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Proteomic Characterization of Plasma-derived Clotting Factor VIII – von Willebrand Factor Concentrates

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Abstract

Proteomic methods were used to identify the levels of impurities in three commercial plasma-derived clotting factor VIII-von Willebrand factor (FVIII/VWF) concentrates. In all three concentrates, significant amounts of other plasma proteins were found. In Octanate and Haemoctin, two concentrates developed in the 1990s, the major impurities identified were inter-alpha inhibitor proteins, fibrinogen and fibronectin. These two concentrates were also found to contain additional components such as clotting factor II (prothrombin) that are known activators of FVIII. In Wilate, a recently developed FVIII/VWF concentrate, the amount of these impurities was significantly reduced. Batch-to-batch variations and differences between three investigated products were detected using iTRAQ, an isotope labeling technique for comparative mass spectrometry, demonstrating the potential value of this technique for quality control analysis. The importance of thorough proteomic investigations of therapeutic F VIII/VWF preparations from human plasma is also discussed.

Keywords

Clotting factor VIII; von Willebrand factor; Prothrombin; Proteomic analysis

INTRODUCTION

Clotting factor VIII (F VIII) is a glycoprotein of complex structure, consisting of a heavy and a light chain. This glycoprotein plays a key role in the intrinsic pathway of the blood coagulation cascade [1]. Factor VIII circulates in human plasma in a stable complex with von Willebrand factor (VWF), a multimeric glycoprotein, consisting of disulfide-bridge linked dimers of the 225-kDa single-chain molecule. Von Willebrand factor itself circulates blood plasma in form of series of high molecular weight multimers. These multimers have M_r values up to 2×10^4 kDa [2]. In addition to its involvement in both primary and secondary hemostasis, VWF also functions as a carrier and stabilizing protein that protects F VIII from proteolysis and clearance [2–4]. Reduced levels or a missing or dysfunctional F VIII glycoprotein are associated with the disease known as hemophilia A [5]. The absence or reduction of functional VWF also has a dramatic impact on hemostasis and is associated with von Willebrand disease (VWD) [6]. As a consequence of the reduced level of VWF in

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plasma, the level of F VIII is also decreased [3,7]. The normal plasma concentration of F VIII is about 0.2 µg/mL (0.7 nM). At about 10 µg/mL, the average plasma concentration of its carrier protein VWF is fifty times higher [8]. Compared to the most abundant proteins, human serum albumin (HSA) and immunoglobulin G (IgG), the concentration of F VIII in blood plasma is about five orders of magnitude lower [9].

For more than thirty years, the treatment of hemophilia A has been accomplished by infusions of F VIII concentrates. Initially, F VIII concentrates were prepared from human plasma [10]. These concentrates contained relatively high amounts of other plasma proteins. By use of new technologies, mostly chromatography, the purity of plasma-derived F VIII (pd F VIII) concentrates was considerably improved [10,11]. Although the pd F VIII concentrates currently on the market nominally belong to the group of so-called “well-characterized biologicals”, they still contain relatively high amounts of foreign proteins, the exact levels and/or identities of which are often not well characterized [11,12]. Most pd F VIII concentrates contain considerable amounts of VWF. If these concentrates contain VWF protein with a multimer composition similar to that of human plasma, they can also be used for the treatment of VWD [7,11,12].

Factor VIII is sensitive to proteolysis, activation and degradation [11,13], and therefore, it has to be stabilized during the production process and in the final formulation [10,11]. The basic requirements that have to be fulfilled for these very sensitive therapeutics are virus safety, effectiveness, and the absence of side effects [11,14]. The most serious complication in the treatment of hemophilia A is the development of inhibitor antibodies, called inhibitors, most frequently at an early stage of therapy. These antibodies are capable of blocking F VIII procoagulant activity [14,15]. There have been dangerous outbreaks of inhibitors in multitransfused patients in the past, and they seem to be due to the creation of neopeptides in the F VIII molecule during the manufacturing process [12,16,17], a possibility that necessitates careful monitoring of batch-to-batch variations and thorough characterization of the final product [12,18, 19]. In this paper, some double virus-inactivated pd F VIII concentrates containing different amounts of VWF were analyzed by proteomic methods carried out in parallel to biochemical and functional analyses routinely used for the quality control.

EXPERIMENTAL PROCEDURES

Materials

Octanate® (five samples from five different batches containing 1000 IU FVIII/vial) and Wilate® (three samples from three different batches, 1000 IU/vial), two plasma-derived, double virus inactivated F VIII concentrates were obtained from Octapharma Pharmazeutika GmbH (Vienna, Austria). Double virus inactivated plasma-derived F VIII concentrate Haemoctin® (one sample, 1000 IU/vial) was purchased from Biotest Pharma GmbH (Dreieich, Germany). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Biochemical and functional analysis FVIII/VWF concentrates

Biochemical analyses and determination of biological activity were performed as previously described [11,20–24]. Each concentrate containing 1000 IU F VIII/vial was dissolved in 5 mL aqua bidest. Total protein was quantified using a Bradford assay (Pierce, Rockford, IL, USA) with bovine serum albumin as standard. F VIII cofactor activity was determined by use of a commercial kit (Coamatic FVIII:C Testkit, diaPharma, West Chester, OH). F VIII:Ag was quantified by an ELISA method using the Asserachrom ® FVIII:C:Ag (Diagnostica Stago, Asniers, France, distributed by American Bioproducts, Parsippany, NJ,

USA) commercial kit according to manufacturer's instructions. The ability of VWF to agglutinate fixed human platelets in the presence of ristocetin (Dade Behring, Marburg, Germany) was determined according to European Pharmacopoeia. Von Willebrand factor collagen binding activity (VWF:CBA) was determined by use of a commercial ELISA kit (VWF: CBA ELISA kit, Gradipore, French Forest, Australia) according to the manufacturer's instructions. Total fibrinogen, fibronectin and immunoglobulins were quantified using corresponding rabbit polyclonal antisera. IaIp levels were determined using a competitive ELISA with monoclonal antibody (MAb) 69.31 as described by Lim *et al.* [24]. Shortly: 96-well plates (Dynex, Chantilly, VA, USA) were coated with purified IaIp and incubated overnight at 4°C. A serial dilution of purified IaIp in PBS that contained 1% rat serum was used to establish a standard curve. For the quantitative analysis of IaIp content in F VIII concentrate, 50 µL of sample containing 1 µg/µL protein diluted 1:25 in PBS or serially diluted IaIp were added to individual wells of a 96 plate. After addition of 50 µL of MAb 69.31 to each well, plates were incubated for 1 h at 37°C and subsequently washed using an automated plate washer (LabSystem, Derwood, MD, USA). The bound MAb was detected by adding horseradish peroxidase-conjugated goat anti-mouse IgG (Biosource, Wayne, PA, USA) for 1 h at 37°C. After washing, 100 µL of 1-Step ABTS (Pierce, Rockford, IL, USA) was added, and the absorbance was measured on ELISA plate reader (BioTek, North Seattle, WA). Each sample was tested in triplicate.

Proteolytic activity was determined using the synthetic substrate N-benzoyl-Pro-Phe-Arg *p*-nitroanilide hydrochloride according to Ref. [22].

SDS-PAGE

SDS-PAGE under reducing and non-reducing conditions was performed according to Laemmli [25] as described previously [19].

Size-Exclusion Chromatography

For size-exclusion chromatography (SEC) a tandem system containing one Superose 6 column and Superose 12 column (both 300×10 mm I.D., GE Healthcare, Piscataway, NJ, USA) or TSK G3000 SWXL and TSK G4000 SWXL (both 300×7.8 mm I.D., Tosoh Bioscience, King of Prussia, PA, USA) was used. The mobile phase was phosphate-buffered saline, pH 7.4 (PBS). The flow rate during separation was 0.5 mL/min. All separations were performed at room temperature. Proteins were detected at 210 nm. Fractions after chromatographic separation were collected, separated by SDS-PAGE, and used for protein identification by LC-ESI-MS/MS. For separation and fraction collection, a BioLogic Duo Flow chromatographic system containing a fraction collector was used (BioRad Laboratories, Hercules, CA, USA).

Identification of Proteins with LC-ESI-MS/MS

The bands separated by SDS-PAGE were excised and digested with trypsin as described previously [19]. Tryptic digests were separated with an RP column (C-18 PepMap 100, LC Packings/Dionex, Sunnyvale, CA, USA) as previously described, with the column eluate introduced directly onto a QSTAR XL mass spectrometer (Applied Biosystems, Foster City, CA, USA and Sciex, Concord, Ontario, Canada) via ESI [26]. Candidate ion selection, fragmentation and data collection were performed as described previously [26]. Briefly: Half-second MS scans (300–1500 Thompson, Thompson(Th) = Da/z) were used to identify candidate ions for fragmentation during MS/MS scans. Up to five 1.5 s MS/MS scans (65–1500 Th) were collected after each scan. An ion had to be assigned a charge in the range of +2 to +4. The dynamic exclusion was 40. Protein identifications were performed with ProteinPilot (versions 1.0 and 2.0; Applied Biosystems and Sciex), searching the human and “RefSeq” databases from NCBI (<http://www.ncbi.nlm.nih.gov/RefSeq/>). ProteinPilot is the

successor to ProID and ProGroup, and uses the same peptide/protein scoring method [26]. Briefly, given a protein score, S , the likelihood that the protein assignment is *incorrect* is 10^{-S} . Furthermore, scores above 2.0 require that at least two sequence-independent (unique) peptides be identified.

iTRAQ Protein Quantitation of Factor VIII Samples

iTRAQ (isobaric tag for relative and absolute quantitation) was used for comparative analysis of protein levels. iTRAQ is a non-gel based method for comparing proteins from different sources in one single experiment [27]. Following tryptic digestion and N-terminal labeling of each sample with a different mass tag, the samples are pooled, fractionated by nano-LC, and analyzed by tandem mass spectrometry to identify the peptide and simultaneously generate a low mass reporter ion from the mass tag that can be used to determinate the relative amount of the peptide in each sample [27]. This method was applied to the analysis of three F VIII preparations: #1,4-Octanate, #5-Haemoctin, and #8-Wilate. These samples were precipitated with the ReadyPrep 2-D Cleanup Kit (Bio-Rad), according to the manufacturer's instructions. The precipitates were redissolved with 20 μ L of 0.5 M triethylammonium bicarbonate, pH 8.5, and reduced, alkylated and tryptically digested according to the iTRAQ protocol (Applied Biosystems). One μ L aliquot from each were saved for LC-MS/MS verification of the tryptic digestions. The remaining material was labeled with iTRAQ reagents (#1: iTRAQ 114; #4: iTRAQ 115; #5: iTRAQ 116; #8: iTRAQ 117) according to the manufacturer's instructions.

Aliquots (one-tenth volume) from each sample were mixed together and dried in a vacuum centrifuge. The material was twice redissolved with water and dried. It was then twice redissolved in a solution of 0.1% (v/v) formic acid and 20% (v/v) acetonitrile, with vacuum drying. After dissolving in the same solvent, and confirming the pH, the peptides in the iTRAQ labeled mixture were isolated using a strong cation exchange TopTip™ (PolyLC, Inc., Columbia, MD USA) according to the manufacturer's instructions. The ammonium formate eluates were dried and redissolved in formic acid:water:acetonitrile:trifluoroacetic acid (0.1:95:5:0.01) in preparation for LC-ESI-MS/MS analysis. Triplicate LC-MS/MS data collections were performed for quantitation.

Standard information dependent acquisition (IDA) of MS and MS/MS spectra during μ HPLC separation of the peptide mixture was performed as described previously [28]. Suitable collision energies for fragmenting iTRAQ-labeled peptides in the QSTAR mass spectrometer were determined empirically using one of the laboratory's standard peptide mixtures. Peptides and proteins were identified and quantitated using ProteinPilot, using default program settings and searching a human database (as above, *Protein Identifications*). Briefly, peak areas for iTRAQ reporter ions are integrated; the program automatically determines the peptide ratios and their associated errors. The protein ratios are calculated from the weighted (by error) average of all contributing peptide ratios.

RESULTS

Biochemical and functional analysis

Results of biochemical and functional analyses of three investigated FVIII/VWF concentrates are listed in Table 1. All values are given in corresponding units (international units – IU, or milligrams) per vial. According to the manufacturer's declaration, each vial contains 1000 $\pm$ 200 IU F VIII [11,20,21]. As shown in this Table, results of these analyses are very similar for Octanate and Haemoctin. Compared to those two previous concentrates, the contents of VWF (VWF:Ag), and both VWF:RCoF and VWF:CBA in the recently developed concentrate Wilate are 2–3 $\times$ higher.

SDS-PAGE and Size-Exclusion Chromatography

In Figure 1, SDS-PAGE of four different Octanate batches (lanes 1–4), one Haemoctin (lane 5) and three Wilate batches (lanes 6–8) under reducing (Figure 1A) and non-reducing conditions (Figure 1B) are shown. Size-exclusion chromatograms of Octanate (full line) and Wilate (broken line) are shown in Figure 2. The results of SDS-PAGE under non-reducing conditions and SEC show similar results. Octanate, the F VIII concentrate that was purified by only anion-exchange chromatography, shows three peaks in SEC [12,29]. SDS-PAGE of four different batches of this concentrate yields one diffuse band in the very high molecular weight region and different bands in the molecular weight region between 60 and 250 kDa. The F VIII/VWF activity was found only in the high molecular weight region (see Figure 2 and Reference 29). Wilate, the concentrate that was purified by an additional size-exclusion chromatography step [11], shows in SEC only one high molecular weight peak, which corresponds to the diffuse high molecular weight band in SDS-PAGE (see Figure 1B, lanes 6–8, and Figure 2, broken line). In SEC, Haemoctin shows a chromatographic profile identical to Octanate (data not shown). After SEC separation of Octanate and Haemoctin, collected fractions were separated by SDS-PAGE under reducing and non-reducing conditions (cf. Figures 3A and 3B). Under these conditions disulfide bridges in proteins are broken. The diffuse band in the very high molecular weight region practically disappears, and additional bands appear in regions with apparent molecular weights between 50 and about 300 kDa (cf. Figure 1A and 1B). Under reducing conditions, Wilate samples (lanes 6–8 in Figure 1A) show a similar pattern to those of Octanate (lanes 1–4) and Haemoctin (lane 5). However, the band with apparent molecular weight greater than 250 kDa is stronger, and bands with apparent molecular weights of 225, 125 and 80 kDa are missing (cf. Figure 1A, lanes 6–8). Again, for Haemoctin, the pattern in SDS-PAGE was virtually identical to that of Octanate (cf. lane 5).

Protein identification

Protein bands in SEC fractions resolved by SDS-PAGE from Octanate under reducing (Figure 3A) and non-reducing conditions (cf. Figures 3B) were excised, digested with trypsin and identified by LC-ESI-MS/MS. Identified proteins are listed in Tables 2 and 3. Additionally, tryptic digests of protein bands resolved by SDS-PAGE under non-reducing and reducing conditions from unfractionated samples of Octanate, Haemoctin and Wilate were also analyzed by LC-ESI-MS/MS. Several additional proteins were identified in Octanate and Haemoctin (Table 4), but only when samples were separated by SDS-PAGE under reducing conditions. Proteins identified in bands excised from reduced and non-reduced SDS-PAGE of unfractionated Wilate are listed in Table 5.

Quantitative comparison of F VIII concentrates after iTRAQ labeling

The total ion chromatogram of a pool of iTRAQ-labeled tryptic peptides from four different FVIII concentrates (two different Octanate batches and Haemoctin and Wilate respectively) is shown in Figure 4. Figure 5 shows a representative MS/MS spectrum of the peptide YYWGGQYTWDMAK during this experiment. In the insert of Figure 5, a magnified view of the mass range from 113 to 118 shows the iTRAQ reporter ions that were generated during the fragmentation process. Quantitative determination of these signals provides the relative abundance of this peptide in the four samples.

Results from the quantitative comparisons of F VIII concentrates by iTRAQ analysis (Table 6, for all identified proteins see also the Supplement, Table S1) revealed both batch-to-batch and concentrate-to-concentrate variations in F VIII and VWF content and in the levels of contaminating proteins.

Table 6 shows iTRAQ comparisons of 1) two different batches of Octanate designated Octanate 1 and 2; 2) Haemoctin and Octanate; and 3) Wilate and Octanate. Based on ratios of the measured iTRAQ, the content of F VIII and VWF in the two Octanate batches differed by less than 15%. The content of main impurities, the inter-alpha inhibitor proteins (IaIp, identified as subunits H1, H2, H3 and bikunin) showed much higher batch-to-batch variation. The content of clotting factor II in first Octanate (Octanate1) batch was about 2× higher than in the second one (Octanate 2).

In the investigated Haemoctin sample, the VWF content was at about same level as in Octanate. The F VIII content in this sample was approximately 30% higher. The content of IaIp subunits of Octanate 1, and the amount of F II was higher than in this sample.

The content of F VIII in Wilate was at the same level as the F VIII content in Octanate 1. The content of high-molecular proteins VWF (the second active component) and fibronectin and fibrinogen (both impurities) in Wilate were higher than in Octanate. The content of VWF in analyzed Wilate batch more than 2× higher than in Octanate1, and corresponding Haemoctin batch. The level of other contaminating proteins, IaIp, kininogen and F II was significantly lower (cf. Table 6).

Determination of IaIp in F VIII concentrates by competitive ELISA

The amount of IaIp in F VIII concentrates was determined by competitive ELISA using the monoclonal antibody 69.31, directed against the IaI light chain bikunin [24]. As shown in Table 7, the amount of IaIp in Octanate and one Haemoctin sample was between 16.5 and 20%. The amount of IaIp in Wilate was two orders of magnitude lower (0.2 and 0.4% respectively in two samples). In one Wilate sample, the amount of IaIp determined by this method was below detection limit (see Table 7). The protein concentrations and F VIII activity in investigated samples were comparable (between 1.3 and 2.43 mg/mL, and 180–210 IU/mL, cf. Table 7).

DISCUSSION

Virological safety of human plasma-derived therapeutic protein products has been the primary concern for a long time, and thorough validation work is performed to make these products virologically safe [11]. Other aspects of clinical safety were covered by extensive and very expensive clinical trials [11,21,30]. However, there have been unforeseen accidents after some products were introduced to the market: After the introduction of two F VIII concentrates, unexpected development of inhibitors in previously treated patients occurred [12,16,17], resulting in the withdrawal of these products from the market. Some work was done to explain the origin of these unexpected side effects, unfortunately with limited success [12,31]. It was shown that inhibitory antibodies were directed against the C2 domain in the light chain of the F VIII [32], but the peptide(s) responsible for antibody formation could not be conclusively determined. Barrowcliffe *et al.* [33] proposed that the purification process and virus inactivation, specifically heating, may expose new F VIII immunogenic epitopes. This hypothesis was examined further by Raut *et al.* [31], Josic *et al.* [12] and Saenko *et al.* [34] using different chromatographic and electrophoretic methods, combined with surface plasmon resonance (SPR). From the analysis of potentially immunogenic, double virus inactivated F VIII concentrates, they found that some batches showed definitive evidence of elevated F VIII hydrolysis. In SPR measurements, these batches showed impaired binding to phospholipid vesicles [34]. In model experiments, Smales *et al.* [35] demonstrated that heat treatment of F VIII concentrate in the presence of stabilizers, e.g. sucrose, led to the formation of disulfide crosslinks and protein aggregation. They also detected changes in protein glycosylation and the formation of glycation-type modifications. However, a thorough proteomic investigation of commercial F VIII concentrates and other

therapeutic proteins derived from human plasma, all belonging to the group of so-called “well characterized biologicals,” has not yet been performed.

As a part of the product development for a potentially new therapeutic from human plasma, the family of inter-alpha inhibitor proteins (IaIp), we have already performed a proteomic investigation during the whole production process of this substance [19]. To our surprise, we found that the fraction of highly purified IaIp contains a significant amount of clotting factor II (F II – prothrombin). Activated F II, F IIa, better known as thrombin, is potentially harmful and had to be removed from the final product. As shown in Figures 2 and 3B (fractions 5 and 6) and Tables 2,3 and 7, IaIp (heavy chains 1, 2 and 3 and light chain bikunin) are the main impurities in both F VIII concentrates, Octanate and Haemoctin. Unfortunately, the quantitative analysis of these proteins is still not a part of routine quality control of F VIII concentrates [11,23]. Lim *et al.* recently developed a competitive ELISA for determination of IaIp, and we used this test for quantitative determination of these proteins in F VIII concentrates (cf. Table 7). In fraction 5 (see Figures 2, and 3B), in addition to IaIp, we also detected significant amounts of F II (prothrombin; cf. Tables 2 and 3 and 7). In this fraction (apparent molecular weight in SEC ~ 200,000, see Figure 2), prothrombin (MW~70,000) appears to be associated with another protein, possibly with an IaIp family member. The presence of high levels of IaIp may be the reason that no proteolytic activity was detected in investigated FVIII/VWF concentrates (see Table 1).

Isotope labeling has been used to identify quantitative differences in protein composition in closely related samples [27]. Results presented in Table 6 demonstrate that the iTRAQ isotope labeling method can be used for quantitative comparison of active components and impurities in different batches and to quantify batch-to-batch variation for a therapeutic protein preparation. This method is also valuable for comparing the composition of products from different manufacturers or different generation products such as Octanate and Wilate. As shown in Table 6, the content of VWF, an important active component in these therapeutic concentrates, is in Wilate significantly higher than in the F VIII concentrates of the previous generation, Octanate and Haemoctin. Similar results were obtained when biochemical and functional analyses were performed (see Table 1). The higher VWF content, higher content of high-molecular weight multimers, higher VWF:RcoF and VWF:CBA values make this concentrate suitable for the treatment of both hemophilia A and VWD [7,11]. In contrast to Octanate and Haemoctin, we did not identify any F II in Wilate after protein separation by SDS-PAGE and analysis of excised bands by LC-MS/MS (cf. Table 5). However, after iTRAQ labeling and direct comparison between concentrates (Octanate, Haemoctin and Wilate), some F II could be found in all three concentrates. Importantly, the content of this potentially harmful protein was almost two orders of magnitude lower in Wilate (see Table 6). Besides F II, some other potentially harmful impurities, such as kininogen and clotting factor V (cf. Tables 2,3 and Table S1, supplemental material), were also detected. Comparative analysis of F VIII batches produced by similar purification schemes (Octanate and Haemoctin, cf. Table 4) showed that these impurities were present in all samples subjected to analysis. As shown in Table 7, the main impurity in both Octanate and Haemoctin is the family of inter alpha inhibitor proteins. As determined by ELISA with the monoclonal antibody 69.31, the amount of IaIp in these two concentrates is between 16.5 and 20.0%. The amount of IaIp in Wilate is roughly two orders of magnitude lower (0.2 and 0.4% in the first two concentrates, and below the detection limit in the third one, see Table 7). However, with iTRAQ labeling, the content of heavy chains H1, H2 and H3 was only one order of magnitude lower (cf. Table 6). The monoclonal antibody 69.31 is directed against the light chain, bikunin [24]. The content of this part of IaIp molecules determined by LC-ESI-MS/MS after iTRAQ labeling was two orders of magnitude lower in Wilate than in Octanate and Haemoctin. This result is much closer to the data obtained by ELISA. The consequence is that the relative amount of

IaIp heavy chains in Wilate is much higher than the amount of bikunin. The amounts of fibronectin and fibrinogen determined in Wilate by LC-ESI-MS/MS was 1.42 times higher for fibronectin, and between 1.31 and 1.19 times higher for different fibrinogen chains (cf. Table 6 and supplemental material, Table S1). When determined immunochemically, the level of fibronectin was comparable in all FVIII concentrates. The amount of fibrinogen determined by the immunochemical method was below the detection limit. As shown in Table 5, fibrinogen was also detected in bands excised after SDS-PAGE of Wilate. As mentioned above, in both FVIII concentrates developed in the early 1990's, significant amounts of FII was detected. When assayed by other biochemical methods, this protein was not detected [12]. In conclusion, comparative analyses of two first generation products, Octanate and Haemoctin, and the new product, Wilate, show that a reduction in the levels of contaminating proteins IaIp, F II and kininogen 1 can be achieved by SEC (cf. Fig. 2, Table 6 and Reference 11). The contradictory results obtained for fibronectin and fibrinogen, if proteomic and immunochemical methods are applied, need further investigation and validation.

One solvent-detergent treated and pasteurized F VIII/VWF concentrate that was shown to be immunogenic was produced by use of technology similar to Octanate and Haemoctin production processes [12]. After detection of F II in these concentrates, it is very intriguing to speculate that possible F II activation during the production process, possibly during heating in combination with solvent-detergent treatment, could have caused partial degradation of F VIII. The products of this partial degradation could potentially have caused further immunological reactions in previously treated patients that were infused with such double virus inactivated F VIII/VWF concentrates [12,17]. Unfortunately, conclusive proof of this hypothesis is no longer possible.

Most pd F VIII concentrates have been on the market for a long time [10], and intensive virological and clinical investigations have proved that these preparations are safe [11,20]. However, for the next generation of pd F VIII, and also for other therapeutic proteins from human plasma, thorough proteomic investigations, in addition to routine biochemical and functional analyses, during product development [19,36,37] and for quality control and assurance of the final product have been highly recommended [38–40]. The investigations presented in this paper demonstrate the importance of proteomic investigations on F VIII/VWF concentrates produced from human plasma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

F II	clotting factor II
F VIII	clotting factor VIII
pd FVIII	plasma-derived clotting factor VIII
ESI	electro spray ionization
HSA	human serum albumin
IaIp	inter alpha inhibitor proteins
IgG	immunoglobulin G
IU	international unit

SEC	size-exclusion chromatography
SPR	surface plasmon resonance
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:RCof	VWF/ristocetin cofactor

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cp-calibration proteins

Figure 1A

cp-calibration proteins

Figure 1B

Figure 1.
SDS-PAGE of different pd F VIII concentrates.
A – under reducing conditions
B – under non-reducing conditions.
Lanes 1–4 – Octanate (four different batches)
Lane 5 – Haemoctin
Lanes 6–8 – Wilate (three different batches)

Figure 2.

Size exclusion chromatography of the F VIII concentrate Octanate and Wilate.

Chromatographic conditions: Columns – Superose 6 and Superose 12, 500 μ g protein in 500 μ L PBS were applied. For other conditions – cf. Experimental Procedures. In each fraction F VIII and VWF:RCof activities were determined. For Octanate, different fractions (Nos. 1–8) were also separated by SDS-PAGE (see Figure 3).

Figure 3A

Figure 3B

Figure 3.
SDS-PAGE of collected fractions from size-exclusion chromatography of Octanate.
A – under reducing conditions
B – under non-reducing conditions.
The band from fraction 5, where clotting factor II was identified by MS, is labeled.

Figure 4.

Total ion chromatogram of a pool of iTRAQ-labeled tryptic peptides from four different F VIII concentrates (two different Octanate batches and Haemoctin and Wilate respectively).

Figure 5.

A representative MS/MS spectrum collected during the experiment shown in Figure 4. This spectrum was assigned to the peptide YYWGGQYTWDMAK ($m/z=652.98$; $z=3$) with 99% confidence (the maximum allowed). Inset (right, upper corner): magnified view of the mass range from 113 to 118, showing the iTRAQ reporter ions that were generated during the fragmentation process. Quantitative determination of these signals provides the relative abundance of this peptide in the four samples.

Table 1

Biochemical and functional analysis of FVIII/VWF concentrates

Protein	Octanate	Haemoctin	Wilate
FVIII:C	975 ± 75	980 IU/vial	915 ± 35
FVIII:Ag	1075 ± 125	1050 IU/vial	950 ± 50
VWF:Ag	400 ± 100	450 IU/vial	1100 ± 100
VWF:RCoF	300 ± 50	300 IU/vial	850 ± 50
VWF:CBA	235 ± 15	300 IU/vial	750 ± 50
Total Protein	10 ± 2	8.0 mg/vial	7.5 ± 0.5
FVIII Specific Activity	110 ± 10	120 IU/vial	130 ± 10
Fibrinogen	2 ± 0.5	1.2 mg/vial	n.d.
Fibronectin	0.75 ± 0.25	0.5 mg/vial	0.5 ± 0
HSA	n.d.	n.d.	n.d.
IgA	n.d.	n.d.	n.d.
IgG	0.11 ± 0.01	0.09 mg/vial	n.d.
IgM	0.065 ± 0.035	0.03 mg/vial	n.d.
Proteolytic activity	n.d.	n.d.	n.d.

n.d.—not detected

Table 2

Proteins identified in excised bands after SEC of Octanate and SDS-PAGE under nonreducing conditions

Score ¹	% coverage ²	Unique peptides ³	Accession	Protein name
140.30	44.3	84	gi 37947	von Willebrand factor, precursor
46.70	63.4	29	gi 30585049	Fibrinogen, gamma polypeptide
58.40	69.7	35	gi 7924018	Fibrinogen, beta chain
43.70	36.3	25	gi 4503689	Fibrinogen, alpha chain
43.22	20.2	25	gi 2506872	Fibronectin precursor
74.40	49.3	41	gi 4504781	Inter-alpha inhibitor H1
67.10	57.6	42	gi 125000	inter-alpha-trypsin inhibitor H2 prec.
10.92	19.6	5	gi 579676	bikunin
31.51	26.7	21	gi 54400755	inter-alpha inhibitor H3
2.25	7.03	2	gi 386852	kininogen
3.88	8.5	2	gi 30582253	lumican
13.15	25.2	9	gi 30802115	Coagulation factor II, precursor
4.16	7.2	3	gi 18202115	Vitronectin, precursor
2.74	4.5	2	gi 825681	Inter-alpha-trypsin inh., C-terminal

¹ Score: The protein assignment score based on all sequence-unique peptide scores. The likelihood that the assignment is *wrong* is $10^{-\text{SCORE}}$.

² % Coverage: Percent of the protein sequence covered by sequence-unique peptide assignments.

³ Unique peptides: Number of sequence-unique peptide assignments.

Table 3

Proteins identified in excised bands after SEC of Octanate and SDS-PAGE under reducing conditions

Score ^I	% coverage	Unique peptide	Accession	Protein name
112.40	36.5	76	gi 4507907	von Willebrand factor, precursor
40.30	40.5	58	gi 47132549	Fibronectin 1 isoform 6 preprotein
7.41	5.5	4	gi 340361	von Willebrand factor prepropeptide
16.42	19.2	10	gi 7924018	Fibrinogen beta chain
3.61	5.2	2	gi 59939295	Ifapsoriasin
23.07	31.1	15	gi 30583001	Fibrinogen, gamma polypeptide
8.37	11.2	6	gi 71823	Fibrinogen alpha chain precursor
52.01	21.2	40	gi 53791223	Fibronectin 1
44.00	25.5	21	gi 4504781	Inter-alpha inhibitor H1
44.10	42.6	39	gi 55958062	Inter-alpha inhibitor H2
9.70	16.2	4	gi 4699843	Bikunin
34.95	37.2	17	gi 54400755	Inter-alpha inhibitor H3
17.64	28.0	10	gi 179674	Compliment component C4
4.20	9.2	3	gi 30584851	Lumican
2.04	4.2	2	gi 4557385	Compliment component 3 precursor
20.91	31.2	16	gi 6013427	Serum albumin precursor
17.80	22.2	14	gi 125507	Kininogen, precursor
16.61	35.6	13	gi 4503635	Coagulation factor II precursor
12.33	36.3	7	gi 229601	IgG1 H Nie
7.72	12.6	5	gi 72146	Vitronectin precursor
4.77	6.6	2	gi 455970	Vitamin D-binding protein
4.54	7.6	2	gi 177933	Alpha-1-antichymotrypsin

^I Score, % Coverage and Unique peptides are as defined in Table 2.

Table 4

Additional proteins identified in excised bands after SDS-PAGE under reducing conditions (without SEC pre-separation)

Score ^I	% coverage	Unique peptide	Accession	Protein name
4.72	9.2	6	gi 31499	Clotting factor VIII
6.08	12.2	5	gi 32699324	Semenogelin II
4.29	8.6	3	gi 32450797	Semenogelin I
4.27	5.2	3	gi 42716297	Sulfated glycoprotein-2
2.71	14.0	5	gi 123995421	Heparin cofactor II precursor ²

^I Score, %Coverage and Unique Peptides are as defined in Table 2

² Found in Haemectin sample.

Table 5

Proteins identified in the F VIII/vWF concentrate Wilate. The bands after SDS-PAGE separation under reducing and non-reducing conditions were excised.

Total ProtSC	% coverage	Unique peptide	Accession	Protein name
271.10	52.8	134	gi 89191868	von Willebrand factor
20.10	36.4	12	gi 33988372	IgM
21.50	16.6	10	gi 182383	Clotting factor VIII
9.80	14.0	4	gi 4503647	Clotting factor VIII, LC
20.20	23.2	6	gi 18044959	IgM, heavy chain (HC)
20.10	22.2	5	gi 33988372	IgG, HC
29.20	42.0	19	gi 13591823	Fibrinogen, alpha chain
44.20	66.0	28		Fibrinogen, beta chain precursor
66.20	36.7	37	gi 47132553	Fibronectin 1
2.00	6.5	2	gi 46981961	Growth-inh. prot. 25
2.00	4.0	2	gi 88853069	Vitronectin, precursor
2.00	3.2	2	gi 6013427	Serum albumin precursor
6.00	12.0	3	gi 62089410	Thrombospondin 1 precursor
2.00	2.2	2	gi 32450797	Semenogelin I

Table 6

Quantitative Comparison of Factor VIII Concentrates

Score	Accession	Protein	Octanate(2):Octanate(1)			Haemoclin:Octanate			Wilate:Octanate		
			Ratio	PVal	EF	Ratio	PVal	EF	Ratio	PVal	EF
		Active Components									
154.32	gi 89191868	von Willebrand factor	1.11	7.20e-15	1.03	0.96	3.37e-03	1.03	2.34	0.00	1.08
20.07	gi 4503647	coagulation factor VIII isoform a	0.85	1.04e-06	1.06	1.32	3.25e-10	1.07	1.07	6.59e-01	1.05
		impurities									
55.92	gi 70778918	inter-alpha globulin inhibitor H2 polypeptide	0.68	0.00	1.02	1.00	1.00e+00	1.02	0.09	0.00	1.20
31.41	gi 4504781	inter-alpha (globulin) inhibitor H1	0.68	2.6e-37	1.05	0.99	7.3e-01	1.06	0.13	1.36e-16	1.50
22.22	gi 54400755	inter-alpha (globulin) inhibitor H3	0.59	9.71e-11	1.28	1.28	1.6e-11	1.06	0.18	7.31e-13	1.50
9.70	gi 4504893	kininogen 1	0.93	2.76e-03	1.04	0.57	1.13e-14	1.06	0.05	2.20e-11	1.52
6.00	gi 4502067	alpha-1-microglobulin/bikunin	0.78	1.38e-08	1.08	1.15	4.34e-04	1.08	0.06	1.66e-06	2.52
5.00	gi 4503635	coagulation factor II	0.46	1.15e-05	1.18	1.78	7.62e-06	1.12	0.09	1.05e-04	2.00
90.00	gi 47132553	fibrinectin 1	1.30	0.00e+00	1.03	1.59	0.00e+00	1.07	1.42	0.00e+00	1.07
73.19	gi 4503689	fibrinogen, alpha polypeptide	0.74	0.00e+00	1.03	1.59	0.00e+00	1.04	1.31	0.00e+00	1.10
72.86	gi 70906435	fibrinogen, beta chain	0.79	0.00e+00	1.02	1.57	0.00e+00	1.02	1.19	1.40e-45	1.04

Score The protein assignment score based on all sequence-unique peptide scores. The likelihood that the assignment is *wrong* is $10^{-\text{SCORE}}$.

Accession The accession number from the searched database (DB).

Protein The protein name in the DB.

Ratio The ratio of the measured iTRAQ levels, as determined by all contributing peptides.

PVal P-value. Standard statistical measure of significance that the ratio deviates from unity.

EF Error factor. The multiplicative factor to determine ratio range: The true ratio should fall within ratio/EF and ratio*EF

Table 7

Inter-alpha inhibitor proteins in FVIII samples (ELISA)

Sample No.	Protein mg/ml	ITIp mg/ml	ITIp %
1	1.71	0.344	20.0
2	2.18	0.405	18.6
3	1.81	0.299	16.5
4	1.63	0.321	19.7
5	2.43	0.405	16.9
6	1.30	0.003	0.2
7	1.31	0.005	0.4
8	1.39	n.d.	n.d.

Samples 4–5 -Octanate; 6–8 - Wilate