Analytical Techniques for Qualitative and Quantitative Analysis of Monoclonal Antibodies (mAbs)

A Dissertation Report submitted
for the partial fulfillment of the Degree of Master of Science

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[M.Sc. Biotechnology]

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Designation of the Guide

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2022-23

(On letterhead of the Department)

C E R T I F I C A T E

This is to certify that this training report entitled "TITLE" was successfully carried out by Miss <Name of Student> towards the partial fulfillment of requirements for the degree of Master of Science in Biotechnology of Atmiya University, Rajkot. It is an authentic record of his/her own work, carried out by him/her under the guidance of Name of Supervisor for a period of 3 months during the academic year of 2022-23. The content of this report, in full or in parts, has not been submitted for the award of any other degree or certificate in this or any other University.

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With deep thanks…

Mansi Kansagra

D E C L A R A T I O N

I hereby declare that the work incorporated in the present dissertation report entitled "Analytical Techniques for Qualitative and Quantitative Analysis of Monoclonal Antibodies (mAbs)**"** is my own work and is original. This work (in part or in full) has not been submitted to any University for the award of any Degree or a Diploma.

Date : 07/04/23 Mansi Kansagra

1. INTRODUCTION

1.1clonal Antibodies (mAbs):

Monoclonal antibodies (mAbs) are monospecific antibodies. These antibodies are produced from clone of single lymphocyte directed against a single antigenic determinant or epitope [2]. They were first generated in mice in 1975 using a hybridoma technique. The first licensed monoclonal antibody was Orthoclone OKT3 (muromonab-CD3) which was approved in 1986 for use in preventing kidney transplant rejection [2].

Since then, mAb based therapeutics are playing an important role in the treatment or prevention of many important diseases such as cancers, autoimmune disorders and infectious diseases. They are engineered to serve as substitute antibodies that can restore, enhance or mimic the immune system's attack on cancer cells. They are designed to bind to antigens that are generally more numerous on the surface of cancer cells than healthy cells. Cancer cells that are coated in monoclonal antibodies are more easily detected and targeted for destruction [2].

The drug (mAbs) helps the immune system in following ways: Flagging cancer cells, triggering cell membrane destruction, blocking cell growth, preventing blood vessel growth, blocking immune system inhibitors, directly attacking cancer cells, delivering radiation treatment, delivering chemotherapy, binding cancer and immune cells.

Development of mAbs as therapeutic drugs is becoming rapidly emerging area in biopharmaceutical industries. The global mAbs market was 95.1 Billion USD in 2017 and is estimated to reach 131.33 Billion USD by 2023. Even though, the development of mAb products has good prospects, the structure of mAbs are far more complex than those of small molecule drugs including the primary structure, higher order structure, glycosylation and charge variants [2]. Other than these intrinsic heterogeneities, the mAbs introduced to bioprocess manufacturing are susceptible to further chemical modifications and degradation [2]. Thus, to guarantee the quality and consistency of mAbs each step of manufacturing from protein expression to storage phase should be controlled and characterized. Based on the platform knowledge of the Critical Quality Attributes (CQAs) of mAbs an analytical platform is established to support the development of therapeutic mAbs.

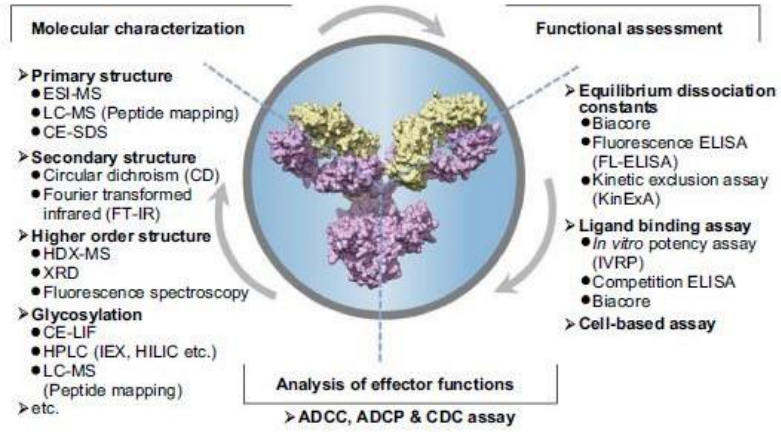

Figure 1: Overview of analytical methods (The example mAb is a representative IgG1) [2]

3.1 Production Processes for monoclonal antibodies:

Nowadays, mAbs are produced mostly from engineered microorganisms at commercial scale. Various systems of production are used, including animal cells, microorganisms, plants and mammary glands [3].

In general, process of commercial production of mAbs begins with generation of mAb by immunizing an animal or by molecular biology methods involving construction and identification of a stable high producing clone. After the clone development, the cell line selection is done which is a critical step to maintain the cells to secrete the desired protein with the correct conformation at high levels. Generally, CHO cell lines are used.

4. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatography (HPLC) is a technique used to separate different

constituents of a compound using high pressure to push solvents through the column. It is the most widely used technique to identify, quantify and separate components of a mixture.

Before the existence of HPLC, LC analysis was carried by gravitational flow of the eluent that takes several hours for the analysis to be completed. These analysis systems use high-pressure pump that generates rapid flow of eluent, and thus results in dramatic improvement in the analysis time.

Components of HPLC System:

1. Solvent Reservoir: A reservoir holds the solvent, the mobile phases.
2. High-pressure pump: It has high-pressured pump (solvent delivery system or solvent manager) is used to generate and provide a specified flow rate of mobile phase, typically ml/min.
3. Injector: An injector (sample manager or autosampler) can introduce (inject) the sample into the continuously flowing mobile phase stream that carries the sample in the HPLC column.
4. Column Compartment: The compartment has the HPLC column. Column contains the chromatographic packing material needed for the separation. The packing material is called the stationary phase because it is fixed in the column hardware.
5. Detector: The detector is used to give the output in the form of the chromatogram. It is needed to see the separated compound as they elute form the HPLC column. The detector is wired to the computer data station, which records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample components.

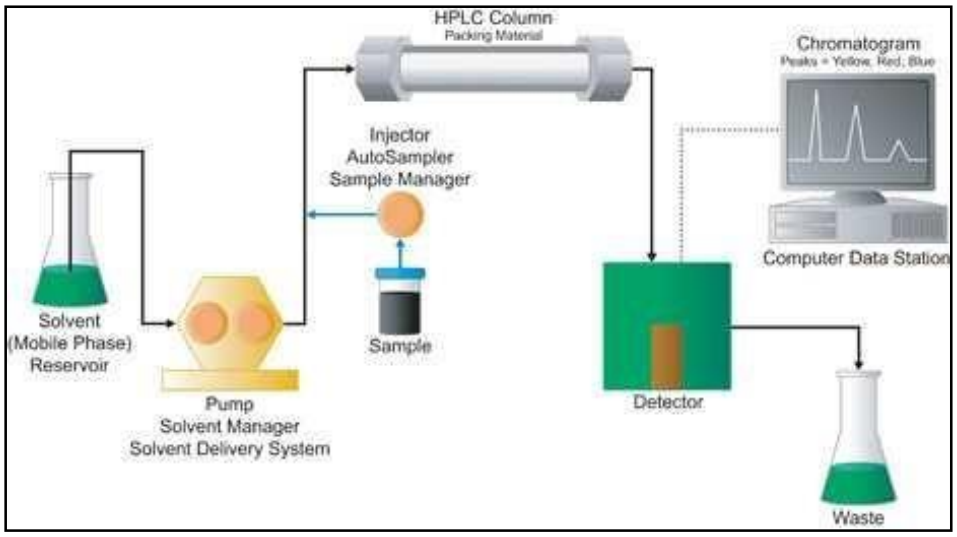

Figure 4: High Performance Liquid Chromatography (HPLC) system

Since sample compound characteristics can be very different, several kinds of detector have been developed.

5. TYPES OF HPLC & EXPERIMENTAL ANALYSIS

5.1 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

5.1.1 ***Objective***: To determine purity of recombinant proteins (monoclonal antibodies) by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

5.1.2 ***Principle:*** Given the complexity of protein-based therapies, a quantitative assessment of the impurities including oxidized variants of the active protein, is of concern given their potential effect on efficacy and immunogenicity. Reverse Phase chromatographic method depends on the reversible adsorption of proteins, which have varying degrees of hydrophobicity, to a hydrophobic stationary matrix. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent. The solute mixture is initially applied to the sorbent in the presence of aqueous buffers, and the solutes are eluted by the addition of organic solvent to the mobile phase. This brings about a high resolution between protein and related impurities.

RP-HPLC is a very powerful technique for the analysis of peptides and proteins because of several factors listed below:

- Excellent resolution for very closely related molecules as well as for highly distinct molecules
- The experimental ease with which chromatographic selectivity can be manipulated through changes in mobile phase characteristics.
- The generally high recoveries and, hence, high productivity
- The excellent reproducibility of repetitive separations carried out over a long period of time, which is caused partly by the stability of the sorbent materials under a wide range of mobile phase conditions
- Limitations: RP-HPLC can cause the irreversible denaturation of protein samples thereby reducing the potential recovery of material in a biologically active form.

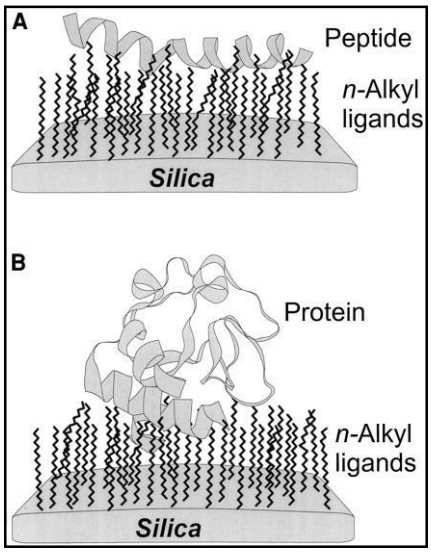

Figure 6: Schematic representation of the binding of **(A)** a peptide and **(B)** a protein, to an RP-HPLC silica-based sorbent. The peptide or protein interacts with the immobilized hydrophobic ligands through the hydrophobic chromatographic contact region.

5.1.3 Materials

1. Instrument: - Alliance WATERS e2695 series HPLC system
2. Equipment and materials: - Pipettes, vortex, MabPac RP Column (4 µM pore size, 2.1 ID*100mm length)
3. Glass-wares: Measuring cylinder, Glass bottles, Beakers
4. Chemicals: - Reference Standard, test sample, Milli Q Water, Analytical grade Acetonitrile (CH_3CN, ACN), Trifluoroacetic acid (TFA)

5.1.4 Method

5 mcg protein was injected onto HPLC column and eluted using an organic solvent gradient of acetonitrile at 0.4 mL/min and monitored by UV absorbance at 214 nm. Purity was evaluated by determining the peak area of each variant that eluted separately as a percentage of the total peak area.

A. <u>Sample/reference standard preparation:</u>

- *Reference standard preparation:* The reference standard was diluted to a concentration of 0.5mg/mL with Milli Q water.

$$Reference\ standard\ \ (\frac{mg}{ml}) = \frac{Targeted\ concentration\ (\frac{mg}{ml})}{labelled\ concentration}\ x\ Total\ Volume$$

- *Sample preparation:* The test sample was diluted at a concentration of 0.5mg/ml in MilliQ based on the concentration obtained from OD 280nm.

B. Chromatographic conditions:

Error! Reference source not found. Table 2: RP HPLC chromatographic conditions

Sr. No	**Parameters**	**Specifications**
1.	Mobile Phase A	0.1% TFA in 10 % ACN
2.	Mobile Phase B	0.1% TFA in CAN
3.	Column specifications	MabPac RP Column (4 µM pore size, 2.1 ID*100mm length)
4.	Flow rate	0.4 mL/min
5.	Elution condition	Gradient Elution
6.	Detection wavelength	214 nm
7.	Column temperature	80 ℃
8.	Sample temperature	5 ° C ± 3
9.	Injection Volume	10 µL
10.	Run Time	56 min

Table 3: Gradient for the RP HPLC Method

S.no	Time (min)	Flow (mL/min)	% A	% B
1.	0.01	0.4	77.5	22.5
2.	3.25	0.4	77.5	22.5
3.	20	0.4	68.5	31.5
4.	26	0.4	59.5	40.5
5.	41	0.4	19	81
6.	46	0.4	10	90
7.	51	0.4	10	90
8.	52.5	0.4	77.5	22.5
9.	56	0.4	77.5	22.5

- *Sample Injection and analysis:* Injected 10 µL of sample and used slow gradient from 0 to 100 % B over 56 min to elute the sample.

C. Setting up of HPLC System:

- Before injecting the system for analysis, the system was cleaned properly. This should be done to avoid the chances of inaccuracy during the analysis.
- Following system cleaning procedure was followed for proper cleaning of the system during routine analysis:

(i) Switched Off the system and the detector.

(ii) Only switched on the system.

(iii) Dry Prime: Kept all the lines in hot water. Carried out dry prime by selecting "Dry prime" option from the system. An empty syringe was attached to the prime/vent valve to pull out any air bubbles in the solvent lines.

(iv) Wet Prime: Wet prime was done first by hot water, followed by magic solution (25:25:25: 25::water: ACN: Methanol: IPA)

(v) Seal Wash: Seal wash is done to lubricate the plunger and flush away any solvent or dry salts deposited onto the plunger. It was done with 10 % Methanol using "Prime Seal wash" option from the system.

(vi) Needle Wash: Needle wash is done to prevent the carryover from previous injection. 90 % methanol is used as needle wash.

(vii) Detector cleaning: Detector cleaning was carried out by first passing hot water followed by magic solution. Detector was switch on after 2-3 minutes of flow time. Detector is auto calibrated after switching it on.

(viii) Sampler Defrosting: The buildup of frost degrades the cooling efficiency of sample heater/cooler. To prevent this, defrosting is to be done. Defrosting was done through Defrost sample heater/cooler option from the system.

5.1.5 Observation and Results:

Chromatogram was obtained and analyzed by manual integration method. Various peaks were obtained corresponding to the different components of the test sample.

Retention time of main peak in test sample and reference standard was comparable. (Refer Table 4)

Purity of the test sample was determined in terms of area percent. Purity was observed to be 97.5 %. (Refer Table 4)

Table 4: Results of purity by RP-HPLC

Sample name	Retention time (min)	% pre peaks	% main peak	% post peaks
Reference standard	17.104	3.32	96.02	0.66
Test sample	17.245	2.04	97.49	0.47

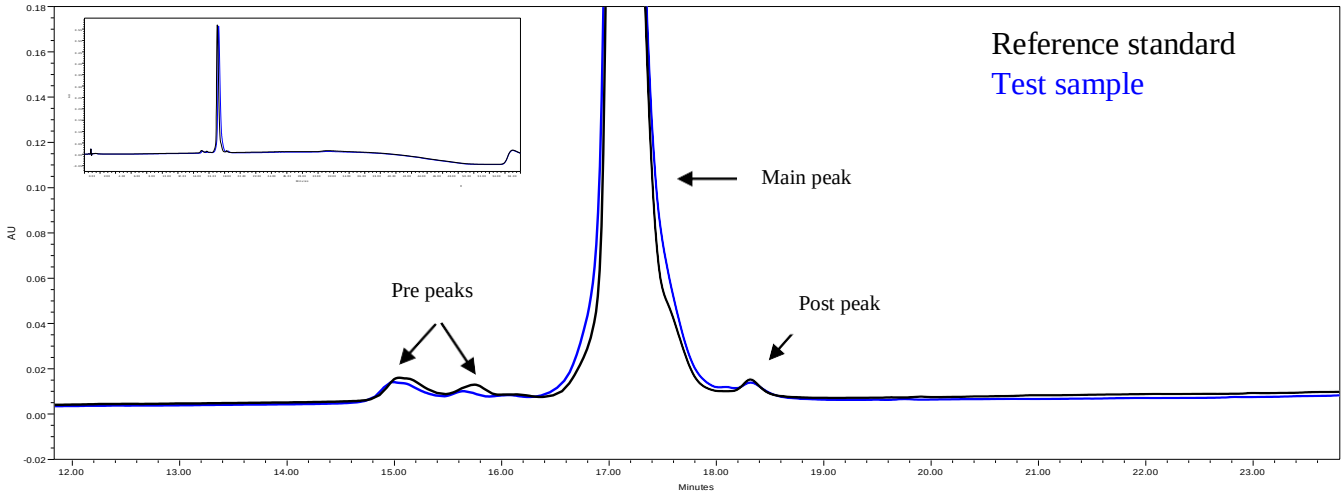

Figure 7: RP chromatogram overlay profile of reference standard and test sample

5.2 Size exclusion chromatography (SE-HPLC)

5.2.1 ***Objective:*** To determine high molecular weight (HMW) and low molecular weight (LMW) impurities of recombinant proteins (monoclonal antibodies) by Size-Exclusion High Performance Liquid Chromatography (SE-HPLC).

5.2.2 ***Principle:*** Molecular weight related impurities constitute the key product-related impurities as they typically exhibit a high degree of immunogenicity. Typically, 1–3 % mAb aggregates are observed. This can be monitored by SE-HPLC technique.

The size exclusion separates on the basis of the molecular size. The molecules smaller than the fractionation range enter the pores of the resin while molecules larger than the fractionation range are excluded from entering the pores, separating the HMW and LMW impurities.

SEC is classified into Gel Permeation Chromatography (GPC) and Gel Filtration Chromatography (GFC). GFC is used widely to measure the molecular weight distribution of molecules (proteins and polysaccharides).

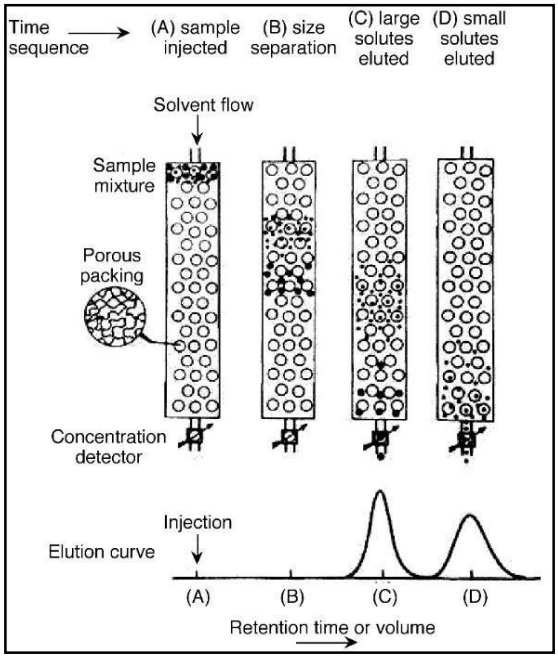

Figure 8: Schematic representation of separation of LMW and HMW impurities

SEC is an important technique for the impurities removal and consist of following factors:

- Allows contaminating material to be removed in relatively small volume.
- It is different from dialysis, as it has the advantage of speed, which is necessary for certain experimental situations.
- Separates on the basis of molecular size.
- Stationary phase is composed of uncharged porous particles.
- Molecules smaller than fractionation range can enter the pores of the resin, while molecules larger than the fractionation range are excluded from entering the pores.
- Small molecules (buffer salts, small molecules) enters the pores and macro molecules (proteins, nucleic acids, DNA) pass the resin bed so we can collect desalting fraction at the end.
- For effective separation, resin bed should be sufficiently tall and voluminous to fully separate the emergence from the end of the column.

5.2.3 Materials

1. Instrument- Alliance WATERS e2695 series HPLC system
2. Equipment and materials- Pipettes, vortex, TSK Gel SEC Column (5µM pore size, 7.8mm ID*30cm length), pH Meter

   Glass-wares: Measuring cylinder, Glass bottles, Beakers, PVDF membrane filter (0.2µM), Solvent filtration apparatus
3. Chemicals- Reference Standard, test sample, Milli Q Water, Analytical grade Sodium phosphate monobasic monohydrate, Sodium phosphate dibasic dihydrate, Sodium Chloride

5.2.4 Method

SE-HPLC was performed using TSK Gel SW Type steel column. The method follows isocratic elution. Chromatogram was acquired and processed (integrated) for purity determination.

A. Sample and Standard Preparation

- *Preparation of reference standard:* The reference standard (5mg/ml) was diluted at a concentration of 1 mg/ml in Milli Q water and injected. Reference standard is considered as the criteria for system suitability.

$$Reference\ standard\ \left(\frac{mg}{ml}\right) = \frac{Targeted\ concentration\ \left(\frac{mg}{ml}\right)}{labelled\ concentration}\ x\ Total\ Volume$$

- *Sample preparation:* The test sample was diluted at a concentration of 1mg/ml in MilliQ based on the concentration obtained from OD_{280nm}. After dilution, the samples were filled in HPLC vials for analysis.
- *Sample Injection and analysis:* Injected 10 µL of sample and used isocratic flow from 0 to 100 % over 30 min to elute the sample.

B. Chromatographic conditions:

Table 5: SE-HPLC chromatographic conditions

S. No	Parameters	Specifications
1	Mobile Phase	100 mM phosphate buffer+200 mM NaCl, pH 6.7
2.	Elution Condition	Isocratic elution
3.	Column specifications	TSK Gel SW Type (5µM pore size, 7.8mm ID*30cm length)
4.	Flow rate	0.5 mL/min
5.	Detection wavelength	217 nm
6.	Column temperature	25 ℃
7.	Sample temperature	5 ° C ± 3
8.	Injection Volume	10-100 µL
9.	Run Time	30 min

C. Setting Up of HPLC System: Refer section 5.1.4 C

5.2.5 Observation and Results:

Chromatogram was obtained and analyzed by manual integration method. LMW and HMW peaks were identified and purity determines in terms of area %.

Retention time of main peak in test sample and reference sample was comparable. (Refer Table 6)

Purity of the test sample was observed to be 99.23 %. (Refer Table 6)

Table 6: Results of purity by SE-HPLC

Sample name	Retention time (min)	% HMW	% main peak	% LMW
Reference standard	15.370	0.24	98.59	1.18
Test sample	15.356	0.73	99.23	0.03

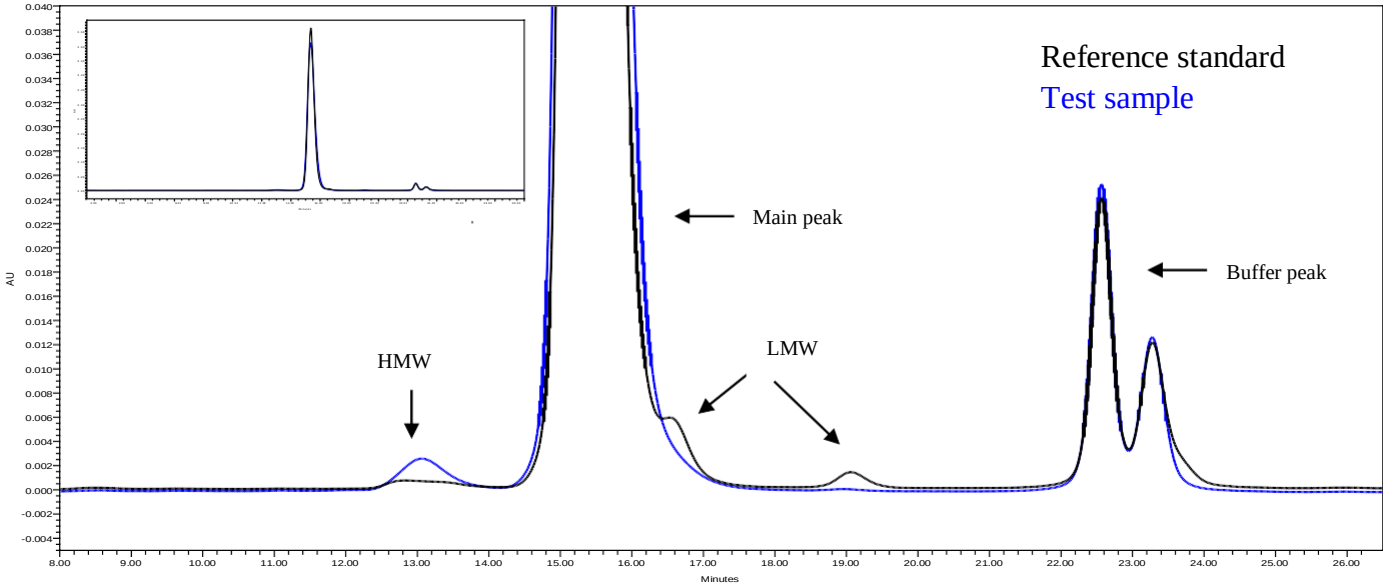

Figure 9: SE chromatogram overlay profile of reference standard and test sample

5.3 Protein A Chromatography (Protein-A HPLC)

5.3.1 ***Objective:*** To determine the total protein concentration of monoclonal antibody using Protein-A High Pressure Liquid Chromatography (Protein A-HPLC).

5.3.2 ***Principle:*** Protein A affinity column chromatography is a widely used method for the purification of antibodies. It is widely used for the purification of Fc containing proteins like mAbs, however, it can be used for other molecules as well. It is simple and highly selective method based on the strong and specific interaction between protein A and the crystallizable fragment (Fc) of the antibody.

Protein A is a 56 kDa surface protein of *Staphylococcus aureus*. It is composed of five immunoglobin binding domains each of which can bind proteins from many mammalian species, most notably IgG through the heavy chain within Fc region. Nowadays, recombinant Protein A is used, produced in *E. coli*.

Protein A resins are the most frequently used affinity resins in bio-manufacturing. Today it is the standard technique for capturing recombinant monoclonal antibodies. Protein A chromatography is a very robust purification procedure and is used as a capture step due to its specificity. Depending on the intended use for the target molecule (antibodies for

diagnostic testing) Protein A capturing might be the only chromatographic step required to achieve adequate product purity. Resins used for the protein A has hydrophilic polymer based on polyvinylether and ligand as recombinant protein A.

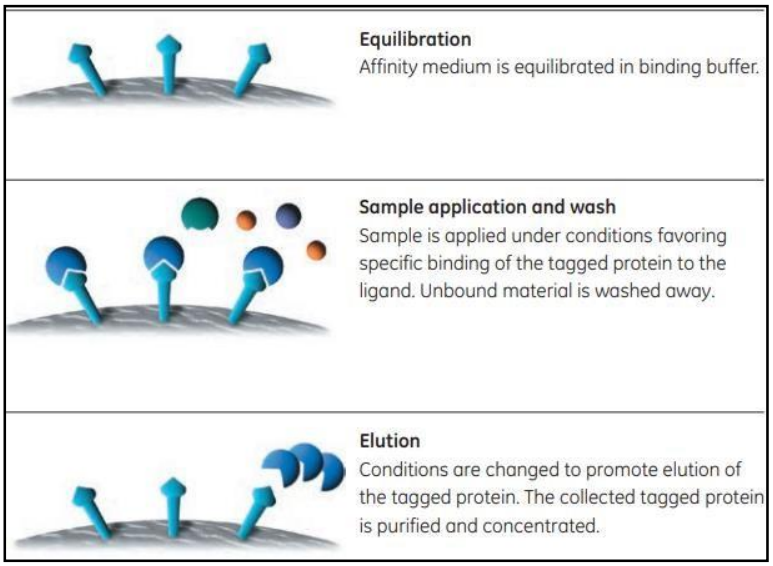

Figure 10: General affinity purification workflow of tagged recombinant proteins

Product molecules are eluted from protein A resins by lowering the pH. A typical elution buffer is 0.1 M sodium citrate, pH 3.3. Low pH is often maintained for a period for the purpose of viral inactivation.

5.3.3 Materials

1. Instrument- Alliance WATERS e2695 series HPLC system,
2. Equipment and materials- Pipettes, vortex, POROS protein A column (Thermo fisher), pH Meter, Amicon Ultra 0.5 centrifugal filter devices, Solvent filtration apparatus, Centrifuge, PVDF membrane filter (0.2µM), Measuring cylinder, Glass bottles, Beakers
3. Chemicals- Reference standard, test sample, MilliQ Water, Analytical grade Sodium acetate, Sodium Chloride, Acetic acid, Sodium hydroxide

5.3.4 Method: Protein A was performed using affinity column POROS A (20µM pore size, 2.1mm ID*30mm length) using pH gradient for elution with detection at 280 nm.

A. Sample & Reference standard preparation:

- *Reference standard preparation:*

  The reference standard was diluted at a concentration of 3 mg/mL in Milli Q water and injected. Injected 10µL of 3 mg/mL solution.

$$Reference\ standard\ \left(\frac{mg}{ml}\right) = \frac{Targeted\ concentration\ \left(\frac{mg}{ml}\right)}{labelled\ concentration}\ x\ Total\ Volume$$

- *Test sample preparation:*

  10 µL of test sample was injected directly on to the column and analyzed.

B. Chromatographic conditions:

Table 7: Chromatographic conditions for Protein A-HPLC

S. No	Parameters	Specifications
1	Mobile Phase A	Sodium Acetate + Sodium Chloride, pH 6.7±0.2
2.	Mobile Phase B	Sodium Chloride + Acetic acid, pH 2.5±0.2
3.	Elution Condition	Gradient elution
4.	Column specifications	POROS A (20µM pore size, 2.1mm ID*30mm length)
5.	Flow rate	1 mL/min
6.	Detection wavelength	280 nm
7.	Temperature	25 ℃
8.	Injection Volume	10-100 µL
9.	Run Time	5 min

Table 8: Gradient for Protein A HPLC

Time (min)	% Mobile phase A	% Mobile phase B	Flow rate
0.00	100	0	2.0 mL/min
1.00	100	0	
1.01	0	100	
3.00	0	100	
3.01	100	0	
5.00	100	0	

- *Sample Injection and analysis:* Injected 10 µL of sample and used step gradient from 0 to 100 % B for 5 min to elute the sample.

C. Setting Up of HPLC System: Refer section 5.1.4 C

5.3.5 Observation and Results:

Chromatogram was obtained and analyzed by manual integration method. Protein concentration was determined by slope-intercept method. Slope and intercept was obtained from the calibration curve.

The retention time (RT) for the main peak and reference standard was comparable.

The concentration is calculated by using following equation:

By using equation: $y = mx+c$

where, y- area of the main peak, m- slope of the calibration curve, x- protein concentration (mg/ml), c- intercept (-ve)

Formula: $Concentration, x\ (\frac{mg}{ml}) = \frac{peak\ area - intercept}{slope}$

Table 9: Protein A HPLC results

Sample name	Retention time (min)	Peak area	Concentration (mg/mL)
Reference standard	1.600	1133819	2.9
Test sample	1.601	1963181	5.0

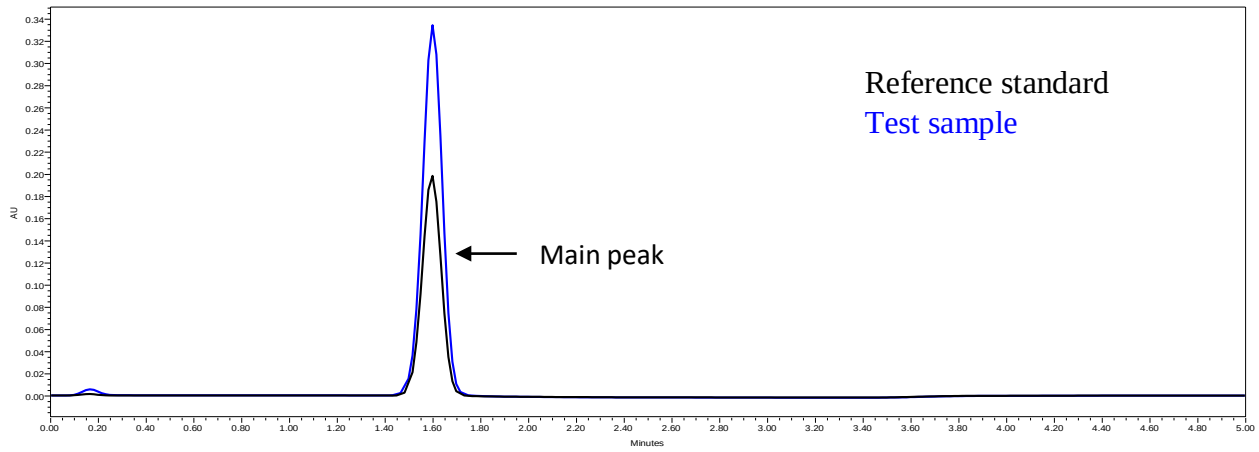

Figure 11: Protein A chromatogram overlay profile of reference standard and test sample

8. REFERENCES

1. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Steven A. Berkowitz1, John R. Engen2, Jeffrey R. Mazzeo3 and Graham B. Jones2, Nature Reviews, July 2012, Volume 11.
2. Molecular and functional analysis of monoclonal antibodies in support of biologics development. Xin Wang, Zhiqiang An, Wenxin Luo, Ningshao Xia, Qinjian Zhao, Protein & Cell, July 2017.
3. The history of monoclonal antibody development- progress, remaining challenges and future innovations. Justin K.H. Liu
4. Production Processes for monoclonal antibodies. Silva Carvalho, Otávio Bravim da Silva, Gabriela Carneiro de Almeida, Juliana Davies de Oliveira, Nadia Skorupa Parachin and Talita Souza Carmo, November 2015
5. A-Mab: A case study in bioprocess development.
6. Methods in Molecular Biology, vol. 251, HPLC of Peptides and Proteins: Methods and Protocols.