



REVIEW ARTICLE

Innovative approach for improved rFVIII concentrate

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Abstract

The development of a new recombinant factor VIII was designed and implemented to answer a number of unmet needs of patients affected by hemophilia A. Turoctocog alfa is bioengineered in a specific Chinese hamster ovary clone to present translational and posttranslational characteristics (sulphation, glycosylation) biosimilar to natural circulating forms of FVIII, with the aim to devoid any minimal change which may impact immunogenicity and antigenicity of recombinant protein. Both producer cell line and media are maintained free of any animal or human plasma derivative. Downstream processes of purification are performed by five steps (immunoaffinity chromatography, ion-exchange chromatography, virus inactivation by means of solvent-detergent treatment and nanofiltration, and to end with gel filtration), to provide the best possible margin of safety from known and unknown infectious agents. Large clinical trials seem to confirm the expectations placed in Turoctocog alfa in terms of high quality and safety of recombinant FVIII toward the goal of overcoming actual and future challenges of hemophilia therapy.

Key words haemophilia A; recombinant factor VIII; Turoctocog alfa; inhibitor; safety

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The coagulation cascade is a tightly regulated and efficient zymogen/protease system. Factor VIII (FVIII) is a cofactor which amplifies of about thousands of times the formation rate of stable clots (1); deficiency or absence of FVIII, due to mutations of chromosome X gene in hemophilia A predisposes to repeated spontaneous or induced bleedings, the latter by trauma or surgery. As a consequence of repeated joint and muscle bleedings, patients suffer of severe debilitating muscle-articular damage and are exposed to life-threatening bleeding episodes (2). Replacement therapy with the missing factor is therefore the mainstay of treatment in patients affected by hemophilia.

Replacement therapy is based on exogenous FVIII infusion at the time of bleeding (on demand therapy) or in a preventive schedule of bleeding episodes (prophylaxis). Since the first transfusion of whole blood in 1840 (3) until the introduction of freeze-dried clotting factor concentrates in 1970s (4), life expectancy of hemophiliacs was increased from 20 to over 60 yr (2, 5); nevertheless, life expectancy had dropped down to <50 yr and the mean age at death to 40 yr in next two decades (6), when more than three quarter

of hemophiliacs were found to be infected with HIV and hepatitis B and C viruses (7). These epidemic burdens accelerated the approval of first recombinant FVIII (rFVIII) products in the 1990s.

The last 20 yr presented new challenges in the treatment of hemophilia such as new infectious threats and the occurrence of neutralizing inhibitors to exogenous FVIII, which raised up to 25–30% of patients with severe hemophilia A treated for the first time with rDNA FVIII concentrates. (10, 11).

Furthermore, hemostatic protection is limited by a too short half-life of exogenous FVIII, and intravenous administration requires maintenance of a good venous access especially in children (12, 13).

There are therefore a number of possible improvements in the current treatment of hemophilia A. FVIII concentrates should be characterized by the following properties:

1. posttranslational structural and functional characteristics similar to natural circulating forms of FVIII (14);
2. devoid of relatively minor changes in manufacturing FVIII which may impact immunogenicity (15);

3. purity with no risk of old or newly emerging infectious agents transmission (16).

Furthermore, phase III clinical trials of new FVIII concentrates with longer plasma half-life to obtain an adequate hemostatic protection by a weekly administration are currently on going (17).

This brief review discusses the manufacturing process of a new third generation B-truncated rFVIII, Turoctocog alfa. Turoctocog alfa is designed to answer the actual unmet needs of hemophilia A patients, and it is bioengineered using innovative technologies to be applied to future FVIII products.

Manufacturing processes linked to immunologic safety of rFVIII

Previous experience with FVIII products pointed potential risks in the development of a new rFVIII concentrate such as immunogenicity (the ability to induce an immune response) and neoantigenicity (ability to bind a secreted or surface antibody) (18).

The production of antibodies against recombinant proteins is influenced by several factors, including structural properties of the protein, formulation, presence of impurities, and the formation of aggregates (19, 20). A large and complex protein such as FVIII requires critical posttranslational additions, particularly sulphation and glycosylation (21). Not all mammalian expression systems may implement all of the changes necessary for proper structure and function of FVIII (21). The choice of a suited cellular environment provides opportunity to minimize inhibitor incidence at the clinical level. Chinese hamster ovary (CHO) cells are the most commonly used mammalian 'recombinant' cell clone for large-scale production of rFVIII (22). Progress in CHO culture technology brought to a substantial optimization in protein production, including improvement in gene expression, culture process, and medium development (23).

N8 protein, the native structure of Turoctocog alfa synthesized in these CHO cells, shows sulphation and glycosylation patterns superimposable to those of plasma-derived FVIII (24).

Analysis of Turoctocog alfa for the degree of sulphation demonstrated that the all six tyrosine sites of FVIII are sulphated: every site performs a peculiar role in functional or antigenic term (24–26).

Turoctocog alfa glycosylation is mainly characterized by the presence of carbohydrate chains ubiquitous in human organism (21, 24) and by absence of non-human glycan epitopes, critically expressed by different culture cells (27, 28). These non-human epitopes carry potential to real implications for inhibitory issues (29, 30).

Furthermore, the characterization of sulphation and glycosylation has been a critical step to assess improvement and consistency of the production process (24).

Amelioration of culture media from non-animal sources is ongoing, and it is important to ensure products of consistent quality and without the potential danger of contamination (31). Plasma-derived concentrates, other than potential danger of contamination, concern about consistent quality, product performance, and reliability of supply (32). There are over 1000 different components in serum, including proteins, peptides, hormones, enzymes, electrolytes, lipids, carbohydrates, vitamins, and other constituents (33). Development of serum-free media must be formulated for each cell line (31). In fact, even different clones of the same CHO cell line may require specific cultivation process and environment (34).

The manufacture of Turoctocog alfa does not require any human- and animal-derived component (35). Turoctocog alfa is produced in CHO cells growing in a medium free of plasma-derived ingredient: a low-protein synthetic medium contains all necessary nutrients and elements, which are filtrated to eliminate contaminants (24).

Moreover, no animal-derived material has been used in the production of any raw material employed in the manufacturing process, including chromatography media, the affinity ligand and filters (24). Hence, technology of plasma and animal free-production of Turoctocog alfa resolve the safety issues of pathogen transmission through serum. In addition, serum-free technology avoids variability of proteins and low-protein medium avoids high protein content exposure. Both elements may contribute to reduce the immunogenicity of rFVIII.

Current challenges of viral safety

Manufacturers of recombinant products have addressed the issue of emerging infectious agents by acting on two fronts: limit up to cancel the use of human plasma in all reagents used for cell culture, purification steps, stabilization, and storage buffers (36); at the same time, virus inactivation and removal measures are adopted.

In the preparation of plasma-derived products, the solvent/detergent process is the most common virus inactivation technique used, because this process is vulnerable to enveloped viruses, from known hepatitis viruses (HBV, HCV) or HIV to recently emerging West Nile virus, Chikungunya virus, new influenza strains and severe acute respiratory syndrome coronavirus (37). Gamma irradiation sized of the target virus is a widely employed method for inactivation of non-lipid-coated viruses, such as parvoviruses, enteroviruses and circoviruses (38). However, a study involving 195 hemophiliacs treated for 5 yr with virally inactivated clotting factor concentrates identified a seroconversion incidence of 1.7%/yr for anti-human parvovirus 4, a transmissible agent that is resistant to viral inactivation via solvent/detergent treatment and to some extent to heat and radiation too (39).

Thus it's necessary to implement further methods for the inactivation and removal of potentially contaminating viruses, including separation/purification techniques (such as

ion exchange and immunoaffinity chromatography) and nanofiltration.

The problem of blood safety remains despite the improvement of viral inactivation methods and plasma-derived products could always cause infectious concerns (40). The risk of contracting novel pathogens, some of which could be actually not detectable, is sustained by continuous emergence of new agents and new strains of existing agents: potential threats are not limited to viruses, but include bacterial, protozoan, and prion agents (41). Among more than 60 such agents, including HIV and hepatitis viruses, some have been identified as the highest-priority agents for which a blood safety intervention should be considered. (Table 1).

Recombinant FVIII concentrates have provided improved safety for patients with hemophilia A as they significantly reduce the risk of transmission of blood-borne infections. Nevertheless, human- or animal-derived plasma proteins are still included at some step in the preparation of first or second generation of rFVIII products, thereby introducing the potential for transmission of human or animal pathogens (50).

Two examples can be briefly treated. Parvovirus B19 is a small, non-enveloped virus that typically causes a benign flu-like illness that occurs most frequently in childhood. The virus is resistant to current viral inactivation steps used in the manufacture of anti-hemophilic factor concentrates. B19V transmission through these products has been documented also after nucleic acid test (NAT) screening of plasma pools has been implemented: children exposed to plasma-derived products were 1.7 times more likely to have antibodies to B19V compared to those unexposed to blood products (46). One first generation recombinant FVIII product containing human serum albumin, added to stabilize rFVIII before lyophilization, was associated to a reliable

seroconversion for parvovirus B19 (36). Even though the study conducted by Centers of Disease Control and Prevention of Atlanta was limited to detect serumconversion, the poor reliability of plasma mini-pool screening by NAT was revealed. Albumin was also the source of TT virus found out in the first generation rFVIII concentrates (49).

Prions are self-replicating infectious proteins that cause currently untreatable and fatal neurodegenerative disorders (51). vCJD, the human form of bovine spongiform encephalopathy, is a prion-associated disease with a lengthy incubation period of 5–15 yr. An unexpectedly high transmission rates by transfusion of 36% for BSE and 43% for scrapie, together with a short and consistent incubation periods in clinically positive recipients have been experimentally demonstrated, suggesting that infectivity titers in blood were substantial and that blood transfusion is a possible vehicle of transmission (44). It is recent the demonstration of abnormal prion protein in a spleen sample at postmortem examination of a United Kingdom elderly hemophilia patient who received coagulation factor concentrates obtained by a pool of donors including a subject incubating vCJD and who subsequently died from vCJD (42, 43). The outcomes of a recent published survey conducted in United Kingdom to establish the prevalence of subclinical infections with prions in normal population showed that 16 of 32441 archived appendix samples were positive for abnormal prion protein (PrP) (52). This prevalence of 0.49/1000 is definitively not trivial taking into account the size of plasma pools (20–3000 L derived from 50–7500 donors!) fractionated to produce clotting factor concentrates. Peripheral lymphoreticular infections seem to be a weak barrier against cross-species transmission of prions (53).

Of note are the challenges which make these infective agents elusive to recognition by surveillance policies. Many emerging blood-borne infectious agents are characterized by long-lasting, silent carrier states in which the pathogen is present in the circulation without causing noticeable symptoms, but the same pathogen is highly infectious when contained in blood or plasma (41). These blood-borne agents have in common the transmission by transfusion, the association with a clinically apparent or fatal disease, and the lack of an effective intervention. Moreover, the prevalence of subclinical diseases is not known, as well as the challenges in dealing with undefined risks requiring the implementation of surveillance and risk management measures. Pathogens with blood-borne stages that are resistant to viral inactivation steps in the manufacturing process must be taken into particular consideration as potential threats.

Turoctocog alfa, a novel advanced category rFVIII produced without the addition of any human or animal plasma proteins, has been developed with the aim of providing the best possible margin of safety to hemophilia patients, because it eliminates the risk of blood-borne infections during lifelong therapy (24).

Table 1 Emerging infectious disease agents associated with potential blood, plasma or FVIII concentrate transmission (39, 42–49)

Parasite	Virus	Prion
Babesia	Dengue viruses	Variant Creutzfeldt-Jakob disease (vCJD), Agent of bovin spongiform encephalopathy (BSE) Scrapie Other 2 strains currently known Other prions not currently known
	Human parvovirus B4 (HPB4)	
	Human parvovirus B19 (HPB19)	
	West Nile virus (WNV)	
	Xenotropic murine leukemia virus-related virus (XMRV)	
	SARS-associated coronavirus	
	Transfusion Transmitted virus or Torque-tenovirus (TTV, circoviruses)	
	HTLV (human T-cell lymphotropic virus)	
	Human herpes virus 8 (causative agent for Kaposi's sarcoma)	

Turoctocog alfa is purified through a series of steps, including an immunoaffinity chromatography process utilizing a recombinant monoclonal antibody produced by cells grown in a plasma-free environment (24).

In any case, as Turoctocog alfa is never exposed to plasma, the risk of transmitting known and emerging blood-borne pathogens is virtually eliminated.

Purification as method of infectious safety

The advanced techniques of purification utilized for the production of Turoctocog alfa result in a final product formulation which is devoid of both albumin and animal/human-derived materials.

A total of five steps are employed to remove host cells, medium components, chemicals used during purification, DNA, and proteins. The key purification step uses a non-animal-derived affinity ligand that is produced in the same CHO cell line used for Turoctocog alfa and is specific for FVIII (24).

The manufacturing process used for purify Turoctocog alfa, including two additional steps other than the minimum regulatory requirement, is depicted in Table 2.

1. Solvent-detergent (SD) treatment is a standardized and well documented process in terms of the spectrum of potential pathogen reduction (54). In addition to inactivate potential viral contaminants, SD process is necessary for product concentration. SD treatment for Turoctocog alfa is conducted in respect to the physicochemical/biological characteristics of rFVIII. Nevertheless, adverse reactions to the final preparation are possible when this technique is used for viral inactivation (24). Therefore, an additional practical step such as chromatography is required to remove the solvent/detergent residual chemicals and their metabolites (24).
2. Immunoaffinity chromatography is a process which isolates only full molecules of rFVIII, that is, with an intact heavy chain. This chromatographic process uses a recombinant version of the monoclonal F25 antibody directed against integral A2 domain of the heavy chain,

which must include residue 740 of the amino acid sequence. The step excludes incomplete molecules, particularly those with a damage on heavy chain at its weak point position 720, which is easily inactivated by proteolysis. Recombinant monoclonal antibody directed against A2 domain is produced in the same cell line used for production of Turoctocog alfa and its manufacturing is realized avoiding any contact with other animal additives (24).

The affinity step is highly specific for FVIII and has been shown to reliably remove essentially all host cell proteins (24).

3. Ion-exchange chromatography removes impurities generated by the producing cell line, using electrical charges to eliminate other impurities, thus obtaining a further purification step of the product. The resin used for the mixed mode capture chromatography step also binds FVIII through multiple interactions, providing an additional tool for the removal of impurities.

Researchers optimized the parameters of the chromatographic process to obtain a highly purified rFVIII concentrate. The best support matrix of ion exchange was selected to obtain the highest purified product, in that all investigated contaminant proteins have been found to be below the detection limit (data on file, personal communication).

4. Nanofiltration: a 20 nm pore size filter has been designed for removal of almost all viruses, included protein enveloped viruses like Parvovirus. Nanofiltration increases the clearance capacities for viruses *via* a membrane-based ultrafiltration system (55). It has been implemented to assure a high virus safety level, especially for non-enveloped viruses, thus complementing the profile of viral reduction treatments active against enveloped viruses such as SD process (56). Non-enveloped viruses, resistant to SD treatment, are too small to be removed if the filter is larger than 30 nm (56). Nanofiltration consists in filtering protein solutions through membranes of a very small pore size under conditions that retain viruses by a mechanism based on size exclusion (55).

Table 2 Purification steps of Turoctocog alfa

Step	Mechanism	Comments
Virus inactivation	Chemical inactivation (solvent/detergent)	In the potential case of enveloped viruses being present, these would be inactivated through chemical disruption of their surrounding lipid membrane
Immunoaffinity purification	mAb chromatography	Purification step removing all host cell proteins, using a non-animal derived FVIII monoclonal antibody
Charge purification	Ion-exchange chromatography	The rFVIII product is concentrated and purified using a charged chromatographic resin
Virus removal	Nanofiltration (20 nm pores)	Pores with a size of 20 nm have the capability to capture all pathogens based on size, including any theoretically present small non-enveloped viruses (e.g. parvoviruses), disrupted enveloped viruses, prions, etc.
Gel filtration	Size exclusion	The rFVIII is formulated without aggregate/agglutinated proteins

Turoctocog alfa is purified using a 20-nm pore size filter for nanofiltration. Such a size is tight enough to remove the smallest viruses as it corresponds to that of Parvovirus, which have been shown to be a model for virus clearance validation method and to be efficiently removed by the 20 nm filter (56, 57). Such technique refinements led to improve viral removal as established by large-scale world wide experience (55).

In the purification process of a rFVIII product, prion removal capacities of 3.8 logs and >5.2 logs have been demonstrated for the immunoaffinity step, resulting in a total clearance of >9 log (24). Also nanofiltration may remove prions and provides a possible safeguard against new infectious agents potentially entering the human plasma pool (55).

Lastly, the technology of membrane nanofiltration process has the major advantage not only in protein-virus separation, but also in protein purification, with a recover of more than 95% of protein activity because the absence of denaturing effect (55, 58).

5. Gel filtration step provides the pure protein Turoctocog alfa, eliminating aggregated/agglutinated forms of protein (24). A high capacity gel filtration system was developed with the purpose of isolating rFVIII in high yields. After implementation of gel filtration, a rFVIII-containing fraction was collected containing exclusively FVIII and filtration was used to monitor the bioactivity and purity of the chromatographic product (24, 35).

In summary, two dedicated virus clearance steps, S/D treatment and nanofiltration through a 20-nm pore size filter, are included to ensure inactivation/removal of potentially present enveloped and non-enveloped viruses. Indeed, in the purification process for Turoctocog alfa, the nanofiltration and the immunoaffinity step do contribute to prion reduction as well.

Clinical evidence of safety

Recombinant FVIII products are now classified into three generations based on the degree of elimination of plasma and albumin from production and/or formulation steps. First generation products use plasma and/or albumin during both the cell culture process and the final formulation steps, while second generation products use serum proteins during the cell culture process only. Plasma-free recombinant proteins are classified as third generation products (59).

Many physicians' organizations in Commonwealth and other developed countries (Denmark, Canada, Japan) encourage switching to recombinant products as a standard for the treatment of hemophilia A, and both FDA and EMA have prompted drug and device manufacturers to find alternatives to the use of human and animal raw materials and additives whenever possible.

Together with viral safety, the main concern in the development of Turoctocog alfa was the attempt to reduce

the formation of inhibitors to FVIII (18). The primary objective of the two Guardian™ phase 3 studies was therefore to evaluate the safety of Turoctocog alfa in terms of incidence of inhibitors (60, 61). Adult patients ($n = 150$) were exposed to Turoctocog alfa for a mean of 85 d (ranging from 11 to 172 exposure days) while young patients ($n = 60$) for a mean of 60 exposure days (range 20–104 d) (60, 61). No patient developed inhibitors during both trials (60, 61). One adult patient was excluded from analysis because had a pre-trial-positive inhibitor test (1BU). Interestingly, this patient received 13 doses of Turoctocog alfa with no occurrence of inhibitors (60).

In a phase 2 study, the observations of 22 patients immediately after the first dose of Turoctocog alfa showed no signs of early inhibitor development. For instance, no inhibitor formation was reported in the 72-h period after administration of a single dose of Turoctocog alfa (50 IU/kg) (18).

Regarding adverse events other than infection and inhibitor development in the Guardian™ studies (60, 61), six non-serious events (hypertension, sinus tachycardia, and insomnia in a 27-yr-old patient, increase of hepatic enzymes in a 37-yr-old patient, and contusion for incorrect dose administration in one pediatric patient) were evaluated as possibly or probably related to Turoctocog alfa by investigators (60, 61).

Overall, treatment with Turoctocog alfa was safe, with none 'infectious concern' (60, 61).

Turoctocog alfa and new rFVIII products

Several new bioengineering technologies are now applied to FVIII to improve the quality of treatment of hemophilia patients. For example, a variety of approaches are under evaluation to increase the half-life of FVIII thus reducing administration frequency. Addition of polyethylene glycol (PEG) and fusion proteins technologies are the most advanced in preclinical and clinical development (62).

The structure of Turoctocog alfa, N8 protein, is so similar to native FVIII to be incorporated in a glycopegylated FVIII (N8-GP) to increase plasma half-life of FVIII (59). Indeed, a novel site-specific PEGylation attaches a 40-kDa PEG to a unique O-glycan in the N8-glycoprotein (N8-GP) (63). When activated by thrombin, the residual part of the B-domain containing the PEGylation is cleaved off, generating active FVIIIa that is similar in structure to native FVIIIa (63).

The N8-GP pharmacokinetic parameters were estimated in different animal models. In hemophilia A dogs, mice, rabbits, and monkeys, a nearly twofold prolongation of half-life was demonstrated (63, 64). In a phase I clinical trial, N8-GP demonstrated a significant increase of plasma half-life. Twenty-six patients received one dose of their previous FVIII product followed by the same, single dose of the N8-GP (65). The mean terminal half-life of N8-GP was 19.0 h (range, 11.6–27.3), 1.6-fold longer than that of the patients'

previous products. The estimated time from dosing of 50 U/kg N8-GP to a plasma activity of 1% was 6.5 d (range, 3.6–7.9) (65). A single dose of up to 75 U/kg N8-GP was well tolerated in patients with hemophilia A, with no safety concerns (65).

Taken together, clinical data show that Turoctocog alfa serves as an optimal tool for a long-acting future FVIII product.

Conclusions

The rationale for producing rFVIII is ultimately the reduction of immunogenic and infectious challenges to patients. For Turoctocog alfa, a manufacturing process using a well-known mammalian cell line suitable for rFVIII production and entirely free from added animal- or human-derived additives was designed and developed. Three complementary approaches were used to minimize the risk of potential contamination: thorough testing of cell lines and close control of medium, the capacity of the purification process to eliminate potential contaminations and systematic analysis of the final product. The safety data obtained to date demonstrate that Turoctocog alfa can be safely used in the clinical practice and that the manufacturing process sets new standards in terms of purity and pathogen safety.

Conflicts of interest disclosure

The Author declares that he served as consultant for Novo Nordisk A/S, CSL Behring and Pfizer and received a grant for research from Baxter and from Bayer.

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