# Development of immobilized von Willebrand factor (VWF) affinity chromatography to characterize a protein fraction unable to bind VWF in recombinant factor VIII products

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## Abstract

All recombinant Factor VIII (rFVIII) products contain protein unable to bind von Willebrand factor (VWF). To analyze this fraction ($FVIII^{FT}$), we developed an affinity chromatography method using immobilized VWF. We demonstrated different content of $FVIII^{FT}$ in various rFVIII products and differences in its biochemical features from the major protein fraction.

## Introduction

Therapeutic products with coagulation factor VIII (FVIII), used to treat FVIII deficiency (Hemophilia A), have a wide range of specific activities, implying presence of FVIII molecules with altered structures. Previous studies showed that recombinant FVIII products contain a fraction unable to bind VWF, reported to lack activity. The level of $FVIII^{FT}$ and its clinical relevance should be examined more closely in rFVIII products.

**Table 1.** List of rFVIII products used in the study

| Product | Molecule name (nonproprietary) | Produce cell line* | Protein length* | Modification* |
|---|---|---|---|---|
| A | Octocog alfa | CHO | FL | - |
| B | Ruriotocog alfa pegol | CHO | FL | PEGylated (EHL) |
| C | Moroctocog alfa | CHO | BDD | - |
| D | Efmoroctocog alfa | HEK | BDD | Fc fusion (EHL) |
| E | Octocog alfa | BHK | FL | - |
| F | Octocog alfa | BHK | FL | - |
| G | Turoctocog alfa | CHO | BDD | - |
| H | Damoctocog alfa pegol | BHK | BDD | PEGylated (EHL) |
| I | Simoctocog alfa | HEK | BDD | - |
| J | Lonoctocog alfa | CHO | BDD | SCh |

Abbreviations: FL, full-length; BDD, B-domain deleted; CHO, chinese hamster ovary; BHK, baby hamster kidney; HEK, human embryonic kidney; PEG, polyethylene glycol; SCh, single-chain; EHL, extended plasma half-life.
* Data from the prescribing information

## Materials and Methods

Protein fractions unable and able ($FVIII^{EL}$) to bind VWF were isolated from rFVIII products ($FVIII^{TP}$) using Immobilized VWF Affinity Chromatography (IVAC) and characterized by gel electrophoresis, immunoblotting, FVIII activity test, surface plasmon resonance, mass spectrometry, and plasma clearance in mice.

## Disclaimer

The information in these materials is not a formal dissemination of information by FDA and does not represent agency position or policy.

## Results

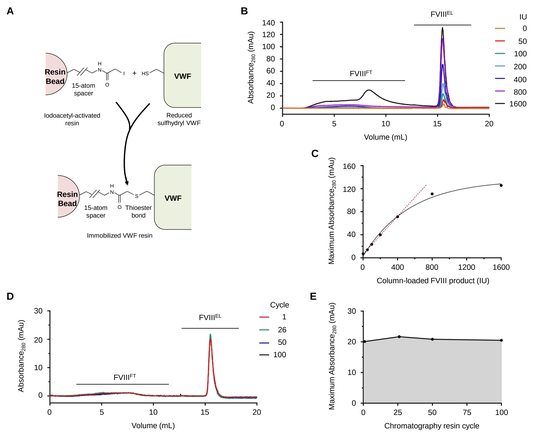


**Figure 1.** Development of immobilized VWF affinity chromatography (IVAC). (A) Schematic illustration of VWF coupling to the resin. (B) Determination of maximum protein load (product A) for IVAC column. $FVIII^{FT}$ corresponds to flow-through fraction with VWF-unbound protein collected during sample loading and column washing. $FVIII^{EL}$ corresponds to fraction with VWF-binding protein eluted from the column. Colored curves correspond to chromatograms monitored at $A_{280}$ upon consecutive running with a various amount of FVIII sample (0-1600 IU). (C) Plotting maximum $A_{280}$ values for $FVIII^{EL}$ fractions versus respective amounts of the loaded total protein ($FVIII^{TP}$) (experiment in panel B). A dashed red line indicates expected absorbance for the loaded amounts of protein. (D) Durability test of the IVAC column. Colored curves correspond to $A_{280}$ recorded at respective column cycle number. Although the column was used for analysis of various rFVIII products, its performance was tested using product A on cycle numbers 1, 26, 50, and 100. (E) Quantification of maximum $A_{280}$ values in $FVIII^{EL}$ fractions on different column cycle (experiment in panel D).

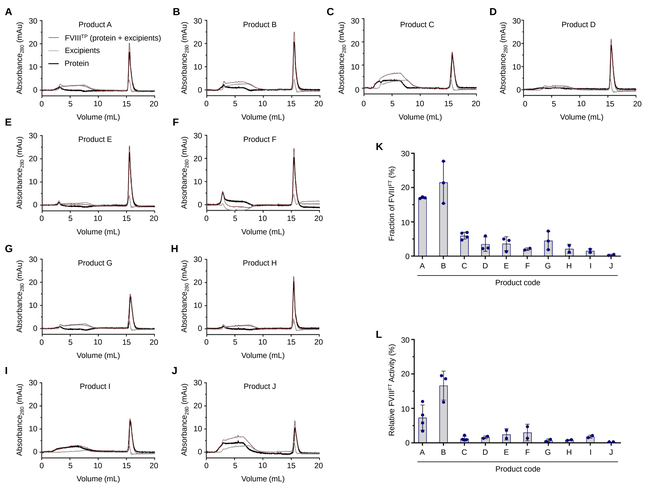


**Figure 2.** Determination of content and activity of $FVIII^{FT}$ in rFVIII products. (A-J) Chromatograms of protein analysis in 10 rFVIII products by IVAC. By subtracting a chromatogram of excipients (gray) from that of 100 IU $FVIII^{TP}$, reconstituted rFVIII products containing excipients (red), a chromatogram of only FVIII protein (black) isolated from $FVIII^{TP}$ by IVAC is calculated. (K) Relative content of $FVIII^{FT}$ in rFVIII products. The content of $FVIII^{TP}$ and $FVIII^{FT}$ was determined by silver-stained gel and MSD-ECL assay. (L) Relative activities of $FVIII^{FT}$ in rFVIII products. The activity was determined by a chromogenic substrate assay. The raw value of each data point in graphs K and L are summarized in Table S2. Error bars in the graphs indicate percentage of $FVIII^{FT}$ in $FVIII^{TP}$ (mean ± standard deviation, n = 2-4). Lot-to-lot variability was not conclusive because limited number of lots were analyzed in this study.

An IVAC methodology was developed and applied for analysis of ten rFVIII products marketed in the USA. $FVIII^{FT}$ was found at various contents (0.4–21.5%) in all tested products. Compared to $FVIII^{EL}$, $FVIII^{FT}$ had similar patterns of polypeptide bands by gel electrophoresis, but lower chromogenic assay activity. In several products, $FVIII^{FT}$ was found to have reduced sulfation at Tyr1680, which is important for VWF binding; decreased interaction with a low-density lipoprotein receptor related protein 1 (LRP1) fragment; and faster plasma clearance in mice.

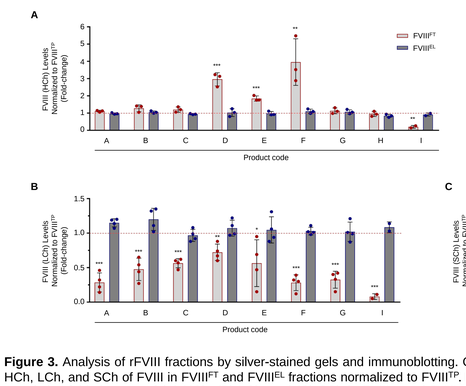


**Figure 3.** Analysis of rFVIII fractions by silver-stained gels and immunoblotting. Quantification of HCh, LCh, and SCh of FVIII in $FVIII^{FT}$ and $FVIII^{EL}$ fractions normalized to $FVIII^{TP}$. Data are shown as relative fold changes of (A) HCh, (B) LCh, and (C) SCh in $FVIII^{FT}$ and $FVIII^{EL}$ compared with corresponding chain of FVIII in $FVIII^{TP}$ (means ± standard deviation) from multiple replicates per condition. Intensity of each chain was measured from SDS-PAGE gels under reducing condition followed by silver staining and densitometry analysis. A dashed red line marks indicated chain levels of FVIII in $FVIII^{TP}$. Statistics: one-way ANOVA for each product, Dunnett's post hoc test (not indicated, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

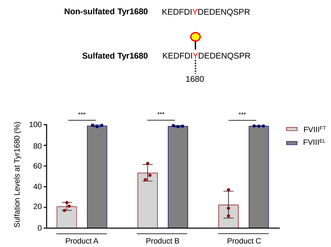


**Figure 4.** Content of sulfated Tyr1680 in rFVIII protein fractions. $FVIII^{FT}$ and $FVIII^{EL}$ fractions, isolated from products A (FL-FVIII), B (EHL FL-FVIII), and C (BDD-FVIII), were analyzed for the content of sulfated Tyr1680 within respective tryptic peptide (shown at top) using nano LC-MS/MS. FVIII samples from product A (lot c), product B (lots a and b), and product C (lot d) were independently purified using IVAC three times and analyzed by MS independently. Data are shown as relative content of sulfated Tyr1680 in total Tyr1680 in the same protein fraction (mean ± standard deviation) from three replicates per condition. Statistics: two-way ANOVA, Tukey's post hoc test (***$P < 0.001$).

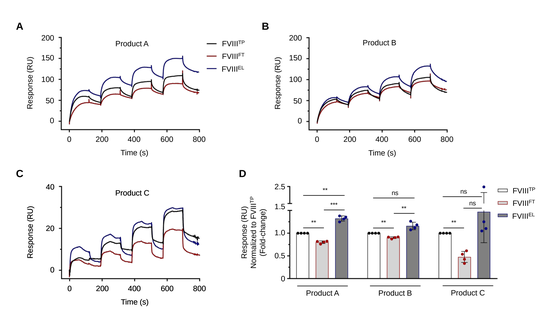


**Figure 5.** Interaction rFVIII fractions with LRP1 cluster II by SPR. (A-C) Increasing concentrations of $FVIII^{TP}$, $FVIII^{FT}$, and $FVIII^{EL}$ (3.8, 7.5, 15, and 30 nM), corresponding to products A (lot b), B (lot a), and C (lot d) were injected over immobilized cluster II of LRP1 (60 nM) in single-cycle kinetics runs. The signals are expressed in resonance units (RU). Three independent experiments with different conditions were performed, and the representative experiment is shown here. (D) Quantification of RU of injected rFVIII fractions (3.8, 7.5, 15, and 30 nM) on an LRP1 cluster II chip in the single experiment (experiments in panels A-C). Relative changes compared with $FVIII^{TP}$ signals are presented as mean ± standard deviation ($n = 4$). Statistics: one-way ANOVA for each product, Tukey's post hoc test (ns, not significant, $P > 0.05$; **$P < 0.01$; and ***$P < 0.001$).

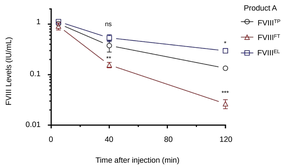


**Figure 6.** Clearance of rFVIII fractions in mice. Eight-week old wild-type mice (male) were administered with a single dose of 65 IU/kg of each FVIII fraction ($FVIII^{TP}$, $FVIII^{FT}$, and $FVIII^{EL}$) of product A via retro-orbital injection. Blood was collected at time points shown on the graph and the levels of injected FVIII protein (antigen) in plasma samples were measured by MSD-ECL assay used relevant anti-human FVIII antibodies. Error bars indicate average ± standard deviation of three different mice samples. Statistics: two-way ANOVA, Tukey's post hoc test (ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

## Conclusion

These findings provide some basic characterization of $FVIII^{FT}$ and demonstrate a potential for IVAC to control $FVIII^{FT}$ fraction in rFVIII products. This may result in improving quality and efficiency of rFVIII products, thus improving care of Hemophilia A.