“Purification of Protein Charge Variants and Determination of their Potency”

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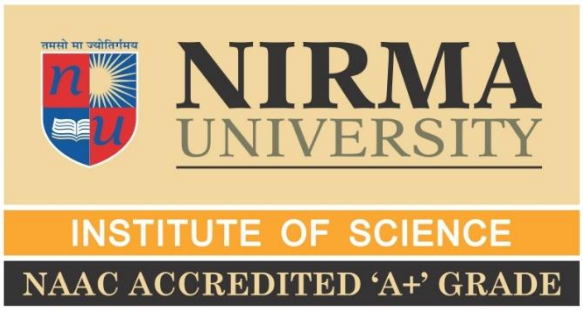
Nirma University

In Partial Fulfillment of the Requirement for
the Degree of

Doctor of Philosophy
In
Science

By

Tarun Kumar
(20PTPHDS79)



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Dedicated to My Parents

Smt. Kusum Varshney

Shri Devendra Pal

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Abstract

Monoclonal antibodies (mAbs) related biological drugs are fastest growing in therapeutic industries across the globe. mAbs were in top best-selling categories of antibody related therapies in 2015. The interest towards mAbs biosimilar development is increasing day by day as there are many patents filed by innovators are supposed to be expire soon. Biosimilars are highly complex and similar biological drugs are developed with different manufacturing processes which are not similar to originator manufacturing process. Due to this, biosimilar product inherently has quality differences in comparison to innovator molecule which may be related to size, charge and glycosylation. Despite these differences they are supposed to demonstrate similar behaviour in safety and efficacy profile to the reference product and these differences should not be clinically meaningful. Charge variants are one of the critical quality attributes and sources of heterogeneity.

Omalizumab (Xolair) is a humanized monoclonal antibody derived by recombinant DNA technology. It binds specifically to immunoglobulin E (IgE) which plays a major role in allergic reaction. In this study, biosimilar product of Xolair was expressed in mammalian cell culture process in laboratory to isolate charge variants (acidic and basic) and main peak. Isolated charge variants were purified with preparative cation exchange chromatography technique and characterized with different analytical tools includes size exclusion chromatography (SEC-HPLC) and cation exchange chromatography (CEX-HPLC). Purity of acidic variants, main peak and basic variants was more than 90% estimated by SEC-HPLC and CEX-HPLC.

Highly purified charge variants of Xolair biosimilar were also assessed for their impact on *in-vitro* potency and stability at different thermal stress conditions (2-8 °C and -20 °C). The study data indicates purified charge variants (> 90%) have no impact on *in-vitro* potency and are stable at different thermal stress conditions up to a week.

This is not only one factor related to charge heterogeneity which shows no impact, but also other factors can affect potency of mAbs. Hence, product safety and efficacy are dependent on other quality parameters those needs to be ensured throughout the product life cycle. This study also showed that biological activity of mAbs is totally dependent on mAb molecule interaction, either Fab or Fc is interacting and providing the drug response

Chapter 1
Introduction

1.1 Biotherapeutics

Biotherapeutics or biologicals are drug products which are produced from a biological system or source. These products include recombinant proteins and hormones, cytokines, growth hormones or factors, monoclonal antibodies (mAbs), gene therapy products, vaccines, cell-based products, stem cell therapies, gene-silencing/editing therapies and tissue-engineered products (Johnson, 2018).

In the biotherapeutic field, large biological molecules such as monoclonal antibodies (mAbs) have brought dramatic benefits to the individualities suffering from a critical illness, where former therapies-antibiotics to statins and small molecules were not too effective or towards non-existent (Oskouei et al., 2021; Bhunia et al. 2013; Joshi et al., 2021; Reslan et al., 2020). Now, mAbs based biotherapeutic drugs are the face of new drug development in biopharmaceutical industries. Starting with first approval in 1986, now more than a hundred marketed mAbs are serving patients with previously unmet medical needs (Beck et al., 2010; Perobelli et al., 2018; Martin et al., 2023). mAbs are developed very fast against numerous diseases not only due to their specific targets in different areas related to immunology, neurology, metabolic disorder, and oncology, *etc*. but also attributed to their accessibility and cost-effectiveness (McAtee et al., 2012; Kadkhoda et al., 2021). Information gathered in Table 1 discussed about Food and Drug Administration (FDA) approved marketed monoclonal antibodies and their details like brand name, manufacturer, molecular weight, and year of approval (Oskouei et al., 2021; Lu et al., 2020). These mAbs are used for different disease target such as Sickle cell disease, Macular degeneration, Rheumatoid arthritis, Psoriasis and different types of cancers.

mAbs are large biological molecules with complex biological structures; hence the manufacturing of mAbs is very challenging due to their complicated structure, functions, and *in-vitro* media and feed conditions inside the bioreactor (Berkowitz et al., 2012). Biosimilars, which are very similar to the originator molecule, has contributed to the availability of comparatively low-cost treatment due to its developmental and investment cost. Biologic patent expiration (2006) has supported biosimilars development globally. Biosimilar approval pathway was established in 2014. The first biosimilar developed in oncology and approved by FDA was filgrastim for neutropenia (Brinckerhoff et al., 2015; Colwell, 2015).

1.2 Biosimilars

Biosimilars are "generic" versions of "originator" with respect to the amino acid sequence, but they are produced with different cell clones, production processes, and parameters. As biosimilars are produced with different production process, they may have different glycosylation and charge variants patterns which may significantly affect the product quality and safety (Vlasak et al., 2009; Khawli et al., 2010; Shen et al., 2021).

Biosimilars provide support to the healthcare system and provide savings in the budget due to their economics. The lower cost of biosimilars is due to less expenditure on research and development, clinical trials and marketing (Simoens et al., 2021). In the biosimilar era patients, clinicians and payers are benefiting from a choice of biologics and due to cost reduction, access to treatment with biosimilars is increased.

Table 1. FDA approved monoclonal antibodies (Lu et al., 2020).

Monoclonal antibody	Brand	Manufacturer	Molecular weight (~kDa)	Antigen target	Indication	Approval year
Crizanlizumab	Adakveo	Novartis Pharmaceuticals Corp.	146	P-selectin	Sickle cell disease	2019
Brolucizumab	Beovu	Novartis Pharmaceuticals Corp.	26	VEGF-A	Macular degeneration	2019
Romosozumab	Evenity	Amgen/UCB	145	Sclerostin	Osteoporosis in	2019
Ravulizumab	Ultomiris	Alexion Pharmaceuticals Inc.	144	C5	Paroxysmal nocturnal hemoglobinuria	2018
Galcanezumab	Emgality	Eli Lilly	144	CGRP	Migraine prevention	2018
Sarilumab	Kevzara	Regeneron Pharmaceuticals Inc./ Sanofi	144	IL-6R	Rheumatoid arthritis	2017
Avelumab	Bavencio	Merck Serono International S.A./ Pfizer	143	PD-L1	Merkel cell carcinoma	2017
Obiltoxaximab	Anthim	Elusys Therapeutics Inc.	145	*B. anthrasis*	Prevention of inhalational anthrax	2016
Atezolizumab	Tecentriq	Roche, F. Hoffmann-La Roche, Ltd./ Genentech Inc.	144	PD-L1	Bladder cancer	2016
Ixekizumab	Taltz	Eli Lilly	146	IL-17α	Psoriasis	2016

The two qualities of biosimilars, such as cost-effectiveness and easy availability, make biosimilars affordable and accessible which confirms the easy adoption of biosimilars into the market (Oskouei et al., 2021). A typical comparison between biological and biosimilar drug development in terms of timeline and cost is shown in Figure 1, which reflects biosimilar development is efficient and faster available in the market (Agbogbo et al., 2019). The typical biological molecule approval timeline from identification to Phase III is approximately twelve years. Molecules following the standard biologics approval pathway must perform all clinical phases (Phase I to Phase III). However, biosimilars follow a shortened regulatory route. As biosimilars are copies of innovator molecules with known quality attributes, hence discovery phase or initial clinical trial Phase 2 is not required, and thus development path is reduced to eight years (Agbogbo et al., 2019).

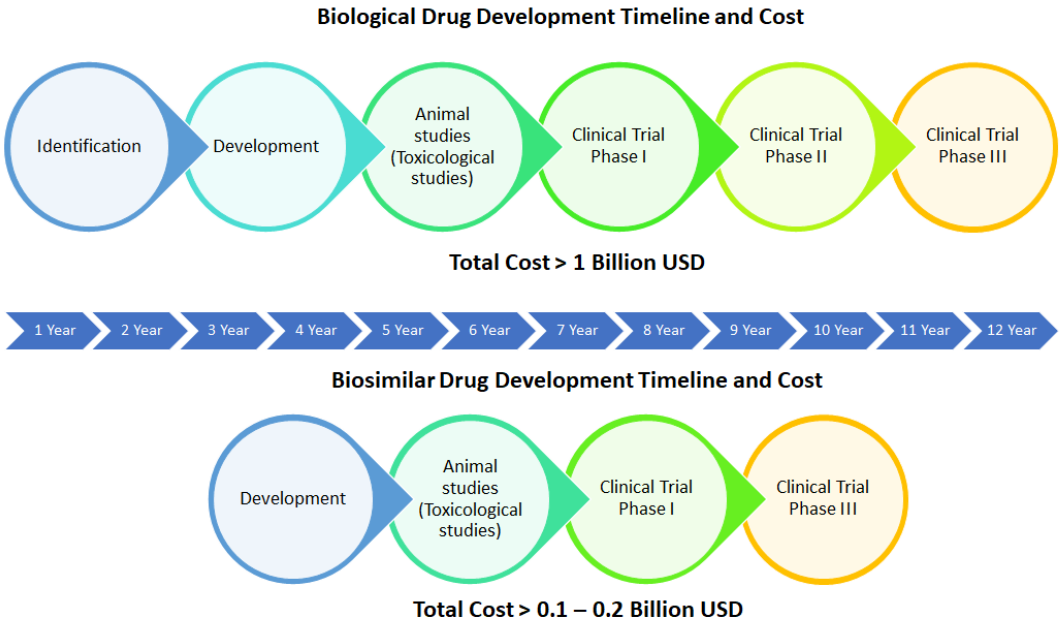

Figure 1. Biological and biosimilar drug development (Agbogbo et al., 2019).

Biosimilar drugs are regulated and approved by different regulated agencies like Food and Drug Administration (FDA), The International Council for Harmonization (ICH), European Medicines Agency (EMA), and World Health Organization (WHO). The first biosimilar, Filgrastim-sndz (Zarxio) approved by FDA in 2015, since then it has received tremendous attraction into the biological market, following which a total of 27 biosimilar mAbs have been approved by FDA (three in 2024, five in 2023, four in 2022, two in 2021, two in 2020, nine in 2019, three in 2018, five in 2017 and three in 2016) (Agbogbo et al., 2019). Table 2 describes approved biosimilar mAbs in the regulated market from 2016 to 2024 and highlights the biosimilar manufacturer, delivery mode and route of administration.

Table 2. Approved biosimilar mAbs in regulated market (https://www.fda.gov/drugs/biosimilars/biosimilar-product-information).

Innovator molecule	Biosimilar	Manufacturer	Regulated market	Year	Mode of delivery	Route of administration
Tocilizumab	Tyenne	Fresenius Kabi USA	USA	2024	162 mg/0.9 mL; 80mg/4 mL; 200 mg/10 mL; 400mg/20mL	Subcutaneous, Intravenous infusion
Denosumab	Jubbonti and Wyost	Sandoz Inc	USA	2024	60 mg/1 mL and 120 mg/1.7 mL	Subcutaneous
Adalimumab	Simlandi	Alvotech USA Inc	USA	2024	40 mg/0.4 mL	Subcutaneous
Bevacizumab	Avzivi	Bio-Thera Solutions Ltd	USA	2023	100 mg/4 mL (25 mg/mL) or 400 mg/16 mL (25 mg/mL)	Intravenous infusion
Ustekinumab	Wezlana	Amgen Inc.	USA	2023	45 mg/0.5 mL or 90 mg/mL 130 mg/26 mL (5 mg/mL)	Subcutaneous, Intravenous infusion
Tocilizumab	Tofidence	Biogen MA	USA	2023	80 mg/4 mL (20 mg/mL), 200 mg/10 mL (20 mg/mL), 400 mg/20 mL (20 mg/mL)	Intravenous infusion
Natalizumab	Tyruko	Sandoz Inc	USA	2023	300 mg/15 mL (20 mg/mL)	Intravenous infusion
Adalimumab	Yuflyma	Celltrion, Inc.	USA	2023	40 mg/0.4 mL	Subcutaneous
Adalimumab	Idacio	Fresenius Kabi USA	USA	2022	40 mg/0.8 mL	Subcutaneous
Bevacizumab	Vegzelma	Celltrion, Inc.	USA	2022	100 mg/4 mL (25 mg/mL) or 400 mg/16 mL (25 mg/mL)	Intravenous infusion
Ranibizumab	Cimerli	Coherus BioSciences, Inc	USA	2022	0.5 mg (0.05 mL of 10 mg/mL solution); 0.3 mg (0.05 mL of 6 mg/mL solution)	Intravitreal
Bevacizumab	Alymsys	Amneal Pharmaceuticals LLC	USA	2022	100 mg/4 mL (25 mg/mL) or 400 mg/16 mL (25 mg/mL)	Intravenous infusion
Adalimumab	Yusimry	Coherus BioSciences, Inc.	USA	2021	40 mg/0.8 mL	Subcutaneous
Ranibizumab	Byooviz	Samsung Bioepis Co., Ltd.	USA	2021	0.5 mg (0.05 mL of 10 mg/mL solution)	Intravitreal
Rituximab	Riabni	Amgen Inc.	USA	2020	100 mg/10 mL (10 mg/mL) and 500 mg/50 mL (10 mg/mL)	Intravenous infusion

Innovator molecule	Biosimilar	Manufacturer	Regulated market	Year	Mode of delivery	Route of administration
Adalimumab	Hulio	Mylan Pharmaceuticals Inc.	USA	2020	40 mg/0.8 mL, 20 mg/0.4 mL	Subcutaneous
Infliximab	Avsola	Amgen Inc.	USA	2019	100 mg of lyophilized drug in a 20 mL single-dose vial	Intravenous infusion
Adalimumab	Abrilada	Pfizer Inc.	USA	2019	40 mg/0.8 mL, 20 mg/0.4 mL, 10 mg/0.2 mL	Subcutaneous
Adalimumab	Hadlima	Samsung Bioepis Co., Ltd.	USA	2019	40 mg/0.8 mL	Subcutaneous
Rituximab	Ruxience	Pfizer Ireland Pharmaceuticals	USA	2019	100 mg/10 mL (10 mg/mL) and 500 mg/50 mL (10 mg/mL)	Intravenous infusion
Bevacizumab	Zirabev	Pfizer Inc.	USA	2019	100 mg/4 mL (25 mg/mL) or 400 mg/16 mL (25 mg/mL)	Intravenous infusion
Trastuzumab	Kanjinti	Amgen Inc.	USA	2019	420 mg lyophilized powder in a multiple-dose vial for reconstitution	Intravenous infusion
Etanercept	Eticovo	Samsung Bioepis Co., Ltd.	USA	2019	25 mg/0.5 mL and 50 mg/mL	Subcutaneous
Trastuzumab	Trazimera	Pfizer Ireland Pharmaceuticals	USA	2019	420 mg lyophilized powder in a multiple-dose vial for reconstitution	Intravenous infusion
Trastuzumab	Ontruzant	Samsung Bioepis Co., Ltd.	USA	2019	150 mg lyophilized powder in a single-dose vial for reconstitution	Intravenous infusion
Trastuzumab	Herzuma	Celltrion, Inc.	USA	2018	420 mg lyophilized powder in a multiple-dose vial for reconstitution	Intravenous infusion
Rituximab	Truxima	Celltrion, Inc.	USA	2018	100 mg/10 mL (10 mg/mL) and 500 mg/50 mL (10 mg/mL)	Subcutaneous
Adalimumab	Hyrimoz	Sandoz Inc.	USA	2018	40 mg/0.8 mL	Subcutaneous
Infliximab	Ixifi	Pfizer Ireland Pharmaceuticals	USA	2017	100 mg of lyophilized drug in a 15 mL vial	Intravenous infusion

Innovator molecule	Biosimilar	Manufacturer	Regulated market	Year	Mode of delivery	Route of administration
Trastuzumab	Ogivri	Mylan GmbH	USA	2017	420 mg lyophilized powder in a multiple-dose vial for reconstitution	Intravenous infusion
Bevacizumab	Mvasi	Amgen Inc.	USA	2017	100 mg/4 mL (25 mg/mL) or 400 mg/16 mL (25 mg/mL)	Intravenous infusion
Adalimumab	Cyltezo	Boehringer Ingelheim Pharmaceuticals, Inc.	USA	2017	40 mg/0.8 mL	Subcutaneous
Infliximab	Renflexis	Samsung Bioepis Co., Ltd.	USA	2017	100 mg of lyophilized drug in a 20 mL single-dose vial	Intravenous infusion
Adalimumab	Amjevita	Amgen Inc.	USA	2016	40 mg/0.8 mL, 20 mg/0.4 mL	Subcutaneous
Etanercept	Erelzi	Sandoz Inc.	USA	2016	25 mg/0.5 mL and 50 mg/mL	Subcutaneous
Infliximab	Inflectra	Celltrion, Inc.	USA	2016	100 mg of lyophilized drug in a 20 mL single-dose vial	Intravenous infusion

Biosimilar drug candidates are supposed to be similar to the originator molecule in terms of physiological and functional characteristics- purity, efficacy and potency (Niazi, 2019). However, differences between innovators/originators and biosimilar candidates can be observed, which are majorly found in charge variants, hydrophobicity, glycoforms, post-translational modifications (PTMs) and biological functions. These differences are typically generated by the host cell used in the upstream process, which is the first process step. Subsequently, during purification, different process conditions, such as buffers, formulations, storage conditions, *etc*. also generate several modifications, translated into structural and functional heterogeneity (Vanam et al., 2015; Neill et al., 2015; Zheng et al., 2006; Brown et al., 2019; Majumder et al., 2011; Majumder et al., 2014).

1.3 Charge Variants

The presence of differences related to either charge or size is ubiquitous during the biosimilar manufacturing process. A protein alteration either due to post-translational modification or chemical degradation modifies the isoelectric pH (pI) values, leading to charge heterogeneity. Generally, the process generates two different charge variants, either acidic in nature or basic in nature with the main species. Differences in charge variants can alter mAbs properties which affect the tissue penetration, distribution and pharmacokinetics (PK) of the mAbs. Hence, development of an effective process and drug formulation is critical to understand those differences and their chemical nature. Due to multiple variations in the charge variants, complete understanding is very challenging and likely to be discovered based on current database and knowledge (Du et al., 2012).

Since the first biosimilar development, remarkable improvements such as cell culture productivity, high-yielding purification steps and improved guidelines have been discovered to make more effective and robust biosimilar processes (Cramer et al., 2011). However, current

manufacturing practices should provide similar profiles in terms of charge heterogeneity with improved process yield for successful product development.

In this study, we have worked on Xolair biosimilar molecule. Xolair (Generic Name: Omalizumab) is a recombinant immunoglobulin G1 (IgG1) monoclonal antibody manufactured by Genentech, USA. It binds specifically to free IgE and reduces the circulatory IgE level to control different allergic diseases. Allergic rhinitis, atopic dermatitis, food allergy and allergic asthma are chronic disorders related to immune system. These disorders are increasing all over the world and impacting a large population of patients (Prakash et al., 2006; Mannino et al., 2002; Ford et al., 2003). Asthma is very common respiratory ailment impacting billions of people and rising day by day around the globe (Liu et al., 2020; D'Amato et al., 2014; Barnes, 2012). Due to asthma quality of life is significantly reduced and accordingly impacting economic and social life balance. Medicines are present in market to treat these reactions but those are either nonspecific or only for symptomatic relief. IgE plays a key role in different allergic reaction or diseases which needs to be controlled to a normal level. Xolair is the best available drug option for allergic diseases (Belliveau, 2005).

We have investigated impact of charge variants on biological activity (*in-vitro* potency) for the biosimilar of Omalizumab by enriching individual charge variants. Acidic, basic charge variants and main peak variants were isolates and purified from Xolair biosimilar product by using different preparative chromatographic purification techniques. These highly purified (> 90%) individual charge variants were formulated in stable formulation buffer to check their impact on stability at different temperature conditions.

Chapter 2
Review of Literature

2.1 Therapeutic Monoclonal Antibodies

Advancement of mAbs development and manufacturing is taking place very fast in both upstream and downstream processing at different scales in various biopharma industries. Since the last decade, the upstream process with respect to cell lines, clone selection strategies, media, feed and batch operating parameters have changed significantly (Chon et al., 2011; Shukla et al., 2010).

The representative production process of mAbs is shown in Figure 2 (Rathore et al., 2010). Upstream process development initiates with clone selection and finalization, media and feed screening/optimization followed by process parameters optimization and finalization at different small-scale models includes 96-well plates followed by shake flask and small-bioreactor models, ensuring speedy screening and process finalization (Li et al., 2006).

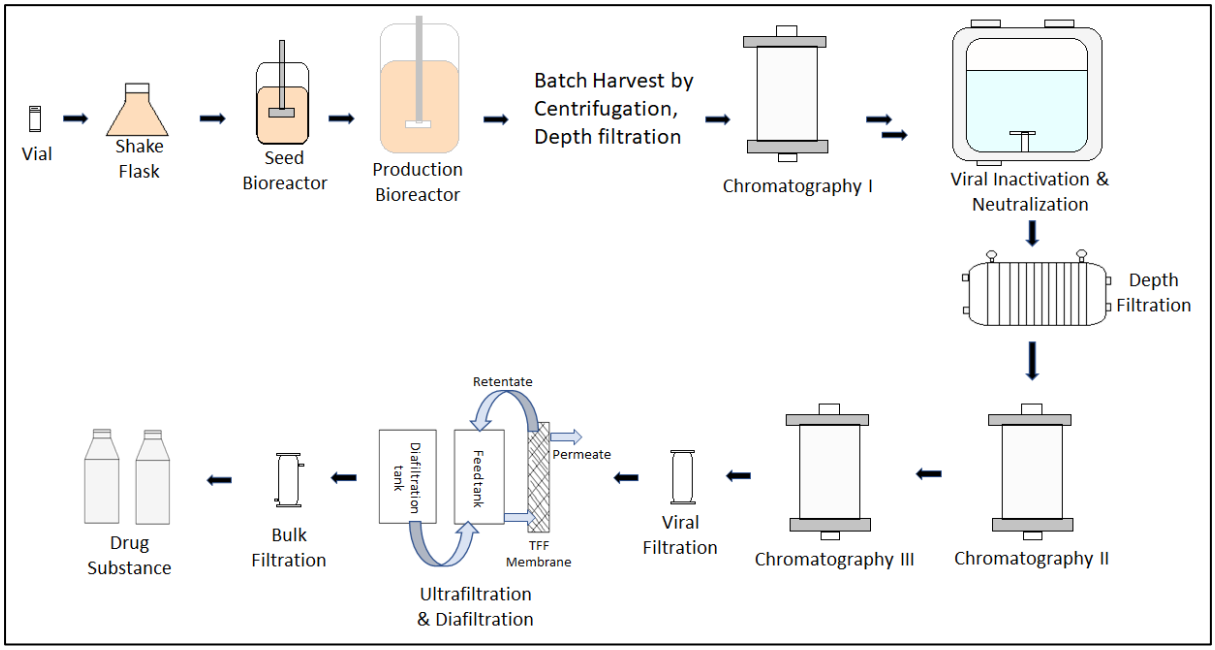

Figure 2. Production process of monoclonal antibody (Rathore et al., 2010).

Most commercially available mAbs are manufactured from Chinese Hamster Ovaries (CHO) and NS0 cells, but CHO cells are the preferred choice across industries due to their easy scale up, adaptation to serum free conditions, productivity and similar PTM profile as humans (Yoo et al., 2002).

Cell lines and clones are selected based on high productivity and PTMs. Still, other factors such as growth pattern, stable and consistent production, serum-free media amplification and possible risk assessment also need to be considered. Media and feed selection are key factors in improving productivity and cell growth, but they also affect product quality. Besides media and feed screening, upstream process parameters optimization is also essential to get desired product expression and quality (Li et al., 2006; Costa et al., 2010). These process parameters are further divided into three categories- physical parameters such as temperature, agitation speed and gas flow rate, chemical parameters such as dissolved oxygen and carbon dioxide, redox potential, osmolality, pH, and metabolite levels including substrate, amino acid, and waste by-products) and biological parameters such as viable cell concentration, viability and a variety of intracellular and extracellular measurements such as nicotinamide adenine dinucleotide, cell cycle analysis, lactate dehydrogenase levels, and mitochondrial activity) in behavior (Jordan et al., 2013; Li et al., 2010).

The downstream process must produce a consistent and purified product suitable for human use. During purification, process-related impurities such as adventitious/endogenous viruses, endotoxin, host cell DNA, host cell proteins and product-related impurities such as size variants and other species should be controlled with an acceptable process productivity. Additionally, contaminants generated during downstream processing should also be controlled, such as

residual leached protein A, extractable from chromatography resins and filters, process buffers and reagents like detergents that may be used for the virus inactivation (Liu et al., 2010).

Protein purification, including mAbs is generally performed using different chromatography resins to separate the molecule according to their physical and chemical properties. Protein A based chromatography resins are most commonly used for the purification of most of the mAbs due to resin specificity, which only provides high purity and step yield during single chromatography step (Fahrner et al., 2001).

A typical mAbs purification process is depicted in Figure 3. mAbs purification generally starts with Protein A chromatography by loading clarified cell culture harvest, yields pure product compared to other capture resins and removes process and product-related impurities in small proportion. After Protein A chromatography, one or two additional chromatography step (anion exchange [AEX] or cation exchange [CEX] chromatography) requires a polishing step based on the requirements to get desired purity. Based on the purity requirement, the polishing step can be selected with different chromatography resins, such as hydrophobic interaction and mixed-mode chromatography (MMC) (Liu et al., 2010).

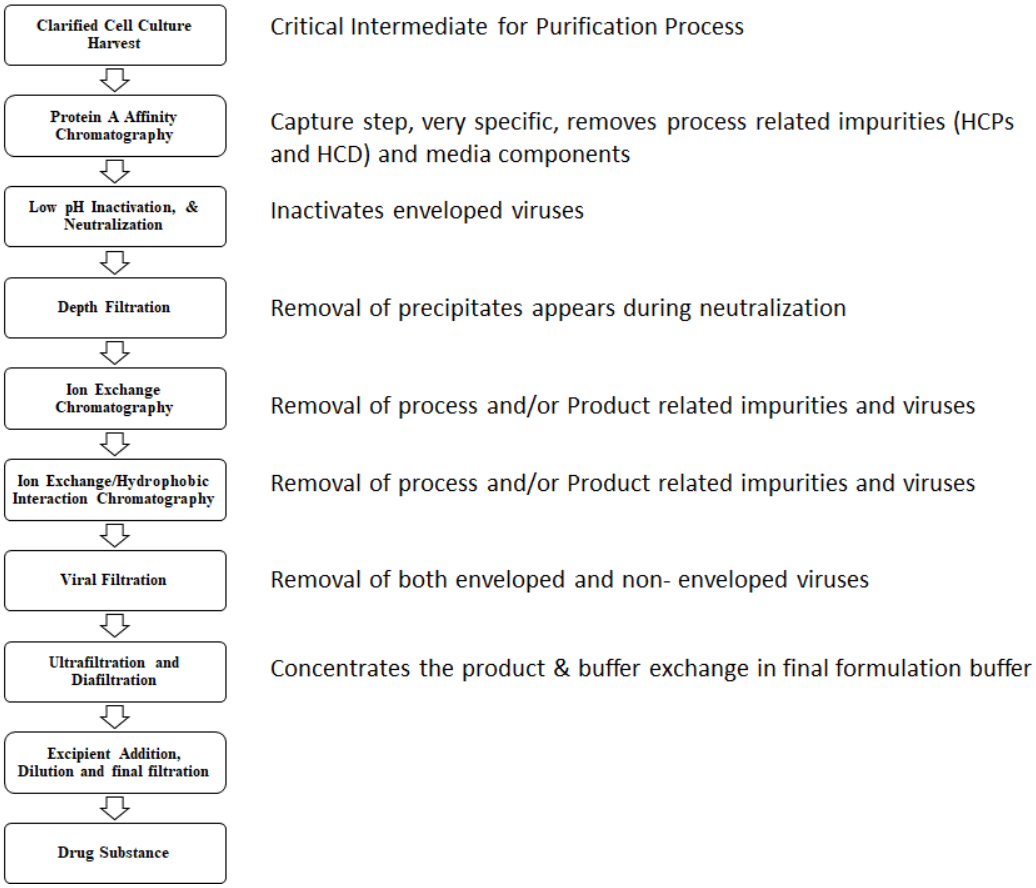

Figure 3. Typical mAb purification process (Liu et al., 2010).

These purification steps provide process and product-related clearance, including host cell proteins, host cell DNA, size or charge variants and viral-like particles. Additionally, to get required viral clearance, viral inactivation by either a low pH incubation or detergent treatment and viral filtration steps are generally incorporated in the mAbs purification process. Finally, purified protein is concentrated and diafiltered with a final formulation buffer to get a stable bulk product for further use (Liu et al., 2010).

Challenges during mAbs production those are associated with upstream and downstream processes are enlisted in Table below (Table 3). Upstream processing challenges includes stable cell line selection, production cell culture longevity with viable cell count and high

productivity. On the other hand, downstream process challenges are high process yield, product purity and cost (Gupta et al., 2023).

Table 3. Challenges in mAbs production: upstream and downstream process (Gupta et al., 2023).

Process step	Challenges
Upstream	Stable cell line selection
	Media and feed optimization
	Production cell culture longevity with viable cell count
	High productivity
	Process contamination
	Product CQA
	Process scale up
Downstream	Process optimization
	Product stability
	High process yield and product purity
	Final product CQA
	Process scale up
	Stable formulation
	Cost of production

After finalization of all process parameters, the process is usually scaled up to a suitable higher scale to manufacture material for toxicological studies and followed by technology transfer to manufacturing for further scale-up (if required) to produce clinical trial material under current Good Manufacturing Practices (cGMP) conditions. Once the process is finalized for commercial production scale, the final process triggers other activities such as process characterization studies and validation of the manufacturing process (Li et al., 2006).

2.2 Charge Variants of Therapeutic Antibodies

Over the last two decades, biopharmaceutical market growth has highlighted the great success of therapeutic proteins and related drugs. The robust and controlled purification process of the drug substance manufacturing also ensures the safety and efficacy of therapeutic drugs.

However, the final product contains multiple protein species (Manning et al., 2010). During mAbs manufacturing, the final product generally shows variations from the desired structure. These variations provide charge and size heterogeneity to the molecule and may be due to either known or novel types of PTMs or spontaneous, non-enzymatic protein degradations (Talebi et al., 2013).

Several mAbs variations have been discussed in the past twenty years, primarily related to PTMs and physical and chemical degradations (Manning et al., 2010). These modifications or variations may affect the surface charge of mAbs by altering the overall surface charge distribution or the number of charge residues. Typical mAbs modifications is shown in Figure 4 (Wagner-Rousset et al., 2017).

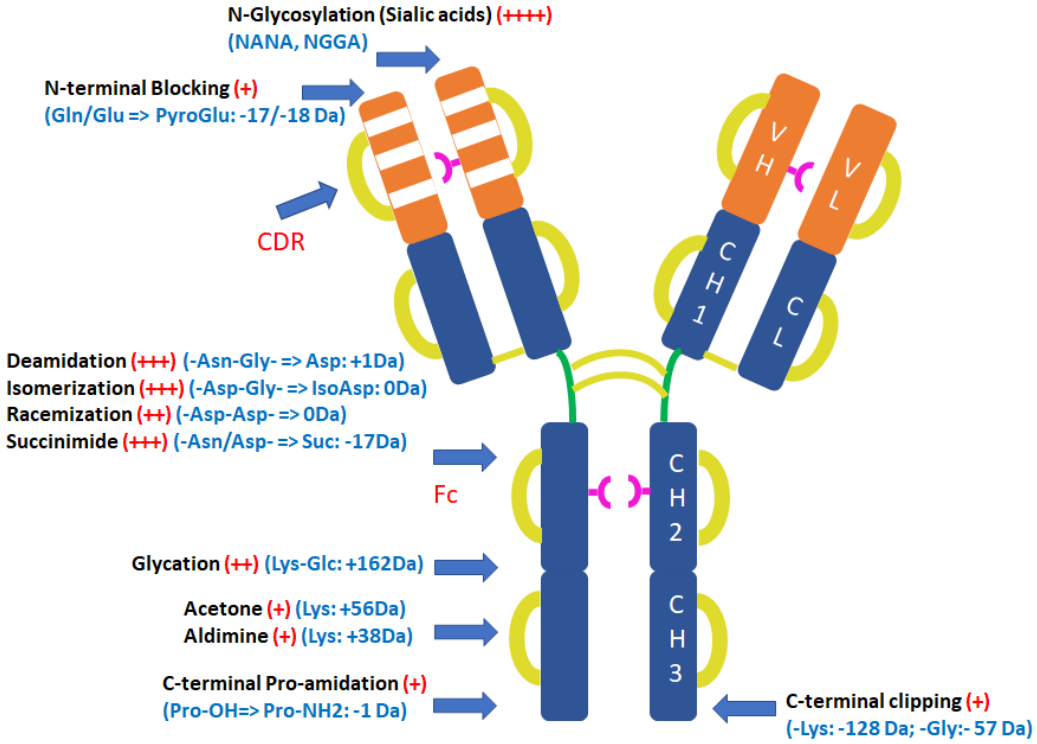

Figure 4. Typical mAb modifications (Wagner-Rousset et al., 2017).
++++ indicates the highest level of importance; + indicates the lowest level of importance.

During mAbs manufacturing process, charge heterogeneity is commonly observed, and this charge heterogeneity has a potential influence on the stability and biological activities of the

molecule. This heterogeneity can be generated by several factors linked with either extracellular or intracellular processes. It can also be generated by protein incubation with different buffers, protein storage and protein purification. These enzymatic and non-enzymatic charge related modifications include disulfide bonds formation, glycosylation, N-terminal glutamine cyclization, C-terminal lysine processing, oxidation, deamidation, glycation, and peptide bond cleavage (Khawli et al., 2010; Liu et al., 2008).

There are various known chemical and enzymatic methods to generate acidic variants such as deamidation (removal of the amino group), sialylation which involves the addition of sialic acid, glycation (glucose or lactose reaction with the primary amine of a lysine residue) and basic variants such as C terminal lysine or glycine amidation, succinimide formation, amino acid oxidation or removal of sialic acid (Liu et al., 2008; Dick et al., 2008).

Charge variants or modifications may impact *in-vitro* and *in-vivo* characteristics of the antibodies, as demonstrated by using modified antibodies. This shows that charge variation can alter mAbs properties which affect the tissue penetration, distribution and PK of the mAbs (Du et al., 2012).

It has been studied that a low percentage of acidic and basic species did not impact potency, binding to FcRn, and PK, when compared with the main species or the unfractionated material (Khawli et al., 2010). The effects of the variants heavily depend on the nature, location and degree of PTMs that initiate the formation of acidic and basic species.

2.3 Analytical Methods for mAb Charge Variants and Their Profile

mAbs charge heterogeneity comes from chemical and biological modifications, which leads to changes in molecule characteristics in terms of isoelectric point (pI), total net charge and charge distribution on the molecule surface (Yüce et al., 2021).

Charge variants like acidic and basic variants are generated due to several modifications that result in a shift in the isoelectric point (pI) of the molecule. The formation of acidic variants is due to changes, such as increased sialic acid, deamidation, high mannose content, fragmentation, glycation and disulfide structural heterogeneity. These modifications show a decrease in the pI of the molecule and impart acidic properties to them. Similar way, basic variants form owing to C-terminal lysine truncation (Khawli et al., 2010), incomplete cyclization of N-terminal glutamine or glutamic acid, succinimide formation, methionine oxidation, amidation aglycosylation, incomplete removal of the leader sequence and aggregation which shows an increase in the pI of the molecule resulting in basic properties to them (Khawli et al., 2010; Perkins et al., 2000; Yan et al., 2009; Singh et al., 2016). A typical cation exchange-high pressure liquid chromatography (CEX-HPLC) profile of the charge variants elution pattern depending on their charge and interaction with resin is shown in figure below (Figure 5). This profile shows different charge variants; acidic elute first before main peak, followed by the prominent peak (main peak) and basic variants (B0, B2), including lysine variants (K1 and K2) elutes after main peak.

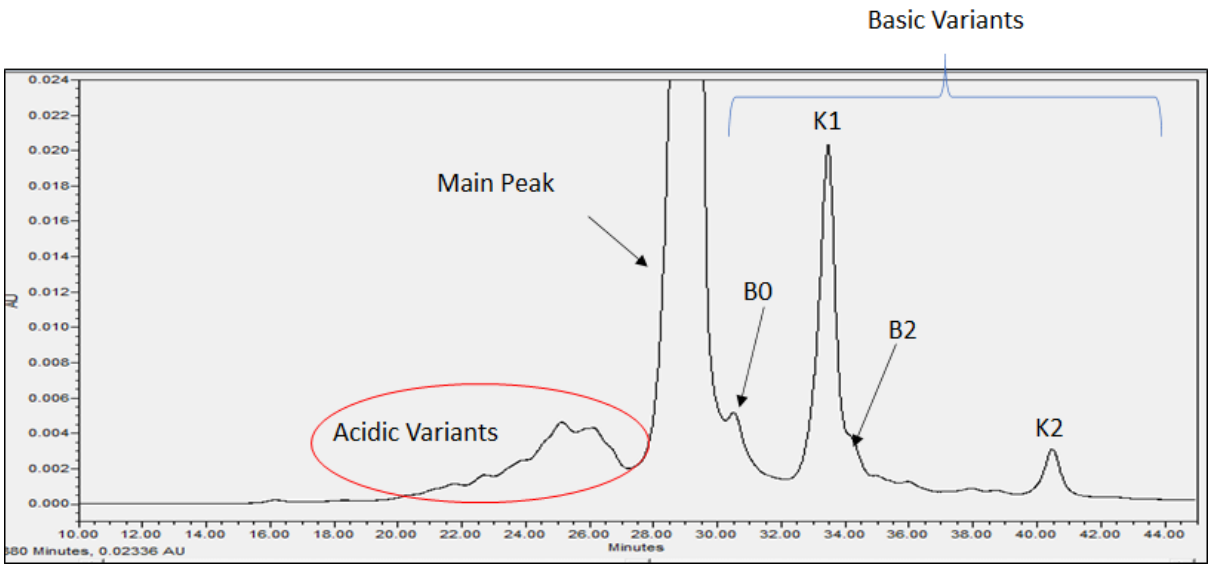

Figure 5. Charge variants profile for monoclonal antibody (CEX-HPLC).

During analysis by different chromatography-based methods, acidic species used to elute first in comparison to the main peak when analyzed by CEX. In contrast, they elute later by AEX due to their charge and binding chemistry with resin (Du et al., 2012).

2.4 Charge Variants: Impact on Biological Functions

Antibody charge variants may influence product stability and biological activity. Due to this attention towards charge variants, studies in the biotech industries increased with time. During routine manufacturing or process scale-up, differences in the percentage of charge variants are commonly observed that creates challenges to show product comparability or bio-similarity with innovator molecules (Khawli et al., 2010). These charge variant differences between the innovator's product and biosimilar candidate can impact their potency (*in-vivo* and *in-vitro*) for some biological products. Thus, to comply with mAbs critical quality attributes, *in-vitro* and *in-vivo* potency of the biosimilar candidate with differences in charge variants percentage should be characterized (Du et al., 2012; Hintersteiner et al., 2016). Table 4 describes charge variants and their impact on biological functions (Chung et al., 2018). These charge variants

changes can potentially affect antigen binding (Kadkhoda et al., 2021; Huang et al., 2005), half-life (Gaza-Bulseco et al., 2008), and complete inactivation (Rehder et al., 2008), and no significant impact on binding, half-life or PK (Alt et al., 2016) is also observed.

Table 4. Charge variants impact on biological functions (Chung et al., 2018).

Charge modifications	Affected amino acids	Impact on biological functions
Deamidation	Asn, Gln	14-fold reduced antigen binding
Oxidation	Cys, Met, Trp, His, Tyr	Reduced binding with Protein A and FcRn Reduced half-life Loss of target binding and activity
Glycation	Lys	No significant impact on half-life or potency May illicit response with AGE pathways
Isomerization	Asp	Complete inactivation
Succinimide	Asn, Asp	May illicit immune response
C-terminal Lys/Arg	Lys, Arg	No significant impact on binding, PK, or half-life
C-terminal amidation	Gly	No known impact in mAbs
N-terminal pyroGlu	Gln, Glu	Potency not significantly impacted

To further understand the impact of charge variants heterogeneity on biological activity and PK, effective charge variants of recombinant humanized IgG1 were isolated, and *in-vivo* and *in-vitro* PK properties were compared (Khawli et al., 2010). mAb has pI of 8.7 to 9.1 with 20% acidic variants, 68% main peak and 12% basic variants used as starting material and CEX displacement chromatography was used to isolate charge variants for animal studies.

All the isolated charge variants were analyzed and characterized, and *in-vitro* potency was also tested before being injected either subcutaneously or intravenously in animals (rats). All isolated variants showed similar potency and FcRn binding compared to starting material. Also, no difference was observed in the serum PK study, which indicates that modifications and pI differences among charge variants were not enough to show differences in PK profile. Outcome

of this study depicted that charge variants heterogeneity does not affect the *in-vivo* and *in-vitro* potency, FcRn binding affinity, or PK profile in rats (Khawli et al., 2010).

SB5, Humira biosimilar, and reference material (Adalimumab) have C-terminal Lysine residue below the detection limit after the carboxy peptidase (CPB) treatment. To access the effect of C-terminal Lysine heterogeneity on biological activities, charge variants of SB5 and reference material were isolated and fractionated by CEX-HPLC. Each fraction was tested for biological activities such as tumor necrosis factor (TNF) binding, FcRn, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC) assays. The biological activities for both were not significantly different, which reveals that C-terminal heterogeneity has no potential impact on biological activity of both the SB5 and the reference product (Lee et al., 2019).

In other study, the investigator studied charge variants impact on Bevacizumab's structure, stability and biological activity. Five primary and one acidic charge variant were isolated by using semipreparative CEX chromatography with linear pH gradient elution achieving a purity of 85%. One acidic variant, two basic variants, and the main peak, were selected and used for further study and none of the charge heterogeneities were due to glycosylation. Based on this, it was concluded that different charge variants show distinct behavior with respect to their structure and bioactivity (Singh et al., 2021). Charge variants or isoforms were characterized to understand mAb safety, potency and bioavailability. However, very few information is available about their role in stability and viscosity properties, which controls immunogenicity and delivery.

To study this, acidic variants level was varied as a function of cell culture harvest time. With these changes, antibody was purified and formulated and no impact on aggregation behavior was observed at high protein concentrations concerning acidic variant level. Additionally, it was observed that enriched acidic variants protein fraction do not impact viscosity, colloidal or conformational stability. Interestingly, variants that are most acidic in nature contribute to the formulation color (Sule et al., 2017).

The deamidated variant of an immunotoxin was isolated by using analytical ion exchange HPLC to understand the impact. Immunotoxin charge variants were fractionated using analytical ion exchange HPLC. Isolated charge variants were analyzed by different analytical methods such as peptide mapping and liquid chromatography-mass spectroscopy (LC-MS) to identify the site of modification. Cell-based bioassay study revealed that deamidation led to reduction in biological activity and hence needs to be controlled during batch manufacturing. The process could further reduce the deamidated form up to the desired level to achieve acceptable biological activity (Linke et al., 2012). Charge variants difference which impacts biological functions or not, is totally depends on the location and percent of differences.

2.5 Hypothesis

Monoclonal antibodies (mAbs) are now well-established therapeutic modalities with key targets involved in inflammatory, oncologic and autoimmune diseases. Heterogeneity of purified antibodies (immunoglobulins, Ig) based on the simple chemical modifications of selected amino acids sites is of considerable importance in the biotechnology field. Although substantial knowledge and experience with the degradation pathways that are active during production in cell culture, purification, formulation and storage of therapeutic mAbs has accumulated, the biopharmaceutical industry continues to characterize microheterogeneity thoroughly in order to demonstrate batch-to-batch consistency and predict shelf-life of these complex protein molecules.

The current challenge is to understand that mAb microheterogeneity (charge or size) may have impact on efficacy, potency, immunogenicity and clearance profile.

Therefore, we hypothesized that highly purified Omalizumab biosimilar protein charge variants may have impact on *in vitro* potency. This study will provide scientific approach to decide up to what percentage of charge variants should be present in the final drug formulation. Outcome of this study will open a systematic approach/protocol for evaluation of other similar molecules.

2.6 Objectives

The main objectives of the study were as follows:

1. To generate material for purification with optimized cell culture process, Isolation and purification of protein charge variants.
2. To show on hold stability of protein charge variants (cluster of acidic, main peak and cluster of basic).
3. To understand the role of protein charge variants on *in vitro* potency.

Chapter 3
Materials and Methods

3.1 Cell Culture Process: Material Generation for Purification

Omalizumab biosimilar cell line was developed by contract research organisation (CRO) from a proprietary Chinese hamster ovary (CHO)-M cell line. The CHO-M parental cell line (ATCC Cat# CCL-61) was derived from a CHO-K1 cell line adapted to serum free media and improved for recombinant protein production. The Omalizumab heavy and light chain plasmids were designed and generated by CRO. Parental CHO-M cells were initially transfected by microporation with puromycin resistant plasmids separately, encoding the light and heavy chains. After initial selection, clones were transfected by microporation again (Super transfection) with hygromycin resistant plasmids separately encoding the light and heavy chains. All raw materials and excipients used in upstream and downstream process were of multicompendial grade.

The Omalizumab biosimilar manufacturing process was started by thawing a vial of the cell bank into the inoculum medium supplemented with glutamine, poloxamer 188. The culture is propagated in a series of shake flask cultures in order to generate a sufficient viable cell to inoculate a production bioreactor. The production bioreactor is operated in fed-batch mode. Additions are made to the bioreactor over the 12-day run including scheduled feeding with glucose solution and antifoam added as needed. The Production culture was harvested on Day 12 or when viability is below 70%, whichever comes first. Overall process flow is mentioned in below Figure 6.

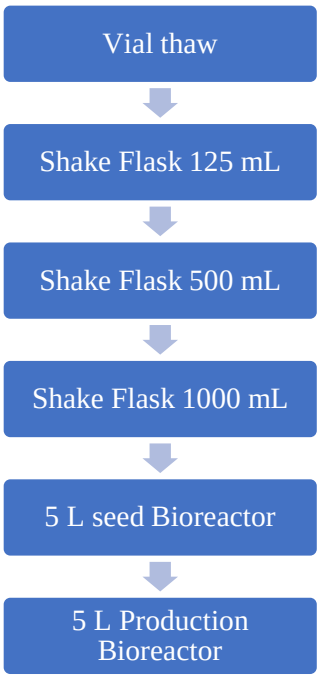

Figure 6. Cell culture process flow

3.2 Purification to Generate Intermediate Biosimilar Product

Omalizumab biosimilar purification process starts with loading of clarified harvest (CH) on Protein A chromatography to capture the product of interest and get rid of other process related impurities. Protein A chromatography eluate is then neutralized for subsequent depth filtration (DF) step. The depth filtrate is further purified by anion exchange chromatography (AEX) in flow through mode. A polishing step is performed by mixed mode (CHT) chromatography followed by Tangential flow filtration (TFF) and filtration step to generate final purified drug substance. The overall purification process flow is provided in Figure 7.

Chromatographic resins used in this process were from Cytiva (Protein A affinity chromatography), Thermo (Poros HQ), Biorad (CHT Type I), Merck (Eshmuno CPX). Chromatographic column hardware used for purification was from Cytiva and Millipore.

Tangential flow filtration used for concentration and buffer exchange was from Merck Millipore. Analytical column used for size variants analysis was from TOSOH biosciences and charge variants analysis was from Thermo Scientific.

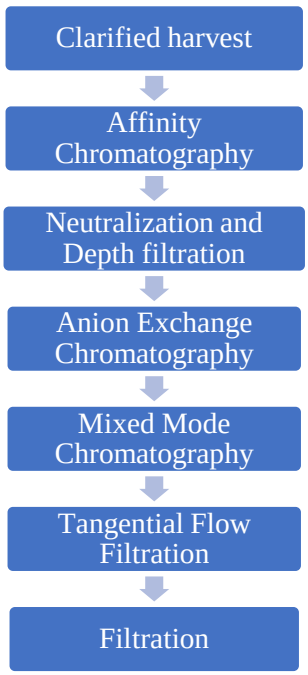

Figure 7. Purification process flow for intermediate biosimilar product

3.3 Isolation and Purification of Charge Variants

The charge variants were isolated from intermediate purified sample of biosimilar product and purified using Eshmuno CPX chromatography resin which was selected based on better resolution capacity from other screened resins as mentioned in Figure 8. Cytiva Akta Pure 150 chromatography purification system was used for each purification step. Intermediate purified sample of biosimilar product first buffer exchanged by using Merck Millipore 30 kDa (88 cm^2) with 20 millimolar (mM) Tris acetate pH 5.5 buffer to prepare the loading sample.

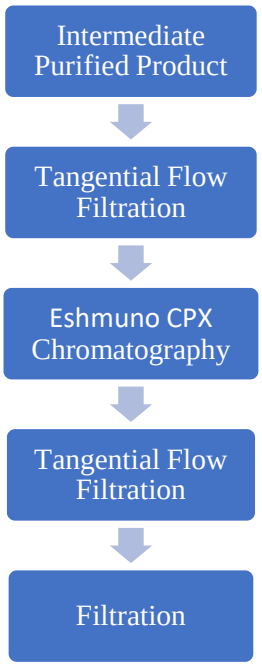

Figure 8. Isolation and Purification of charge variants

After load sample preparation it was loaded on to pre equilibrated (20 mM Tris acetate pH 5.5) Eshmuno CPX chromatography column and bound charge variants were eluted with linear gradient 40 % to 100 % B in 30 column volume (CV). Elution buffer was 20 mM Tris acetate pH 9.0. Protein elution was monitored at 280 nm wavelength and collected in equal volume fractions. After fractions collection different representative pools were prepared and analysed by weak cation exchange and size exclusion analytical column to determine the purity of different charge variants.

All raw materials and excipients used to formulate charge variants were of multicompendial grade. Tangential flow filtration (TFF) system used for concentration and buffer exchange was from Merck Millipore. TFF membrane, 88 cm^2 and Amicon Ultra-15 centrifugal filter devices, 30 kilodalton (kDa) were also from Merck Millipore. Centrifuge and 0.2 micron filter were from Beckman Coulter and Pall Lifesciences respectively. Analytical column used for charge variants analysis was from Thermo Scientific. Human IgE used in ELISA bioassay was from

Abbiotec. Phosphate buffered saline, Tris buffered saline and carbonate-bicarbonate buffer were from Sigma- Aldrich. Recombinant Protein A/G peroxidase conjugate, Tetramethylbenzidine (TMB) substrate, stop solution, 96-well dilution plate and plate sealer were from Thermo Scientific. Microplate with 96 wells and lid was from Corning.

3.4 Formulation of Charge Variants

The purified charge variants (acidic and basic) and main peak were first concentrated, and buffer exchanged with 88 cm^2, 30 kDa TFF membrane. After equilibration of TFF membrane with 20 millimolar (mM) Tris acetate pH 5.5, charge variants were concentrated and buffer exchanged with formulation buffer by doing diafiltration in continuous mode till 8 to 10 diafiltration volumes. Diafiltration activity completion was verified by checking pH and conductivity of permeate sample. Samples were retrieved from TFF membrane and further individually concentrated with 30 kDa amicon centrifugal filter devices at 4500g for 20 minutes at 15 °C temperature using Beckman Coulter's centrifuge.

Each individual charge variants were concentrated to get more than 10 mg/mL concentration. After completion of concentration step, individual charge variants were recovered from devices with the help of pipettes and collected in polypropylene tubes. After collection individual charge variants were filtered through 0.2 micron (µm) filter under controlled conditions to avoid contamination and filled separately in small Polypropylene (PP) container.

3.5 Thermal Stress

The filled bottles were kept at different temperature conditions (5 ± 3)°C and (-20 ± 5)°C to check thermal stress impact on the quality of charge variants. Sampling was done after defined time interval (0 day and 7 days) from each temperature conditions and provided for CEX-

HPLC analysis. Sampling for analysis was done under controlled conditions to avoid any microbial contamination.

3.6 *in-vitro* Potency Analysis by Enzyme Linked Immunosorbent Assay (ELISA)

Omalizumab binds specifically to human immunoglobulin E (IgE). Coating was done with 100 microliters (µL) of working solution of IgE (0.8 microgram/millilitre in carbonate-bicarbonate buffer) prepared with diluent (Tris buffer saline + 1% BSA, pH 8.0) into the plate well and was incubated overnight at 4°C. After overnight incubation plate was washed three times with 300 µL of wash solution (Phosphate buffer saline + 0.05% Tween 20, pH 7.4). After that 300 µL of blocking solution (diluent) was added and it was incubated at room temperature for 60 minutes or longer. After incubation plate was washed one time with 300 µL of washing solution. Reference standard and sample solution (300 µL each) was added into the respective well and incubated at room temperature for 2 hrs followed plate was washed three times with 300 microliters of washing solution. Peroxidase conjugated recombinant protein A/G working solution (100 µL) was added into respective well and incubated at room temperature for 1 hr followed by this plate was washed three times with 300 µL of wash solution. 100 µL of Tetramethyl benzidine (TMB) solution was added to respective well and kept for 20 to 25 min at room temperature in dark conditions. After incubation 100 µL of stop solution was added into respective well and reading was done using 450 nm and 650 nm wavelength.

3.7 Analytical Cation Exchange (CEX-HPLC) Analysis

Isolated charge variants and input material purity was estimated by weak cation exchange chromatography with a ProPac Elite WCX analytical column (4 mm × 250 mm). Mobile phase A used was 20 mM 2-(N-Morpholino) ethanesulfonic acid (MES) buffer, pH 6.5, and mobile phase B was 20 mM MES, 200 mM sodium chloride buffer, pH 6.5. The method was started

with 90% of phase A and run till 2 min followed by a linear gradient from 90 % to 72 % phase A till 45 minutes. After that 100 % phase B was implemented till 53 minutes followed by 90 % phase A till 75 minutes with flow rate of 0.6 mL/min. The samples (standard and charge variants) were diluted with mobile phase A to approximately 2.0 mg/mL. The analytical column was cleaned and equilibrated with respective buffers until the baseline was stable. Peak areas were calculated, and elution profile was detected at 214 nm.

3.8 Analytical Size Exclusion (SEC-HPLC) Analysis

The size variants purity was estimated by analytical SEC using a TSKgel G3000 SWXL, 7.8 mm ID × 30 cm, 5µm column and detected by UV at 215 nm. Mobile phase of 100 mM phosphate and 100 mM sulfate buffer, pH 6.5 was used. The sample was diluted to get 1.0 mg/mL by using the mobile phase and was used for analysis. The elution profile was analysed by integrating the area and percentages of aggregate and monomer was calculated.

Chapter 4
Results and Discussion

4.1 Cell Culture Process: Material Generation for Purification

Omalizumab biosimilar monoclonal antibody was cultured *in vitro* in bioreactor at 5 L scale with defined process to generate material for further processing. Three independent 5 L scale bioreactor batch were completed. Cell culture process performance parameters such as viable cell count (VCC), viability and other parameters glucose, lactate, pH and osmolality were compared. As shown Figure 9 all the process related parameters were found comparable and process consistency was established. Additionally, all three-batch harvest productivity was analysed by protein A HPLC and also harvest was captured on affinity chromatography and analysed by SE-HPLC to check product quality which also found consistent as shown in Table 5. After cell culture process consistency material has been generated for further processing.

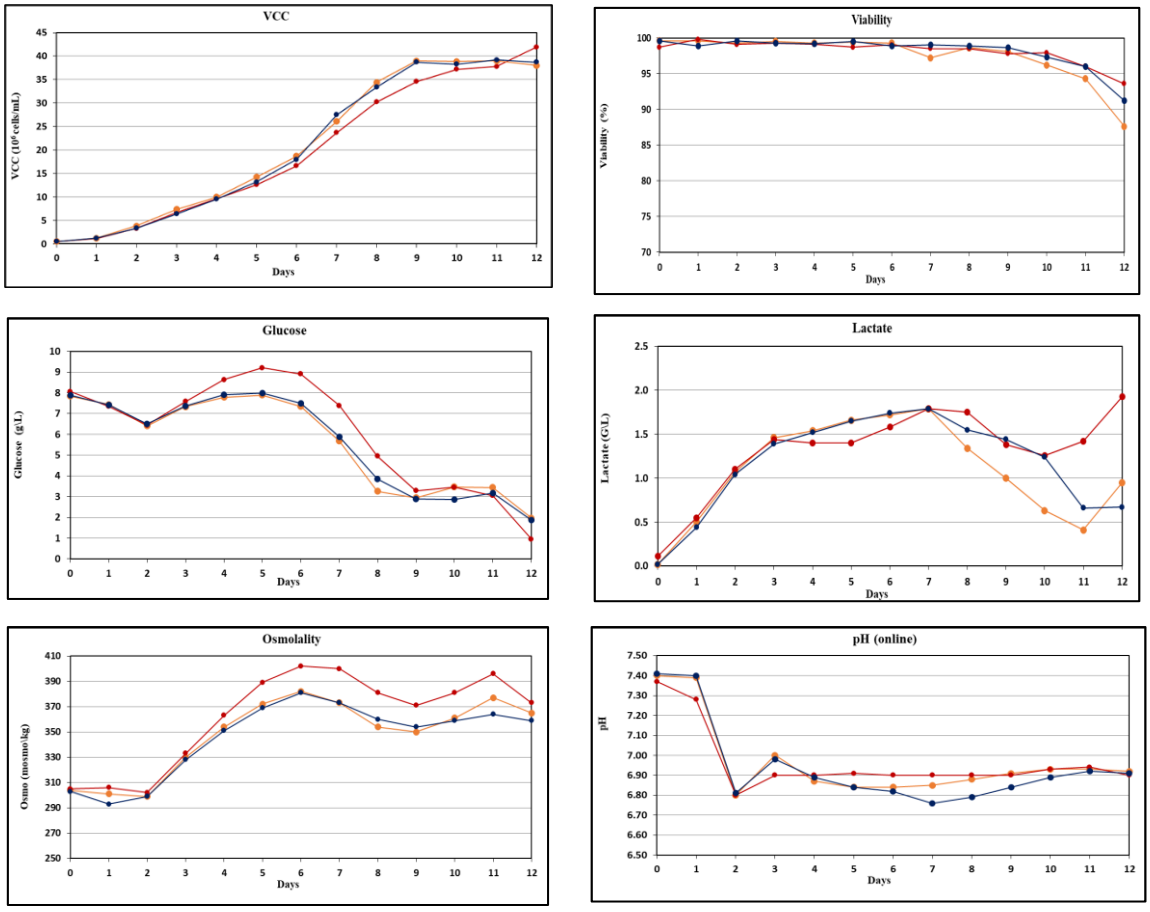

Figure 9. Cell culture process consistency profile

Table 5. Cell Culture process consistency productivity and quality data

Sample Name	Productivity (g/L)	Product Quality after affinity chromatography		
		HMW (%)	Monomer (%)	LMW (%)
Batch 1	4.38 ± 0.02	4.62 ± 0.02	94.55 ± 0.03	0.84 ± 0.01
Batch 2	4.21 ± 0.04	5.07 ± 0.02	94.07 ± 0.02	0.86 ± 0.01
Batch 3	4.13 ± 0.04	5.92 ± 0.03	93.20 ± 0.06	0.88 ± 0.01

All experiments and samples were done in triplicate and average ± SD values are reported.

4.2 Purification to Generate Intermediate Biosimilar Product

Cell culture harvest material which was generated from consistent process was further used for purification to generate intermediate biosimilar product. As described in previous section, intermediate biosimilar product was produced. This material was analysed by SE-HPLC and CEX-HPLC as shown in Figure 10 and Table 6. Purified material was also confirmed by LC-MS and peptide map. Intermediate biosimilar purified product was found comparable with innovator product and used for further charge variants isolation and purification.

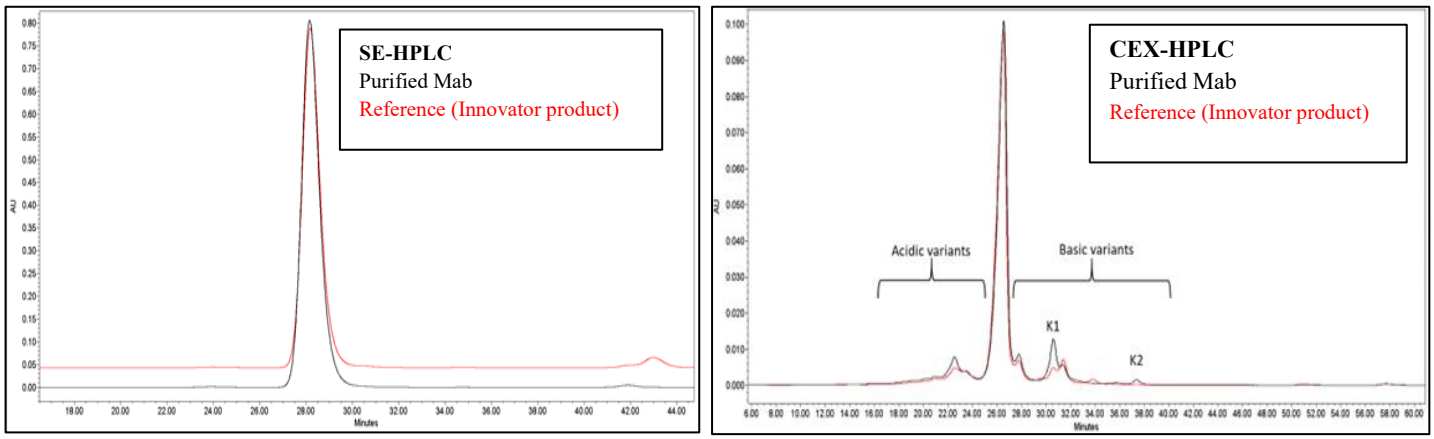

Figure 10. SE-HPLC and CEX-HPLC profiles of purified product

Table 6. Analytical SE-HPLC and CEX-HPLC results of purified product

	SE-HPLC			CEX-HPLC		
Sample Name	**Purity (%)**	**HMW (%)**	**LMW (%)**	**Main Peak (%)**	**Total Acidic (%)**	**Total Basic (%)**
Purified intermediate product	99.5 ± 0.08	0.23 ± 0.02	0.30 ± 0.01	77.1 ± 0.07	12.86 ± 0.03	10.01 ± 0.02
Reference (Innovator product)	99.0 ± 0.07	0.34 ± 0.02	0.70 ± 0.01	76.3 ± 0.04	11.17 ± 0.01	12.54 ± 0.02

All experiments and samples were done in triplicate and average ± SD values are reported.

4.3 Isolation and Purification of Charge Variants

Charge based heterogeneity is generally found in recombinant mAbs which is due to multiple modifications takes place at various stages during development of molecule (Khawli et al., 2010). Charge variants were purified by using intermediate biosimilar product with 10.56 % acidic variant, 68.63 % main peak and 20.81 % basic variant as shown in Table 7. All charge variants (acidic, main peak and basic variants) were purified with preparative chromatography method using Eshmuno CPX packed resin. Intermediate purified biosimilar product was first buffer exchanged with 20 mM Tris acetate buffer pH 5.5 which is suitable for loading onto the Eshmuno CPX column and binding of all charge variants. After completion of loading, column was washed with equilibration buffer (20 mM Tris acetate, pH 5.5) to remove loosely bound protein followed by elution phase. Bound variants were eluted with elution buffer (20 mM Tris acetate, pH 9.0) in linear pH gradient in 30 CV. Linear gradient changed pH from 5.5 to 9.0 in increasing order which helps bound variants to elute in distinct peaks (Fekete et al. 2015). Elution peak as mentioned in Figure 11 was resolved in three distinct parts. The major peak was considered as main peak. Pre and post peaks with respect to main peak were considered as acidic and basic variants respectively (Khawli et al., 2010). Each peak was collected in fractions for further analytical testing (CEX-HPLC and SEC-HPLC).

Table 7. Analytical CEX-HPLC results for Eshmuno CPX resin.

Sample	Main Peak (%)	Total Acidic Variants (%)	Total Basic Variants (%)
Eshmuno CPX Input	68.63 ± 0.03	10.56 ± 0.03	20.81 ± 0.02
Eshmuno CPX Acidic Pool (Fr 1 to Fr 9)	4.10 ± 0.10	94.25 ± 0.06	1.64 ± 0.03
Eshmuno CPX Main Peak (Fr 19 to Fr 20)	95.58 ± 0.05	1.09 ± 0.03	3.33 ± 0.03
Eshmuno CPX Basic Pool (Fr 25 to Fr 43)	1.72 ± 0.03	6.96 ± 0.02	91.33 ± 0.02

All experiments and samples were done in triplicate and average ± SD values are reported.

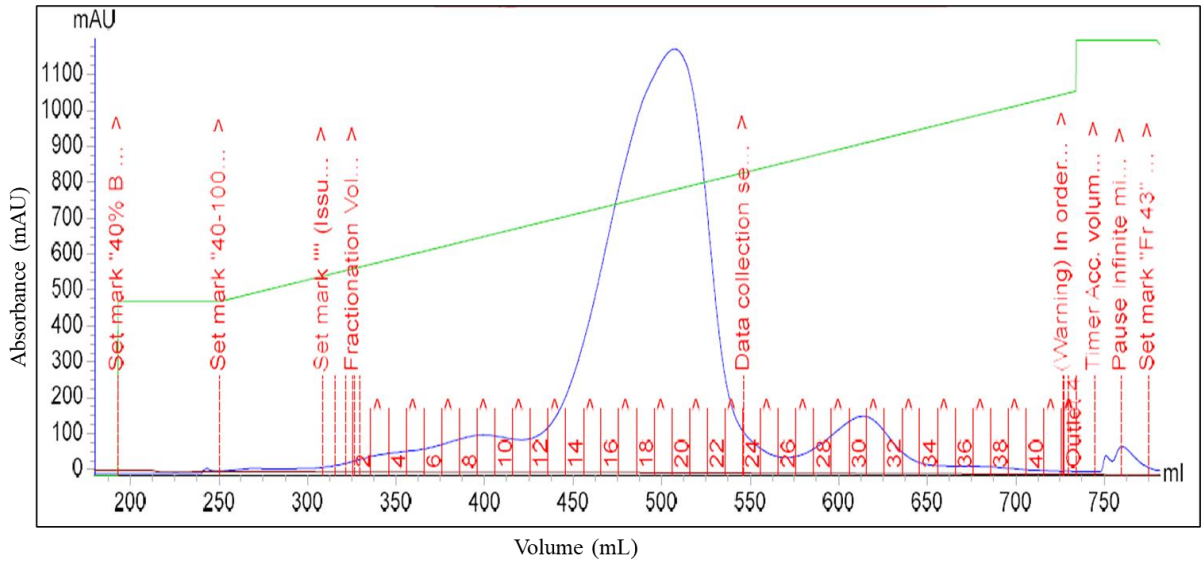

Figure 11. Chromatographic profile of Eshmuno CPX resin showing elution profile

Based on CEX-HPLC results, fractions were pooled separately to get different charge variants. The resolved peaks of Eshmuno CPX column were identify as three different variants due to resin bead size and tentacle technology. Pre and post eluting parts of cation exchange column were identified as acidic and basic charge variants respectively. However, middle part of the peak was identified as main peak based on residence time with respect to innovator profile analysed by CEX-HPLC. Each charge variants species was collected in sufficient amount and used for further studies.

The purity of the biosimilar product, individual charge variants and Xolair (liquid formulation) was estimated by the weak cation-exchange (CEX-HPLC) analytical method. The purity of acidic, main peak and basic variants were 94.25 %, 95.58 % and 91.33 % respectively as shown in Table 7. The isolated individual variants were eluted in acidic, main and basic retention time when compared with biosimilar product as shown in Figure 12.

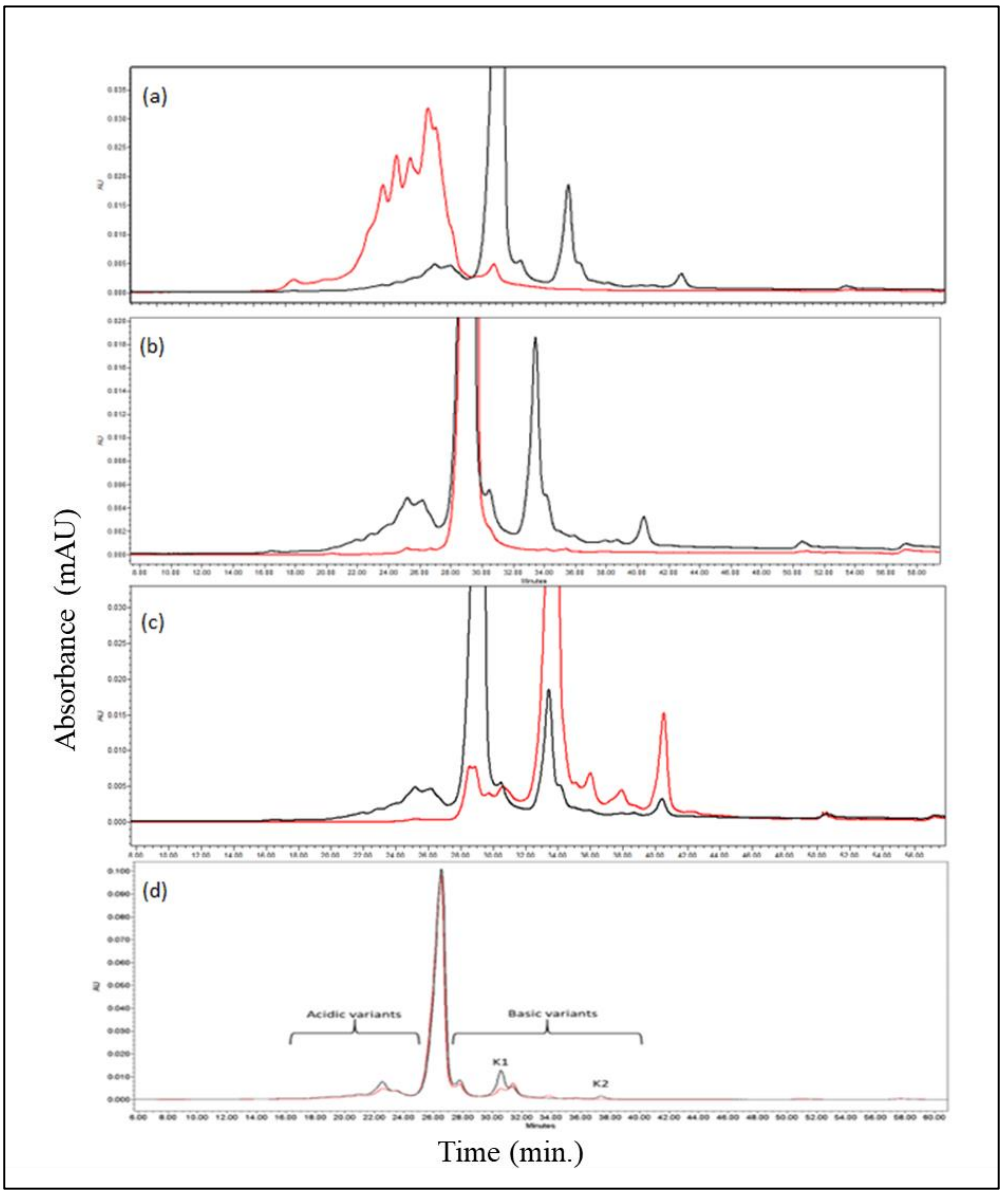

Figure 12. Analytical CEX-HPLC profiles of a. acidic charge variants; b. main peak; c. basic charge variants; d. Comparative profile with biosimilar product (red line) and Reference Standard (black line)

Size variants analysis for isolated charge variants and biosimilar product was completed with analytical SEC-HPLC. Purity of 99.58 %, 99.98 % and 98.64 % was achieved for acidic, main peak and basic variants respectively as shown in Table 8. Basic variants were with lesser purity in comparison to other variants was due to higher aggregation level which eluted selectively in basic region, have higher binding of aggregated with Eshmuno CPX. SEC-HPLC chromatogram is shown in Figure 13.

Table 8. Analytical SEC-HPLC results for Eshmuno CPX resin

Sample	**Main Peak (%)**	**HMWs (%)**	**LMWs (%)**
Eshmuno CPX Input	99.84 ± 0.05	0.00 ± 0.05	0.16 ± 0.02
Eshmuno CPX Acidic Pool (Fr 1 to Fr 9)	99.58 ± 0.05	0.06 ± 0.02	0.37 ± 0.02
Eshmuno CPX Main Peak (Fr 19 to Fr 20)	99.98 ± 0.01	0.02 ± 0.01	0.00 ± 0.01
Eshmuno CPX Basic Pool (Fr 25 to Fr 43)	98.64 ± 0.10	1.18 ± 0.02	0.18 ± 0.02

All experiments and samples were done in triplicate and average ± SD values are reported.

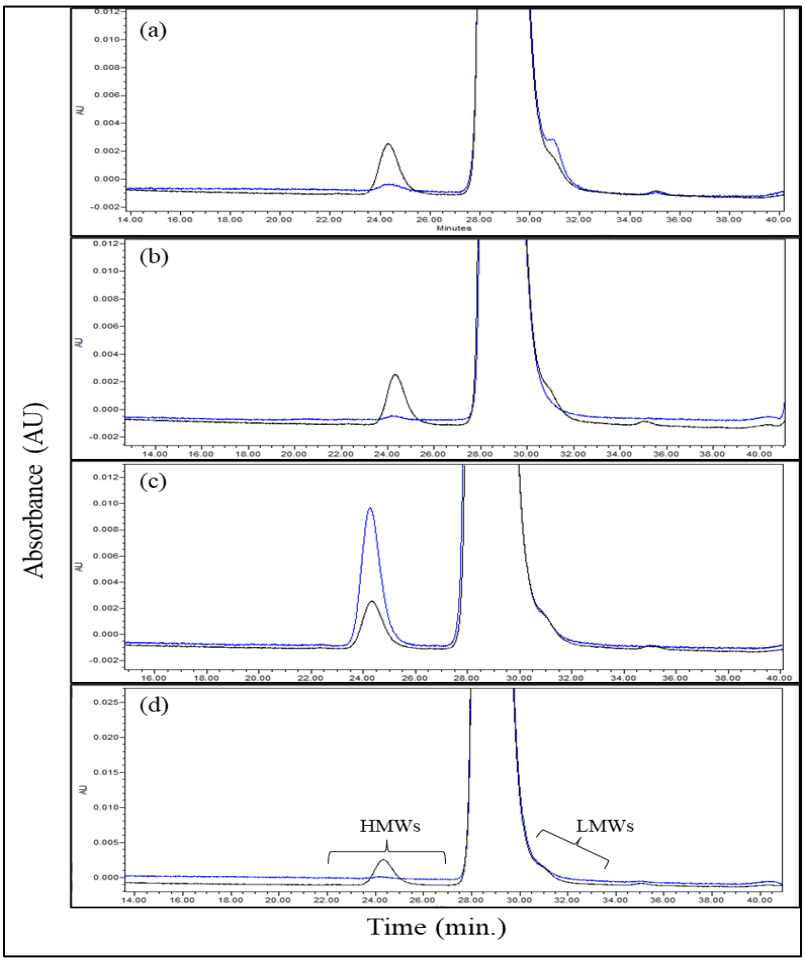

Figure 13. Analytical SEC-HPLC profiles of a. acidic charge variants; b. main peak; c. basic charge variants; d. Comparative profile with biosimilar product (blue line) and Reference Standard (black line)

Analytical results of CEX-HPLC and SEC-HPLC presented in Table 7 and Table 8 showed that isolated charge variants were highly pure in terms of charge and size. Hence, these purified charge variants were further used for studies such as stability at different temperature conditions, in-vitro potency estimation etc. (Singh et al., 2021). There may or may not be significant impact on in-vitro potency and in-vivo kinetics study of purified charge variants. Zhao et al. found that heterogeneity of charge variants of the Avastin biosimilar molecule shows no impact on the in-vitro potency and identical PK in rats was demonstrated (Zhao et al., 2016). However, Dakshinamurthy et al. demonstrated with Trastuzumab biosimilar, charge variants do have impact in the binding and potency assay (Dakshinamurthy et al., 2016).

4.4 Formulation of Charge Variants and Thermal Stress

Purified charge variants (acidic, main peak and basic) were formulated individually in stable buffer containing 20 mM phosphate and 200 mM arginine, pH 6.0 and conductivity 15 mS/cm. Final concentration of acidic, main peak and basic variants was 13.9 mg/mL, 17.5 mg/mL and 12.5 mg/mL respectively. Respective charge variants were dispensed, labelled and charged at different thermal stress conditions (2-8 °C and -20 °C) for 7 days. Stability and control samples were analysed further by CEX-HPLC analytical methods.

At each time point (0 day and 7 days) and stress condition, the purity of charge variants, was estimated by the cation-exchange (CEX-HPLC) analytical tool. The purity of acidic pool is comparable and not significantly changing (91.15% at 2-8 °C Day 7, 91.81% -20 °C Day 7 vs 94.25% Day 0) at two different thermal stress conditions up to 7 days in comparison to starting material as shown in Table 9. Similar trend has been observed in case of main peak (93.85% at 2-8 °C Day 7, 95.08% -20 °C Day 7 vs 95.58% Day 0) and basic pool (91.19% at 2-8 °C Day 7, 91.62% -20 °C at Day 7 vs 91.33% at Day 0) during different thermal stress conditions as shown in Table 10 and Table 11 respectively. The respective variants stability samples were eluted at same retention time when compared with initial samples as shown in Figure 14.

Table 9. Analytical CEX-HPLC results for acidic pool at thermal stress (2-8 °C and -20 °C)

Sample	Main Peak (%)	Total Acidic Variants (%)	Total Basic Variants (%)
Acidic Pool (Day 0)	4.10 ± 0.04	94.25 ± 0.05	1.64 ± 0.02
Acidic Pool (2-8 °C Day 7)	5.60 ± 0.08	91.15 ± 0.04	3.24 ± 0.06
Acidic Pool (-20 °C Day 7)	4.63 ± 0.02	91.81 ± 0.04	3.57 ± 0.07

All experiments and samples were done in triplicate and average ± SD values are reported.

Table 10. Analytical CEX-HPLC results for main peak at thermal stress (2-8 °C and -20 °C)

Sample	Main Peak (%)	Total Acidic Variants (%)	Total Basic Variants (%)
Main Peak (Day 0)	95.58 ± 0.03	1.09 ± 0.03	3.33 ± 0.04
Main Peak (2-8 °C Day 7)	93.85 ± 0.06	0.99 ± 0.03	5.17 ± 0.04
Main Peak (-20 °C Day 7)	95.08 ± 0.06	1.03 ± 0.03	4.28 ± 0.08

All experiments and samples were done in triplicate and average ± SD values are reported.

Table 11. Analytical CEX-HPLC results for basic pool at thermal stress (2-8 °C and -20 °C)

Sample	Main Peak (%)	Total Acidic Variants (%)	Total Basic Variants (%)
Basic Pool (Day 0)	1.72 ± 0.04	6.96 ± 0.04	91.33 ± 0.05
Basic Pool (2-8 °C Day 7)	1.78 ± 0.03	7.04 ± 0.06	91.19 ± 0.03
Basic Pool (-20 °C Day 7)	1.65 ± 0.04	6.72 ± 0.04	91.62 ± 0.03

All experiments and samples were done in triplicate and average ± SD values are reported.

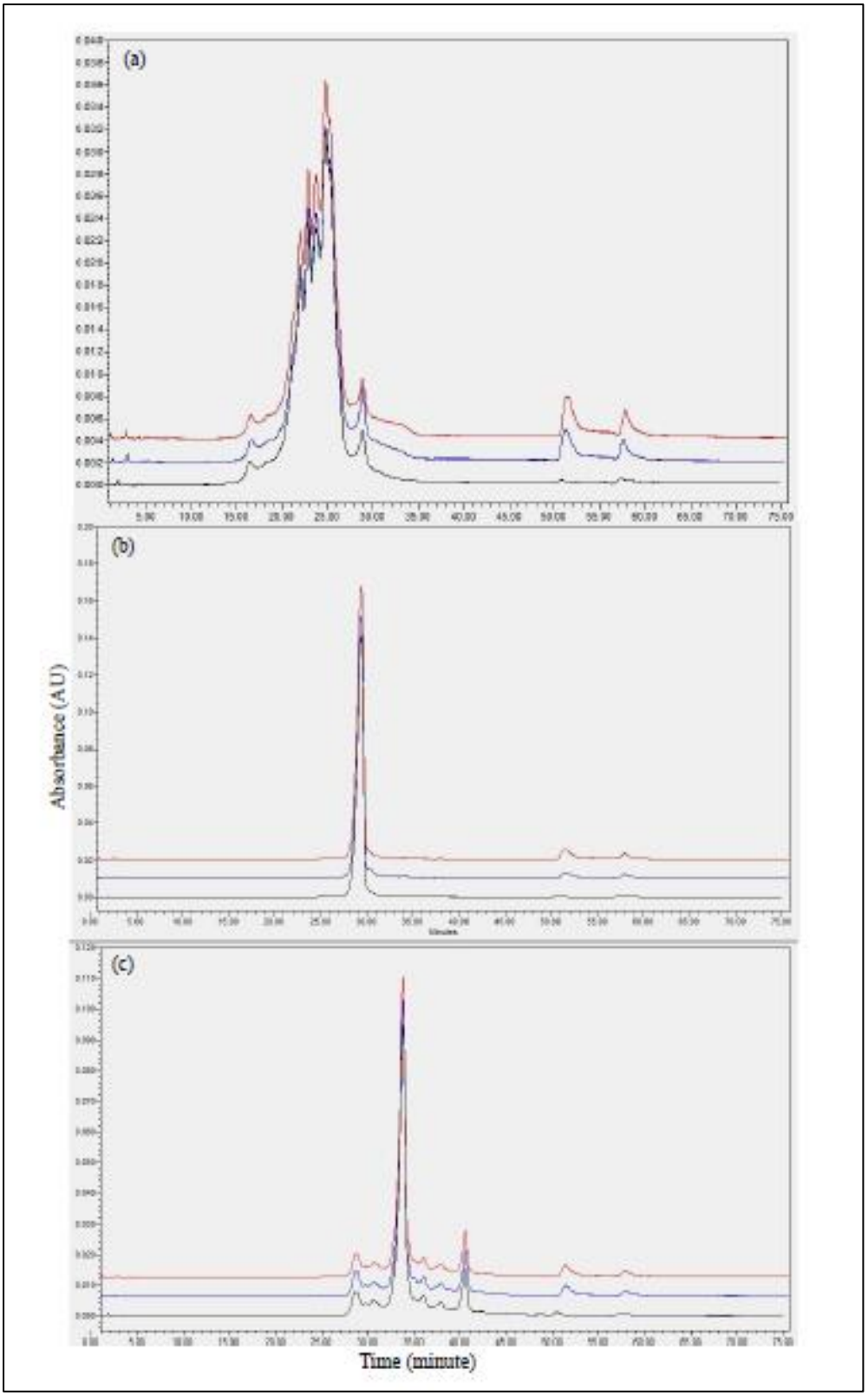

Figure 14. Analytical CEX-HPLC profiles of a. acidic charge variants; b. main peak; c. basic charge variants at different thermal stress conditions up to 7 days (Blackline- 0 day; Blue line 7 days at 2-8 °C; Redline- 7days at -20 °C)

Analytical results of CEX-HPLC presented in Table 9, Table 10 and Table 11 showed that respective charge variants (acidic, main peak and basic) were highly stable up to 7 days at two different thermal stress conditions (2-8 °C and -20 °C). These two temperature conditions are mostly used for therapeutic protein solution storage and temperature conditions such as 25°C and higher affects the protein solution stability due to molecular motion and kinetic energy (Zheng et al., 2024). Different temperatures are also playing an important role in molecule stability but mAbs shows good stability towards thermal stress (Wang et al., 1999; Paul et al., 2012; Le Basle et al., 2020).

4.5 *in-vitro* Potency analysis by Enzyme Linked Immunosorbent Assay (ELISA)

These purified charge variants were further used for *in vitro* potency estimation by ELISA method to check whether they have any impact on *in vitro* potency or not in comparison to control sample where less pure forms are present respectively. As shown in Table 12, all the purified charge variants have no impact on *in vitro* potency as all variants shows similar potency (acidic: 108.5%, maim peak: 121.8% and basic: 119.6%). This variance observed in potency is the assay variation and falls within the assay specification of 75% to 130%. This observation was also confirmed with orthogonal cell-based bioassay which also showed identical results (acidic- 99.7%, maim peak- 112.4% and basic pool- 87.1%) and confirms that purified charge variants doesn't have any impact on *in vitro* potency as shown in Table 13.

Table 12. Relative Potency by ELISA method for all charge variants

Sample	Relative Potency (%)
Acidic Pool	108.5 ± 7.91
Main Peak	121.8 ± 5.93
Basic Pool	119.6 ± 9.62

Confidence limits: 85% to 115% and specifications: 70% to 130% of reference standard

Table 13. Relative Potency by cell-based bioassay for all charge variants

Sample	Relative Potency(%)
Acidic Pool	99.7 ± 11.5
Main Peak	112.4 ± 7.8
Basic Pool	87.1 ± 3.0
Reference	94.5*

*Purity of Reference – Acidic varants-11.10%, Main peak- 73.64 and basic variants-15.25%
Confidence limits: 85% to 115% and specifications: 70% to 130% of reference standard

Yanchao et al., 2022 had worked on omalizumab biosimilar (named KA) where charge variants (acidic, main peak and basic) differences were observed in comparison to the innovator molecule (Xolair). Acidic and basic variants percentage was found higher in biosimilar molecule KA than Omalizumab reference product. They have also shown that these differences were due to post translational modifications and glycosylation which have no impact on biological activity of biosimilar molecule KA (Yanchao et al., 2022). In another study with different biosimilar molecule, Zhao et al. also found that heterogeneity of charge variants of the Avastin biosimilar shows no impact on the potency and identical pharmacokinetics (PK) profile in rats was demonstrated (Zhao et al., 2016).

In the present study it has been observed that all charge variants of omalizumab biosimilar with high purity of 90% with single respective species have no impact on *in vitro* potency as omalizumab Fab region plays an important role in providing the drug response. These results shows that biological activity of mAbs is totally dependent on mAb molecule interaction which part of mAb (either Fab or Fc region) is interacting and providing the drug response. This kind of more characterization studies will shed new light on the impact of charge heterogeneity on potency and stability (Gupta et al., 2023; Gupta et al., 2024a; Gupta et al., 2024b) . These

studies will also provide scope of process yield improvement and justification for product quality differences which does not have any impact on safety, efficacy and potency.

Chapter 5
Summary and Conclusion

The current biopharmaceutical industry practices during biosimilar product development are to closely match its charge variants to the originator up to the possible extent. The composition for originator and biosimilar products quality attribute should be based on cluster of respective variants and not for individual charge variant. This study discussed about the isolation and purification of Xolair biosimilar charge variants purification with preparative chromatography resin. These purification methods are robust and developed with aqueous buffer system. The isolated charge variants were highly purified for charge and size distribution determined by cation and size exclusion analytical methods respectively. These purified charge variants were also used to determine the impact on *in-vitro* potency and on hold stability at different thermal stress conditions.

Based on above mentioned results we conclude that there are no significant differences in *in vitro* potency with highly purified (> 90%) charge variants which is responsible for charge heterogeneity in many mAbs. This observation was also confirmed by different studies on mAbs biosimilars where percentage changes in charge variants have no impact on potency and PK profiles. We also found that highly purified charge variants are very much stable at different thermal stress conditions up to a week.

This is not only one factor related to charge heterogeneity which shows no impact, but also other factors can affect potency of mAbs. Hence, product safety and efficacy are dependent on other quality parameters those needs to be ensured throughout the product life cycle. This study also showed that biological activity of mAbs is totally dependent on mAb molecule interaction, either Fab or Fc is interacting and providing the drug response (Dakshinamurthy et al., 2017; Singh et al., 2016; Yanchao et al., 2022; Zhao et al., 2016). Based on that mAbs bio

similarity needs to be demonstrated and it may differ from mAbs to mAbs. These highly purified charge variants can be used for multiple studies needed for product life cycle such as impurity characterization by liquid chromatography-mass spectrometry, impurity spiking to know the purification capabilities.

Future Scope

These highly purified charge variants can be used for multiple studies needed for product life cycle such as Impurity characterization by liquid chromatography-mass spectrometry, Impurity spiking to know the purification capabilities, *in-vivo* efficacy and safety studies.

Abbreviation

ADCC	Antibody-dependent cellular cytotoxicity
AEX	Anion exchange
AGE	Advanced Glycation End-Products
BSA	Bovine serum albumin
CDC	Complement-dependent cytotoxicity
CDR	Complementarity-determining region
CEX	Cation exchange chromatography
CEX-HPLC	Cation exchange-high pressure liquid chromatography
cGMP	current Good Manufacturing Practices
CGRP	Calcitonin gene-related peptide
CH	Clarified harvest
CH	Constant Heavy
CHO	Chinese Hamster Ovaries
CL	Constant Light
cm	Centimetre
CPB	Carboxypeptidase B
CQA	Critical quality attribute
CRO	Contract research organisation
CV	Column volume
DF	Depth filtration
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
Fab	Fragment antigen-binding

Fc	Fragment crystallizable
FcRn	Neonatal fragment crystallizable (Fc) receptor
FDA	Food and Drug Administration
Fr	Fraction
HCD	Host cell DNA
HCP	Host cell protein
HMW	High molecular weight
ICH	International Council for Harmonization
Ig	Immunoglobulin
IgE	Immunoglobulin E
IgG1	Immunoglobulin G1
IL-17α	Interleukin 17 alfa
IL-6R	Interleukin 6 receptor
kDa	Kilo Dalton
LC-MS	Liquid chromatography-mass spectroscopy
LMW	Low molecular weight
mAbs	Monoclonal antibodies
MES	2-(N-Morpholino) ethanesulfonic acid
mg	Milligram
mL	Millilitre
mM	Millimolar
MMC	Mixed-mode chromatography
mS	Milli Siemen
NANA	N-Acetylneuraminic acid
NGNA	N-Glycolylneuraminic Acid

NS0	Non-secreting cell line
PD-L1	Programmed death-ligand 1
pI	Isoelectric point
PK	Pharmacokinetics
PP	Polypropylene
PTMs	Post-translational modifications
SEC-HPLC	Size exclusion chromatography
TFF	Tangential flow filtration
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
USA	United States of America
USD	United State Dollars
UV	Ultraviolet
VCC	Viable cell count
VEGF-A	Vascular Endothelial Growth Factor A
VH	Variable Heavy
VL	Variable Light
WHO	World Health Organization

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List of Publications

Research Papers

Tarun Gupta, Anuj Kumar, Sriram Seshadri (2023) Bioprocess Challenges in Purification of Therapeutic Protein Charge Variants. Biotechnology and Bioprocess Engineering, (Springer) E 28, 493–506. https://doi.org/10.1007/s12257-023-0078-4. (Impact Factor 3.2)

Tarun Gupta, Sriram Seshadri (2024) Charge variants of proposed biosimilar to Omalizumab: Isolation, purification and analysis by HPLC methods. Annales Pharmaceutiques Françaises, (Elsevier) 82: 64-71. https://doi.org/10.1016/j.pharma.2023.09.003. (Impact Factor 3.2)

Tarun Gupta, Sriram Seshadri (2024) Highly purified charge variants of a proposed biosimilar to Omalizumab: impact on in vitro potency and stability under thermal stress. Bioprocess and Biosystems Engineering, (Springer) 47: 57-64. https://doi.org/10.1007/s00449-023-02944-8. (Impact Factor 3.8)

Publications other than PhD work

Om Narayan, **Tarun Gupta**, Mayankkumar Thakkar, Rupen Bhavsar, Kishor Galani, Sudharti Gupta, Kaushal Joshi, Hetal Katrodiya, Shalini Sharma, Vivek Dave, Anuj Kumar, Divya Tailwani, Chandramauli Rawal (2023) Novel Purification Process of Omalizumab Biosimilar for Industrial Production. International Journal for Research Trends and Innovation, Volume 8, Issue 6.

Om Narayan, Kaushal Joshi, Sumit Shah, Sudharti Gupta, Kiran Avadhani, Bruce Weaver, **Tarun Gupta**, Shalini Sharma, Chandramauli Rawal, Divya Tailwani (2023) Development of High Concentrated IgE Antibody for Anti-Asthmatic Therapy. International Journal for Research Trends and Innovation, Volume 8, Issue 6.

Om Narayan, Kaushal Joshi, **Tarun Gupta**, Mayank Thakkar, Utpal Deori, Chandramauli Rawal (2023) Monoclonal Antibody Purification with Reduced Turbidity by Using High-Salt Elution Buffer During Protein A Chromatography. International Journal for Multidisciplinary Research, Volume 5, Issue 3.

Oral Presentations

Tarun Gupta, Anuj Kumar, Sriram Seshadri. Therapeutic biosimilar protein charge variants purification and challenges. International conference **IIChE - CHEMCON 2023** on 27-30th December 2023, Kolkata, India. **(Abstract book page no.- 404 of 441).**

Tarun Gupta, Anuj Kumar, Sriram Seshadri. Isolation, purification and analysis of charge variants of proposed biosimilar to Omalizumab. International conference **IIChE - CHEMCON 2023** held on 27-30th December 2023, Kolkata, India**. (Abstract book page no. 404 of 441).**

Platinum Jubilee Celebration

Of

Indian Institute of Chemical Engineers

An International Conference on

Energy Transition: Challenges and Opportunities

IIChE-CHEMCON 2023

December 27 - 30, 2023

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Soumi Gupta

has presented a paper (Oral / Poster) in **IIChE-CHEMCON 2023,** *held at Heritage Institute of Technology, Kolkata, during 27 - 30 December 2023.*

Title: Isolation, purification ..... Omalizumab
(Isolation, purification and analysis of charge variants of proposed biosimilar to Omalizumab)

Co-authored by:

Prof. Anil Kumar Saroha
President, IIChE

Dhawal Saxena
Honorary Registrar, IIChE

Dr. Avijit Ghosh
Organizing Secretary, IIChE-CHEMCON 2023

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ORIGINAL ARTICLE

Charge variants of proposed biosimilar to Omalizumab: Isolation, purification and analysis by HPLC methods

Variants de charge du biosimilaire proposé à l'omalizumab isolement, purification et analyse par des méthodes HPLC

Tarun Gupta[a,b], Sriram Seshadri[a,*]

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HIGHLIGHTS

- Charge heterogeneity of Xolair biosimilar by Cation exchange chromatography.
- Charge variants were characterized by SEC-HPLC and CEX-HPLC.
- Purity of acidic, main peak and basic variants were 99.58%, 99.98% and 98.64% respectively by SEC-HPLC.
- Purity of acidic, main peak and basic variants were 94.25%, 95.58% and 91.33% respectively by CEX-HPLC.
- Purified charge variants could be used to determine the impact on in-vitro potency.

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Tanmoy Gupta

has presented a paper (Oral / Poster) in IIChE-CHEMCON 2023, *held at Heritage Institute of Technology, Kolkata, during 27 – 30 December 2023.*

Title: Therapeutic Biosimilar Protein Drugs: Purification and Challenges

Co-authored by:

Prof. Anil Kumar Saroha	Dhawal Saxena	Dr. Avijit Ghosh
President, IIChE	Honorary Registrar, IIChE	Organizing Secretary, IIChE-CHEMCON 2023

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REVIEW PAPER

Bioprocess Challenges in Purification of Therapeutic Protein Charge Variants

Tarun Gupta, Anuj Kumar, and Sriram Seshadri

Received: 28 March 2023 / Revised: 19 April 2023 / Accepted: 23 April 2023

Abstract Biopharmaceuticals are complex therapeutic protein molecules produced in living cells and have been a major driving force for drug development in the pharmaceutical sector in recent years. Monoclonal antibodies (mAbs) are biological macromolecules used for treating life-threatening and rare illnesses. mAbs with post-translation alterations can be observed during the assessment of charge variants. Controlling the charge variant profile of therapeutic protein is a regulatory requirement to confirm that the macromolecule complies with the quality parameters to ensure patient safety. Unfortunately, manufacturing these biopharmaceuticals is very expensive. However, the emergence of biosimilars has reduced developmental cost across the biopharmaceutical industry. The advent of biosimilars has constrained the development of more efficient downstream bioprocesses that are mainly considered the bottleneck of the manufacturing process. This review focuses on the existing methods for charge variants separation and process optimization and indicates new approaches for future developments. It also provides a comprehensive summary for the biological community about the impact of charge variants.

Keywords: biosimilar, monoclonal antibodies, chromatography, process development, pharmacokinetics

1. Introduction

In the biotherapeutic field, large biologics such as monoclonal antibodies (mAbs) have brought dramatic benefits to the individuals suffering from a critical illness, where previous therapies were not too effective or towards non-existent [1-4]. Now, mAbs based biotherapeutic drugs are the face of new drug development in biopharmaceutical industries [5,6]. mAbs are very fast developed against numerous diseases, not only due to their specific targets in different areas related to immunology, neurology, metabolic disorder, and oncology, *etc.* but also attributed to their accessibility and cost-effectiveness [7,8]. Information gathered in Table 1 discussed Food and Drug Administration (FDA) approved marketed antibodies and their details like brand name, manufacturer, molecular weight, isoelectric point (pI) and year of approval [1,9].

mAbs are biological macromolecules with complex biological structures; hence the production of either new mAbs or their biosimilar is very challenging due to their complicated structure, functions, and *in-vitro* media and feed conditions inside the bioreactor [10]. The introduction of biosimilars, which are very similar to the original molecule, has contributed to the availability of comparatively low-cost drugs due to its developmental and investment cost.

Biosimilars are "generic" versions of "originator" with respect to the amino acid sequence, but they are produced with different cell clones, production processes, and parameters.

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RESEARCH PAPER

Highly purified charge variants of a proposed biosimilar to Omalizumab: impact on in vitro potency and stability under thermal stress

Tarun Gupta[1,2] · Sriram Seshadri[1]

Received: 22 August 2023 / Accepted: 8 November 2023 / Published online: 29 December 2023

Abstract
Biosimilars are highly complex and similar biological drugs are developed with different manufacturing processes which are not similar to originator manufacturing process. Due to this, biosimilar products inherently have quality differences in comparison to innovator molecule which may be related to size, charge and glycosylation. Despite these differences they are supposed to demonstrate similar behaviour in safety and efficacy profile to the reference product and these differences should not be clinically meaningful. Charge variants are one of the critical quality attributes and sources of heterogeneity. In this study, highly purified charge variants cluster (acidic, main peak and basic) of biosimilar product of Xolair were assessed for their impact on in vitro potency and stability at different thermal stress conditions (2–8 °C and – 20 °C). The study data indicating purified charge variants (> 90%) have no impact on in vitro potency and are stable at different thermal stress conditions up to a week.

“Purification of Protein Charge Variants and Determination of their Potency”

A THESIS SUBMITTED TO
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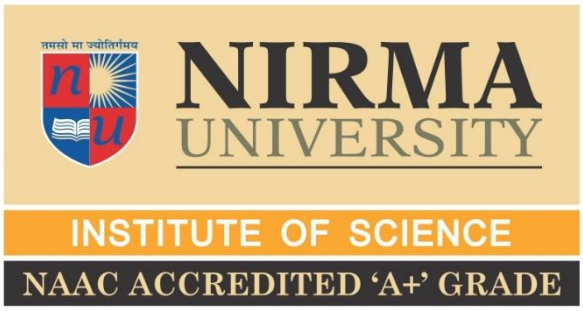
In Partial Fulfillment of the Requirement for
the Degree of

Doctor of Philosophy
In
Science

By

Tarun Kumar
(20PTPHDS79)



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May 2024

Conclusion and Future Scope

The current biopharmaceutical industry practices during biosimilar product development are to closely match its charge variants to the originator up to the possible extent. The composition for originator and biosimilar products quality attribute should be based on cluster of respective variants and not for individual charge variant. This study discussed about the isolation and purification of Xolair biosimilar charge variants purification with preparative chromatography resin. These purification methods are robust and developed with aqueous buffer system. The isolated charge variants were highly purified for charge and size distribution determined by cation and size exclusion analytical methods respectively. These purified charge variants were also used to determine the impact on *in-vitro* potency and on hold stability at different thermal stress conditions.

Based on above mentioned results we conclude that there are no significant differences in *in vitro* potency with highly purified (> 90%) charge variants which is responsible for charge heterogeneity in many mAbs. This observation was also confirmed by different studies on mAbs biosimilars where percentage changes in charge variants have no impact on potency and PK profiles. We also found that highly purified charge variants are very much stable at different thermal stress conditions up to a week.

This is not only one factor related to charge heterogeneity which shows no impact, but also other factors can affect potency of mAbs. Hence, product safety and efficacy are dependent on other quality parameters those needs to be ensured throughout the product life cycle. This study also showed that biological activity of mAbs is totally dependent on mAb molecule interaction, either Fab or Fc is interacting and providing the drug response (Dakshinamurthy et

al., 2017; Singh et al., 2016; Yanchao et al., 2022; Zhao et al., 2016). Based on that mAbs bio similarity needs to be demonstrated and it may differ from mAbs to mAbs. These highly purified charge variants can be used for multiple studies needed for product life cycle such as impurity characterization by liquid chromatography-mass spectrometry, impurity spiking to know the purification capabilities.

Future Scope

These highly purified charge variants can be used for multiple studies needed for product life cycle such as Impurity characterization by liquid chromatography-mass spectrometry, Impurity spiking to know the purification capabilities, *in-vivo* efficacy and safety studies.