Comparative analysis of marketed factor VIII products: recombinant products are not alike vis-a-vis soluble protein aggregates and subvisible particles

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To cite this article: Anzengruber J, Lubich C, Prenninger T, Gringeri A, Scheiflinger F, Reipert BM, Malisauskas M. Comparative analysis of marketed factor VIII products: recombinant products are not alike vis-a-vis soluble protein aggregates and subvisible particles. J Thromb Haemost 2018; 16: 1176–81.

Essentials
- Aggregation is a critical quality attribute of protein therapeutics influencing immunogenicity.
- Aggregates and subvisible particles in 9 recombinant factor VIII (rFVIII) products were analyzed.
- Major differences in aggregate and particle concentrations were detected after reconstitution.
- rFVIII product quality determined aggregation propensity under use-relevant stress.

Summary. Background: Recombinant protein technologies have facilitated the development of novel factor VIII (FVIII) therapeutics with improved production efficiency, potency and half-life, and a low risk of viral transmission. The increasing number of recombinant FVIII (rFVIII) products and information on their efficacy, safety and cost allow patients and healthcare professionals to adjust treatment to individual needs. Nonetheless, 20–32% of previously untreated patients (PUPs) with severe and 3–13% PUPs with moderate and mild hemophilia A develop inhibitory antibodies to rFVIII following treatment. The root cause of the immunogenicity of rFVIII products is not well understood. Data for human interferon and human insulin products suggest that critical quality parameters such as soluble protein aggregates (SPAs) and subvisible particles (SVPs) influence the immunogenicity of protein therapeutics. Therefore, we analyzed SPA and SVP concentrations in commercially available rFVIII products and determined how these parameters change upon exposure of rFVIII products to relevant stress conditions. Objectives: Compare critical quality parameters such as SPA and SVP concentrations in rFVIII products under intended use and use-relevant stress conditions. Methods: Nine rFVIII products (≥ 3 lots each) were analyzed by high-performance liquid chromatography-size exclusion chromatography (HPLC-SEC) and flow cytometry-based particle analysis. Results/conclusions: SPAs and SVPs were present at different concentrations in all freshly reconstituted rFVIII products: SPA concentrations ranged from 0.2% to 11.6%; SVPs were $0.7 \times 10^6$ to $114.0 \times 10^6 / 1000$ IU. Under use-relevant stress conditions (agitation and shear stress) the products formed additional SPAs and SVPs to different degrees. The collected data indicate that product quality determines its propensity to form SVPs and SPAs, and highlights differences between marketed rFVIII products.

Keywords: factor VIII; hemophilia A; protein aggregates; protein stability; recombinant proteins.

Introduction
With the first recombinant factor VIII (rFVIII) concentrate approved for sale in the US in 1992, treatment of hemophilia A changed forever. Over the next 25 years, advances in science and technology allowed development of new rFVIII products with improved safety profiles. Despite all the progress made, 20–32% of previously untreated patients (PUPs) with severe and 3–13% PUPs with moderate and mild hemophilia A develop inhibitory antibody responses to rFVIII treatment [1,2]. Anti-drug antibody formation is not unique to hemophilia A treatment, but is a known phenomenon linked to protein-based therapies [3,4]. The presence of aggregates in protein drug products has been shown to play a role in inducing adverse immune responses in patients [5,6].
Aggregates in protein therapeutics can be classified into two main groups: (i) small soluble protein aggregates (SPAs), which are assemblies of protein molecules other than monomers and up to 100 nm in size, and (ii) subvisible particles (SVPs), which are undissolved species 0.1 to 50 μm in size, containing protein or non-protein components [7].

In 1998, changes in the formulation of an epoetin product, Eprex, led to the formation of cross-reactive antibodies that were able to neutralize human erythropoietin. As a result, over 200 patients developed pure red cell aplasia. An increased tendency of epoetin to aggregate in the new formulation was considered a root cause of this antibody response [8].

Moreover, the presence of SPAs in therapeutic formulations of recombinant human beta interferon and human insulin products was shown to correlate with the incidence of anti-drug antibody formation in clinical use [9–14]. In addition, the presence of SVPs in interferon beta and in monoclonal antibody therapeutics correlated with increased immunogenicity in in vitro animal and in vivo models [15,16].

Some recent large cohort studies have suggested that rFVIII products differ in their propensity for immunogenicity [1,17], which raises the question of potential root causes. Here we present data on SPA and SVP concentrations in marketed rFVIII products and demonstrate how product quality parameters such as SPAs and SVPs change upon exposure to use-relevant stress.

Material and methods

Sample preparation

Nine marketed rFVIII products were included in this study (Table 1). At least three lots of each product were investigated. All products were reconstituted as indicated in the product insert. The products were analyzed by high-performance liquid chromatography-size exclusion chromatography (HPLC-SEC) within 20 min of reconstitution. Remaining samples were aliquoted and stored at −80 °C. Before SVP analysis, all aliquots were thawed at +4 °C and subjected to flow cytometry-based particle analysis.

Conditions for use-relevant stress for rFVIII products were created by subjecting all freshly reconstituted products, in their original vials, to agitation and shear stress. All materials were agitated by hand for 10 min to simulate potential mishandling of the products prior to intravenous injection. Shear stress resulting from the injection was simulated by injecting the materials through ‘Winged infusion injection. Shear stress resulting from the injection was simulated by injecting the materials through ‘Winged infusion

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Size exclusion chromatography

An Agilent 1260 HPLC system equipped with a diode array detector was used for all SEC analyses. The signals were monitored at an absorbance of 280 nm with a reference wavelength of 550 nm. Independent of protein concentration, a 100-μL sample volume was loaded onto the column. Each sample was analyzed in triplicate and a mean value for SPAs calculated. Products and lots were analyzed in random order.

Peaks eluting with the void volume (retention times, 16–22 min) were designated as SPAs. The amount of soluble aggregates was calculated as a percentage of the total area of all peaks in the chromatogram. The HPLC system was equipped with a TSK SWXL 4000 size exclusion column preceded by a SWXL guard column (Tosoh Bioscience, Tokyo, Japan). The samples were run at a flow rate of 0.3 mL min⁻¹ for a total of 55 min in a mobile phase consisting of 400 mM NaCl, 50 mM Tris, 5 mM CaCl₂, 0.05% NaN₃ and pH 7. All non-specific interactions between the column and the test item were minimized by saturating unspecific binding sites by loading the column with mixtures of FL-rFVIII and BDD-rFVIII material. A protein-based gel filtration standard (Bio-Rad, Hercules, CA, USA) was analyzed in between samples to monitor optimal column performance.

Flow cytometry-based particle analysis

A flow cytometry-based particle analysis method was used to detect and characterize SVPs in the samples. The flow
cytometry-based method involved a combination of size calibration beads (Fluoresbrite® YG Carboxylate Size Range beads, Polyscience Inc., Warrington, PA, USA), counting beads (CountBright™ Absolute Counting Beads, Invitrogen Corp., Carlsbad, CA, USA) and fluorescent probes to characterize the SVPs. To distinguish protein and protein-containing particles from non-protein SVPs, samples were stained with the fluorescent dye 4,4'-Dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt (Bis-ANS; Sigma Aldrich, St Louis, MO, USA). The method is detailed in a recent publication [18].

Results and discussion

Soluble protein aggregates in rFVIII products

SPAs were detected in all lots of all tested rFVIII products in varying quantities (Fig. 1A). The highest concentrations were detected in Product E (mean value, 6.4%) and the lowest in Product F (mean value, 0.6%). SPA concentration varied also between lots of the same product. The highest lot-to-lot variability was determined for Product E and the lowest for Product A. Tested lots were of different potencies and contained different amounts of rFVIII; however, no correlation was observed between the labeled potency of the products and SPA concentrations. The largest difference in concentration between two lots was more than 49-fold.

Under use-relevant stress conditions (agitation and shear stress), higher SPA concentrations were measured in most of the tested products, with a clear difference between the products. In one group, consisting of products A, D, F, G, H and I, SPA concentrations were detected at 0.2–2.7% overall (Fig. 1B). For these products, not only the protein aggregate concentrations remained low, but also the absolute increase in concentration was below 0.6%. In the other group, consisting of products B, C and E, SPA concentrations increased on average to above 7.6% (range, 5.9–9.9%) (Fig. 1B). For these products, use-relevant stress resulted in absolute increases in SPA concentration of 1.6–3.0% (Fig. 1B).

This segregation correlated well with the SPA concentrations determined in the tested rFVIII products under intended-use conditions. These data suggest that the main determinant of the product’s stability upon use-relevant stress is the quality of the product under intended-use conditions. Pre-existing SPAs may act as seeds that nucleate further protein aggregation upon exposure to stress [19–21]. The presence of FVIII SPAs may play a critical role in the development of unwanted immune responses to FVIII, as previously shown in murine models [22]. No difference was observed in the initial presence or de novo formation of SPAs that could be attributed to the presence or lack of the B-domain in FL-rFVIII and BDD-rFVIII products, respectively.

Fig. 1. Soluble protein aggregates (SPAs) in different lots of rFVIII under intended-use conditions (A), and after exposure to use-relevant stress (B) measured by high-performance liquid chromatography-size exclusion chromatography (HPLC-SEC).
Subvisible particles in rFVIII products

Subvisible particles are commonly defined as particles 0.1–50 μm in size [7]. Such particles are beyond the analytical range of HPLC-SEC; therefore, their measurement requires special tools. We recently developed a flow cytometry-based analytical method for SVPs of 0.75–70 μm. The method quantifies SVPs and distinguishes proteins and protein-containing particles from non-protein particles (e.g. silicone oil) [18]. Samples taken from the same vials as used for SEC analysis were subjected to the flow cytometry-based particle analysis.

Particle counts for the tested products are shown in counts/1000 IU in Fig. 2 to allow better comparison of the products with different volumes and potencies.

The average SVP concentration in the rFVIII products varied from 1.1 to 61.7 × 10^6 counts/1000 IU. The highest concentration was determined in product E, 61.7 × 10^6 counts/1000 IU (range, 35.6–114.0 × 10^6 counts/1000 IU), and the lowest in product A, 1.1 × 10^6 counts/1000 IU (range, 0.7–1.7 × 10^6 counts/1000 IU). A 158-fold difference in SVP concentration between the lots with the lowest and the highest particle counts highlighted the difference in quality of the rFVIII products.

Fig. 2. Concentration of non-protein and protein-containing subvisible particles (SVPs) of 0.75–70 μm in different rFVIII product lots under intended-use conditions (A) and after exposure to use-relevant stress (B).
majority (53–99%) of SVPs was protein or contained protein. Lots of some products (e.g. B, C, D and E) were found to contain increased concentrations of non-protein particles (Fig. 2A). No differences in SVP size distribution were observed between products (Fig. 3A). Under intended-use conditions, an average of 53.3% of SVPs of 0.75–1 µm were detected, 23.9% that were 1–2 µm, and 18.0% that were 2–4.5 µm. Only 4.7% of SVPs were 4.5–70 µm in size. These results indicate that similar mechanisms are involved in the formation of SVPs in all rFVIII products. The data also suggest a continuum of protein aggregates in rFVIII products, which cannot be fully assessed using only one analytical method (e.g. HPLC-SEC).

After exposure to use-relevant stress, SVP concentrations increased in all tested rFVIII products (Fig. 2B), reaching similar levels in products B, C, D, F and G (i.e. 7.6–43.4 × 10^6 counts/1000 IU). Product H contained the lowest concentration (mean value, 4.5 × 10^6 counts/1000 IU; range, 3.5–6.0 × 10^6 counts/1000 IU) and product E the highest (mean value, 144 × 10^6 counts/1000 IU; range, 71.1–225 × 10^6 counts/1000 IU). The influence of the applied freeze/thaw procedure during sample preparation on the formation of SVPs was investigated for product A. The difference in SVP concentration after freeze/thawing compared with freshly reconstituted material was within assay variability [18] and within an acceptable analytical error (data not shown). Furthermore, the difference was negligible compared with the increase in SVP concentration observed after applying use-relevant stress conditions.

The size distribution of SVPs in rFVIII products after exposure to use-relevant stress was similar to that determined in the products under intended-use conditions, albeit with a slight increase in larger SVPs (on average 45.8% 0.75–1 µm, 26.4% 1–2 µm, 21.7% 2–4.5 µm and 6.1% 4.5–70 µm in size) (Fig. 3B). The size distribution of SVPs in antibody drugs was previously shown to be dependent on the type of applied stress [23]. After exposure to agitation, the rFVIII products in this study showed a similar SVP size distribution. Although their efficacy after exposure to use-relevant stress was unaffected as determined by in vitro chromogenic FVIII activity assay (unpublished data), increasing amounts of SVPs might increase safety risks. A link between protein/protein-containing SVP concentrations and the immunogenicity of monoclonal antibody therapeutics has previously been demonstrated [16].

In conclusion, we provide evidence that rFVIII products are susceptible to protein aggregation and formation of SVPs. In our study, commercially available rFVIII products showed different concentrations for the quality parameters of SPAs and SVPs, although all are effective in controlling bleeding in hemophilia A patients. The quality of the products under intended-use conditions
determined their stability upon use-relevant stress in controlled laboratory conditions. Thus, it remains to be elucidated if and how SPAs and SVPs contribute to unwanted immune responses against rFVIII. Immunogenicity of artificially generated FVIII aggregates in in vivo animal models indicates that not only the size and quantity, but also the nature and structure, of FVIII aggregates are important in initiating unwanted immune responses towards native FVIII [22]. How this translates to humans and if and how the identified protein aggregates in FVIII products interact with components of the human immune system are subjects for further investigation. Nevertheless, our findings confirm that rFVIII products are not alike. Because of potential short or long-term safety concerns, the choice of rFVIII for treatment of individual hemophilia patients should take into account all quality attributes of the drug.

Addendum

M. Malisauskas and J. Anzengruber performed HPLC-SEC and flow cytometry experiments, analyzed and interpreted data, prepared figures and wrote the manuscript. C. Lubich and T. Prenninger performed flow cytometry experiments and analyzed data. M. Malisauskas designed the research. A. Gringeri, F. Scheiflinger and B. M. Reipert revised the intellectual content and all authors approved the final manuscript.

Acknowledgements

The authors thank K. Benamara and B. Dass for carefully reviewing the manuscript.

Disclosure of Conflict of Interests

This work was funded by Shire. All authors were employees of Shire at the time this work was performed.

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