Laboratory science

Validation of the manufacturing process used to produce long-acting recombinant factor IX Fc fusion protein

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Summary. Recombinant factor IX Fc (rFIXFc) fusion protein is the first of a new class of bioengineered long-acting factors approved for the treatment and prevention of bleeding episodes in haemophilia B. The aim of this work was to describe the manufacturing process for rFIXFc, to assess product quality and to evaluate the capacity of the process to remove impurities and viruses. This manufacturing process utilized a transferable and scalable platform approach established for therapeutic antibody manufacturing and adapted for production of the rFIXFc molecule. rFIXFc was produced using a process free of human- and animal-derived raw materials and a host cell line derived from human embryonic kidney (HEK) 293H cells. The process employed multi-step purification and viral clearance processing, including use of a protein A affinity capture chromatography step, which binds to the Fc portion of the rFIXFc molecule with high affinity and specificity, and a 15 nm pore size virus removal nanofilter. Process validation studies were performed to evaluate identity, purity, activity and safety. The manufacturing process produced rFIXFc with consistent product quality and high purity. Impurity clearance validation studies demonstrated robust and reproducible removal of process-related impurities and adventitious viruses. The rFIXFc manufacturing process produces a highly pure product, free of non-human glycan structures. Validation studies demonstrate that this product is produced with consistent quality and purity. In addition, the scalability and transferability of this process are key attributes to ensure consistent and continuous supply of rFIXFc.

Keywords: haemophilia B, human cell line, manufacturing, recombinant, rFIXFc, viral clearance

Introduction

Current haemophilia B management involves replacement therapy with coagulation factor IX (FIX) concentrates, either given episodically (on demand) to treat bleeding episodes or prophylactically to maintain minimum FIX activity and to prevent bleeding [1–4]. FIX replacement therapy has been available since the 1970s, initially utilizing plasma-derived FIX concentrates [1,2]. Development of recombinant FIX (rFIX) in the 1990s circumvented viral safety concerns associated with plasma-derived concentrates and greatly reduced supply shortages [5] and continues to be important in the treatment of haemophilia B [6].

Due to viral contamination issues with previous plasma-derived FIX products, it is important to characterize the manufacturing process of any new haemophilia therapy and to conduct validation studies that evaluate product quality and demonstrate the ability of the process to remove process-related impurities and viruses. Similar characterization has been done for other rFIX products [7,8]. Recombinant FIX Fc Fusion Protein (rFIXFc; Alprolix; Biogen Idec, Cambridge, MA, USA) is the first of a new class of bioengineered long-acting clotting factors. rFIXFc is composed of a single molecule of rFIX covalently fused to the Fc domain of human immunoglobulin G1 (IgG1). The presence of Fc in rFIXFc enables binding to endogenous neonatal Fc receptor (FcRn), a naturally occurring pathway that delays lysosomal degradation of Fc-containing proteins by cycling them back into circulation [9,10]. The enhanced pharmacokinetic properties, prolonged half-life, safety and efficacy of rFIXFc have been described in animal models [11] and clinical studies [12,13].

The objective of this work is to describe the rFIXFc manufacturing process and validation studies demonstrating the capacity of this process to produce a highly pure product free from virus.
Materials and methods

Manufacturing process: development of the rFIXFc cell line, cell bank creation and cell line confirmation

The coding sequences for human FIX, the Fc region of the human IgG1 (hinge and CH2 and CH3 domains) and PC5 were obtained by a polymerase chain reaction (PCR) from human liver mRNA, human lymphocyte cDNA and human liver mRNA, respectively, and were used to establish the rFIXFc manufacturing cell line [11]. Human embryonic kidney (HEK) 293H cells (Life Technologies, Grand Island, NY, USA), pre-adapted for serum-free suspension culture, were stably transfected with a plasmid containing a dual expression cassette for a FIXFc and Fc chain, a second plasmid expressing Fc alone, and a third plasmid expressing PC5, an enzyme used to ensure complete processing of the FIX propeptide [11,14]. Transfected HEK 293H cells were grown in serum-free medium. Clonal cell lines were derived and the optimal cell line selected was based on considerations of rFIXFc monomer productivity, rFIXFc activity [measured by a one-stage clotting assay based on activated partial thromboplastin time (aPTT)], propeptide processing, cell growth properties and stability. The cell lines with the optimal characteristics were then subcloned by limiting dilution and further characterized, and the clonal production cell line was chosen for manufacturing.

The clonal cell line that was selected for manufacturing was expanded to create a research cell bank (RCB). The RCB was expanded to create the master cell bank (MCB), from which a working cell bank (WCB) was derived. The WCB was created by thawing one MCB vial that was serially expanded to obtain sufficient cells for banking. The MCB and WCB were tested for identity, purity and freedom from adventitious agents. The cell banks were stored under liquid nitrogen to ensure long-term stability. The transgene coding sequence, copy numbers and gene integration patterns of the MCB and a cell bank produced from a cell culture that was propagated beyond the end of the manufacturing process [extended end-of-production cell bank (EEPCB)] were compared. The comparison was used to assess and confirm transgene integration and stability of the cell line over the course of the manufacturing process.

Production of rFIXFc

A WCB vial was used to produce a single batch of rFIXFc in a multi-step manufacturing process outlined in Fig. 1 and described below. The inoculum

| Process step no. | Step description |
|-----------------|------------------|
| Step 0          | Thaw of one working cell bank vial |
| Step 1          | Inoculum expansion (culture expansion in shake flasks) ↓ |
| Step 2          | Seed train bioreactors (culture expansion in bioreactors) ↓ |
| Step 3          | Production bioreactor ↓ |
| Step 4          | Harvest by centrifugation ↓ |
| Step 5          | Clarification (depth filtration) ↓ |
| Step 6          | Capture chromatography (MabSelect SuRe™) ↓ |
| Step 7          | Product neutralization ↓ |
| Step 8          | Anion exchange chromatography ↓ |
| Step 9          | Pseudo affinity chromatography ↓ |
| Step 10         | Virus reduction filtration (Planova 15N) ↓ |
| Step 11         | Ultrafiltration/diafiltration ↓ |
| Step 12         | rFIXFc product formulation, filtration, bottling, and storage |

Fig. 1. Overview of the recombinant factor IX Fc fusion protein (rFIXFc) manufacturing process.

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preparation phase includes thawing a WCB vial (Step 0) and expansion of culture in shake flasks (Step 1). Shake flasks were then pooled and used to inoculate the first seed train bioreactor (Seed Train Bioreactor Phase) for further culture expansion (Step 2). The seed train bioreactors were operated in batch mode, with agitation, pressure, temperature, pH and dissolved oxygen controlled during the process. The expanded culture was used to inoculate a large-scale production bioreactor (Step 3), the function of which is to express and produce rFIXFc prior to subsequent harvest and purification operations. The production bioreactor was operated in fed-batch mode, with agitation, pressure, temperature, pH and dissolved oxygen controlled. Harvest of the production bioreactor was initiated at a set time following completion of the final nutrient feed.

Following completion of the cell culture process in the production bioreactor, cells and cellular debris were removed by centrifugation (Step 4) and subsequent depth filtration steps (Step 5) to produce a clarified cell culture harvest containing the rFIXFc product. The product was then initially captured and purified from the clarified cell culture harvest using a protein A affinity capture chromatography step (MabSelect SuRe™; GE Healthcare Bio-Sciences AB, Björksgatan, Uppsala, Sweden) (Step 6), which binds to the Fc portion of the rFIXFc molecule with high affinity and specificity. After elution from the MabSelect SuRe adsorbent and neutralization (Step 7), rFIXFc was further purified using an intermediate anion exchange chromatography step (Step 8) and a polishing anion exchange chromatography step performed in pseudo affinity mode (Step 9). After these three chromatography steps, rFIXFc was passed through a 15 nm pore size virus removal filter (Planova 15N; Asahi Kasei Bioprocesses, Inc., Glenview, IL, USA) (Step 10). The product was concentrated and buffer exchanged using an ultrafiltration step (Step 11) to form the bulk product. The bulk product was then filtered into bottles (Step 12) and stored at −70°C.

**Table 1.** Analytical tests used to assess identity, purity, activity and safety of rFIXFc.

| Test                                | Method description                                                                 |
|-------------------------------------|------------------------------------------------------------------------------------|
| Polyacrylamide gel electrophoresis  | Polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulphate (SDS PAGE) under both reducing and non-reducing conditions. Gels were stained using Colloidal Coomassie Blue staining |
| (reducing and non-reducing)         |                                                                                     |
| Size exclusion chromatography       | Resolution of aggregated forms from the monomeric form of rFIXFc using high performance liquid chromatography (TSKgel G3000SWXL column) |
| Coagulation activity                | One-stage aPTT method performed in accordance with Pharmacopeia guidelines (USP<32> and Ph.Eur. 2.7.11) with respect to WHO International Standard for Factor IX concentrate |
| FeRn binding                        | FeRn competitive binding measured using an amplified luminescent proximity homogenous assay |
| Activated rFIXFc                    | Enzyme-linked immunosorbent assay (ELISA) based on the binding of hATIII to activated factor IX for the detection of the activated form of rFIXFc |
| Bioburden                           | Microbial enumeration test performed in accordance with Pharmacopeia guidelines (USP<61> and Ph.Eur. 2.6.12) |
| Endotoxin                           | Kinetic turbidimetric method in accordance with Pharmacopeia guidelines (USP<85> and Ph. Eur. 2.6.14) |

aPTT, activated partial thromboplastin time; FeRn, neonatal Fc receptor; hATIII, human antithrombin III; rFIXFc, recombinant factor IX Fc fusion protein; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; USP, United States Pharmacopeia; WHO, World Health Organization.

**Manufacturing process validation studies**

Process validation studies were performed to confirm identity, purity, quality and activity of the rFIXFc product. A summary of the analytical tests used in these assessments is shown in Table 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) gels obtained under reducing and non-reducing conditions and stained with colloidal Coomassie were used for validation studies for purity assessment and identity confirmation. The potency [in International Units (IU)] was determined using the one-stage (aPTT) clotting assay against the World Health Organization (WHO) international standard for FIX concentrate, and was used to calculate the specific activity of rFIXFc. The level of activated rFIXFc was monitored for all batches, as activated FIX (FIXa) has been implicated with increased thrombogenic risk [15], and therefore, a sensitive FIXa enzyme-linked immunosorbent assay (ELISA) was developed utilizing an anti-thrombin III capture step [11]. Safety determination was based on testing for the presence of bioburden and endotoxin.

The purification process was designed to provide a pure product with a high level of viral clearance. To demonstrate the capacity and robustness of the manufacturing process to remove adventitious viruses, the purification process was evaluated for the capacity for removal of enveloped and non-enveloped viruses using four model viruses [xenotropic murine leukaemia virus (X-MLV), mice minute virus (MMV), mammalian orthoreovirus 3 (Reo-3; also known as reovirus serotype 3) and suid herpes virus 1 (SuHV-1; also known as pseudorabies virus)]. These studies were conducted according to the International Conference on Harmonisation Q5A-E Guidelines and the US Food and Drug Administration Points to Consider [16,17] and in accordance with Good Laboratory Practice [18]. The virus clearance results were compared to those previously reported for a rFIX product produced in Chinese hamster ovary (CHO) cells [8].

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The rFIXFc manufacturing process was also evaluated for the capacity to remove process-related impurities. Process-related impurity clearance validation studies were performed both at the manufacturing scale and in scaled-down spiking studies at the laboratory scale. The clearance validation studies performed at the manufacturing scale consisted of direct measurement of the impurities obtained from the manufacturing process intermediates, in which clearance was calculated from the amount removed during the process step. Scaled-down impurity clearance validation studies (performed at the laboratory scale) consisted of the addition of an impurity to the process intermediate, followed by purification of the spiked intermediate using a scaled-down chromatography step. Scaled-down clearance validation studies were employed for several of the impurities for cases in which the impurity was below detectable levels in the manufacturing process intermediates, and were used to demonstrate the capacity and robustness of the process to provide additional clearance.

**Results**

**Cell line safety**

MCB and WCB were manufactured in accordance with current good manufacturing practice procedures, with purity, safety and identity test results demonstrating no detectable virus or adventitious agents. Table 2 shows all test results for the MCB, WCB and EEPCB. Testing confirmed the cell bank origin and that they are free of bacteria, fungi, mycoplasma and adventitious viruses.

**Manufacturing process validation studies: assessment of product quality and process consistency**

The rFIXFc manufacturing process (Fig. 1) produced a product of consistent purity, quality and activity. All steps in the manufacturing process were successfully validated for consistency based on process performance and product quality data from four batches. The manufacturing process validation study demonstrated the consistency of the rFIXFc process through evaluation of in-process measurements, in-process tests and product quality. Results from four validation batches are shown in Table 3.

Overall, results from the analytical testing demonstrated that the manufacturing process consistently produced a highly pure and active rFIXFc product. The results illustrate that the rFIXFc product possesses a high degree of purity, with all batches demonstrating >97% purity, when measured by non-reducing SDS PAGE (Fig. 2) and size exclusion chromatography [SEC (Fig. 3)]. Results of the SDS PAGE analysis of the rFIXFc product (under non-reducing conditions) illustrate a highly pure product, with a single band present with an observed molecular weight of 100 kDa (Fig. 2). As shown in Fig. 3 and Table 3, SEC was used to confirm that very low levels of aggregates (≤1.0%) were present in all of the batches, further illustrating that a highly pure rFIXFc product was manufactured using the described process.

Table 3 also shows that the specific activity of rFIXFc was consistent with validation batches possessing specific activity values of 6.5–7.1 IU nmol⁻¹ of rFIXFc, similar to levels reported previously [11]. In addition, binding of rFIXFc to FcRn was consistent across the validation batches, with relative binding levels ranging from 104 to 124%. Using a sensitive ELISA for assessment, the level of activated rFIXFc was found to be consistently low (0.002–0.004 mol%) in the validation batches (Table 3), which were significantly lower than levels reported for an approved rFIX product [11]. In addition, no bioburden was detected in any of the batches, while endotoxin levels were all below detectable levels. Additional analytical characterization was performed with the purified product (data not shown), which further illustrated...
that product quality was consistent across the validation batches.

Validation studies: virus- and process-related impurity clearance

The results of virus clearance studies support that the manufacturing process demonstrates robust clearance of viruses possessing different physical and chemical properties.

The capture affinity chromatography step (MabSelect SuRe) and the virus filtration step (Planova 15N) each removed at least 3.5 logs of the model retrovirus (X-MLV) (Table 4). For the virus filtration step, potential virus species are removed from the product stream based upon a size-based mechanism of separation, in which the virus is retained by the filter while rFIXFc passes through it. Importantly, the small pore size (15 nm) of the Planova 15N virus filter provides an effective barrier for a wide variety of virus types. As shown in Table 4, using the Planova 15N filtration, 4.5 logs of clearance was obtained for MMV, a non-enveloped virus with an approximate diameter of 20 nm. The size differential between the relatively large enveloped viruses (≥100 nm) and 15 nm pores of the Planova filter provides an absolute barrier, with retrovirus levels below quantitation following filtration (Table 4).

The overall total virus clearance claimed for the rFIXFc purification process is >16.0 log10 for X-MLV, >14.1 log10 for SuHV-1, >16.0 log10 for Reo-3 and 11.9 log10 for MMV. These results demonstrate that the rFIXFc manufacturing process can effectively clear a broad spectrum of adventitious virus types, ranging from retroviruses to small parvoviruses.

For comparison, the virus clearance of a rFIX product produced in mammalian (CHO) cell culture is shown in Table 5 [8]. The rFIX product manufactured in CHO cells reported virus clearance with two chromatography steps and one virus filtration step. As shown in Table 5, the CHO-based rFIX process achieved >11.8 logs for Amphotropic-MLV, >14.0 logs for Herpes simplex virus, 11.3 logs for Reo-3 and 12.5 logs for Bovine parvovirus.

Table 6 summarizes the clearance validation results for several process-related impurities, including host cell proteins, host cell DNA and recombinant protein A ligand leachate. The results demonstrated that the

| Product attribute | Test method | Results |
|-------------------|-------------|---------|
| Identity          | Non-reducing and reducing SDS PAGE; colloidal Coomassie staining | rFIXFc single chain and Fc single chain were identified under reducing conditions. Non-reducing conditions showed one single band of rFIXFc |
| Purity            | Non-reducing gel electrophoresis (%) | 98.3 98.5 98.5 97.6 |
| Size exclusion chromatography (%) | 99.3 99.1 99.0 99.0 |
| Activity          | Coagulation activity based on aPTT specific activity (IU/mmol rFIXFc) | 7.1 6.9 6.8 6.5 |
| FcRn binding relative potency* (%) | 118 110 104 124 |
| Safety            | Bioburden (CFU 10 mL^-1) | 0 0 0 0 |
|                   | Endotoxin (EU mL^-1) | <0.20 <0.20 <0.20 <0.20 |

aPTT, activated partial thromboplastin time; CFU, colony-forming units; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin units; FcRn, neonatal Fc receptor; rFIXFc, recombinant factor IX Fc fusion protein; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

*One good manufacturing practice (GMP) batch manufactured using the same process, scale and facility has been designated as a reference standard. Relative potency was determined against this reference standard.
rFIXFc purification process provides more than four logs of clearance for protein A leachate, HEK 293H host cell proteins and HEK 293H host cell DNA. In particular, host cell DNA levels were <1 pg mg⁻¹ protein, well below that considered acceptable by the WHO [19]. Clearance factors were also determined for other process-related impurities, such as cell culture media additives. The results (data not shown) demonstrate sufficient degrees of removal of these impurities to ensure product safety.

Discussion
rFIXFc is the first in a new class of long-acting clotting factors and is approved for the control and prevention of bleeding episodes, perioperative...
management, and routine prophylaxis in adults and children with haemophilia B (Alprolix prescribing information, Biogen Idec, Cambridge, MA). Manufacturing process validation studies demonstrated that rFIXFc possessed consistent purity, quality and safety. The validation studies also demonstrated the manufacturing process provides robust removal of potential adventitious viruses and process-related impurities. No human- or animal-derived raw materials are used during the rFIXFc cell culture and purification processes, minimizing the risk of pathogenic contamination. The process described in this study uses a well-characterized human cell line and is built upon a process developed and commonly used for the large-scale production of monoclonal antibody products [20,21]. State-of-the-art chromatographic and nanofiltration technologies are used to enhance product purity and to provide robust viral clearance. These attributes collectively support a robust and reproducible manufacturing process that can support continuous supply and production of rFIXFc.

A key feature of the rFIXFc manufacturing process is the use of a human cell line (Table 7). HEK 293 cells have been chosen for the production of a number of human recombinant protein therapeutics [22–26]. HEK 293H cells reportedly produce FIX more efficiently than animal cell lines, with full biologic activity and greater capacity for γ-carboxylation and propeptide processing [25]. The HEK293 cell line is an established expression tool that has been used extensively over the past 30 to 40 years to produce recombinant proteins for research [27]; its features include amenability to transfection and high efficiency [22,23,28,29]. An attribute of relevance to haemophilia is that the use of this cell line produces a rFIXFc molecule that displays nonglycosylated variants of α-Gal antigens [33].

Table 6. Summary of process-related impurity clearance for the rFIXFc manufacturing process for several process-related impurities.

| Process-related impurity | Impurity clearance validation scale | Overall reduction factor (log10)* | HCPs, host cell proteins; HEK, human embryonic kidney; rFIXFc, recombinant factor IX Fc. |
|-------------------------|-----------------------------------|---------------------------------|---------------------------------------------------------------------------------|
| HEK 293H HCPs | Manufacturing | 4.6 | *Overall reduction factor (log10) is the sum of the reduction factor values for each of the process steps validated for removal of the respective process-related impurities. |
| HEK 293H DNA | Manufacturing | >7.9 |
| Recombinant protein A leachate | Laboratory | 4.1 |

Using human cell lines ensures that the correct translational machinery and subcellular components are present to generate a product with a human pattern of PTMs [22,27]. Thus, use of a human cell line to produce recombinant proteins may protect against immunogenic epitopes, and potentially reduce the risk of developing neutralizing or non-neutralizing antidrug antibodies [22,23,26–28]. In a recent study, α-Gal and N-glycosylation [33], acid, two entities that are potentially immunogenic if present in recombinant proteins administered to humans, were not detected in rFIXFc [33–35]. In contrast, characterization of several commercially available FIX and FVIII products produced in CHO or BHK cells showed detectable levels of both NNGN and α-Gal antigens [33].

The impurity clearance validation studies demonstrated the capacity of the manufacturing process to remove viruses of various sizes and biophysical properties, along with process-related impurities from rFIXFc, with a total reduction factor of 11.9 log10 or greater for each of the four model viruses evaluated. Reducing the pore size during nanofiltration has been shown to greatly enhance viral clearance without affecting the purified biologic [36]. The Planova 15N filter provides robust clearance (≥4.5 logs of removal) for all four model viruses evaluated in the clearance studies.

In addition to the purification process demonstrating a high capacity for removal of adventitious viruses, the cell culture harvest for every batch is tested and con-
firmed to be free of adventitious viruses. In-process controls and tests are in place during cell culture and purification to ensure that they are well controlled and performing consistently, robustly and reproducibly.

The manufacturing process for rFIXFc is built upon an established, well understood manufacturing process that has been routinely and successfully applied to large-scale production of monoclonal antibodies [20, 21]. For preparation and production of this Fc fusion molecule, the process has been adapted to support the use of the HEK 293H cell line. Scale and continuity of production are essential features of any manufacturing process for therapeutic biologicals, and the methods described here are scaled to manufacture in a large-scale (15 000 L) production bioreactor. Furthermore, by utilizing a platform process approach, the product can be produced within any of the manufacturer’s large-scale facilities, which will help to mitigate potential supply risks [37].

Conclusion

The manufacturing process described here produces a novel, fully human, active and highly purified product free from viral contaminants or other impurities as supported by validation studies. Importantly, the manufacturing process is readily transferable and scalable to ensure consistent supply of the rFIXFc product.

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Author contributions

Justin McCue, Doug Osborne, Robert Peters and Kazumi Kobayashi wrote the first draft of the manuscript. Kazumi Kobayashi analysed the data. All authors contributed to the interpretation of the data, manuscript revisions and approval of the submitted version.

Disclosures

All authors are employees of and hold equity interest in Biogen Idec.

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