Development of High Concentrated IgE Antibody for Anti-Asthmatic Therapy

1Om Narayan, 2Kaushal Joshi, 3Sumit Shah, 4Sudharti Gupta, 5Kiran Avadhani, 6Bruce Weaver, 7Tarun Gupta, 8Shalini Sharma, 9Chandramauli Rawal, 10Divya Tailwani

1 Sr. Vice-President- R&D, Kashiv BioSciences Private Limited, Ahmedabad, India
2 Sr. Director-IP and R&D, Kashiv BioSciences Private Limited, Ahmedabad, India
3 Ast. Director- R&D, Kashiv BioSciences Private Limited, Ahmedabad, India
4 Ast. Vice President- R&D, Kashiv BioSciences Private Limited, Ahmedabad, India,
5 Research scientist II- R&D, Kashiv BioSciences Private Limited, Ahmedabad, India
6 Director- R&D, Kashiv Biosciences, LLC, New Jersey, USA
7 Sr. Director- Kashiv BioSciences Private Limited, Ahmedabad, India
8 Ast. Vice President- R&D, Kashiv BioSciences, LLC, New Jersey, USA
9 COO, Kashiv Biosciences, LLC, New Jersey, USA
10 Executive- IP, Kashiv BioSciences Private Limited, Ahmedabad, India

Abstract: Asthma, allergic asthma and chronic idiopathic urticaria are almost invariably accompanied by elevated levels of IgE. Omalizumab is a recombinant DNA-derived humanized monoclonal antibody that selectively binds to human immunoglobulin (IgE). The antibody has a molecular weight of approximately 149 kDa. The liquid formulations of omalizumab are pharmacologically effective at high concentrated formulation, results in substantial increase in the protein-protein interaction and thereby increasing the viscosity of the formulation. The present study attempts to develop and optimize high concentration liquid formulation for omalizumab having high monomer, low aggregates, and desirable viscosity. The formulations were prepared by using different buffers, amino acids, and surfactants. All formulations were analysed for viscosity at fixed protein concentration. Based viscosity data, formulations were short listed and charged for stability at 37℃ up to one month. Stability samples at different time points were analysed for aggregation behaviour. Samples were also analysed for acidic and basic species. Based on data trend, the formulation was finalized and applied in the treatment of Asthma, allergic asthma and chronic idiopathic urticaria as subcutaneous administration.

Keywords: Omalizumab, anti-IgE antibody, high concentration.

Introduction:

Allergies also called as allergic reactions or diseases relates to the hypersensitivity of one’s immune system to any substance that is present in their environment. Allergies include food allergy, allergic asthma, atopic dermatitis, and anaphylaxis. When a person comes contact/inhales an allergen e.g., Pollen, peanuts, dust etc. These allergens enter his body and are engulfed by the antigen presenting cells namely macrophages and basophils and the peptide episode of these allergens are displayed on the surface of these antigen presenting cells[1]. This initiates the production of antibody immunoglobulin E/IgE in large amount. These IgE comprises of high affinity IgE receptor (FccRI) binding site which will bind to the high affinity IgE receptor (FccRI) present on the surface of Basophils and macrophages. This binding of IgE and Basophils triggers the degranulation of the basophils, resulting in the release of inflammatory mediators and cytokines (such as IL-4, IL10, IL-33 and B cell activating factor (BAFF)[2]). These mediators initiate early response and manifest mucous production nasal congestion itching sneezing, bronchial spasm and mucosal edema[3].

In regular practices, Oral corticosteroids are used to treat allergic reactions. However, it is seen that with the use of these corticosteroids for a long time can result in patients suffering from osteoporosis[4,5], increases the risk of increase the risk of cardiovascular disease[4,6], muscle weakness, stomach ulcers, increased blood sugar (glucose) and delayed growth in children[4,7]. As antibodies are considered as a powerful and important therapeutic agent due to their specificity towards the variety of host cells and their limited side effects, a monoclonal antibody is developed to treat allergic asthma and allergy. Omalizumab is a biologically engineered molecule, more specifically it is a monoclonal antibody recombinant DNA-derived humanized IgGlK that binds to the free IgE, released by the patient’s body, and thereby prevents the binding of free IgE on high affinity IgE receptor (FccRI) on mast cells, and basophiles. It prevents early and late phase allergic reactions of skin and lungs[8].

In earlier times antibody formulations were prepared and administered in solid form and were stabilised with lyophilisation of antibody and were reconstituted prior to use with appropriate solvent system. However, with time, the requirement of stable high concentrated liquid formulation is increased because it can be administrated subcutaneously in shorter time compared to intravenous injections. Further, patient can self-administer subcutaneously at home or with the help of health care giver which avoids hospitalization and treatment costs, and a lower level of tissue invasiveness[9]. However, high concentrated antibody formulation comprises high amount of antibody approximately more than 100mg/ml in small volume which pose challenges due to their susceptibility towards protein aggregation and their tendency to form viscous solutions due to their high concentration and potential of inter-molecular interactions[9]. Viscosity is not only an issue regarding the biophysical and biochemical properties of the therapeutic protein, but also for the delivery and manufacturing of such highly concentrated protein solutions. Higher the viscosity of the solution the longer it takes to inject such a viscous solution via syringe.
and needle. In addition, protein-protein interaction in high concentrated antibody formulation tends to form oligomerization, aggregation and thereby makes unstable formulation.[10].

Several problems associated with development of high concentration monoclonal antibody formulations such as aggregation, viscosity, fragmentation, insolubility, and degradation of antibodies. The term used “aggregates” are classified based on types of interactions and solubility. Soluble aggregates are invisible particles and cannot be removed with a filter. Insoluble aggregates can be removed by filtration and are often visible to the human eye. Both types of aggregates cause problems in biopharmaceutical product development. Covalent aggregates arise from the formation of a covalent bond between multiple monomers of a given peptide. Disulphide bond formation of free thiols is a common mechanism for covalent aggregation. Oxidation of tyrosine residues can lead to formation of tyrosine which often results in aggregation. Reversible protein aggregation typically results from weaker protein interactions they include dimers, trimers, multimers among others.[10].

Further, aggregation of antibodies affects their stability in storage, including shelf-life. It has been well recognised that there is no standard formulation strategy which can work for all types of antibodies to provide stable and concentrated solution for pharmaceutical use. Therefore, it is well established the need of the present study is to develop and provide with a liquid formulation which shows substantial increase in the stability and provides antibody in its desired form throughout shelf-life of the product.

2. Materials and Methods:

2.1 Materials:

Omalizumab was manufactured at Kashiv Biosciences Pvt Limited. and same is being used for formulation development. Reagents for preparing such as amino acids and salts excipients were of biopharma grades and obtained from commercial vendors as follows: Sodium phosphate monobasic monohydrate (Merck), Sodium phosphate dibasic heptahydrate (Merck), Arginine HCl (J T Baker), L-Lysine HCl (J T Baker), L-Histidine (J T Baker), L-Histidine HCl monohydrate (J T Baker), L-Lysine (J T Baker), Poloxamer -188(Merck), Polysorbate – 20 (Croda), Supor syringe filter of 32 nm 0.2 µm ( Pall life sciences), 1 mL long glass syringes, centrifugal filter units with the molecular weight cut-off of 30 kDa (Amicon Ultra, Merck Millipore Ltd., Germany). 1 mL long florotect coated plunger stopper and plunger rod.

In this in vitro study, the pharmacologically active antibody is Omalizumab, is present in high concentration in the range of 50mg/ml-200mg/ml. Histidine and phosphate buffer are utilized in the concentration range of 5 mM-20 mM. High concentration of the antibody in the formulation leads to protein-protein interaction which tends to form protein aggregates and results in the increase of viscosity of the formulation.

2.2 Methods:

2.2.1 Preparation of high concentration omalizumab solution

A high concentration omalizumab solution was prepared using Amicon Ultra 50K (15 mL) centrifugal filter devices for desalting and concentration. The buffer has been fully exchanged because of three times 10-fold dilution. The solution was desalted with Histidine and phosphate buffer are utilized in the concentration range of 5 mM-20 mM (pH 5.9 to pH 6.2) by centrifuging at 2,500 g for 45 min at 25°C. Lysine, arginine and/or arginine hydrochloride or lysine hydrochloride being used as protein aggregation inhibitors in the concentration range of 100 mM - 200 mM. For surfactant poloxamer 188 are being used in the concentration range of 0.02% - 0.04% of the solution. After repeating this process twice, the sample was collected from the filter. The concentration of omalizumab was adjusted by concentration with centrifugation or dilution with the His buffer, so that the final concentration was 150 mg/mL. Prepared formulations were analysed for protein concentration using a UV-vis spectrophotometer (Shimadzu UV2600), and the extinction coefficients for the omalizumab were measured at 280 nm (1.55 cm-1). The formulations were charged for stability at different temperature conditions as per ICH guidelines.

<table>
<thead>
<tr>
<th>Table 1. Different formulation compositions. (M – Molar concentration; mM – Millimolar concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.No.</td>
</tr>
<tr>
<td>F1</td>
</tr>
<tr>
<td>F2</td>
</tr>
<tr>
<td>F3</td>
</tr>
<tr>
<td>F4</td>
</tr>
<tr>
<td>F5</td>
</tr>
<tr>
<td>F6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. Formulations with Poloxamer (0.04%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.No.</td>
</tr>
<tr>
<td>F7</td>
</tr>
</tbody>
</table>
2.2.2 Viscosity Measurements

Viscosity measurement was carried out for different high concentration omalizumab formulations by BROOKFIELD CAP 1000+ Viscometer at ambient temperature (Data were given in Table 1).

2.2.3 Size-Exclusion HPLC

Separations were performed using an Agilent 1100 HPLC system (Agilent, California) equipped with a binary or quaternary pump, an automated sampler cooled to 4°C, a heated column compartment at 25°C and a diode array detector all controlled by Empower 3 software (Dionex, California). All samples were run on a TSKGel G3000SWXL column, 7.8 ×300 mm² (Tosoh Bioscience, California), TSKGel Guard SWXL column, 6.0 ×400 mm² at a flow rate of 0.3 mL/min over 45 min. A 10 mm Semi-Micro flow cell and in some cases, as an option to increase the linear range of detection, a 6 mm Micro flow cell (Agilent) was used. Data were acquired at 215, 235, and 280 nm [12].

2.2.4 Cation-exchange chromatography at pH 6.5

The CEX method was run on an UltiMate 3000 Series (Dionex, Sunnyvale, CA) HPLC system. Chromatography was performed on a ProPac Elite WCX analytical column (weak cation exchange, 4 mm X 250 mm; Dionex) preceded by a ProPac WCX-10G guard column (weak cation exchange, 4mm X 50 mm; Dionex) at 40°C. Formulation sample was loaded onto the column and analyzed at a flow rate of 0.6 mL/min. The column was equilibrated with Buffer A (20 mM MES, pH 6.5), and protein was eluted with a linear gradient of Buffer B from 0 to 100% (20 mM MES, 200 mM sodium chloride, pH 6.5) over 75 min. Absorbance was measured at UV 280 nm [13].

2.2.5 Differential scanning fluorimetry (DSF)

The DSF method is the conventional method utilized to measure the protein unfolding based on the changes in fluorescence due to temperature. Hence DSF is used to analyse protein folding and thermal stability. It is a fast, reliable and robust tool to examine protein stability, thermal protein unfolding by controlled heating (e.g., 0.5 °C / min) of protein in a range of increasing temperature (e.g., 25°C to 95 °C). Protein concentrations of samples were normalized before analysis.

3. Results and Discussion:

3.1 Viscosity Measurements

Viscosity is one of key parameter for optimization of high concentration antibody formulations. Viscosity behaviour of monoclonal antibody formulations that determine the solubility, aggregation, protein molecule interaction and adsorption of protein. Apart from molecule, there are several challenges from manufacturing to clinical path [14]. Hence, it is important to select the formulation with lowest viscosity from different formulations. Several experimental approaches to reduce the viscosity of monoclonal antibody formulations such as formulating with amino acids, different buffer and stabilizers existed in current formulation field [15]. In the present study, viscosity results for eight omalizumab drug product formulations with concentration of approximate 150 mg/mL are summarized in Table 1 and table 2. Viscosity was measured for all formulations at pH 6.2 (F1-F6) and pH 5.9 (F7 and F8) at ambient temperature as shown in Table 1. Formulation (F6) with 200 mM L-Arginine HCl, 8.8 mM L-Histidine, 12.2 mM L-Histidine HCl, Polysorbate 20 (0.04%) considered as control formulation. From the viscosity data, highest viscosity was found with F1 (200mM L-Histidine-Histidine), F3 (200 mM Lysine-HCl, 20mM histidine buffer) and F4 (200 mM Lysine-HCl, 20mM acetate buffer). Viscosity for control formulation (F6) was found to be 12.83 cPs. Formulation (F5) was very close to the control formulation and considered as top selected formulation. It was formulated with poloxamer of 0.04% (F7) and viscosity was found to be 15 cPs. L-Arginine HCl and phosphate buffer composition along with poloxamer has given reduced viscosity formulation.

3.2 Size-Exclusion HPLC

Aggregates are measured in terms of % high molecular weight proteins (% HMW). On the other hand, % low molecular weight proteins (% LMW) indicates prone to fragmentation. These are critical quality attribute which can directly affect the safety and efficacy. Several gaps found in quantitation of aggregates with currently existed formulations in the market. It was acknowledged by widely used SEC-HPLC to determine the aggregates. It is simple method and determine the % HMW and % LMW without stressing the molecule. In the present study, formulations were short listed based viscosity criteria. From F1 to F6 formulations, F5 was short listed. Later F7 and F8 were charged for stability and SEC-HPLC analysis was done at different time points and trend was considered to optimize the formulation. In F7 formulation, it was found that L-Arginine along with phosphate buffer and poloxamer 188 was able to control the % HMW (dotted line in the Figure 1 and figure) and acts as tonicity adjusting agent [16]. In case of formulation with % L- Lysine along with phosphate buffer and poloxamer 188, % HMW was increased at 1M stability study. But in case % LMW was same for both formulations F7 and F8. There is no impact of L-Arginine and L-Lysine on % LMW. Hence formulation F7 has been considered as optimized formulation.

**Figure 1.** Aggregation (% HMW) for formulation F7 and F8 has shown. F7 formulation (with 200 mM L-Arginine, phosphate buffer and poloxamer 188) showing better results with reduces % HMW on stability at 37 °C up to 1 month (dotted lines) when compared with F8 formulation (200 mM L-lysine, phosphate buffer and poloxamer 188).
3.3 Cation-exchange chromatography at pH 6.5

Acidic variants refer to the variants of a protein, which are characterized by an overall acidic charge. For example, in monoclonal antibody (mAb) preparations, such acidic species can be detected by various methods, such as ion exchange, for example, WCX this is HPLC (a weak cation exchange chromatography), or IEF (isoelectric focusing). Acidic variants of antibodies are formed through Chemical and enzymatic modifications such as deamidation and sialylation, respectively, resulting in an increase in the net negative charge on the antibodies and causing a decrease in pI values, thereby leading to formation of acidic variants. C-terminal lysine cleavage results in the loss of net positive charge and leads to acidic variant formation. Another mechanism for generating acidic variants is the formation of various types of covalent adducts, e.g., glycation, where glucose or lactose can react with the primary amine of a lysine residue during manufacturing in glucose-rich culture media or during storage if a reducing sugar is present in the formulation. The basic variants refer to variants that results from the presence of C-terminal lysine or glycine, amidation, succinimide formation, amino acid oxidation or removal of sialic acid, which introduce additional positive charges or removal of negative charges; both types of modifications cause an increase in pI values. From the present CEX data, % acidic species for F7 formulation (with 200mM L-Arginine, phosphate buffer and poloxamer 188) at 37 °C up to 1 month (dotted lines) and F8 formulation (200mM L-lysine, phosphate buffer and poloxamer 188) are following same trend (as shown in Figure 3 and 4). There is no change in % acidic trend across both formulations F7 and F8 when compared to reference product data (data has not shown). Similarly, % basic species for F7 formulation (with 200mM L-Arginine, phosphate buffer and poloxamer 188) at 37 °C up to 1 month (dotted lines) and F8 formulation (200mM L-lysine, phosphate buffer and poloxamer 188) are almost comparable with reference product data (data has not shown).
3.4 Differential Scanning Fluorimetry (DSF)

DSF is a method which is used to determine the temperature at which proteins melt, wherein the protein mixture solution is exposed to a temperature gradient that results in the unfolding of the proteins, present in the solution. The intrinsic fluorescence of the protein solutions is mainly from the aromatic side chain of tyrosine and tryptophan, and upon unfolding these residues gets exposed to the solvent and the fluorescence intensity changes. The temperature gradient and change in fluorescence intensity can be used to get the melting temperature (Tm). The Tm is measured by measuring the change in heat associated with molecule thermal denaturation when the molecules are heated at the constant rate. The high the Tm value the more stable the molecule. Therefore, herein DSF is used to analyse protein folding state and thermal stability. It is a fast, reliable and robust tool to examine protein stability, the normal protein unfolding by controlled heating (e.g., 0.5 °C / min) of protein in a range of increasing temperature (e.g., 25°C to 95 °C). In the present study, the F6, F7 and F8 were analysed through Differential Scanning Fluorimetry (DSF) to analyse protein folding and thermal stability and the results are shown in Table 3. The Tm value of F6 was observed as Tm3 = 78.6, on contrary the Tm value for F7 and F8 was observed as 81.5 and 81.1 respectively. It is evident from Table 3 that onset temperature of both the formulations (F7 and F8) is higher than F6. Which indicates that both the formulations F7 and F8 are more thermally stable than F6 formulation. Further it is observed that Tm value of F7 (Tm3 81.5) is even higher than the value of F8 that is 81.1. Which indicates that among F7 and F8, formulation F7 is more thermally stable.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Onset temperature</th>
<th>Tm1</th>
<th>Tm2</th>
<th>Tm3</th>
</tr>
</thead>
<tbody>
<tr>
<td>F6</td>
<td>58.4</td>
<td>65.1</td>
<td>74.7</td>
<td>78.6</td>
</tr>
</tbody>
</table>

Table 3. Thermal stability of formulations through DSF.
4. **Conclusion:**

Novel high concentration monoclonal antibody formulation containing amino acids, phosphate buffer and poloxamer were successfully prepared and optimized using critical parameters such as viscosity, protein concentration, % HMW and % LMW by SEC-HPLC analysis and % acidic species and % basic species by cation exchange chromatography. The optimized formulation containing L-Arginine, phosphate buffer and poloxamer 188 has shown lower viscosity, higher stability against aggregation formation and maintaining the acidic species and basic species as comparable with reference drug product at 37°C for one month. This study demonstrates the use of 200 mM L-Arginine, 20 mM phosphate buffer and poloxamer 188 (0.04%) for stabilizing the omalizumab at 150 mg/mL concentration under stress condition.

**Acknowledgements (if applicable)**

We are thankful to Mr. Kaushal Joshi and Mr. Sumit Shah for their valuable suggestions during this work.

**Conflict of interests:**

Authors declare that there is no conflict of interest.

**References**