“IMPURITY PROFILLING OF MONOCLONAL ANTIBODIES”

A PROJECT SUBMITTED TO

NIRMA UNIVERSITY

In partial fulfillment of the requirement for the degree of

Bachleor Of Pharmacy

By

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SEMESTER VIII

UNDER THE GUIDANCE OF

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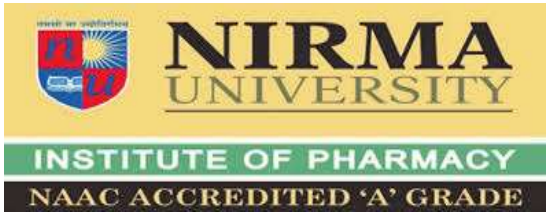

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APRIL 2020

"IMPURITY PROFILLING OF MONOCLONAL ANTIBODIES"

CERTIFICATE

This is to certify that ***"Impurity Profiling of Monoclonal Antibody"*** *is the bonafide work carried out by* ***POOJA PARMAR (16BPH069)****, B. Pharm semester 8 under guidance and supervision in the Institute of Pharmacy, Nirma University, Ahmedabad during the academic year 2019 to 2020. This work is up to my satisfaction.*

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"IMPURITY PROFILLING OF MONOCLONAL ANTIBODIES"

CERTIFICATE OF SIMILARITY OF WORK

This is to undertake that the B.Pharm Project work entitled ***"Impurity Profiling of Monoclonal Antibody"*** *submitted by* ***POOJA PARMAR (16BPH069)*** *of B.Pharm semester 8 is a bonafide review work carried out by me at the Institute of Pharmacy, Nirma University under the Guidance of "Dr. Priti Mehta". I am aware about the rules and regulation of Plagiarism policy of Nirma University, Ahmedabad. According to that, the review work carried out by me is not reported anywhere as per best of my knowledge.*

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"IMPURITY PROFILLING OF MONOCLONAL ANTIBODIES"

DECLARATION

*I, **POOJA PARMAR (16BPH069)**, student of VIII[th] Semester of B.Pharm at Institute of Pharmacy, Nirma University, hereby declare that my project entitled **"IMPURITY PROFILING OF MONOCLONAL ANTIBODY"** is a result of culmination of my sincere efforts. I declare that the submitted project is done solely by me and to the best of my knowledge, no such work is done by any other person for the award of degree or diploma for any other means. I also declare that the information was collected from various primary sources (journals, patents etc.) has been duly acknowledged in the project report.*

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1) INTRODUCTION:

Impurity profiling is identification, detection or structural elucidation and quantitative determination of degradation and impurity of drug substances and formulations. Impurity profiling has acquired significance in modern pharmaceutical research because unknown, potentially toxic impurities are harmful to the health and impurities should be detected and evaluated by specific techniques in order to improve the safety of drug therapy.

1.1) Impurity:

An impurity in Pharmaceutical is defined as an unwanted substance or chemical that is present in the API or is d1eveloped during the manufacturing process or upon ageing. This unwanted chemical known as impurity if present even in trace quantity hinders the safety and efficacy of pharmaceutical product.

Limits of impurities found in API or formulation of allowable level are incorporated by Different pharmacopeia's such as United State Pharmacopeia (USP) & British Pharmacopeia (BP).

An impurity defined by ICH as **'any component of the drug substance that is not the chemical entity defined as the drug substance'.** (KIRAN R. DHANGAR, jun. 2017)

The International Conference on Harmonization (ICH) has published guidelines on impurities present in new drug substances, products and residual solvents.

These guidelines are:

Table 1: ICH impurity guidelines

Q3A (R2)	Impurities in New Drug Substances
Q3B (R2)	Impurities in New Drug Products
Q3C (R6)	Maintenance of Guideline for Residual Solvents
Q3C (R8) Maintenance EWG	Maintenance of the Guidelines for Residual Solvents
Q3D (R1)	Guidelines for Elemental Impurities
Q3D (R2) Maintenance EWG	Revision of Q3D(R1) for cutaneous & transdermal products
Q3D Training	Implementation of Guidelines for Elemental Impurities
Q3E Informal WG	Impurity: Assessment and Control of Extractables & Leachable for Pharmaceuticals and Biologics

(Quality guidelines, n.d.)

1.2.1) Types of impurities:

Types of impurity found in Monoclonal Antibodies are mainly divided into two groups:

A. Process impurities:

It includes:

I. Adventitious microorganisms:

These are the microorganisms that may have been introduced unintentionally. These organisms enter in the manufacturing process through many different ways like; personals, raw ingredients, cells and environment. These microorganisms include fungi, bacteria, parasites, rickettsia etc.

II. Host Cell Proteins:

They are process related impurities. In monoclonal antibodies productions, the protein drugs are expressed at high concentrations. Based on the expression

method, and the process of protein purification, HCPs level and form can be significantly different.

The antibodies then undergo purification steps to expel all hosts cellular components. However, a large no. of other proteins expressed by host cells can contaminate the monoclonal antibodies and left behind making host cell proteins (HCP) an impurity.

Serious immunogenicity is caused in humans due to the presence of host cell protein impurity in the finished product even in trace amounts (1.00-100.00 ppm), can lead to serious immunogenic response in humans. So, it is very crucial to detect, identify and quantify host cell protein impurity in ensuring patient safety.

It threatens patient safety in 3 major ways:

1. HCP even in small concentration (1-100 ppm) are immunogenic as they are foreign to human body.
2. Aggregation of HCP to form soluble or insoluble aggregates of varying size is a problem as they may cause adverse effects when administered and even act as adjuvants.
3. HCP if not removed or inactivated may cause fragmentation of protein in monoclonal antibody and reduces the stability, efficacy and yield of mAb due to proteolytic activity of HCP.

III. Host cell DNA:

Host cell DNA is an impurity that occur during manufacturing of mAb and must be controlled and monitored to ensure drug safety and purity.

A conventional method for removal of host residual DNA requires extraction of DNA from monoclonal antibody drug substance by using various techniques like hybridization, Pico green, threshold assay and qPCR.

IV. Leached protein A:

Protein A is a protein of bacterial cell wall [Staphylococcal Protein A, SpA]. It is important to free the antibodies from protein A because it can cause anaphylactic reactions by reacting with IgG. It can be removed by hydrophobic interaction membrane chromatography

B. Product related impurity:

I. Aggregates:

Aggregates are large tangled clusters of denatured antibody molecules that are irreversibly formed during product expression in cell culture, during storage or during purification. The cycle of aggregate formation is affected by monoclonal antibody biophysical and biochemical characteristic and atmosphere wherein monoclonal antibody was introduced in purification and storing.

Aggregates are responsible for exposing epitopes which can cause increase in immunogenicity. Apart from this they even cause formation of sub visible particles which are too small to be seen by naked eye but are larger in size than 0.1µm. Shear forces, extreme pH, temperature, concentration, ionic strength, and further conditions causes increase in aggregate formation. So, it is very important to monitor aggregation during the purifying and manufacturing process.

The protein is subjected to various stresses during the processing of protein therapeutics. Secretion of protein in the medium which contains buffer constituents, dissolved oxygen, cells, cell nutrients, bacteria and host cell proteins (including proteases) is increased in the cell culture stage of the monoclonal antibody manufacturing process. Suspension of cells is kept for several days above 30°C and neutral pH.

once the reasonable quantity of protein is secreted. For viral inactivation the resultant reservoir has always been maintained at low pH value i.e. acidic. The

usually followed is cation exchange chromatography conducted at high conductivity condition and conditions, and anion-exchange (AEX) chromatography conducted at basic pH. Ultrafiltration/ diafiltration process is used to prepare protein and is filled in the ultimate form. In the protein manufacturing process, the solution is mixed, pumped, filtered and then exposed different materials like glass, stainless steel and plastic. This condition may contribute to the creation of aggregates.

Removal of aggregates: It is difficult to remove aggregates as due to the physical and chemical similarity of the aggregates to the drug product itself, which is chemically monomer, thus the aggregate particles will have higher surface charge or hydrophobicity. Therefore, to separate the aggregate particles from monomers hydrophobic interaction & ion exchange chromatography technique. Ion exchange chromatographic method will separate aggregates based on molecular charge, while hydrophobic interaction method based on hydrophobicity and mixed mode chromatography based on both charge and hydrophobicity. (Aggregation of Monoclonal Antibody Products: Formation and removal , n.d.)

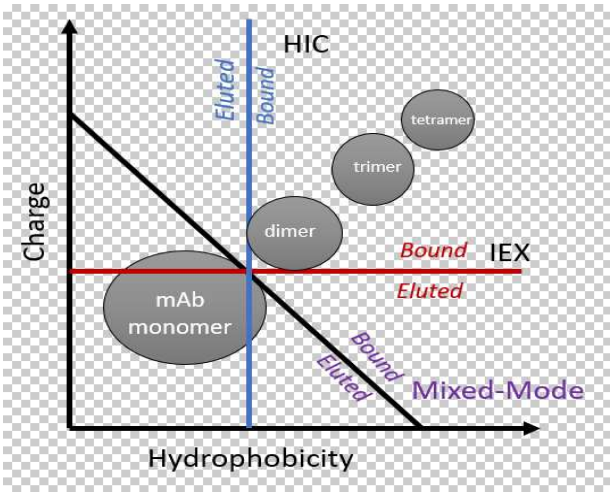

Figure 1: Chromatographic techniques used for removal of aggregates

II. Endotoxins:

Endotoxins are lipopolysaccharides found in most Gram-negative bacteria's cell wall. They are continuously released in environment by dead bacteria. Endotoxins are pyrogens that is they induces fever so it becomes essential to remove endotoxins from drugs as if consumed by human it can be dangerous. Chromatographic technique like ion exchange, gel filtration, affinity adsorbent, ultrafiltration & sucrose gradient centrifugation etc.

III. Product Variants:

It includes changes occurred in the product due to oxidation, deamination, loss of C terminal Lys, denaturation etc.

(HMW) Higher molecular weight substances like antibody dimers and
(LMW) lower molecular weight substances such as protein backbone truncated remains are the process related impurities. These high molecular weight species are produced due to the aggregation of protein in the therapeutic monoclonal antibodies and thus affects the drug efficacy and safety.
Low molecular weight species frequently have reduced or significantly lower activity compared to the antibody's monomer form, thus revealing new antigen-antibody binding site that can contribute to immunogenicity or can affect in vivo pharmacokinetic properties. Consequently, all the higher molecular weight species and lower molecule weight species are called essential quality characteristics and so they are regularly monitored in drug manufacturing during & during processing as a part of release monitoring of distilled substance.
Impurities which are introduced in the process of purification should be removed as well which can be process buffers, leached protein A, extractables, process buffers, extractable from the resins, and detergents.
This can be possible through chromatographic methods which include protein A chromatography, Column chromatography, affinity chromatography and so on.

2) MONOCLONAL ANTIBODIES:

"Mono" stands for 1 & clone stand for "duplicate". Thus. monoclonal antibodies are duplicate copies of 1 form another antibody. They are manmade antibodies derived from the identical immune cell s made from a single B-cell clone. Monoclonal antibody binds to single binding site. In contrast, polyclonal antibodies which are usually made by several different plasma B-cell lineages, binds to multiple epitopes.

Bispecific monoclonal antibodies which is an artificial protein can also be modified, by increasing the therapeutic targets of one single monoclonal antibody to two epitopes.

It is possible to design or produce monoclonal antibodies from any substance which specifically binds to that particular substance; they can then serve to purify or detect that substance.

In the past two decades monoclonal antibodies have been used successfully to ta rget a broad range of therapies. (MONOCLONAL ANTIBODY, n.d.)

2.1) Cell line used in monoclonal antibodies production:

Choosing the cell line is one of the most important steps in production of monoclonal antibody. The cell should be healthy & protein of interest must be secreted to a higher degree having proper conformation greater level. On the basis of criteria, for the development of monoclonal antibody cells of mammals are mostly selected. A mammalian expression mechanism has the main benefit that the cell mechanism is optimized for manufacturing, processing and excretion of extremely complex substances.

Various human cell lines and still further unusual expressions like genetically engineered plant cells, genetically engineered microbe cells, and genetically altered pathogen cells have also been used in the development of monoclonal antibody. Pathogens altered by genomic manipulation techniques have gained

significant interest in commerce, because these cells are simple to manage or to alter compared to mammalian cells. Many benefits to methods of production utilizing genetically engineered microbes are that this cell has excellently-defined transcription mechanisms, so the manufacturing process is repeatable and simple to confirm.

Modified yeast cells, such as Pichia pastoris, has tremendous usage potential as these cells are capable of achieving high rates of heterologous protein secretion. Yeast growing systems for the manufacturing of monoclonal antibodies are simpler and affordable compared with human cell culture schemes. They may be grown in daily stirred bioreactors tanks, in batch mode, or in feed-batch mode. In general, microbes cannot have biological & physicochemical feature of proper transection or post-translational production of monoclonal antibody. Thanks to the fact that plants are easy to grow and spread, modified plants have gained attention. Other growing benefits including affordable medium, lower maintenance costs and higher production yields allow a cheaper alternative to plant cultivation in comparison to mammalian cell culture. There are a few drawbacks, such as-different patterns of glycosylation and post-translation cycling may also allow.

2.2) What Monoclonal Antibodies are made of?

The antibody are proteins prepared by humans and behave as humanized antibodies in immune. These antibodies are prepared & named by four ways.

- **Murine:** These are made from protein of mouse and the names of treatment ends in -omab.

- **Chimeric:** These are a combination of a part of mouse and a part of human and the names of the treatment ends in -ximab.

- **Humanized:** Humanized proteins are made from small These are made from small fragments of mouse proteins attached to human proteins and the names of the treatment ends in -zumab.
- **Human:** These are the fully human proteins and the names of treatment ends in -umab.

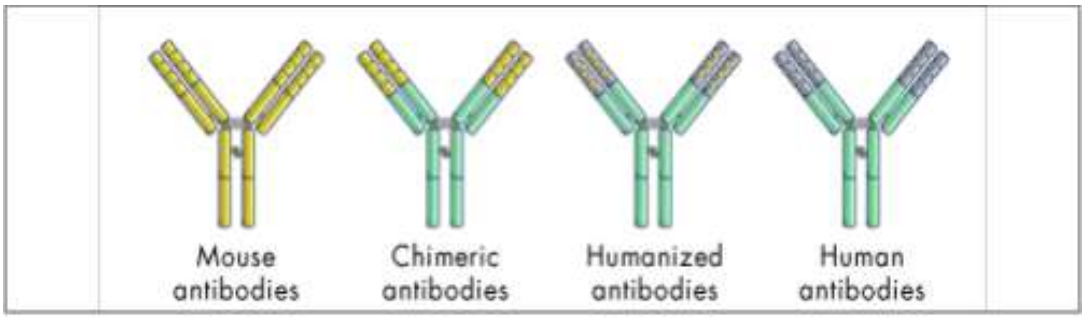

Figure 2: What monoclonal antibodies consist of

2.3) Structure of monoclonal antibody:

Monoclonal antibodies have a covalent heterotetrametric structures consisting of 2 disulphide-linked heavy chains, each linked covalently through a disulphide bond to a light chain forming a "Y" shape. The amino acid sequence in Y region varies in different antibodies. This variable region consists of 110 to 130 amino acids.

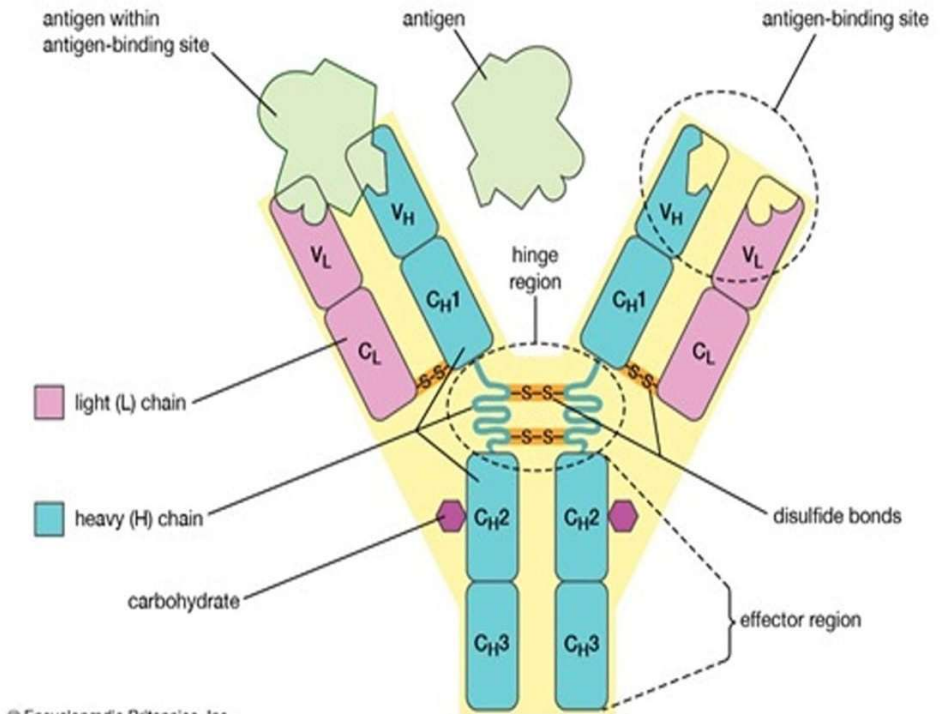

Figure 3: Structure of Monoclonal Antibody

2.4) Monoclonal antibody production:

The production process of monoclonal antibodies can be divided into 6 steps:

STEP I-> BALB/c mice immunization:

In this step a mouse is vaccinated with an antigen for e.g. BALB/c (Bagg albino) mice immunized with Fba- dendritic cell injection to produce antibody against Fba peptide.

This antigen introduced into mice can be a cell or a molecule etc. Various techniques like Enzyme Linked Immunoassay can be used to screen serum of mice.

Mouse vaccination is necessary to introduce an antigen in a way that is sufficient to cause the humoral immune reaction to the organism. This crucial move may lead to antibody secretion from the cell against the antigen selected.

The mice are sacrificed when enough title is reached. Spleen of the mice is separated aseptically and mechanical or enzymatic methods are used to disrupt it to the spleen cells. Gradient centrifugation techniques is used to isolate the spleen's monocytes cells from remaining cells for fusion.

STEP II-> Myeloma Cells Preparation:

Myeloma cells are cells that can divide indefinitely and lack HGPRT enzyme. To ensure the sensitivity of myeloma cells to hypoxanthineaminipterin- thymidine medium, the cells are reacted with 8-azaguanine which is a triazole guanine analogue which is purine nucleotide biosynthesis inhibitor.

8-azaguanine can be introduce in the normal cells by formation of 8-azaguanosine monophosphate through the enzyme hypoxanthine-guanine phosphoribosyl transferase thus it blocks the synthesis of purine nucleotide. Thus, the cells which do not contain the enzyme HGPRTase are not able to incorporate 8-azaguanine and so they continue to function normally.

STEP III-> Spleen cells and myeloma cells fusion:

Myeloma cells are fused with the spleen cells of the immunized mice. Polyethylene glycol is used for the fusion for very short time period as PEG is toxic and so it is important to remove it which can be done by washing. The fused cells are then placed in selection media Hypoxanthineaminipterin-thymidine (HAT). This media contains aminopterin which is an inhibitor of dihydrofolate reductase enzyme responsible for the nucleotide synthesis thus aminopterin inhibits the nucleotide synthesis. Due to this only the cells which are fused will cultivate on HAT. These cells are then spread on the feeder cells containing murine bone marrow thus promoting the growth of hybridoma cells only.

STEP IV-> Cloning of the hybridoma cells:

There are 2 widely used methods for cloning. Isolation and cloning of hybrid cell is done to obtain the desired antibody.

Limiting dilution method:

This method involves serial dilution of suspension containing hybridoma cells. Then the part of each dilution is transferred to culture wells in such a way that only one hybrid cell is present in each well. This is done so that monoclonal antibody is produced.

Soft agar method:

In soft agar method hybrid cells are grown on soft agar medium and due to this technique, many cells can be cultured in medium to produce colonies at once.

To attain maximum manufacturing of monoclonal antibodies combination of both methods is practiced.

STEP V-> Screening:

Screening of the hybridoma cells is done to obtain the antibody of the wanted specificity. So, from each culture of hybridoma the culture medium is tested to determine the specificity. Screening is usually done by 2 techniques Enzyme-linked immunosorbent assay and Radioimmunoassay.

In both techniques, the desired antibody attaches to the particular antigen which is coated on the plastic plates and the remaining free antibody and other medium components are removed by washing. Thus, screening enables us to identify hybridoma cells that produce the needed antibody. This antibody which is secreted by hybridoma cells is known as monoclonal antibody.

STEP VI-> Cloning of antibodies:

Cloning of desired antibody is performed in vivo by culture bottle method.

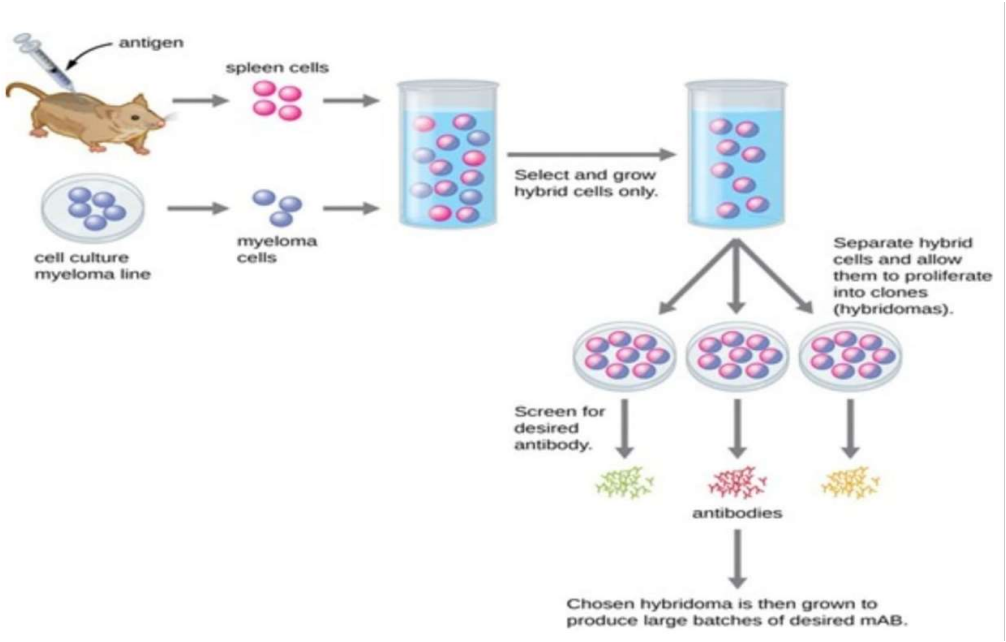

Figure 4: Monoclonal Antibody production process

3) CHROMATOGRAPHIC METHODS FOR PURIFICATION AND IDENTIFICATION:

3.1) Affinity Chromatography:

The most selective chromatographic technique is the affinity separation. It separates proteins covalently attached to the matrix of chromatography on base of reversible interaction among analyte & the ligand.

Affinity's higher antibody to the Immunoglobulin G-type antibody lays the foundation for purification of Immunoglobulin G, IgG fragments etc. The protein A chromatography technique involves a transfer of a smooth, translucent supernatant via the column at pH around 6 to 8 where the antibody binds and the

contaminants including media constituents, host cell proteins (HCP) of cell culture float via the column. An additional intermediary cleaning step can be performed so that un-specifically bounded impurities can be extracted from the column, followed by the material being eluted at pH of 2.5 to 4.

Affinity Chromatography technique is usually employed as initial phase in the cleansing because of the ability of protein A chromatography technique to remove the process related impurity like DNA, host cell protein, media components of cell culture, endogenous and price effective binding efficiency. The substance obtained after this step is robust and pure because of the removal of proteases enzyme and other constituents of media which can contribute to degradation.

Protein A resin is categorized in 3 major groups on the basis of resin backbone composition:

1. Glass or silica based: for e.g. Prosep Va Ultra
2. Agarose based: for e.g. Protein A Sefarose fast flow MabSelect
3. Organic based polymer: for e.g. MabCapture, polystyrene divinylbenzene Poros A

All these 3 resins are resistant towards urea, low pH, reducing agents and guanidinium hydrochloride high concentration. The height of the column's bed is between 10 and 30 cm, depending upon the resin's properties like particle depth, pores depth & compressibility. Dimensions of the flow rate and column decides the time until an antibody will reside on column. The standard linear speed used for measures for affinity varies from 300.00 to 500.00 cm per hr. Binding potential varies from 15 to 50 gm antibody/litre resin, thus depending on the antibody to be purified, rate of flow and matrix used.

Fahrner et al (Hui F. Liu, 2010) have mentioned a tool for assessing the complex binding ability of Protein A resins. A lesser loading rate of flow raises the

residence time of antibody & facilitates binding higher capacity of binding. This leads to a lengthier processing time/ cycle, needs less cycles and per cycle, requires fewer cycles and fewer buffer is used for each batch of collected CCF.

Many considerations like cost of resin, flow rate, measurements of the columns which are available length of buffer solution and the process time should be measured for designing or developing a protein A chromatography procedure. If large amount of CCF are to be extracted, more importance is given to the high price of resin rather than the number of cycles and the size of column. The resin's lifespan is over 200 cycles. A variety of factors influence the lifespan of Protein A resin that is available. One element is the amount of leached protein A at a time. The cleaning procedure also affects the functional lifetime of Protein A resin, as both chemical ligand degradation and inadequate cleaning are significant. Acidic solution with a low pH of less than three and solutions containing chaotropic chemicals like urea, guanidine HCL can usually utilized to remove some impurities and even some antibodies. A sophisticated method of cleaning will guarantee that the components and contaminants are extracted which weakens the resin's Protein A and retain useful period.

After each purification step, agarose and polymer-based resins are washed with a dilute solution of sodium hydroxide (0.05–0.2 N) for a 30 min contact period. A ligand derived from Protein A which is base-stable can resist harsh alkali-stabilized Protein A that can withstand extreme cleaning solution like 0.1to 0.5N NaOH is developed in agarose-based technique.

A typical issue with the resins of Protein-A is the un-specific binding of impurities like HCP, host cell DNA and other such impurities obtained from the culture of cell and the binding impurities level depends upon the resin, extracted CCF's composition and the conditions of loading and washing of the columns. Many intermediate clean solutions which contain additional components or salts have been developed. For example, numerous lipophilic electrolyte diluents like

tetramethylammoniumchloride & tetraethylammonium chloride (TEAC) were developed as efficient glass-based resin wash reagents to decrease the host cell proteins that are bound and co-elute with the product of antibody. The buffer solution composed of polymer and salt showed a similar result upon the elimination of impurities. In a framework purification process of antibody, 2 polishing steps of a chromatography are used frequently, nonetheless an efficient transition washing step may remove the requirement for 1 on these procedures.

Removal of antibody from affinity chromatography involves pH 2.5 to 4 where enclosed viruses like murine retrovirus may be efficiently inactivated; but such an extreme environment may theoretically change the antibody's biological function or trigger aggregation of antibody. Several components of the buffer, like arginine hydrochloride, citric acid, glycine HCl, acetic acid and phosphoric acid had been tested for the consistency of the antibody and the elution pool profile. The elution pH choice is based on the antibody's binding affinity to the resin. There are few antibodies having more binding affinity compared to others, allowing a lesser elution pH. Increase binding affinity is mostly contributed through linking the fAB region to ligand of protein-A; however, the base-stable protein-A obtained ligand have demonstrated a peculiar binding-affinity for many monoclonal antibodies tested, and the contribution via the Fab region is assumed to be reduced. Thus, the production and optimisation of Protein-A Chromatography method must be specialized in determining the binding efficiency at operating flowrate used on the production scale, determining the number of cycles needed to handle the clarified CCF lot, and developing an intermediate wash stage that can effectively remove host cell protein, DNA and other non-specific bonded impurities and optimize elution conditions for wanted elution pool features like pH, low capacity and pools conductivity is achieved as well as to attain the surety of the product quality. Thus, it is very important that method is to make sure that the method is expandable and continuous.

3.2) Ion-Exchange Chromatography:

Exchange chromatography step is performed in every monoclonal antibody purification process. Ion exchange technique is relatively effective in the separation of monoclonal antibody. Not so expensive resins are used in the technique and thus may be added earlier or later in purifying stage. Cation-exchange chromatography is used as a primary collection phase for an antibody having a precise isoelectric point but ion exchange chromatography is more commonly used as polishing stage following Affinity chromatography.

This chromatography process is ideal to reduce high molecular weight aggregates, charging shapes, residual DNA and host cell protein, leached protein A and viral particles. This ion-exchange technique is suitable in minimizing heavy molecular weight aggregates, residual DNA, charging forms, leached protein A, HCP and viruses.

There are many widely used ion exchange resins suitable for commercial use. The backbone resin matrix contains agarose and dextran, glycidyl methacrylate, divinylbenzene polystyrene, and polymethacrylate. Such resins can be washed and sterile by using sodium hydroxide having higher concentration. The speed of flow broadly varying between 100.00 – 500.00 cm/h. There are known ligands which contain strong & weak cation as well as anion and exchange. The selection of resin framework and ligand are mainly driven via the capacity of binding, resolution required be guided by the required resolution, binding capacity, and flexibility of use at manufacturing scale. Anion-exchange chromatographic technique utilizes an immobilized set that is positively charged to the resin. This is a very effective method for eliminating impurities found during process like endotoxin, DNA, leached protein, retroviruses, host cell protein, dimer / aggregates and adventitious microorganisms. The technique is used in bind & elute mode or flow-through mode, based upon the impurity to be eliminated and the antibodies value of pI. For the antibodies with a pI over

7.50, which involves the human antibodies to immunoglobulinG1 & immunoglobulin G2, flow-through mode is safer option to eliminate impurities. The impurities bound with the resin in flow-through mode, thus allowing desired product to flow through. Loading potential of base, that is the antibody mass to resin mass, is always very high, as only impurities fill the sites where binding occurs on the resin. Antibodies including humanized immunoglobulinG4 which have pI value between acid and neutral is purified by eliminating drug and process related impurities using bind & elute mode from the drug.

3.2.1) Anion-Exchange Chromatography:

This chromatographic technique utilizes resin immobilised to positive charge group like diethyl aminoethyl, dimethyl aminoethyl which are basic in nature or quaternary aminoethyl, trimethyl ammonium ethyl as strongly basic. This is an effective method for eliminating endotoxin, HCP, host cell deoxyribose nucleic acid as well as product-related contaminants which include microorganism as parvovirus pseudo rabies and aggregating molecules.

This may be used in 2 modes flow-through/ bind & elute mode, depending upon pI of impurity and antibodies to be eliminated. To eliminate impurities present in antibodies having pI over 7.500 value like immunoglobulins G1 and G2 then (flow-through) mode is considered as safer alternative. The impurities bind to the resin in flow-through mode, and pass through the substance of interest. The weight a base can occupy that is the weight by weight of antibody to resin can be very higher as only the contaminants can hold the binding-site on resin. To eliminate both the types of impurities from desired drug for antibody which have pH value between acid to neutral this involves several immunoglobulinsG4.

Flow-through mode: This mode is widely used as highlightening step to purify the monoclonal antibodies developed to eliminate residual contaminants like

microorganisms, HCP, secreted proteins, deoxyribose nucleic acid with 2 to 3 units. Requirements are selected to prevent the substance from sticking to the base, and acid contaminants such as nucleic acid and host cell proteins are chosen. Based on the resin, load and charging condition of the antibody drug, the volume of the drug filled will reach 10g/l of resin without influencing the drug performance. The quantity of the material filled in a mode of transmission normally relies on the impurities substances as well as the quantities to be removed. A decreased degree of impurity will result in a larger amount of the substance being prepared.

Bind & elute mode: In the production of monoclonal antibodies anion exchange chromatography has also been applied in bind & elute mode. Firstly the product mass is filled upon column then the desired product is removed in phase or linear gradient with higher salt concentration, leaving the most impurities bound to the column. The impurities are removed from column in the purifying or regeneration stage.

In developing and optimizing of this technique in (bind& elute mode) the pH must be around the product pI to achieve overall negative charge upon antibody's surface, thus achieving more linking ability in the chromatographic method. Optimum condition of removal must be accomplished with linear gradient or phase removal. This provide little volume of drug concentrates as well as more concentration, whereas good control can be achieved by linear gradient elution method on the reproducibility & monitoring of the process. If phase elution is selected for the production method, detailed research of many batch of chosen resin should be performed to demonstrate the robustness of process efficiency. The result of linear gradient may be considered as initiation step when designing step-elution condition, starting from the concentration of salt where the large clusters starts to reduce. This exact rule is applicable even if faster or slower linear gradient system is carried out, to

the selection of primary NaCl solution & buffer's conductivity as used to create a phase elution programme.

3.2.2) Weak partition chromatography:

It is a type of anion-exchange chromatography developed recently. It will allow for a two- chromatography recovery cycle involving the exchange of anions & protein A for several antibodies. The procedure can be performed isocratic ally nonetheless, compared to (flow-through) mode the pH as well as conductance both are selected in such a way as to improve the drug & contaminants binding, achieving partition coefficient of antibody from 0.100 to 20.00 & ideally from one to three. It benefits from the statistic that the substances are less acidic than impurities which needed to be eliminated. The impurities are strongly attached compared to the flow through mode thus enhancing the removal of impurities though antibodies & impurities have bound to resin of anion-exchange. Thus, in circumstances where value of partition coefficient is raised, impurities which bound weakly and are inefficient extracted through flowthrough mode may be extracted at higher level.

The amount of product obtained can be increased via performing a washing step at last stage of process & an average of ninety percent of series of product being developed clinically. The capacity of loading should very higher often approaching one thousand five hundred grams per litre which can go outside the value that small columns are efficiently used in the recovery phase, thus over production cannot be used every time. Because of increase in removal of product-related impurities, HCP and microorganism in (flow through) mode compared to anion exchange chromatography, 2 column recovery cycle is allowed for several products by poor partition chromatography. Removing larger aggregates less efficiently as the recovery cycle using 3rd

chromatographic step like cation exchange thus not making it possible for all products is challenging.

Weak portioning chromatography's aspect is need for optimization of the pH and counterion conditions for all products. To identify minimum operating requirements for weak partitioning chromatography high throughput test are designed. A robotic framework that uses resin-filled 96-well filter plates enable optimization in a batch mode thus permitting partition coefficient to be calculated for drug substances & contaminants over variety of condition. Subsequently, this is optimized by column chromatography or when the HTS functionality not usable, so it can be achieved by using filled columns. The conditions of flow through mode was used as initiation step for obtaining optimum composition of mobile-phase.

3.2.3) Cation exchange chromatography:

This technique uses modified-resin having functional groups that are having negative charge. The resins may be highly acidic like sulphoisobutyl & sulphoethyl and weak acid ligands like carboxy groups. It can purify monoclonal antibody having neutral to basic range of pI. Most of the genetically engineered immunoglobulin 1 and 2 sub types are ideal targets in cation-exchange techniques, where in loading stage the antibodies are attached to resin and by raising the conductivity or the pH of buffer can help in removing these antibodies. In load and wash fraction the most negatively charged process-related impurity like HCP, DNA, endotoxins and leaked proteins are eliminated. Cation exchange chromatography may also have separation capacity to remove varieties of antibodies from the targeted drug, like deamidated drugs, oxidized species & truncated forms of N-terminals and also higher molecular weights.

The dynamic binding ability of monoclonal antibodies to the resins used in this chromatography depend on the conductivity & pH. Effect of these factors on

monoclonal antibodies adsorption to 2 separate interchange media of commercial cation. Confocal laser scanning microscopy showed that the compounds are not able to enter deep into beads of chromatography under the conditions tested Proteins accumulated on porous vessels on the membrane's outer layer are likely to prevent certain substances from reaching the openings so this avoidance is reduced at higher electrical conductivity, resulting in a specific interaction with the overall charge & conductivity of solution that yields optimal dynamic ability.

Based upon resin, requirements of loading and ligand the average binding efficiency obtained may be greater than 100.00 gram per litre of resin volume but the elimination of impurity depend strongly on density. Strong resin load usually leads to greater rates of contaminants in concentrates of elute but various ligands & resin bead dimension having a major effect upon the impurity. Hence, to choose a right resin is done by performing screen test. The correct resin can be the resin having good linking ability and highest selectivity as well as resolution should be carried out. This study is connected to the creation of elute state.

The same principles defined for anion exchange chromatography is established to cation- exchange as regards the creation of the elution system. The creation of elution criteria is related to the elimination of impurities and drug reservoir characteristics that may be quickly extracted through consequent activity of the device. In general, the best elution condition can be calculated by performing pH gradient process.

3.3) Hydrophobic interaction chromatography:

Depending upon its hydrophobicity, hydrophobic interaction chromatography is an effective method for isolating the molecule and enhances certain technologies which separate proteins on the basis of size, charge or affinities. Generally, specimen is mounted on column in buffer having high salt concentration. These sodium present in the buffer then interferes with water species. The salt in the

buffer then interacts with water molecules to decrease protein substances solution solvation, thus revealing water insoluble parts in the test protein which adheres to hydrophobic interaction chromatography resin. Less amount of salt is required in facilitating the binding if the substances are more lipophilic in nature and to remove the sample from the column a gradient of reducing amount of salt is generally used. The penetration of substance hydrophobic area rises if ionic-strength reduces and so to enhance hydrophobicity the substance is washed from the column.

In liquid chromatography hydrophobic interaction chromatography is considered as highly flexible technique and often employed as reasonable purifying approach. Hydrophobic interaction chromatographic resin having ligands containing phenyl or butyl groups used in purifying of monoclonal-antibody, but because of good productivity of affinity chromatography, hydrophobic interaction chromatography is therefore used as transition stage after affinity or after ion exchange chromatography. Hydrophobic interaction chromatography is effective in eliminating higher % of dimers which provide very high output. Hydrophobic interaction chromatography in (bind & elute) mode separates process & product impurity effectively through the monoclonal antibody. Most of the impurities are separated from the substance by choosing a correct concentration of salt from buffer by using gradient removing process.

3.4) Hydrophobic Charge-Induction Chromatography:

It focuses upon ligand ionized at small pH values that depends upon the Ph. HCIC uses a high-density heterocyclic ligand such that the adsorption can take place through a hydrophobic interaction without the requirement of high lyotropic salt level. Reduction of pH encourages desorption so that the repulsion is produced between binding protein and ionized ligand to overcome extreme elution requirements problems used with extreme water hating resins.

Because of the massive price of resin of protein-A & its poor resistance to harsh condition, the resin used in this technique has proposed as a possible option to protein-A resins for purifying immunoglobulin antibodies. Effective elution and binding of antibody free of salt have been shown at far advanced pH range than affinity chromatography; however, one important downside of this method is possessing strong un-specific binding and may be less successful in lowering impurities like host cell protein compared to Affinity chromatography.

Therefore, it can be difficult to use this technique in purifying of antibodies. Constant analysis of hydrophobic charge induction chromatography in conjunction with some cleaning measure can offer adequate elimination of the remaining impurities like chromatography of ion-exchange, precipitation, crystal formation, would decide if it can perform an important part in the future growth of antibody purification process.

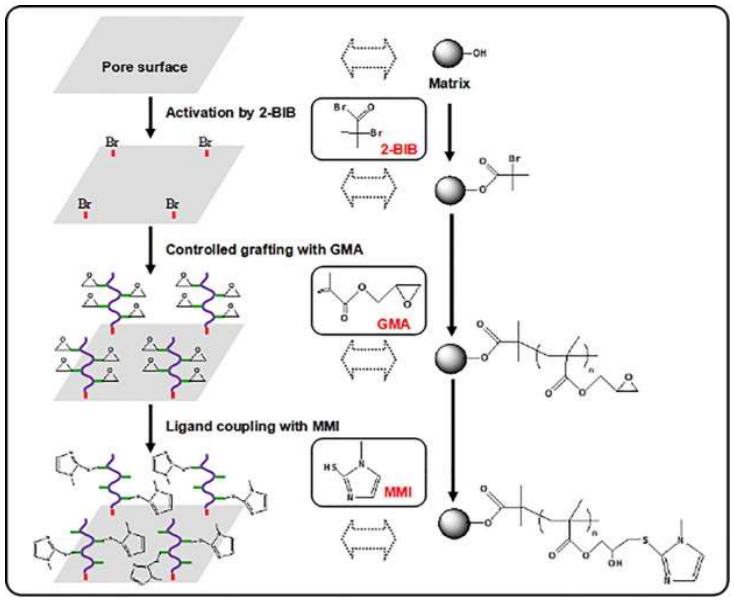

Figure 1: Hydrophobic charge induction chromatography

3.5) Ceramic- Hydroxyapetite Chromatography:

Ceramic hydroxy apetite chromatography is type of chromatography used for isolation & purification for nucleotides, protein, microorganism, enzymes and some substances or molecules. Originally made up of hydroxyapatite by the Tiselius method in the 1950s. Thereafter modifications also led to surplus phosphor found. Due to the brittle, square shaped crystals formation with characteristics of poor flow, strain, & stability. Hydroxyapatite has various properties of separation, and a particular selectivity and resolution. It can distinguish proteins which appears homogeneous to chromatographic and electrophoretic technologies.

For eliminating dimers or aggregates and leached protein-A, ceramic hydroxyapatite chromatography along gradient elution of sodium phosphate is used in the purifying of monoclonal antibody. Gagnon et al. illustrated effective use of linear sodium chloride gradient in chromatography to the human antibody immunoglobulin Ceramic hydroxyapatite n column operation can be used as a powerful polishing stage for big-scale monoclonal antibody development in bind or elution mode; however, a number of factors remain, like resin batch to batch variation, resin lifespan and virus clearing capacity.

3.5.1) Advantages of CHT:

HAP's is also known affinity to DNA has made HAP an important method for eliminating and purifying DNA. DNA binds HAP to crystal calcium by the metal interaction between DNA phosphates. The net binding strength is decreased by electrostatic repulsion among crystals of calcium & deoxyribose phosphate. The latter effect is dampened by adding NaCl, which causes increased retention of DNA.

As with endotoxin, even deoxyribose nucleic acid bounds ceramic hydroxyapatite mainly due to the molecule of phosphate found in the lipopolysaccharides. It is

major constituent of gram-negative bacterial cell wall, like Salmonella Tryphosa, E. coli, Pertussis Bordetella and Salmonella Typhimurium. Lipopolysaccharide in humans is responsible for severe pathological reactions and thus the medications which are administered through intravenous route should be free from lipopolysaccharide.

Throughout the case of drug compounds, removal of these must be important due to the potential toxicity for protein A & higher incidence for the neutral antibodies. Protein A is a complicated associated, with monoclonal antibodies which is elucidated afterward simplified antibodies. Dimers of proteins are eliminated as a result of their concentration on CHT increased. Monomeric IgG can be effectively isolated from the sum with careful control of the pH and phosphate conditions. (Monoclonal Antibody Purification with CHT, 2006)

With regard to the way in which immunoglobulin G binds to the Ceramic Hydroxyapatite, earlier research showed preservation of HAP via combining 2 key methods: exchange of Phosphorous cations involving the interaction between phosphate atom which is negative charged with anion amino groups on the matrix & calcium metal interaction involving among carboxy group, positive charging groups While the initial attraction of the latter mechanism is electrostatic, the actual bond among group of calcium and carbon protein is very strong.

mechanism is electrostatic, the actual bond of coordination amongst the group of calcium and carboxyl proteins is far strong.

3.6) Multimodal Chromatography:

Multimodal gums with ligands e.g. Capto-adhere can be used to purify the antibodies. Varying forms of interactions of multimodal resins can be mixed like ionic contact, bonding with hydrophobic interactions and hydrogen interactions.

Selectivity which is offered due to the performance of multimodal resins is contrasting to the normal ion-exchange ligands, thus making multimodal resin ideal for solving lower- and higher-conductivity or pH purification problems. A multimodal anionic resin is tested as a 2nd stage in the purification of antibody after affinity chromatography to extract host cell proteins, protein A which is leached and the aggregates. An extra careful experimental technique is required to achieve optimum separation efficiency because of the complexity of the chemistry. Factors like variation in batch-to-batch, lifetime of the resin, viral clearance, operative cleaning & renewal, suitability of platform and the chances of identifying an alternative of resin for obtaining next resin effort should be taken into consideration for other types of chromatographic resins.

3.7) Membrane and Filtration Chromatography:

To purify and isolate monoclonal antibodies and other recombinant deoxyribose nucleic acid from primary broth cell- culture to last sterile filtration the clean bulk solution is achieved using membrane and filtration technique.

3.8) Membrane Chromatography:

Membrane chromatography operates likewise to the packed column chromatography in standard module filtration format. Membrane chromatography uses microporous membranes, typically throughout the membrane structure in many layers containing ligands which are functional and are linked to the surface of inner pore.

Over traditional bed chromatography the advantage of membrane chromatography is removal of diffusive pore. Binding site in membrane chromatography are not nestling inside large pores but are located along or with

the pores. Consequently, the mass-transport to the binding site of biomolecules on of biomolecules relies on the capillary action rather than diffusion, so membrane chromatography's binding capacities are mostly not dependent on the flowrate.

Membranes of polymer substrate bound chemically to specific ligand and multilayers of polyether sulfone, polyvinylidene fluoride and regenerated cellulose membrane make up the polymer substratum for membrane chromatography. Among the commercially available adsorptive membranes, the Q membrane absorber has attracted considerable interest from industry, particularly in purification processes of monoclonal antibody. As in traditional bed chromatography, Q membranes are usually used in the mode flow-through as polishing steps for eliminating minute impurities. At neutral to mild pH & low conductivity, impurities like endotoxin, host-cell protein, host cell DNAs, secreted protein a & bacteria binds to membrane Q, while generally simple molecules of antibodies pass without bound through the matrix of membrane. Due to the commercial availability of new anionic mixed-mode membranes the ability to bind impurity or remove impurity at increased range of conductivity and extended operation conditions of anionic membrane is improved.

This technique is widely used at lab scale and pilot scale for purification of protein but there are few drawbacks which should be solved first and then the technique can be used at process scale production. Main drawbacks include irregular supply of flow distribution of flow, irregularities in the thickness of membranes, low capacity to binding, uneven distribution of membrane size, accessibility to appropriate scale-down tools. Poor flow distribution and low capacity of binding are attributed to low surface area. By optimizing the thickness of membrane or the number of layers of membrane, membrane interaction, pore size and use of flexible ligands binding capacities can be enhanced. Additionally, other essential factors, including the time period of membrane used, quality checking, batch-to-

batch variation and method economy, should also be considered for this technology.

3.9) Ultrafiltration

Ultrafiltration is a technique which is controlled by pressure and is primarily used transfer of buffer and protein concentration. It works on the principle of separation of substances on the basis of size, where minor particles move across ultrafiltration membrane and the larger particles are collected on membrane. Ultrafiltration can achieve separation by variation in the speed of filtration of the molecules across membrane under a pressure-controlled force. Buffer exchange is accomplished via process called defiltrations wherein desired amount of buffer is applied on device at a similar speed of the filtrate which is being extracted thus ensuring constant volume.

It is possible to separate substances of molecular weight 500.00D to 1000.00 kD by ultrafiltration's membrane having pore size of 1.0-20.0 nm as this membrane possess a special "skinned structure". This thin film of skin regulates selectivity of membrane which is having thickness of 0.5µm and a thicker macroporous substratum provides the mechanical energy. This allows trapping at top of membrane and not in the structure of filter. The membranes are made of broad range of polymers like polyether sulfone, polysulfone, regenerated cellulose and polyvinylidene fluoride. The polymers made by synthetic route show great tolerance towards the alcohols, bases, acids and increase temperature, permitting efficient washing of the membrane. Membranes of ultrafiltration may be recycled in this fashion deprived of flow rate degradation & cross-contamination. These polymers can have greater stability towards high temperatures and chemical substances however they are vulnerable towards fouling proteins. Polymer membranes made of cellulose by comparison, have poor binding to proteins and thus they may be harmed due to the rough cleaning process. Novel membranes of regenerated cellulose are less foul to proteins, easier to clean & shows superior

mechanical resistance. Despite of the favourable effects, the system's ability to penetrate and retainaing properties of cellulose membranes for the diafiltration & ultrafiltration of proteins are superior to other membranes.

Tangential flow filtration method is generally used in the ultrafiltration process wherein the liquid passes from the filter (cross-flow), peripheral to surface of filter as shown in the figure 6. The main benefit of tangential flow filtration is continuous cross-flow sweeping of the filter surface, minimizing the effectiveness of filtration and extent by which substances accumulate on the surface of filter. Usually, ultrafiltration processes & the diafiltration process can be are establish by means of continuous pressure which is retented, continuous filtration flux or the continuous pressure of trans-membrane. As it is evident that these monitoring mechanisms cannot consider the impact on membrane surface of the protein gel layer, a technique was developed to ensure the steady concentration of protein on surface of membrane. Improved product output, reduced surface area of membranes, consistent loading- time while monitoring the changes in the characteristics of membrane and feed are the possible advantages of this approach.

During diafiltration step the optimum concentration of antibodies is chosen so that the loading time and surface area is reduced. Ultrafiltration processes having increase concentration also result in the solutions being sticky and thick which restricts the final concentration. Current research has confirmed that increase in the temperature can be considered as approach to secure control on the formulations possessing higher concentration of rheological properties, and also to improve the complete transfer of mass.

The last sterile filtration or batch filtration of the substance is one significant process improvement feature of a final UFDF formulation phase. Typically speaking, sterile filtration is a major issue for each transitional pools of purification, then even high thus during the end of cycle wherein maximum

amounts of protein is found & the maximum interest was imparted to the substance. Proper scale-up methods for using machinery representative is important to production. Additionally, manufacturing companies have even produced novel type of asymmetric and composite membranes having advanced capacity performance, throughput capacities, recognizing the necessity for budget- effective, stable sterile filtration.

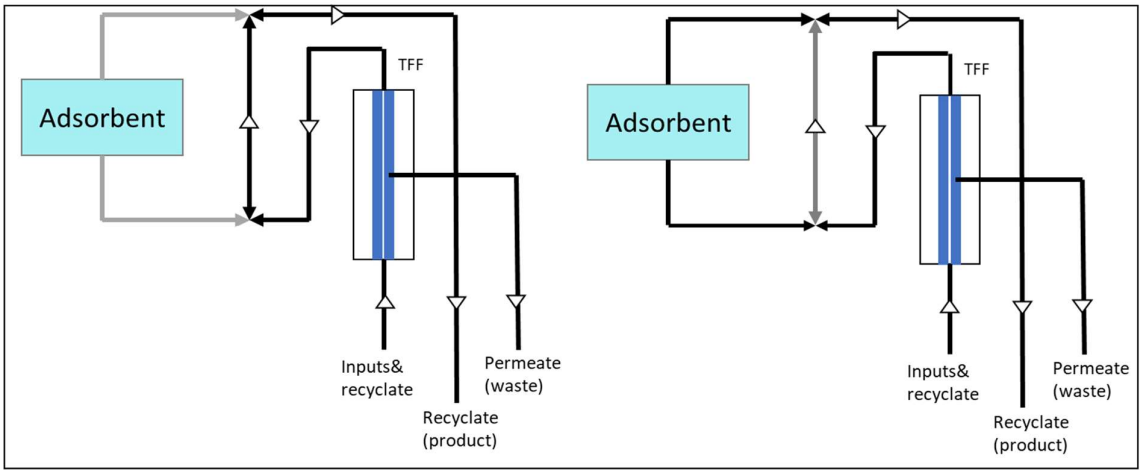

Figure 2: Utrafiltration

3.10) High performance tangential flow filtration

Ultrafiltration has a high throughput and low-resolution character inherent in it. Recent experiments have shown that ultrafiltration systems can be used on the basis of variations in protein charge to distinguish proteins of similar and somewhat different sizes. By manipulating the electrostatic interactions among molecules of protein and membrane apertures, separation is achieved. New High-Performance tangential flow filtration (HPTFF) technique is a 2D unit process where variations in the charge & size are used in the cleansing and separation processes. Additionally, the concentration of proteins & the replacement of buffers may be carried out in the identical unit method.

For electrolyte formulations, active proteins are covered by a dispersed electron cloud or electric dual sheet because of electrostatic reaction with the ground-ions and co-ions. In his technique, by optimizing buffer pH and ionic strength, the variation in hydrodynamic size among substance and contaminants may be decreased. The hydrodynamic size rises with ions and reduces with ionic strength due to higher protein conductivity shielding charges. High selectivity is achieved by rising the electrostatic isolation of the more strongly charging protein as compared to balanced proteins. Furthermore, membrane charging could further improve resolution among charge compounds and neutral ones. Using charged membranes gives the method a level of greater solidity relative to uncharged membranes. Higher retention is observed in positive charge membrane compared to the membrane that is negatively charged. High-performance flow filtration technique involves of membrane pore-size influences specificity effects by modifying the solute solvent extraction coefficient and filtrate liquid distribution. Bigger flaws of HPTFF membrane should be eliminated and pore size control should be controlled to improve performance.

Like in traditional methods of ultrafiltration, the efficient application of high-performance tangential flow filtration processes depends upon optimisation the operating flux, transmembrane strain, module and flow route architecture for increase specificity and protein chocking of the membrane for optimal performance. HPTFF has been reported for the elimination of infected cells protein and infected cells DNA contaminants in monoclonal antibody cleansing processes utilizing non-affinity chromatography methods coupled with high performance tangential flow filtration. This technique is even used in diafiltration process, wherein substance is preserved and impurities are extracted from retentate.

3.11) Virus Filtration:

In the production process of monoclonal antibody & other recombinant therapeutic proteins using mammalian cells produces endogenous retroviruses & thus sometimes get sicked with the adventitious microorganisms with the process.

Products obtained from mammalian can hold less than 1 virus/ million doses, because of safety concerns. An integral component of food safety security techniques is the use of elimination and inactivation of virus measures in developing method of purification. This is usually necessary that orthogonal measures to clear the virus using matching methods, thus the virus not eliminated via one technique the different methods may use to eliminate the virus.

Separation of virus may include virus removal based on size that complements certain measures to remove the virus. Only a limited number of strange wide ports can cause excess leaking of viruses, so it is important to produce virus filters to remove these major porous faults. It is usually achieved by using composite membranes to ensure the retaining of virus & mechanical steadiness that is required. The filters used in this technique are classified into retrovirus filters and parvovirus filters on the basis of the virus size distributed of the viruses which are then eliminated. To ensure successful virus retention and the passage of the monoclonal antibody it is essential for parvovirus filters for having small distribution of size. They are therefore usually prone toward impurities found in feed solution.

Chocking is usually caused during virus filtration due to the by the DNA; protein aggregates, partly denatured substance, and other fragments present in the filters. The use of suitable prefilters will greatly decrease this, and pre-filtration of feed may produce drastic effect on the performance of virus-filtration. Microfilters having 0.1 to 0.2 μm pore size can be used to extract out large impurities, however the slightly bigger impurities than protein is difficult to eliminate on the base of size. Prefiltration was observed across depth filters and charged membranes to support the viral filters with substantial defence. (Hui F. Liu, 2010)

4) CASE STUDIES:

4.1) Adalimumab:

The 1st accepted monoclonal antibody by Food and Drug Administration of USA, made of human proteins was adalimumab. It is a recombinant, IgG1 antibody which particularly binds to tumour necrosis factor-α. It is formed by recombinant DNA technique in the mammal cells and consist of heavy chain & light chain variable regions and human immunoglobulin G1k continuous zone.

Tumour necrosis factor – α is released by immune as well as non-immune cells and is an endogenous pyrogen and thus is responsible for producing fever, cell death, inflammation. Thus, it is important to control the release of TNF in the body which is achieved by adalimumab which is TNF blocker and can block both the biologically active soluble and membrane bound TNF α. This TNF are capable of interacting with the 2 receptor - TNF R1 and TNFR2 and thus cause harmful effects out of which one of the main causing its various effects. One of the prominent effects include beginning of pro-inflammatory cascade through rapid cytokine initiation and then results into damage to cells and destruction. Thus, adalimumab is used to cure Rheumatoid Arthritis, crohn's disease and ulcerative colitis.

4.1.1) Mechanism of Adalimumab:

There are 2 ways through which adalimumab acts. Firstly, adalimumab specifically attaches directly to the tumour necrosis factor α and thus inhibits the interaction with tumour necrosis factor p55 and p75 cell surface receptor. Secondly in the existence of complement cell lysis is induced due to the tumour necrosis factor. (Deshmukh, 2019)

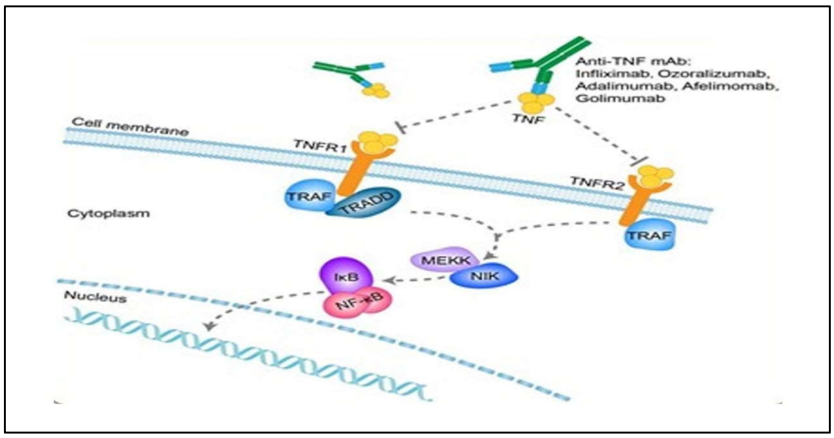

Figure 3: Mechnism of action of Adalimumab

Impurities present in adalimumab include:

A. API:

1. Synthetic impurities (starting materials, intermediates, reagents, ligands etc.)
2. Residual solvents
3. Inorganic and heavy metals

Other impurities:

1. Microbial impurity like viral, bacterial.
2. Polymorphs
3. Isomers
4. Host cell DNA, protein
5. Herbal substance

B. Drug product:

1. Organic synthetic impurities
2. Process related impurities like granulating solvents, Protein A, PEG, Antifoam etc.
3. Organic degradants i.e. Hydrolysis, oxidation, decarboxylation, racemisation etc.

4. Package impurity like leaches and extractables.
5. Microbial contaminants
6. Product related impurities like Host cell DNA and Protein, aggregation.

4.1.2) Testing methodologies:

Different Spectroscopic and Chromatographic methods are used to identify and characterize the impurities, either alone or along with other methods. Some of them include HPTLC, TLC & HPLC. The frequently methods in impurity profiling include GC-MS, LC-NMR, LCMS-MS, and LCNMR-MS.

Table 2: Impurities in adalimumab & techniques to remove it

Type of Impurity	Technologies
Inorganic/ trace elements	ICP-MS, ICP-OES
Residuals	GC-FID/ MS
Bioburden	BET, Microbial
Biologicals	RNA, DNA, stray proteins, Viruses, Peptide sequence, Immunogenicity
Extractables/ Leachable	GC/HPLC/ICP
Organic	FTIR, HPLC-UV/RI/MS, UV, NMR, SFC, Capillary electrophoresis, preparative LC, degradation products, extraction and purification.
Genotoxicity	Repeat dose toxicity

(Simon, 2014)

4.2) Trastuzumab:

Trastuzumab is IgG1 kappa humanized monoclonal antibody. It is prepared by recombinant DNA technology. Trastuzumab is used to treat different cancer such

as gastric cancer and metastatic breast cancer. US FDA in 1983 approved trastuzumab in treating metastatic breast cancer in humans. Trastuzumab may be used alone or together with other chemotherapy medication. It is given by slow injection in the vein or just under the skin.

4.2.1) Mechanism of Action:

In a cell-based assay trastuzumab binds specifically to the extracellular domain of a receptor known as Human Epidermal Growth Factor 2 protein. Trastuzumab antibody human structure comprise of immunoglobulin G1 kappa region while the murine antibody comprises of complementarity- determining regions. This region in the cell-based assay bounds through increase affinity to the extracellular framework of human beings and prevent cancer cell growth, proliferation and survival. Trastuzumab is an antibody-dependent cellular cytotoxicity (ADCC) mediator that prevents human epidermal-growth-factor receptor dimerization thus mainly used for treating positive breast cancer.

4.2.2) Testing Methodologies:

Table 3: Impurities in trastuzumab & techniques to remove it

Type of impurity	Techniques
Endotoxins	Affinity & Anion exchange Chromatography
Unwanted Proteins	Affinity Chromatography
Aggregates & sub-visible particles	Cation exchange, Hydrophobic interaction chromatography
Retrovirus	Anion Exchange Chromatography
DNA	Affinity Chromatography
Host cell protein	Affinity Chromatography

Protein A	Affinity Chromatography

Other Techniques:

1. ***Size Exclusion Chromatography by HPLC (SEC-HPLC):***

Impurities can be determined by size exclusion chromatography and the result complies with limits approved for the product during market authorization.

2. ***Ion-Exchange Chromatography by HPLC (IEC-HPLC):***

Ion Exchange Chromatography is used for determining impurities. To enhance separation the sample is run with correct gradient software. It complies with the limits accepted during marketing authorisation for the specific product.

3. ***SDS by Capillary Electrophoresis (CE-SDS):***

Capillary Electrophoresis determines the impurities. This study was carried out in both reduced and non-reducing condition. Analyse the sample and check against the molecular weight marker using a capillary electrophoresis system capable of separating between 10 and 250 kDa and detecting UV. (Herceptin, INN-trastuzumab)

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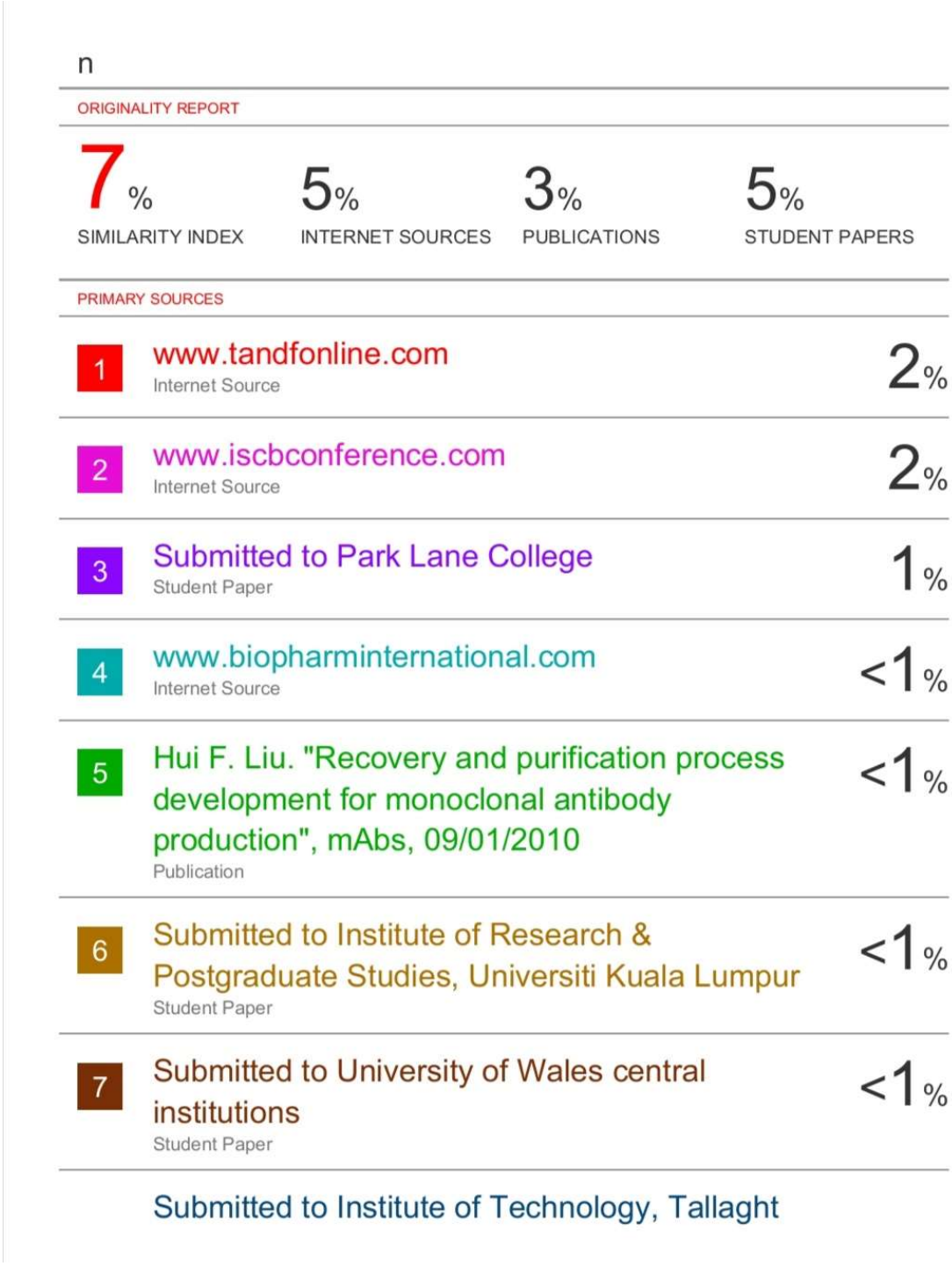

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