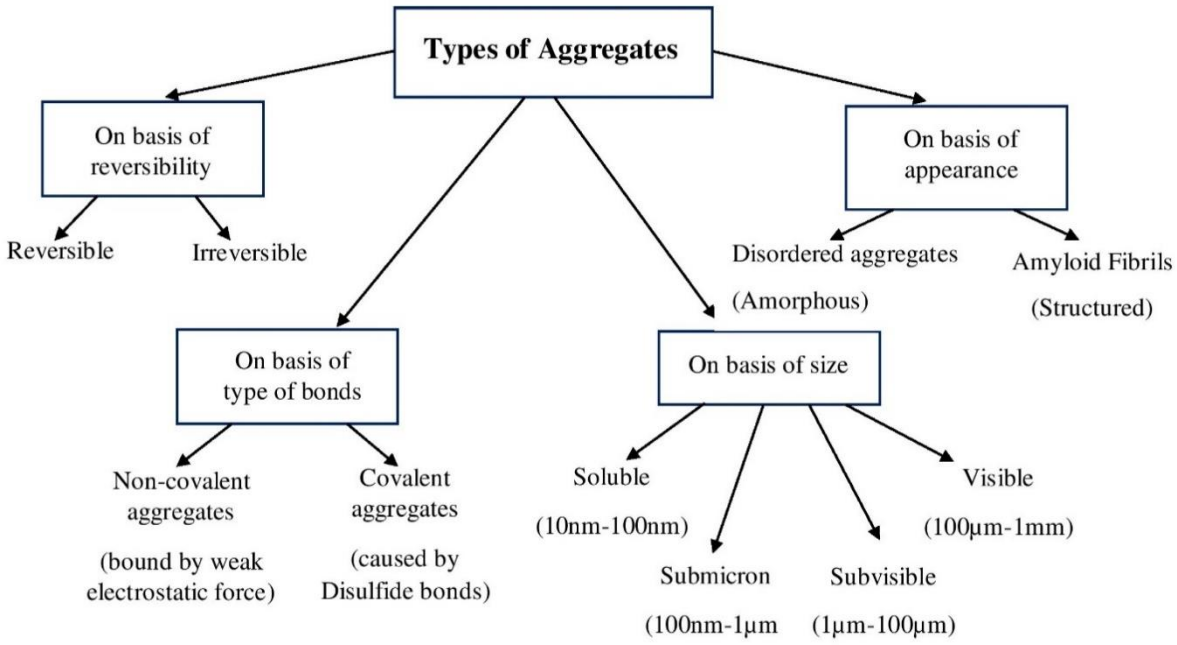

Figure 1.6 : Types of Aggregates

1.6.5 Factors influencing aggregation of mAbs

Various factors such as temperature, protein concentration, pH, mechanical stress including shaking, freezing may induce aggregation. Partially unfolded proteins are also part of mAb molecules in their native state, so aggregation can also occur under non-stress condition. [Mahler et al., 2009]

1.6.5.1 Effect of processing steps

1.6.5.1.1 Fermentation/expression: Fermentation or cell culturing is the first step for large scale mAb production. A mAbs are expressed in various cell systems under processing conditions where they can aggregate and form inclusion bodies during expression in bacterial systems. [Espargaro et al., 2008] Protein aggregation during fermentation/expression can be inhibited by controlling conditions such as fermentation temperature [Hau et al., 2007], use of surfactants or other additives [Bahrami et al., 2009]

1.6.5.1.2 Purification: Purification is the step wherein residues of host cell proteins and contaminants are removed. This purification process can induce aggregation if conditions used are too harsh. Protein-A affinity chromatography is an efficient method used for

purification of protein and requires low pH. A study [Shukla et al., 2007] reported that low pH may induce

aggregation. A study [Heavner et al., 2007] reported that due to unfavourable solution conditions, protein aggregation increased from 1.4% to 3.6%. Aggregation can be controlled by adjusting purification conditions and by use of proper additives. [Cromwell et al., 2006]

1.6.5.1.3 Freezing and Thawing: Freezing is often required for stability of proteins. The freezing may cause aggregation depending upon type, concentration of protein and presence of other additives. Aggregation induced by freezing can occur due to low temperatures, solute concentration, formation of ice-water interface, pH changes and phase separation. Freezing affects the size of ice crystals and surface area of water-ice interface and influences crystallization of other components. Freezing induced pH change is major cause of mAb aggregation while other include configuration of containers and pH before freeze/thaw. Use of appropriate additives can help minimize this problem. Among the additives, surfactants are seen to be most effective. [Wang et al., 2010]

1.6.5.1.4 Shaking and Shearing: Shaking and shearing are mostly encountered during production and transportation. Shaking and shearing can initiate aggregation by creating air-water interfaces. The impact of shaking and shearing depends upon the extent and duration of exposure. Additives such as surfactants are seen to be effective in reducing the aggregation by shaking and shearing by competing with the protein for hydrophobic surfaces by binding directly to protein or by increasing the viscosity of protein solution. [Wang et al., 2010]

1.6.5.1.5 Drying: Drying methods are used for preparation of solid products, including vacuum drying, freeze-drying, spray drying and all of these can potentially lead to aggregation at different extent, as they remove hydration layer of protein causing to disrupt its native state.[Wang et al., 2010]

1.6.5.2 Effect of pH

Solution conditions play critical role in controlling aggregation of mAbs, Among these, the pH of solution is the important one. The pH of solution determines type and distribution of charges on surface of mAbs which has effect on both intermolecular folding and intramolecular interactions. For appropriate pH required for mAb, it is important to understand zeta potential. If the outer charge on mAb is high, it can possibly avoid aggregation. Change in pH may cause change in net charge of mAb and is zero at iso-electric point.

The pH of solution can affect mAb through one or more mechanism:

a) Neutral pH may favour hydrophobic interactions which leads to aggregation

b) At pH above pI, acidic functional group get ionized increasing their solubility whereas at pH lower than pI, protonation of basic functional group occurs

c) pH change may lead to chemical degradation

The total charge on any mAb depends upon the pH so an appropriate pH range should be determined. Monoclonal antibodies are resistant to aggregation over a narrow pH range and so if mAb is placed in a solution where pH is not maintained may lead to aggregation and that is why addition of buffers is done to resist the pH change.

1.6.5.3 Effect of temperature

Temperature increase may accelerate chemical reactions like oxidation and deamidation which may lead to aggregation of mAb. Thermal stability of mAb can be determined by its melting temperature (T_m) at which 50% of molecule is unfolded. Melting temperatures of mAb usually lie in between 40-80°C. Monoclonal antibodies are usually stored well below their $T_{m,}$ at 2-8°C. [Mahler et al., 2009]

1.6.5.4 Effect of protein concentration

The increase in protein concentration has been reported to induce mAb aggregation for many proteins. At high protein concentration, overcrowding of macromolecules occur, and thus aggregation may take place. Increase in protein concentration has also shown to increase the size of aggregates formed. On the other hand, reducing protein concentration by dilution has been shown to affect aggregation as aggregate form due to weak reversible interactions.

1.6.5.5 Effect of Light and irradiation

Monoclonal antibodies may be sensitive to light/UV exposure. Light/UV exposure has been observed to promote aggregation by inducing unfolding mAb molecules [Redecke et al., 2009] or by inducing photo cross-linking reactions [Qi et al., 2009]. A study [Davies., 2003] reported that UV radiation can influence cross-linking reaction between Cysteine and Tyrosine and causing photolysis of native disulfide bonds to form new disulfide bonds. Therefore, protection from light is necessary for mAbs.

Size exclusion chromatography is widely employed as a standard technique for study aggregation. It is also a useful tool in identification and quantification of protein dimers, trimers and oligomers which could help predict aggregation and its prevention. The use of mild conditions offers an advantage for characterization of protein molecules because of minimal effect on protein structure and the local environment.

1.6.6 Key consideration for method development in SEC-HPLC

Size exclusion chromatography has proved its advantages over techniques like SDS-PAGE, analytical ultracentrifugation and has created its own popularity for characterization and monitoring of attributes of monoclonal antibodies. This high level of separation, identification and quantification by size exclusion requires some components to be considered and optimised to provide required results

Some of the key components are listed below:

1. Column: Size exclusion is based on continuous partitioning of mAb molecules and hence resolution to be obtained is directly related to column length. Preparative columns are long and thin ranging from 70-100 cm. Analytical columns are shorter than preparative and have shorter run rime with excellent resolution. Most of the SEC columns are of 30 cm and provide optimum resolution. Increasing the inner diameter of SEC columns can improve peak capacity and resolution. Column having internal diameter of 7.5mm-7.8mm are used to maximize peak capacity.

2. Matrix for separation: The beads used should have controlled pore size having high physical and chemical stability. They should be inert and minimize any chemical interaction between solutes and matrix itself. Earlier, starch-based gels were used as separation matrix but now they are substituted by cross-linked dextran gels e.g., Sephadex. Polystyrene-based matrix is used for separation when nonaqueous solution are used. For low molecular weight species, polyacrylamide gels such as superdex gels are suited, in which dextran chain are bonded to crosslinked agarose for fast size exclusion.

3. Particle size: Separation in size exclusion chromatography is greatly affected by particle size. Smaller particles size has been seen to be advantageous over larger particle size. Various studies have shown that smaller particle size offer improved resolution and high

efficiency. The particle size of 1-2µm are believed to be optimum particle size for SEC separation.

4. Mobile phase: The selection of mobile phase mostly depends on protein. Organic solvents such as acetonitrile may dissociate the protein and may cause partial protein unfolding when used in high concentrations. While salts may enhance the hydrophobic association but they also inhibit electrostatic association. SEC has been found to be compatible with the aqueous buffers. Buffers help to minimize the secondary interaction between the protein and stationary phase. Using low pH buffer is to be avoided as it may result in breakdown of protein.

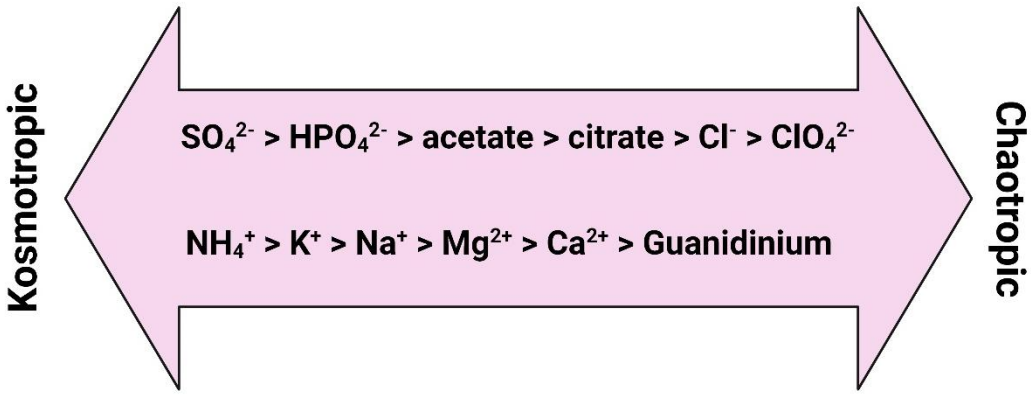

Figure 1.7 : Hoffmeister series

Mobile phase buffers used in SEC-HPLC can be selected on basis of Hoffmeister series. Mobile phase buffers like K+, Na+, which are kosmotropic in nature encourage hydrophobic interactions and are used commonly. Phosphate buffer is most commonly used mobile phase for SEC at pH 7.4 because it tends to stabilize the proteins. [Ma et al., 2020, Hu et al., 2020]

1.7 Analytical Method Development

Analysis of any drug product in pharmaceutical industry is vital because it involves life. Methods used for analysis of drug ensure its identity, purity, potency and performance. A HPLC method should be able to separate, detect and quantify drugs and drug related impurities, degradants that may be introduced during synthesis, manufacturing and storage and therefore, a proper method development is required to get consistent, realistic and correct information.[Sabir et al., 2013] A method should be used in environment where GMP and GLP are followed and must be developed using protocols and acceptance criteria stated in ICH Q2 R1 guideline. The primary requirements for method development are as follows:

1. Qualified and calibrated instrument
2. Documented methods
3. Reliable reference standards
4. Qualified analysts
5. Sample selection
6. Change control

In development of analytical procedure, the choice of method should be based on its intended purpose and its scope. A method development is based on combination of understanding on basics of method and prior experience. Experimental data from early procedures can be used as a guide for further development.

The steps for method development are as below:

1. Characterization of standard and analyte
2. Establishing method requirements
3. Literature search
4. Selecting the method
5. Instrumentation setup and initial studies
6. Optimization of method parameters

7. Documentation

8. Evaluation of developed method with sample

9. Determination of percent recovery of sample

10. Demonstration of quantitative sample analysis

A method development details the steps and techniques necessary to perform an analysis which may include: preparation of samples, standards and reagents, use of apparatus, generation of calibration curve, use of formulas for calculation etc.[Chauhan et al., 2015]

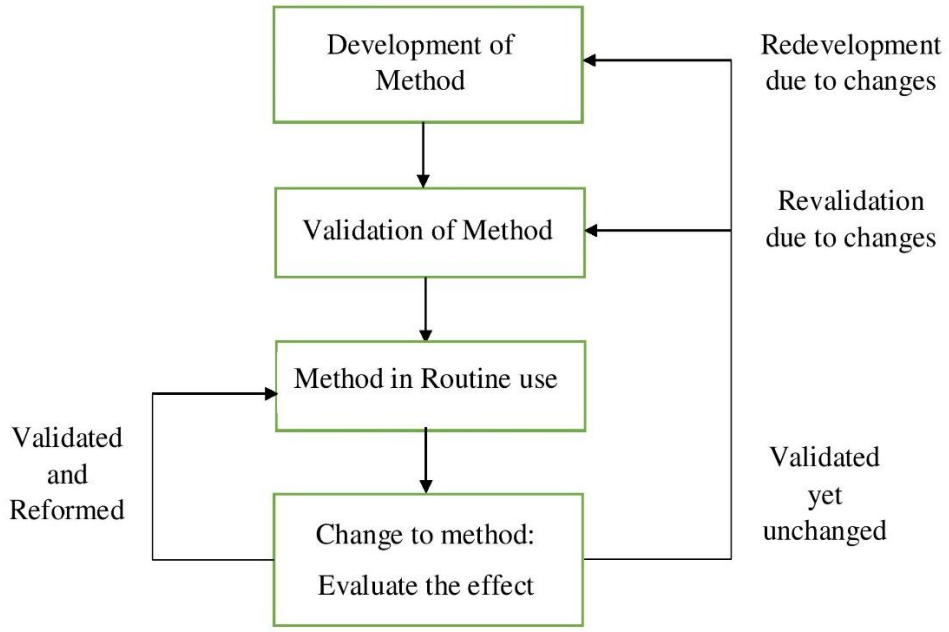

Figure 1.8 : Life-cycle of a method

1.8 Analytical Method Validation

Validation of method is one of the key components of quality assurance. Validation assures that a developed method is suitable for its intended use. A validation is usually required to be done:

1. When a new method is developed

2. When new equipment is used

3. When procedure and equipment have been adjusted to altered needs

A method validation is process of establishing a documented evidence, providing high degree of assurance that a developed method will meet the pre-determined attributes and provide results as expected. The extent of validation is determined by the type of analytical technique. The most common methods for validation are identification, assay and determination of impurities. [Chauhan et al., 2015]

The validation parameters as per ICH guideline are as below:

1.8.1. Accuracy

Accuracy can be defined as closeness of agreement between a value which is accepted as true value or reference value and a value which is found. It is also called as trueness. It is determined by applying method to samples to which known amount of analyte is added and analysed against a standard.[Sabir et al., 2013]. It can be determines using 9 determination of minimum 3 concentrations over a specified range

1.8.2. Precision

Precision can be defined as closeness of agreement between values obtained from multiple samplings from a uniform sample under a prescribed condition. It is done at three levels:

a) Repeatability: Repeatability is the exactness below same operating conditions but over a brief interval of time. It is also known as intra assay precision.

b) Intermediate precision: It expresses exactness in research laboratories by different analyst, different instrument in distinct days.

c) Reproducibility: It refers to precision between research laboratories.

1.8.3. Linearity

The linearity of an analytical method is its ability to produce results that are directly proportional to the concentration of analyte. For linearity, minimum five concentrations are recommended and should be evaluated calculation of regression co-efficient.[Chauhan et al., 2015]

1.8.4. Range

The range of an analytical method is the interval between upper and lower concentration of analyte for which is found to be accurate, precise and linear.[Chauhan et al., 2015]

1.8.5. Limit of detection

Limit of detection of an analytical method is the lowest amount of analyte that can be detected but no necessarily quantified. A signal to noise ratio of 3 is considered for determining limit of detection.

1.8.6. Limit of Quantification

Limit of quantification of an analytical method is the concentration of analyte that can be detected and quantified accurately and precisely. A signal to noise ratio of 10 is considered for determining limit of quantification.

1.8.7. Specificity

Specificity of an analytical method is ability to assess the analyte of interest in presence of other components that are expected to be present. Specificity measures only the analyte without any interference that might be present and not necessarily separated.[Sabir et al., 2013]

1.8.8. Robustness

Robustness can be defined as ability of an analytical method to remain unaffected with small changes in method conditions like pH, temperature, mobile phase and instrumental settings .[Sabir et al., 2013]

CHAPTER 2

AIM & OBJECTIVE

Aim:

To perform analysis of monoclonal antibody (SUNmAb) by specified analytical techniques.

Objective:

To develop and validate Reversed-phase HPLC method for purity analysis of monoclonal antibody, SUNmAb.

To develop and validate Size-exclusion HPLC method for aggregate analysis of monoclonal antibody, SUNmAb.

CHAPTER 3

EXPERIMENTAL WORK

3.1 Instrument/Equipment

Sr. No.	Name	Make	Model
1.	HPLC	Waters	Alliance e2695
2.	pH meter	Thermo scientific	Orion 4 star
3.	Weighing balance	Sartorius	BT 224 S
4.	Spinwin	Tarson	MC02

Table 3.1: List of Instruments/Equipment

3.2 Chemicals/Reagents

Sr. No.	Components	Make
1.	Potassium Dihydrogen Phosphate	Merck
2.	Potassium Chloride	Merck
3.	Potassium Hydroxide	Merck
4.	Sodium Dihydrogen Phosphate monohydrate	Merck
6.	Sodium Chloride	Fisher Chemical
7.	Sodium Hydroxide	MP Biomedicals
8.	Acetonitrile	Merck
9.	Methanol	Merck
10.	1-Propanol	Fischer
11.	Trifluoroacetic acid	Sigma
12.	Formic acid	Sigma

Table 3.2: List of chemicals/reagents

3.3 Consumables

Sr. No.	Component	Make
1.	BiosepSEC s2000, 5µm, 150Å (300 x 7.8mm)	Phenomenex
2.	TSKgeI G2000 SWxL 5µm, 125Å (300 x 7.8mm)	TOSOH
3.	0.22 µm Filter (PES)	MILLIPORE
4.	Zorbax 3.5µm (150 x 4.6mm)	Agilent
5.	HPLC vials	Waters

Table 3.3: List of Consumables

3.4 Reagents and Solutions for SEC-HPLC

3.4.1 10 NNaOH

40 g of NaOH pellets were weighed and dissolved in 60 mL of WFI. Volume was made up to 100 mL with WFI. The solution can be store at room temperature for furtheruse.

3.4.2 10 NKOH

56.11 g of KOH pellets were weighed and dissolved in 60 mL of WFI. Volume was made up to 100mLwithWFI.Thesolutioncanbestoreatroomtemperatureforfurtheruse.

3.4.3 Preparation of Mobile Phase 1 (0.2 M Potassium Dihydrogen Phosphate+ 0.25 M Potassium Chloride, pH 6.20), 2Litre

54.436 g of Potassium Dihydrogen Phosphate & 37.275 g of Potassium Chloride dissolved in 1900 ml WFI and the pH was adjusted to 6.2 using potassium hydroxide and final volume was made up to 2000 mL and filtered through 0.45 µm membrane filter and degas prior to use. The solution can be used upto 15 days if stored at 2-8 °C.

3.4.4 Preparation of Mobile Phase 2 (0.2 M Sodium Dihydrogen Phosphate Monohydrate +0.25 M Sodium Chloride, pH 6.20), 2 Litre

55.196 g of Sodium Dihydrogen Phosphate Monohydrate & 29.22 g of Sodium Chloride dissolved. in 1900 ml WFI and the pH was adjusted to 6.2 using Sodium hydroxide and final

volume was made up to 2000 mL and filtered through 0.45 µm membrane filter and degas prior to use. The solution can be used up to15 days if stored at 2-8 °C.

3.5 Method Development for SEC-HPLC

3.5.1 Method Development Experiment – 1 (Flow rate selection)

In this experiment, different flow rates were evaluated for aggregate analysis of SUNmAb using TSKgel G2000SWxl HPLC column.

3.5.1.1 Experimental details

Two different flow rates as mention below were evaluated using Mobile Phase-1 on TSKgel G2000SWxl HPLC column.

Flow rate 1: 0.5mL/min

Flow rate 2: 0.75mL/min

3.5.1.2 Preparation of Mobile Phase 1

As per section 3.4.3

3.5.1.3 Preparation of Sample

SUNmAb was diluted to 1mg/mL using mobile phase.

3.5.1.4 Chromatographic system setup

Column	TSKgeI G2000 SWxL 5µm, 125Å (300 x 7.8mm, TOSHOH)
Pump mode	Isocratic
Flow rate	0.5mL/min and 0.75mL/min
Column temperature	25°C ± 5°C
Sample temperature	6°C ± 2°C
Injection volume	25µL
UV wavelength	215nm
Method run time	45 minutes

Table 3.4: Chromatographic system setup for experiment 1

3.5.2 Method Development Experiment – 2 (Column selection)

In this experiment, different columns were evaluated for aggregate analysis of SUNmAb.

3.5.2.1 Experimental details

Two different columns as mention below were evaluated using Mobile Phase-1.

Column1 : TSKgeI G2000 SWxL 5µm, 125Å (300 x 7.8mm)

Column 2: BiosepSEC s2000, 5µm, 150Å (300 x 7.8mm)

3.5.2.2 Preparation of Mobile Phase 1

As per section 3.4.3

3.5.2.3 Preparation of Sample

SUNmAb was diluted to 1mg/mL using mobile phase.

3.5.2.4 Chromatographic system setup

Column 1	TSKgeI G2000 SWxL 5µm, 125Å (300 x 7.8mm, TOSOH)
Column 2	BiosepSEC s2000, 5µm, 150Å (300 x 7.8mm, Phenomenex)
Pump mode	Isocratic
Flow rate	0.5mL/min
Column temperature	25°C ± 5°C
Sample temperature	6°C ± 2°C
Injection volume	25µL
UV wavelength	215nm
Method run time	45 minutes

Table 3.5: Chromatographic system setup for experiment 2

3.5.3 Method Development Experiment – 3 (Mobile phase selection)

In this experiment, different mobile phases were evaluated for aggregate analysis of SUNmAb using BiosepSEC s2000HPLC column.

3.5.3.1 Experimental details

Two different mobile phases as mention below were evaluated using BiosepSEC s2000HPLC column.

Mobile phase 1: 0.2 M Potassium Dihydrogen Phosphate+ 0.25 M Potassium Chloride, pH 6.20

Mobile phase 2: 0.2 M Sodium Dihydrogen Phosphate Monohydrate +0.25 M Sodium Chloride, pH 6.20

3.5.3.2 Preparation of Mobile Phase

As per section 3.4.3 and 3.4.4

3.5.3.3 Preparation of Sample

SUNmAb was diluted to 1mg/mL using mobile phase and incubated at 65°C for 1 hour.

3.5.3.4 Chromatographic system setup

Column	BiosepSEC s2000, 5µm, 150Å (300 x 7.8mm, Phenomenex)
Pump mode	Isocratic
Flow rate	0.5mL/min
Column temperature	25°C ± 5°C
Sample temperature	6°C ± 2°C
Injection volume	25µL
UV wavelength	215nm
Method run time	45 minutes

Table 3.6: Chromatographic system setup for experiment 3

3.6 Method Validation of SEC-HPLC

3.6.1 Specificity

The specificity of method was demonstrated by considering the retention time of SUNmAb and comparing it with blank.

3.6.1.1 Blank solution: Mobile phase was used as a blank solution.

3.6.1.2 Sample preparation: SUNmAb was diluted to 1mg/mL using mobile phase and analysed.

3.6.2 Linearity and Range

Linearity of method was determined by diluting SUNmAb in a concentration range from 2.5µg/mL to 1000 µg/mL using mobile phase (individual sample at each concentration) and analysed. The area of SUNmAb peak was used to plot the calibration curve for different dilutions of SUNmAb following the linear equation (1.e. y = mx + c) to calculate slope (m), intercept (c), and regression co-efficient (R^2).

3.6.2.1 Sample preparation

Sr No.	Targeted concentration (µg/mL)	Volume of SUNmAb (stock concentration 10mg/mL) (µL)	Volume of Mobile phase (µL)	Final Volume (µL)
1.	1000	50	450	500
Sr No.	**Targeted concentration (µg/mL)**	**Volume of SUNmAb (stock concentration 10mg/mL) (µL)**	**Volume of Mobile phase (µL)**	**Final Volume (µL)**
2.	100	50	450	500
3.	50	25	475	500
4.	20	10	490	500
5.	10	5	495	500
6.	5	5	995	1000
7.	2.5	2.5	997.5	1000

Table 3.7: Sample preparation for Linearity of SEC-HPLC

3.6.3 Accuracy

Accuracy was demonstrated by analysing samples of concentration of 2.5µg/mL, 5µg/mL and 100 µg/mL which is equivalent to 0.25, 5 and 10 % of aggregates.

Samples were prepared using mobile phase. Samples were injected in triplicates and peak area of standard was used to obtain concentration of prepared samples.

% Recovery and %RSD were calculated.

3.6.4 Precision

3.6.4.1 Instrument Precision :

Instrument precision was demonstrated by multiple injection of homogenous sample indicating the performance of HPLC instrument under the chromatographic conditions.

Precision was demonstrated by analysing samples of concentration of 2.5µg/mL, 5µg/mL and 100 µg/mL.

Samples were injected in triplicate and %RSD of obtained at each concentration level was calculated.

3.6.4.2 Method Precision:

Method precision was demonstrated by multiple injection of homogenous sample from different vials indicating the performance of HPLC instrument under the chromatographic conditions.

Precision was demonstrated by analysing samples of concentration of 2.5µg/mL, 5µg/mL and 100 µg/mL.

Samples were injected in triplicate and %RSD of obtained at each concentration level was calculated.

3.7 Method Development for Reversed-phase HPLC

3.7.1 Method Development Experiment-1

3.7.1.1 Preparation of Mobile Phase A (100% water 0.1% TFA)

500mL ultra-pure water was taken into 500mL capacity glass bottle. 500µL water was discarded from bottle and them 500µL TFA was added.

3.7.1.2 Preparation of Mobile Phase B (100% ACN 0.1% TFA)

500mL acetonitrile water was taken into 500mL capacity glass bottle. 500µL acetonitrile was discarded from bottle and them 500µL TFA was added.

3.7.1.3 Sample preparation

SUNmAb was diluted to 0.125mg/mL using buffer. Solution was stored at 2-8°C and used within 5 days of preparation

3.7.1.4 Chromatographic system setup

Column	Zorbax 3.5µm (150 x 4.6mm), Agilent
Mode	Gradient
Flow rate	0.6mL/min
Detection wavelength	220nm
Colum temperature	60 ± 5°C
Sample temperature	6 ± 2°C
Injection volume	80µL
Run time	50 minutes

Table 3.8: Chromatographic system setup for Experiment-1

3.7.1.5 Gradient Program

Time (min)	Flow	%A	%B
0.0	0.6mL/min	90.0	10.0
5		90.0	10.0
40		40.0	60.0
40.1		10.0	90.0
45		10.0	90.0
45.1		90.0	10.0
50		90.0	10.0

Table 3.9: Gradient program for Experiment-1

3.7.2 Method Development Experiment-2

3.7.2.1 Preparation of Mobile Phase A (100% water 0.15% TFA)

500mL ultra-pure water was taken into 500mL capacity glass bottle. 750µL water was discarded from bottle and them 750µL TFA was added.

3.7.2.2 Preparation of Mobile Phase B (100% ACN 0.15% TFA)

500mL acetonitrile water was taken into 500mL capacity glass bottle. 750µL acetonitrile was discarded from bottle and them 750µL TFA was added.

3.7.2.3 Sample preparation

SUNmAb was diluted to 0.125mg/mL using buffer. Solution was stored at 2-8°C and used within 5 days of preparation

3.7.2.4 Chromatographic system setup

Column	Zorbax 3.5µm (150 x 4.6mm), Agilent
Mode	Gradient
Flow rate	0.6mL/min
Detection wavelength	220nm
Colum temperature	60 ± 5°C
Sample temperature	6 ± 2°C
Injection volume	80µL
Run time	50 minutes

Table 3.10: Chromatographic system setup for Experiment-2

3.7.2.5 Gradient Program

Time (min)	Flow	%A	%B
0.0	0.6mL/min	90.0	10.0
5		90.0	10.0
40		40.0	60.0
40.1		10.0	90.0
45		10.0	90.0
45.1		90.0	10.0
50		90.0	10.0

Table 3.11: Gradient program for Experiment-2

3.7.3 Method Development Experiment-3

3.7.3.1 Preparation of Mobile Phase A (100% water 0.05% TFA)

500mL ultra-pure water was taken into 500mL capacity glass bottle. 250µL water was discarded from bottle and them 250µL TFA was added.

3.7.3.2 Preparation of Mobile Phase B (100% ACN 0.05% TFA)

500mL acetonitrile water was taken into 500mL capacity glass bottle. 250µL acetonitrile was discarded from bottle and them 250µL TFA was added.

3.7.3.3 Sample preparation

SUNmAb was diluted to 0.125mg/mL using buffer. Solution was stored at 2-8°C and used within 5 days of preparation

3.7.3.4 Chromatographic system setup

Column	Zorbax 3.5µm (150 x 4.6mm), Agilent
Mode	Gradient
Flow rate	0.6mL/min
Detection wavelength	220nm
Colum temperature	60 ± 5°C
Sample temperature	6 ± 2°C
Injection volume	80µL
Run time	50 minutes

Table 3.12: Chromatographic system setup for Experiment-3

3.7.3.5 Gradient Program

Time (min)	Flow	%A	%B
0.0	0.6mL/min	90.0	10.0
5		90.0	10.0
40		40.0	60.0
40.1		10.0	90.0
45		10.0	90.0
45.1		90.0	10.0
50		90.0	10.0

Table 3.13: Gradient program for Experiment-3

3.7.4 Method Development Experiment-4

3.7.4.1 Preparation of Mobile Phase A (100% water 0.1% FA)

500mL ultra-pure water was taken into 500mL capacity glass bottle. 500µL water was discarded from bottle and them 500µL FA was added.

3.7.4.2 Preparation of Mobile Phase B (100% ACN 0.1% FA)

500mL acetonitrile water was taken into 500mL capacity glass bottle. 500µL acetonitrile was discarded from bottle and them 500µL FA was added.

3.7.4.3 Sample preparation

SUNmAb was diluted to 0.125mg/mL using buffer. Solution was stored at 2-8°C and used within 5 days of preparation

3.7.4.4 Chromatographic system setup

Column	Zorbax 3.5µm (150 x 4.6mm), Agilent
Mode	Gradient
Flow rate	0.6mL/min
Detection wavelength	220nm
Colum temperature	60 ± 5°C
Sample temperature	6 ± 2°C
Injection volume	80µL
Run time	50 minutes

Table 3.14: Chromatographic system setup for Experiment-4

3.7.4.5 Gradient Program

Time (min)	Flow	%A	%B
0.0	0.6mL/min	90.0	10.0
5		90.0	10.0
40		40.0	60.0
40.1		10.0	90.0
45		10.0	90.0
45.1		90.0	10.0
50		90.0	10.0

Table 3.15: Gradient program for Experiment-4

3.7.5 Method Development Experiment-5

3.7.5.1 Preparation of Mobile Phase A (100% water 0.1% TFA)

500mL ultra-pure water was taken into 500mL capacity glass bottle. 500µL water was discarded from bottle and them 500µL TFA was added.

3.7.5.2 Preparation of Mobile Phase B (80% 1-propanol 0.1% TFA)

400mL 1-propanol was taken into 500mL capacity glass bottle and 100mL ultra-pure water was added to it. 500µL of solution was discarded from bottle and them 500µL TFA was added.

3.7.5.3 Sample preparation

SUNmAb was diluted to 0.125mg/mL using buffer. Solution was stored at 2-8°C and used within 5 days of preparation

3.7.5.4 Chromatographic system setup

Column	Zorbax 3.5µm (150 x 4.6mm), Agilent
Mode	Gradient
Flow rate	0.6mL/min
Detection wavelength	220nm
Colum temperature	60 ± 5°C
Sample temperature	6 ± 2°C
Injection volume	80µL
Run time	50 minutes

Table 3.16: Chromatographic system setup for Experiment-5

3.7.5.5 Gradient Program

Time (min)	Flow	%A	%B
0.0	0.6mL/min	90.0	10.0
5		90.0	10.0
40		40.0	60.0
40.1		10.0	90.0
45		10.0	90.0
45.1		90.0	10.0
50		90.0	10.0

Table 3.17: Gradient program for Experiment-5

3.7.6 Method Development Experiment-6

3.7.6.1 Preparation of Mobile Phase A (15% 1-propanol 0.1% TFA)

75mL 1-propanol was taken into 500mL capacity glass bottle and 425mL ultra-pure water was added to it. 500µL of solution was discarded from bottle and them 500µL TFA was added.

3.7.6.2 Preparation of Mobile Phase B (80% 1-propanol 0.1% TFA)

400mL 1-propanol was taken into 500mL capacity glass bottle and 100mL ultra-pure water was added to it. 500µL of solution was discarded from bottle and them 500µL TFA was added.

3.7.6.3 Sample preparation

SUNmAb was diluted to 0.125mg/mL using buffer. Solution was stored at 2-8°C and used within 5 days of preparation

3.7.6.4 Chromatographic system setup

Column	Zorbax 3.5µm (150 x 4.6mm), Agilent
Mode	Gradient
Flow rate	0.6mL/min
Detection wavelength	220nm
Colum temperature	60 ± 5°C
Sample temperature	6 ± 2°C
Injection volume	80µL
Run time	50 minutes

Table 3.18: Chromatographic system setup for Experiment-6

3.7.6.5 Gradient Program

Time (min)	Flow	%A	%B
0.0	0.6mL/min	90.0	10.0
5		90.0	10.0
40		40.0	60.0
40.1		10.0	90.0
45		10.0	90.0
45.1		90.0	10.0
50		90.0	10.0

Table 3.19: Gradient program for Experiment-6

3.7.7 Method Development Experiment-7

3.7.7.1 Preparation of Mobile Phase A (15% 1-propanol 0.1% TFA)

75mL 1-propanol was taken into 500mL capacity glass bottle and 425mL ultra-pure water was added to it. 500µL of solution was discarded from bottle and them 500µL TFA was added.

3.7.7.2 Preparation of Mobile Phase B (100% ACN)

500mL of acetonitrile was taken into 500mL capacity bottle.

3.7.7.3 Preparation of Mobile Phase C (80% 1-propanol 0.05% TFA)

400mL 1-propanol was taken into 500mL capacity glass bottle and 100mL ultra-pure water was added to it. 250µL of solution was discarded from bottle and them 250µL TFA was added.

3.7.7.4 Sample preparation

SUNmAb was diluted to 0.125mg/mL using buffer. Solution was stored at 2-8°C and used within 5 days of preparation.

3.7.7.5 Chromatographic system setup

Column	Zorbax 3.5µm (150 x 4.6mm), Agilent
Mode	Gradient
Flow rate	0.6mL/min
Detection wavelength	220nm
Colum temperature	60 ± 5°C
Sample temperature	6 ± 2°C
Injection volume	80µL
Run time	60 minutes

Table 3.20:Chromatographic system setup for Experiment-7

3.7.7.6 Gradient Program

Time (min)	Flow	%A	%B	%C
0.0	0.6mL/min	90.0	5.0	5.0
4		90.0	5.0	5.0
44		78.5	5.0	16.5
48		47.5	0.0	47.5
50		00.0	50.0	50.0
54.10		00.0	50.0	50.0
60		90.0	5.0	5.0

Table 3.21: Gradient program for Experiment-7 of

3.8 Method Validation of Reversed-phase HPLC

3.8.1 Specificity

Specificity was demonstrated by considering retention time of SUNmAb and comparing it with that of blank

3.8.1.1 Blank: Buffer was used a blank solution

3.8.1.2 Sample: SUNmAb was diluted to 0.125mg/mL using buffer

3.8.2 Linearity and Range

Linearity of method was determined by diluting SUNmAb in a concentration range from 500µg/mL to 10µg/mL using mobile phase (individual sample at each concentration) and analysed. The area of SUNmAb peak was used to plot the calibration curve for different dilutions of SUNmAb following the linear equation (1.e. y = mx + c) to calculate slope (m), intercept (c), and regression co-efficient (R^2).

3.8.2.1 Sample preparation

Sr No.	**Targeted concentration (µg/mL)**	**Volume of SUNmAb (stock concentration 10mg/mL) (µL)**	**Volume of Mobile phase (µL)**	**Final Volume (µL)**
1.	1000	50	450	500
Sr No.	**Targeted concentration (µg/mL)**	**Volume of SUNmAb (stock concentration 10mg/mL) (µL)**	**Volume of Mobile phase (µL)**	**Final Volume (µL)**
2.	500	100	100	200
3.	250	50	150	200
4.	125	25	175	200
5.	50	25	475	500
6.	25	12.5	487.5	500
7.	10	5	495	500

Table 3.22: Sample preparation for Linearity

3.8.3 Accuracy

Accuracy was demonstrated by analysing samples of concentration 0.125mg/mL

Samples were prepared using buffer. Samples were injected in triplicates and peak area of standard was used to obtain concentration of prepared samples.

% Recovery and %RSD were calculated.

3.8.4 Precision

3.8.4.1 Instrument Precision :

Instrument precision was demonstrated by multiple injection of homogenous sample indicating the performance of HPLC instrument under the chromatographic conditions.

Precision was demonstrated by analysing samples of concentration of 0.125mg/mL.

Samples were injected in triplicate and %RSD of was calculated.

3.8.4.2 Method Precision:

Method precision was demonstrated by multiple injection of homogenous sample from different vials indicating the performance of HPLC instrument under the chromatographic conditions.

Precision was demonstrated by analysing samples of concentration 0.125mg/mL.

Samples were injected in triplicate and %RSD of obtained at each concentration level was calculated

CHAPTER 4

RESULTS & DISCUSSION

4.1 Results for Method development SEC-HPLC

4.1.1 Method Development Experiment-1 (Flow rate selection)

4.1.1.1 Observations :

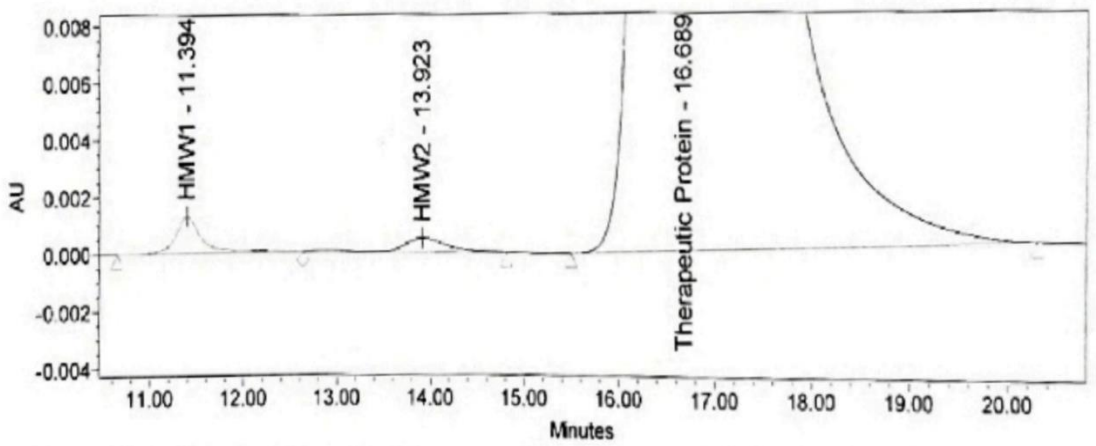

Figure 4.1: Analysis of SUNmAB at flow rate 0.5mL/min using TSKgeI G2000 SWxL

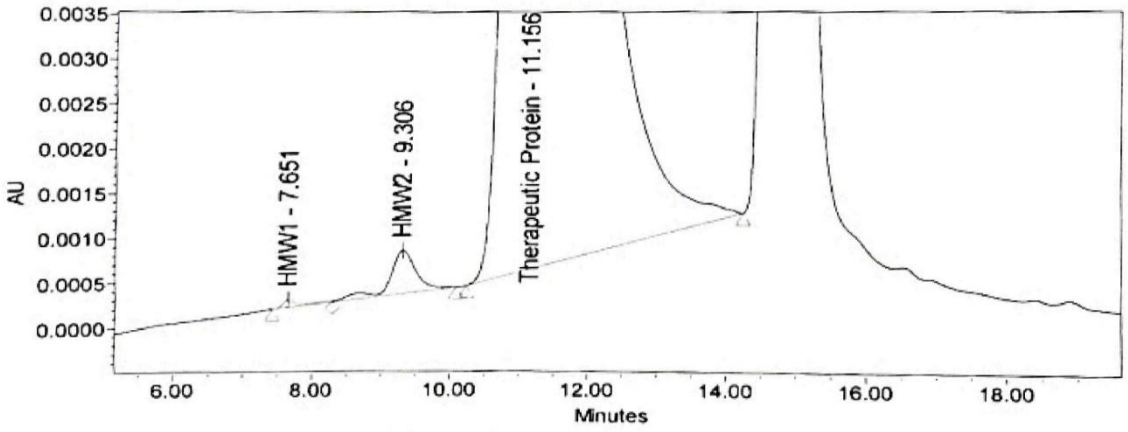

Figure 4.2: Analysis of SUNmAB at flow rate 0.75mL/min using TSKgeI G2000 SWxL

4.1.1.2 Observation table :

Name	Flow rate 0.5mL/min			Flow rate 0.75mL/min		
	Area	% Area	Resolution	Area	% Area	Resolution
HMW 1	26680	0.04	NA	1364	0	NA
HMW 2	17190	0.03	4.4	12521	0.03	3.9
SUNmAb	63087777	99.93	3.6	39391157	99.96	3.1

Table 4.1: Comparison of flow rate 0.5mL/min and 0.75mL/min

4.1.1.3 Result :

From above observation, it was found that flow rate of 0.5mL/min offered more satisfactory results than flow rate of 0.75mL/min and so 0.5mL/min was selected for further method development.

4.1.1.4 Conclusion: Flow rate of 0.5mL/min was selected for aggregate analysis of SUNmAb.

4.1.2 Method Development Experiment-2 (Column Selection)

4.1.2.1 Observations :

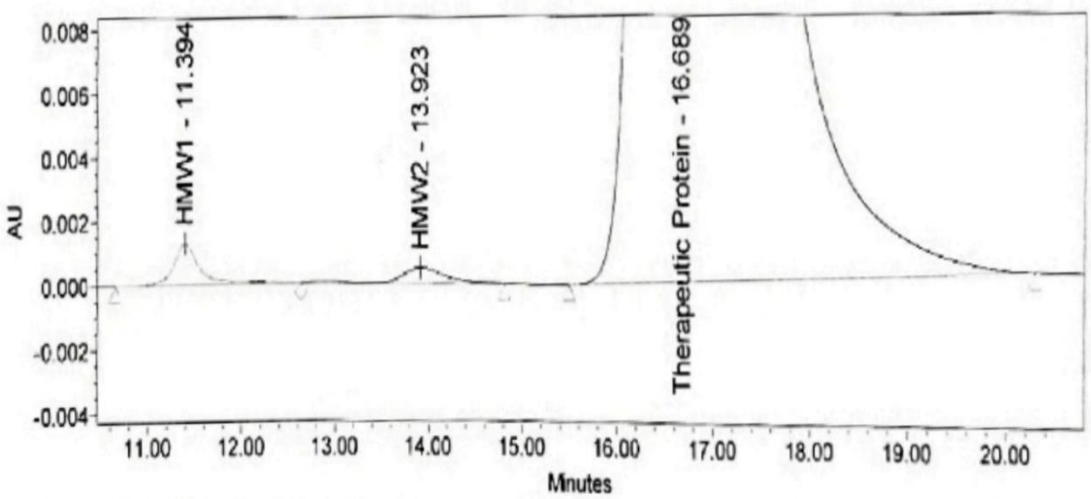

Figure 4.3 Analysis of SUNmAB using TSKgeI G2000 SWxL column

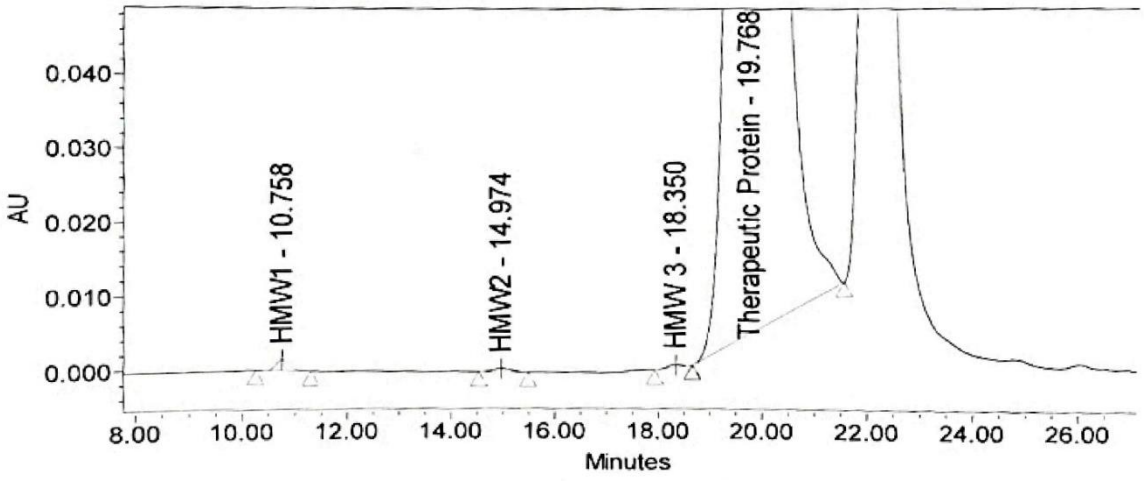

Figure 4.4 Analysis of SUNmAB usingBiosep SECs2000 column

4.1.2.2 Observations table :

Name	TSKgel G2000SW$_{XL}$			Phenomenex Biosep SEC s2000		
	Area	**% Area**	**Resolution**	**Area**	**% Area**	**Resolution**
HMW 1	26680	0.04	NA	31849	0.05	NA
HMW 2	17190	0.03	4.4	11137	0.02	7.7
HMW3	No peak observed			6857	0.01	6
SUNmAb	63087777	99.93	3.6	58572683	99.91	2.3

Table 4.2: Comparison between TOSOH and Phenomenex column

4.1.2.3 Results :

From above observation, it was found that Phenomenex Biosep SEC s2000 HPLC column offered more satisfactory results than TSKgel G2000SW$_{XL}$ HPLC column and so Phenomenex Biosep SEC s2000 columnwas selected for further method development.

4.1.2.4 Conclusion : Phenomenex Biosep SEC s2000 column was selected for aggregate analysis of SUNmAb.

4.1.3 Method Development Experiment-3 (Mobile phase selection)

4.1.3.1 Observations :

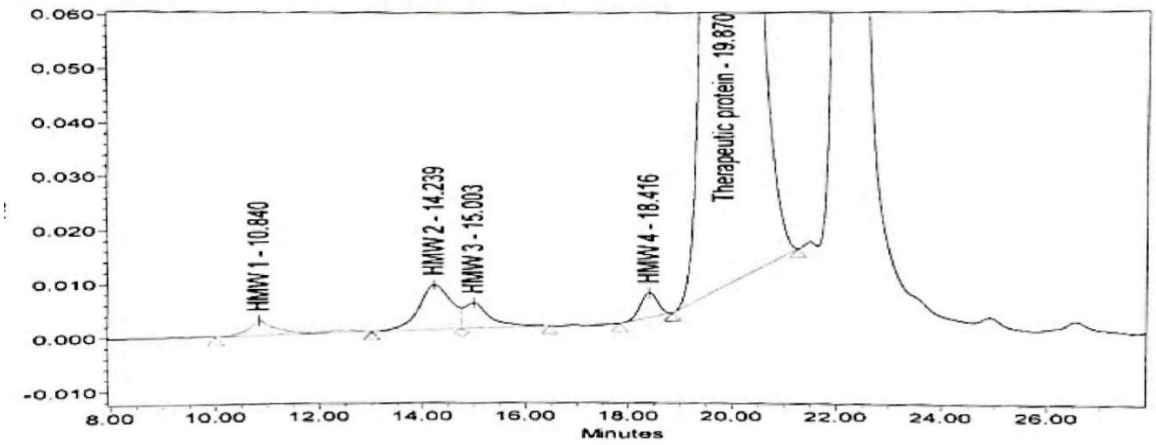

Figure 4.5: Analysis of SUNmAB using Potassium Dihydrogen Phosphate as mobile phase

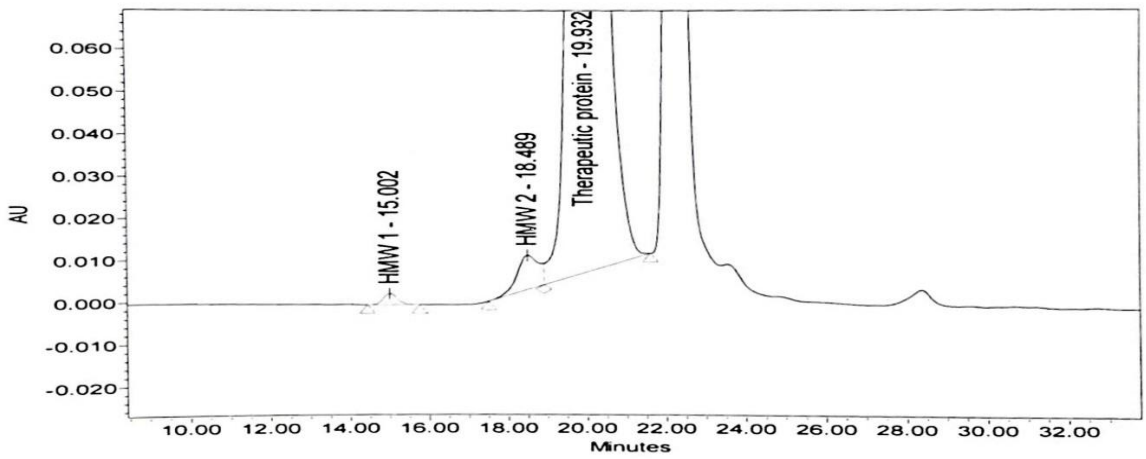

Figure 4.6: Analysis of SUNmAB using Sodium Dihydrogen Phosphate as mobile phase

4.1.3.2 Observation table :

Name	Mobile Phase 1			Mobile Phase 2		
	Area	% Area	Resolution	Area	% Area	Resolution
HMW 1	115266	0.18	NA	66901	0.12	NA
HMW 2	364320	0.58	3.5	308747	0.54	5
HMW 3	162619	0.26	1.1	No peak observed		
HMW 4	108201	0.17	5.9			
SUNmAb	61836767	98.8	2.1	57017294	99.35	2

Table 4.3: Comparison between Potassium dihydrogen phosphate and Sodium dihydrogen phosphate monohydrate

4.1.3.2 Result :

From above observations, it was found that mobile phase 1 offered more satisfactory results than mobile phase 2 and so mobile phase 1 was selected for aggregate analysis of SUNmAb.

4.1.3.3 Conclusion : Mobile phase 1 was selected for aggregate analysis of SUNmAb.

4.2 Final Chromatographic conditions for aggregate analysis of SUNmAb by SEC-HPLC

Column	BiosepSEC s2000, 5µm, 150Å (300 x 7.8mm, Phenomenex)
Mobile Phase	0.2 M Potassium Dihydrogen Phosphate+ 0.25 M Potassium Chloride, pH 6.20
Pump mode	Isocratic
Flow rate	0.5mL/min
Column temperature	25°C ± 5°C
Sample temperature	6°C ± 2°C
Injection volume	25µL
UV wavelength	215nm
Method run time	45 minutes

Table 4.4: Final Chromatographic conditions for SEC-HPLC

4.3 Method Validation for SEC-HPLC

4.3.1 Specificity

4.3.1.1 Observation

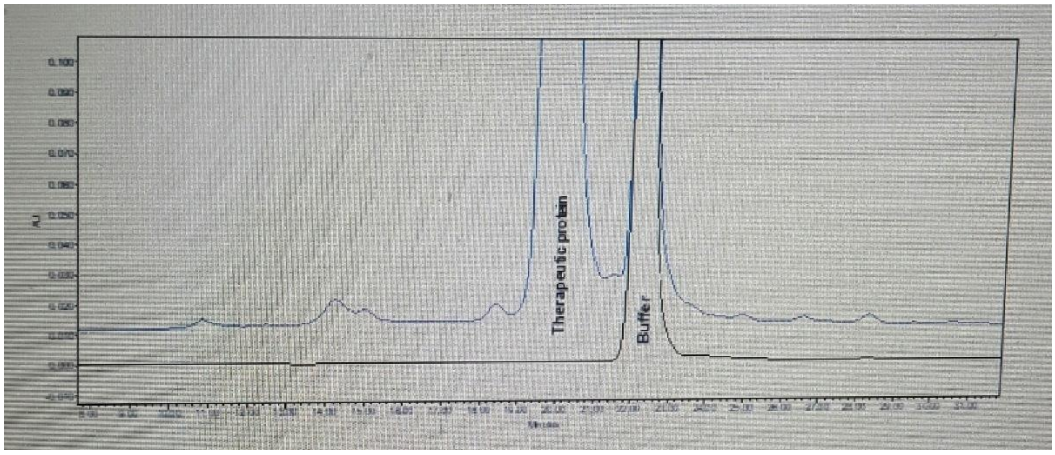

Figure 4.7: Observation for Specificity

4.3.1.1 Results : Absence of peaks in buffer/blank indicated that the matrix did not have interference at retention time of SUNmAb.

4.3.1.3 Conclusion : Method was found to be specific for SUNmAb.

4.3.2 Linearity and Range

4.3.2.1 Observations :

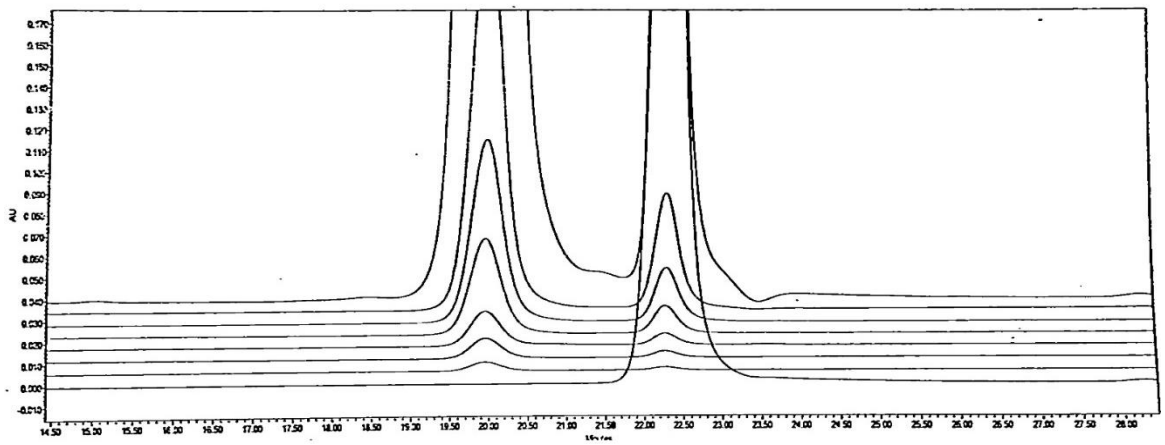

Figure 4.8Overlay of Linearity samples

4.3.2.2 Observation table

Sr No.	Target concentration of SUNmAb (µg/mL)	Peak Area
1.	1000	58319244
2.	100	5723779
3.	50	2547724
4.	20	1330459
5.	10	509031
6.	5	285947
7.	2.5	125677

Table 4.5:Observations for Linearity

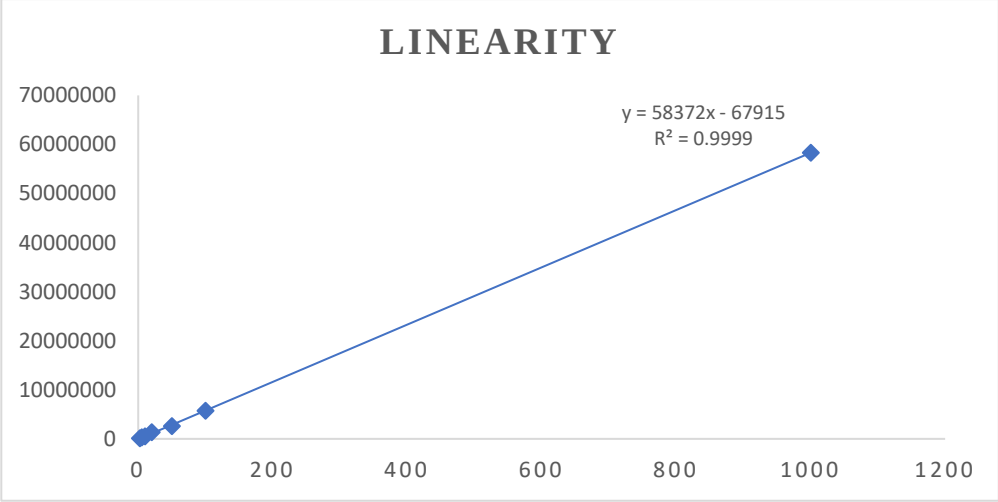

Figure 4.9: Graph of Linearity

4.3.2.3 Results :

From above observations, R^2 was found to be 0.9999 that indicated method gives linear response in range of 2.5µg/mL to 1000 µg/mL.

4.3.2.4 Conclusion : Method was found to be linear in range of 2.5µg/mL to 1000 µg/mL.

4.3.3 Accuracy

4.3.3.1 Observation table :

Conc (µg/mL)	Area	Conc. obtained	% Recovery	% Mean recovery	Std. deviation	% RSD
2.5	125677	2.15	86.2	84.65	2.11	2.5
	124682	2.13	85.5			
	119900	2.05	82.2			
5	285947	4.90	98.06	96.42	1.73	1.79
	281699	4.83	96.6			
	275868	4.73	94.6			
100	5723779	98.14	98.14	98.01	0.37	0.38
	5733472	98.31	98.3			
	5691608	97.59	97.6			

Table 4.6:Observations for Accuracy

4.3.3.2 Results :

From the observations, it was found that %recovery of all samples at each concentration level were in the range of 82.2 to 98.14% which complies with the range of 80-120%.

%RSD at each recovery level also complied with the acceptance criteria.

4.3.3.3 Conclusion : Method was found to be accurate**.**

4.3.4 Precision

4.3.4.1 Instrument precision :

4.3.4.1.1 Observation table

Conc (µg/mL)	Injection No.	Area	Mean	Std. deviation	% RSD
2.5	1	125677	123419.6	3088.45	2.5
	2	124682			
	3	119900			
5	1	285947	281171.3	5060.17	1.79
	2	281699			
	3	275868			
100	1	5723779	5716286.3	0.37	0.38
	2	5733472			
	3	5691608			

Table 4.7:Observations for Instrument precision

4.3.4.1.2 Results : From above observations, %RSD of three injections at each concentration level was found to be 2.50, 1.79, 0.38% which complies with the acceptance criteria.

4.3.4.2 Method Precision :

4.3.4.2.1 Observation table

Conc (µg/mL)	Preparation No.	Area	Mean	Std. deviation	% RSD
2.5	1	104471	103138.3	2296.99	2.22
	2	104458			
	3	100486			
5	1	234937	233627.6	3556.6.	1.52
	2	229602			
	3	236344			
100	1	5010539	5124771	99743.15	1.94
	2	5194616			
	3	5169157			

Table 4.8:Observations for Method precision

4.3.4.2.2 Results : From above observations, %RSD of three injections at each concentration level was found to be 2.22, 1.52, 1.94% which complies with the acceptance criteria.

4.3.4.2.3 Conclusion : Method was found to be precise.

4.4 Results for RP-HPLC

4.4.1 Method development Experiment-1

4.4.1.1 Observation :

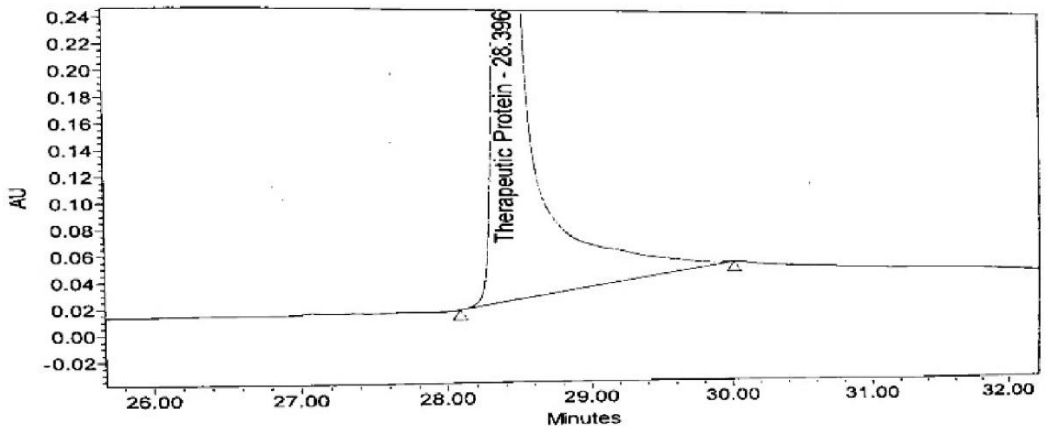

Figure 4.10: Chromatogram for Method Development Experiment-1

4.4.1.2 Observation table :

Name	Retention Time	Area	% Area	Height	USP Tailing
Therapeutic Protein	28.396	8718673	100.00	615957	1.9

Table 4.9: Observations for Experiment-1

4.4.1.3 Result : SUNmAb was eluted at 28min as a sharp peak with tailing factor of 1.9. There was observed a shift in baseline after the main peak.

4.4.1.4 Conclusion : From results, it was decided to change concentration of TFA in next experiment.

4.4.2 Method development Experiment-2

4.4.2.1 Observation

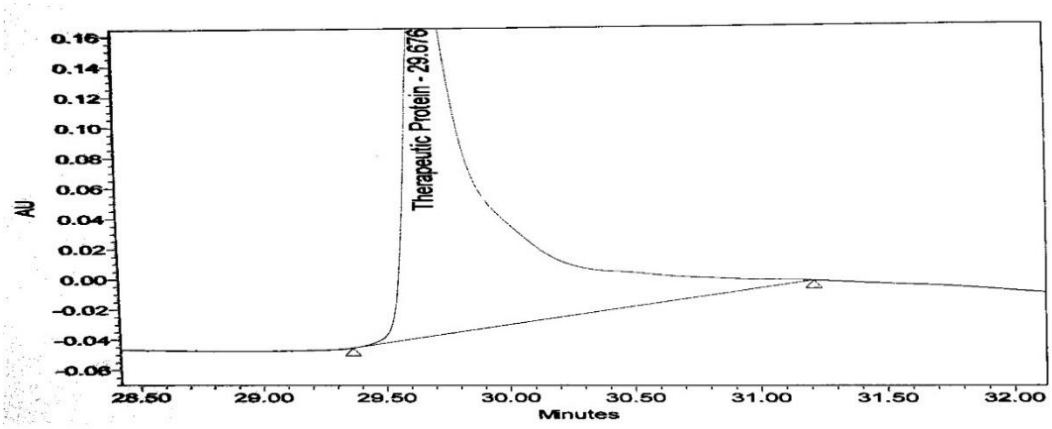

Figure 4.11: Chromatogram for Method Development Experiment-2

4.4.2.2 Observation table :

Name	Retention Time	Area	% Area	Height	USP Tailing
SUNmAb	29.676	5596617	100.00	271712	2.4

Table 4.10: Observations for Experiment-2

4.4.2.3 Result : SUNmAb was eluted at 29min as a sharp peak with tailing factor of 2.4. There was observed a shift in baseline after the main peak and tailing was increased as compared to experiment no. 1

4.4.2.4 Conclusion : From results, it was decided to change concentration of TFA to 0.05% in next experiment.

4.4.3 Method development Experiment-3

4.4.3.1 Observation

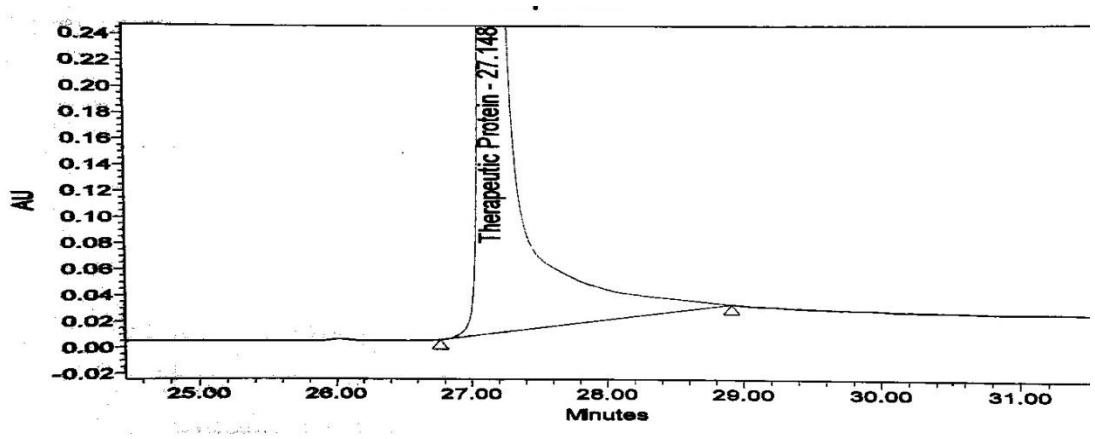

Figure 4.12: Chromatogram for Method Development Experiment-3

4.4.3.2 Observation table:

Name	Retention Time	Area	% Area	Height	USP Tailing
SUNmAb	27.148	11587469	100.00	969121	1.6

Table 4.11: Observations for Experiment-3

4.4.3.3 Result : SUNmAb was eluted at 2min as a sharp peak with tailing factor of 1.6. There was observed a shift in baseline after the main peak and tailing was decreased as compared to experiment no. 1 and 2

4.4.3.4 Conclusion : Although tailing decreased, but there were no impurities resolved so it was decided to change the ion pairing agent to formic acid.

4.4.4 Method development Experiment-4

4.4.4.1 Observation :

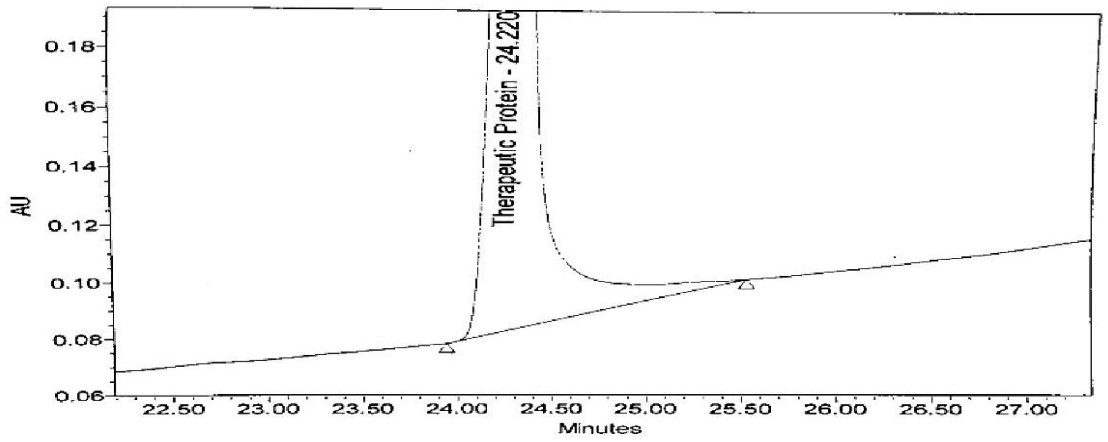

Figure 4.13: Chromatogram for Method Development Experiment-4

4.4.4.2 Observation table :

Name	Retention time	Area	% Area	Height	USP tailing
SUNmAb	24.220	6153075	100	566552	1.3

Table 4.12: Observations for Experiment-4

4.4.4.3 Result : SUNmAb was eluted at 24min as a sharp peak with tailing factor of 1.3. There was observed a shift in baseline after the main peak and tailing was decreased as compared to experiment no. 1, 2 and 3

4.4.4.4 Conclusion : Although tailing decreased, but there were no impurities resolved so it was decided to change the mobile phase.

4.4.5 Method development Experiment-5

4.4.5.1 Observation :

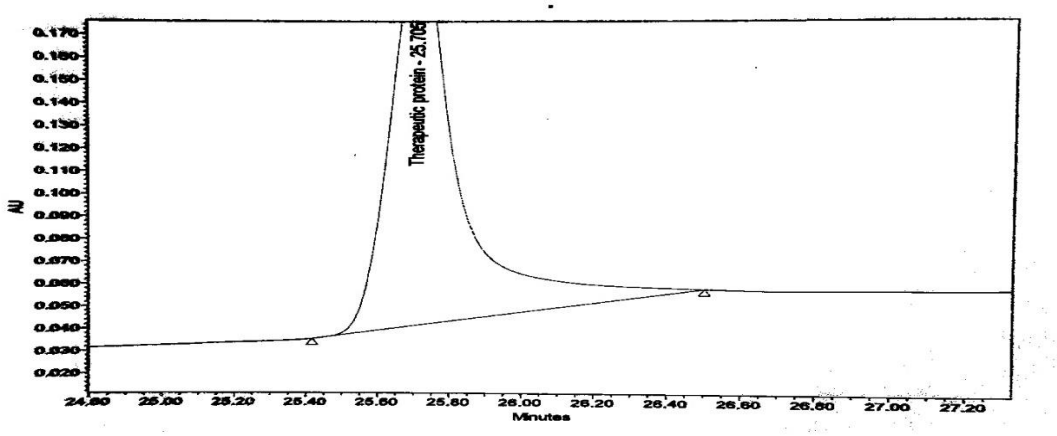

Figure 4.14: Chromatogram for Method Development Experiment-4

4.4.5.2 Observation table :

Name	Retention time	Area	% Area	Height	USP tailing
SUNmAb	25.705	2243935	100	172058	1.3

Table 4.13: Observations for Experiment-5

4.4.5.3 Result : With the introduction of propanol as mobile phase,SUNmAb was eluted at 25min as a sharp peak with tailing factor of 1.3. There was observed a shift in baseline after the main peak and tailing was decreased as compared to experiment no. 1, where the concentration of TFA was same as here.

4.4.5.4 Conclusion : Although tailing decreased, but there were no impurities resolved so it was decided to change the mobile phase.

4.4.6 Method development Experiment-6

4.4.6.1 Observation

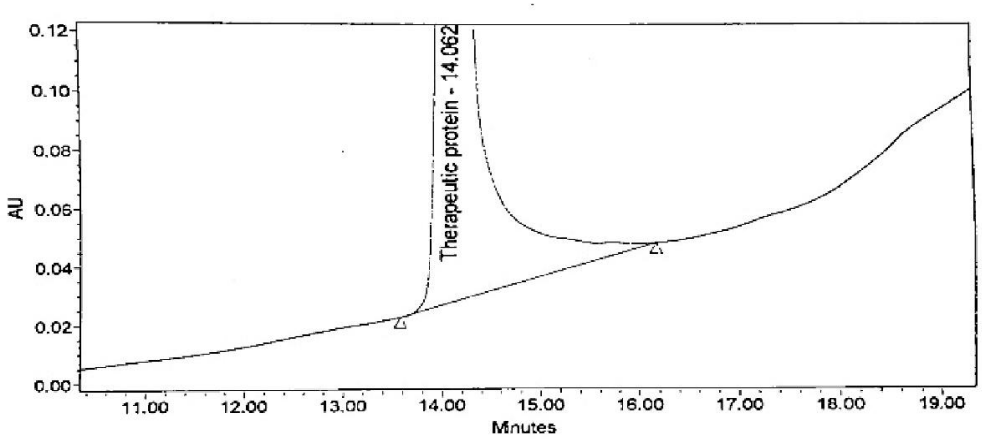

Figure 4.15: Chromatogram for Method Development Experiment-6

4.4.6.2 Observation table :

Name	Retention time	Area	% Area	Height	USP tailing
SUNmAb	14.062	13867454	100	977608	1.3

Table 4.14: Observations for Experiment-6

4.4.6.3 Result : SUNmAb was eluted at 14min as a sharp peak with tailing factor of 1.3. There was observed a very big shift in baseline after the main peak and tailing was decreased as compared to experiment no. 1, where the concentration of TFA was same as here,

4.4.6.4 Conclusion : Although tailing decreased, but there were no impurities resolved with only propanol as mobile phase. So, it was decided to use acetonitrile in next experiment.

4.4.7 Method development Experiment-7

4.4.7.1 Observation :

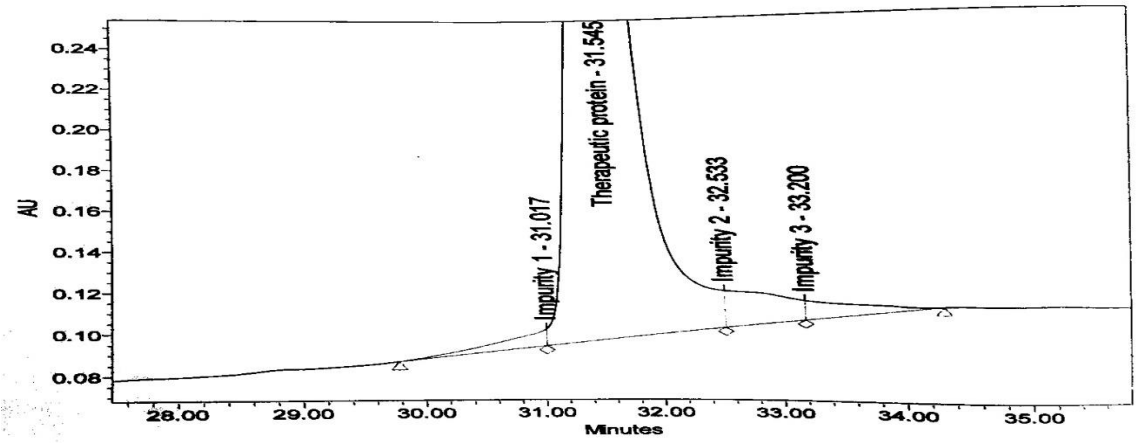

Figure 4.16: Chromatogram for Method Development Experiment-7

4.4.7.3 Observation table :

Name	Retention Time	Area	% Area	Height
Impurity 1	31.017	201904	1.41	8020
SUNmAb	31.545	13213550	92.13	441042
Impurity 2	32.450	638104	4.45	19023
Impurity 3	33.167	288263	2.01	9777

Table 4.15: Observations for Experiment-7

4.4.7.3 Result : SUNmAb was eluted at 31min as a sharp peak with no tailing. There were three impurities observed.

4.4.7.4 Conclusion : In this experiment, alomg with the main peak, there were three impurity peaks observed which can be further identified and quantified. The composition of mobile phases and concentration of TFA used in this experiment were found to provide satisfactory results and were selected for purity analysis of SUNmAb.

4.5 Final Chromatographic conditions for purity analysis of SUNmAb by RP-HPLC

Column	Zorbax 3.5µm (150 x 4.6mm), Agilent
Mobile Phase	A: 15% 1-propanol 0.1% TFA B: 100% Acetonitrile C: 80% 1-propanol 0.05% TFA
Mode	Gradient
Flow rate	0.6mL/min
Detection wavelength	220nm
Colum temperature	60 ± 5°C
Sample temperature	6 ± 2°C
Injection volume	80µL
Run time	60 minutes

Table 4.16: Final Chromatographic conditions for RP-HPLC

Time (min)	Flow	%A	%B	%C
0.0	0.6mL/min	90.0	5.0	5.0
4	0.6mL/min	90.0	5.0	5.0
44	0.6mL/min	78.5	5.0	16.5
48	0.6mL/min	47.5	0.0	47.5
50	0.6mL/min	00.0	50.0	50.0
54.10	0.6mL/min	00.0	50.0	50.0
60	0.6mL/min	90.0	5.0	5.0

Table 4.17: Final Gradient program for RP-HPLC

4.6 Method Validation for RP-HPLC

4.6.1 Specificity

4.6.1.1 Observation

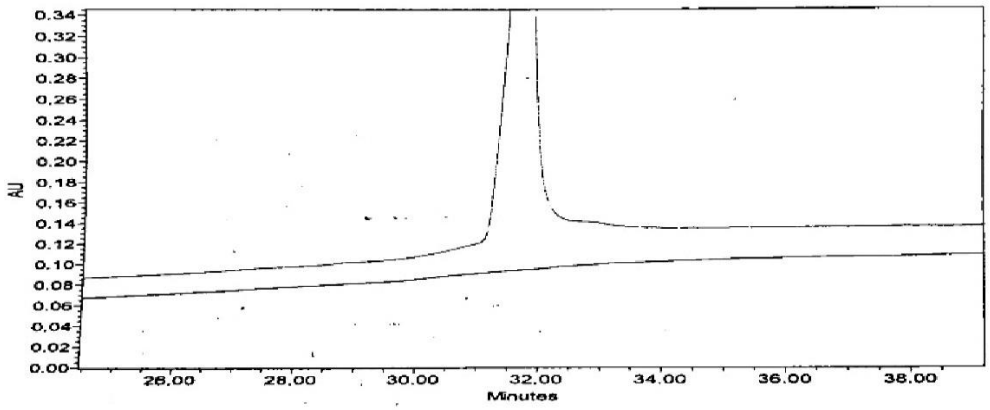

Figure 4.17: Observation for Specificity

4.6.1.2 Result : Absence of peaks in buffer/blank indicated that the matrix did not have interference at retention time of SUNmAb.

4.6.1.3 Conclusion : Method was found to be specific for SUNmAb.

4.6.2 Linearity and Range

4.6.2.1 Observation :

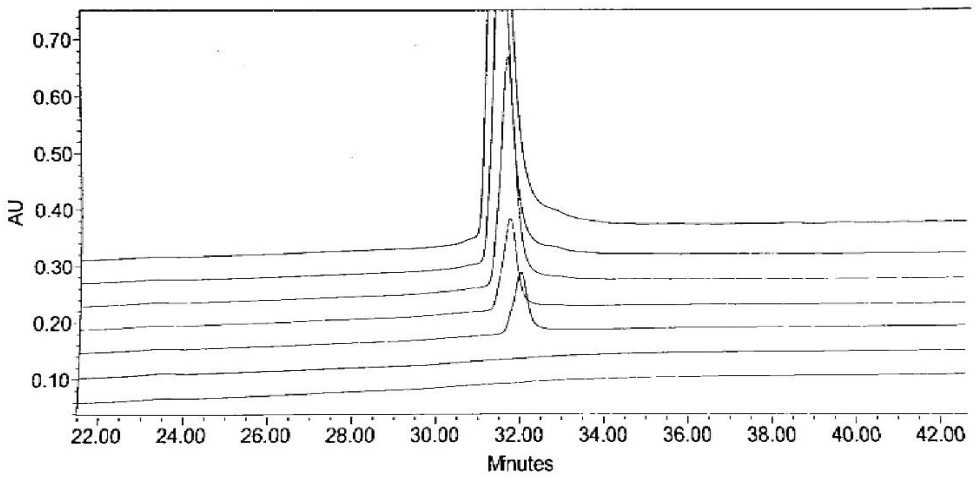

Figure 4.18: Overlay of Linearity samples

4.6.2.2 Observation table :

Sr No.	Target concentration of SUNmAb (µg/mL)	Peak Area
1.	10	536235
2.	25	2545071
3.	50	4476605
4.	125	14061407
5.	250	21271055
6.	500	38272201

Table 4.18: Observations for Linearity

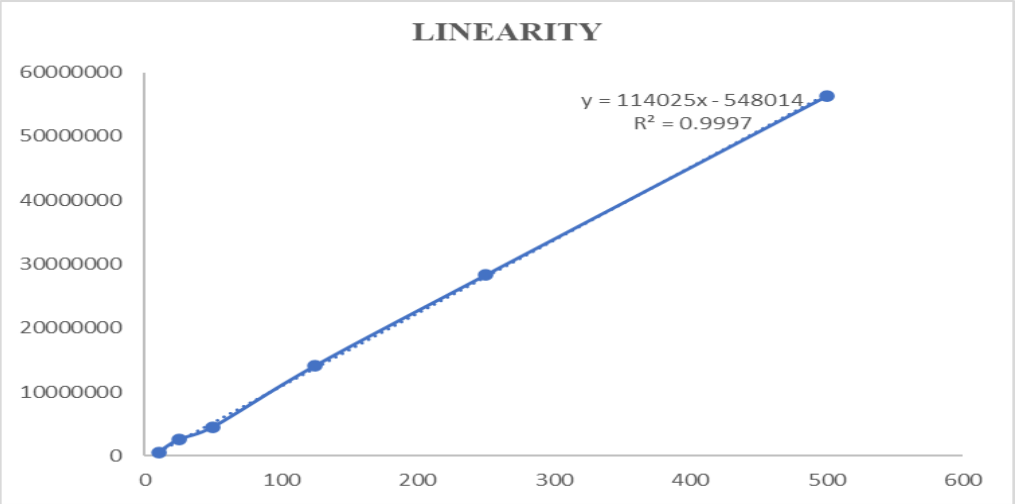

Figure 4.19: Graph of Linearity

4.6.2.3 Results : From above observations, R^2 was found to be 0.9997 that indicated method gives linear response in range of 10µg/mL to 500 µg/mL.

4.6.2.4 Conclusion : Method was found to provide linear response in range of 10µg/mL to 500 µg/mL.

4.6.3 Accuracy

4.6.3.1 Observation table

Conc (µg/mL)	Area	Average	Conc. obtained	% Recovery	Std. deviation	% RSD
125	14242818	14245179	126.57	101.25	171911	1.2068
	14418259					
	14074461					

Table 4.19: Observations for Accuracy

4.6.3.2 Result : From the observations, it was found that %recovery of samples was found to be 101.25%.

%RSD was found to be 1.2 also complied with the acceptance criteria.

4.6.3.4 Conclusion : Method was found to be accurate.

4.6.4 Precision

4.6.4.1 Instrument precision

4.6.4.1.1 Observation table

Conc (µg/mL)	Injection No.	Area	Mean	Std. deviation	% RSD
125	1	14050597	14305408.3	222420.8	1.55
	2	14460642			
	3	14404986			

Table 4.20: Observations for Instrument precision

4.6.4.1.2 Result : From the observations, %RSD for all the injection was found to be 1.5 which complies with the acceptance criteria.

4.6.4.2 Method precision

4.6.4.2.1 Observation table

Conc (µg/mL)	Preparation No.	Area	Mean	Std. deviation	% RSD
125	1	14196219	14142035.33	66431.96	0.469
	2	14067919			
	3	14161968			

Table 4.21: Observations for Method precision

4.6.4.2.2 Result : From the observations, %RSD for all the preparation was found to be 0.46 which complies with the acceptance criteria.

4.6.4.2.3 Conclusion : Method was found to be precise.

CHAPTER 5

CONCLUSION

5.1 Conclusion:

As discussed in this work, the structure of monoclonal antibodies ranges from unordered to highly ordered and multimeric states. Their activity is greatly affected by their composition.

The exact chemical composition of final product is important and is of critical concern as a small change may lead to loss of activity and efficacy of monoclonal antibody.

In this work, two method, Reversed-phase HPLC and Size-exclusion HPLC were developed and validated for purity and aggregate analysis of SUNmAb respectively.

5.1.1 Size-Exclusion HPLC for aggregate analysis of SUNmAb :

A method with 0.2M Potassium dihydrogen phosphate and 0.25M Potassium chloride pH 6.2 in isocratic mode using Phenomenex BIOsep SECs2000 (5µm, 300 x 7.8mm) at flow rate of 0.5mL/min with run time of 45 minutes was developed for aggregate analysis of SUNmAb.

Method was validated and found to be specific, accurate, precise and provided linear response from 2.5µg/mL to 1000µg/mL.

5.1.2 Reversed-phase HPLC for purity analysis of SUNmAb :

A method with 15% 1-propanol, Acetonitrile, 80% 1-propanol as mobile phase in gradient mode using Zorbax 300SB C-18 (3.5µm, 150 x 4.6mm) at flow rate of 0.6mL/min with run time of 60 minutes was developed for purity analysis of SUNmAb.

Method was validated and found to be specific, accurate, precise and provided linear response from 10µg/mL to 500µg/mL.

CHAPTER 6

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