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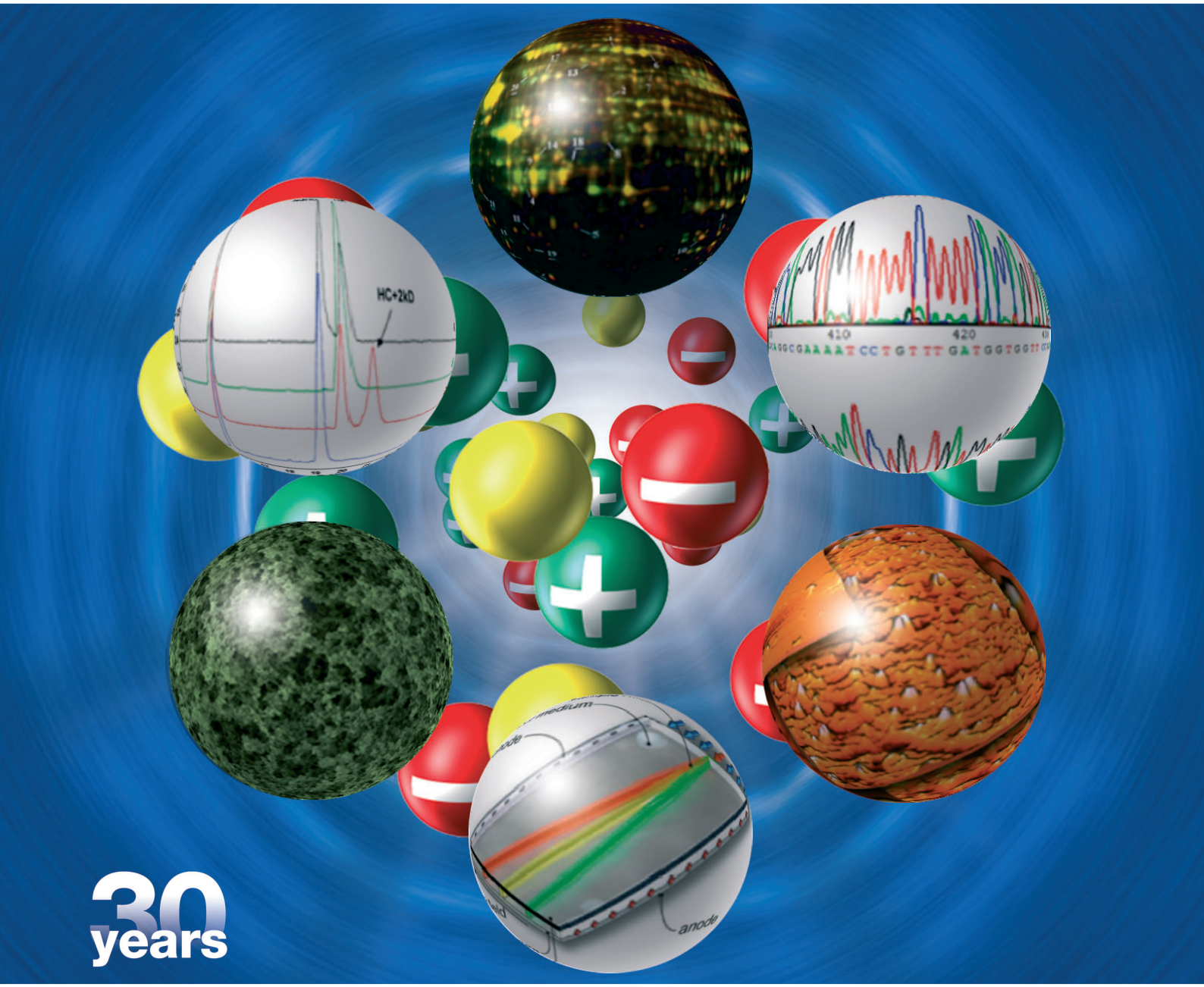

30 years

Emphasis on Fundamentals and Methodologies

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Research Article

Proteomic characterization of plasma-derived clotting factor VIII–von Willebrand factor concentrates

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-α 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 MS, demonstrating the potential value of this technique for quality control analysis. The importance of thorough proteomic investigations of therapeutic FVIII/VWF preparations from human plasma is also discussed.

Keywords:
Clotting factor VIII / Proteomic analysis / Prothrombin / von Willebrand factor
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1 Introduction

Clotting factor VIII (FVIII) 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]. FVIII 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. VWF itself circulates in blood plasma in the form of a 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 FVIII from proteolysis and clearance [2–4]. Reduced levels or a missing or dysfunctional FVIII 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 plasma, the level of FVIII is also decreased [3, 7]. The normal plasma concentration of FVIII is about 0.2 μg/mL (0.7 nM). At about 10 μg/mL, the average plasma concentration of its carrier protein VWF is 50 times higher [8]. Compared to the most abundant proteins, HSA and IgG, the concentration of FVIII in blood plasma is about five orders of magnitude lower [9].

For more than 30 years, the treatment of hemophilia A has been accomplished by infusions of FVIII concentrates. Initially, FVIII 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 FVIII (pd FVIII) concentrates was considerably improved [10, 11]. Although the pd FVIII concentrates currently on

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Abbreviations: FII, clotting factor II; **FVIII,** clotting factor VIII; **IaIp,** inter-alpha inhibitor proteins; **IU,** international unit; **pd F VIII,** plasma-derived clotting factor VIII; **VWD,** von Willebrand disease; **VWF,** von Willebrand factor; **VWF:CBA,** VWF collagen-binding activity; **VWF:RCoF,** VWF/ristocetin cofactor

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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 FVIII 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].

FVIII 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 FVIII 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 neoepitopes in the FVIII 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 FVIII 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.

2 Materials and methods

2.1 Materials

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

2.2 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 FVIII/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. FVIII cofactor activity was determined by use of a commercial kit (Coamatic FVIII:C Testkit, diaPharma, West Chester, OH, USA). FVIII:Ag was quantified by an ELISA method using the Asserachrom® FVIIIC:Ag (Diagnostica Stago, Asniers, France, distributed by American Bioproducts, Parsipanny, 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. VWF 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. Inter-α inhibitor proteins (IaIp) levels were determined using a competitive ELISA with mAb 69.31 as described by Lim *et al.* [24]. Later 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 FVIII 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-well 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, USA). 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].

2.3 SDS-PAGE

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

2.4 SEC

For SEC a tandem system containing one Superose 6 column and Superose 12 column (both 300 × 10 mm id, GE Healthcare, Piscataway, NJ, USA) or TSK G3000 SWXL and TSK G4000 SWXL (both 300 × 7.8 mm id, Tosoh Bioscience, King of Prussia, PA, USA) was used. The mobile phase was PBS, pH 7.4. 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).

2.5 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]. Half-second MS scans (300-1500 Thompson 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 it 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.

2.6 iTRAQ protein quantitation of FVIII 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 MS 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 FVIII 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 μL of 0.5 M triethylammonium bicarbonate, pH 8.5, and reduced, alkylated and tryptically digested according to the iTRAQ protocol (Applied Biosystems). One-microliter aliquot from each was 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 ACN, 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^{TM} (PolyLC, Columbia, MD, USA) according to the manufacturer's instructions. The ammonium formate eluates were dried and redissolved in formic acid:water:ACN:TFA (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 of MS and MS/MS spectra during μHPLC separation of the peptide mixture were 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.

3 Results

3.1 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 (IU or milligrams) *per* vial. According to the manufacturer's

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.

declaration, each vial contains 1000 ± 200 IU FVIII [11, 20, 21]. As shown in this table, results of these analyses are very similar for Octanate and Haemoctin. Compared with those two previous concentrates, the contents of VWF (VWF:Ag), and both VWF:RCoF (VWF/ristocetin cofactor) and VWF:CBA in the recently developed concentrate Wilate are 2–3 times higher.

3.2 SDS-PAGE and SEC

In Fig. 1, SDS-PAGE of four different Octanate batches (lanes 1–4), one Haemoctin (lane 5) and three Wilate batches (lanes 6–8) under reducing (Fig. 1A) and non-reducing conditions (Fig. 1B) are shown. Size-exclusion chromatograms of Octanate (full line) and Wilate (broken line) are shown in Fig. 2. The results of SDS-PAGE under non-reducing conditions and SEC show similar results. Octanate, the FVIII concentrate that was purified by only anion-exchange chromatography, shows three peaks in SEC

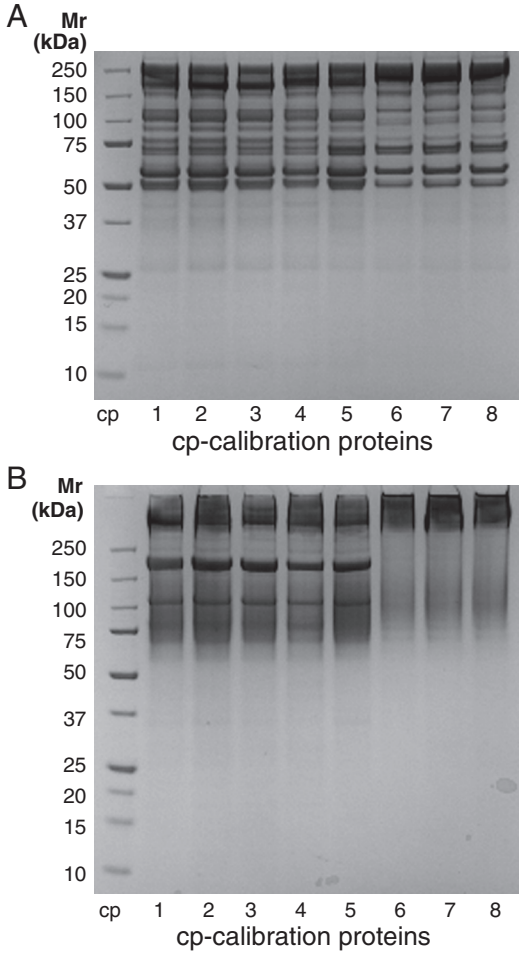

Figure 1. SDS-PAGE of different pd FVIII concentrates. (A) Under reducing conditions. (B) Under non-reducing conditions. Lanes 1–4 – Octanate (four different batches). Lane 5 – Haemoctin. Lanes 6–8 – Wilate (three different batches).

[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 FVIII/VWF activity was found only in the high molecular weight region (see Fig. 2 and Ref. [29]). Wilate, the concentrate that was purified by an additional SEC step [11], shows in SEC only one high molecular weight peak, which corresponds to the diffuse high molecular weight band in SDS-PAGE (see Fig. 1B, lanes 6–8, and Fig. 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. Fig. 3A and B). 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. Fig. 1A and 1B). Under reducing conditions, Wilate samples (lanes 6–8 in Fig. 1A) show a similar pattern to these 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. Fig. 1A, lanes 6–8). Again, for Haemoctin, the pattern in SDS-PAGE was virtually identical to that of Octanate (cf. lane 5).

3.3 Protein identification

Protein bands in SEC fractions resolved by SDS-PAGE from Octanate under reducing (Fig. 3A) and non-reducing conditions (cf. Figs. 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.

3.4 Quantitative comparison of FVIII 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 Fig. 4. Figure 5 shows a representative MS/MS spectrum of the peptide YYWGGQYTWDMAK during this experiment. In the inset of Fig. 5, a magnified view of the mass range from 113 to 118 shows the iTRAQ reporter ions

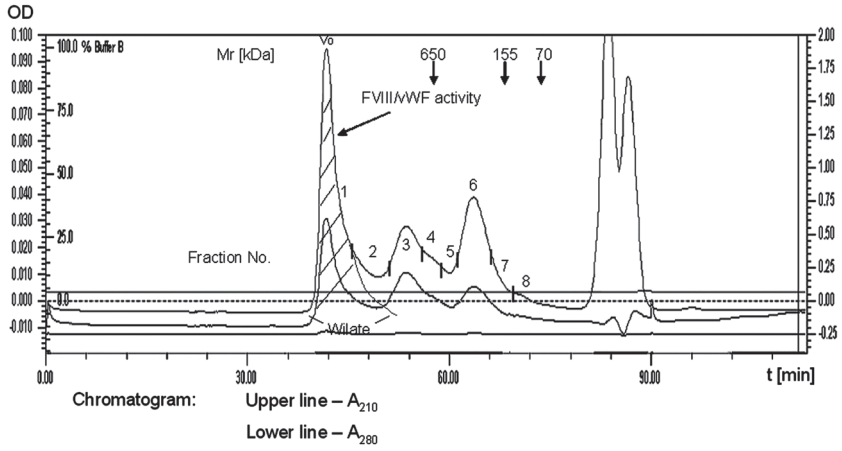

Figure 2. SEC of the FVIII concentrate Octanate and Wilate. Chromatographic conditions: Columns – Superose 6 and Superose 12, 500 μg protein in 500 μL PBS was applied. For other conditions – cf. Section 2. In each fraction FVIII and VWF:RCoF activities were determined. For Octanate, different fractions (Nos. 1–8) were also separated by SDS-PAGE (Fig. 3).

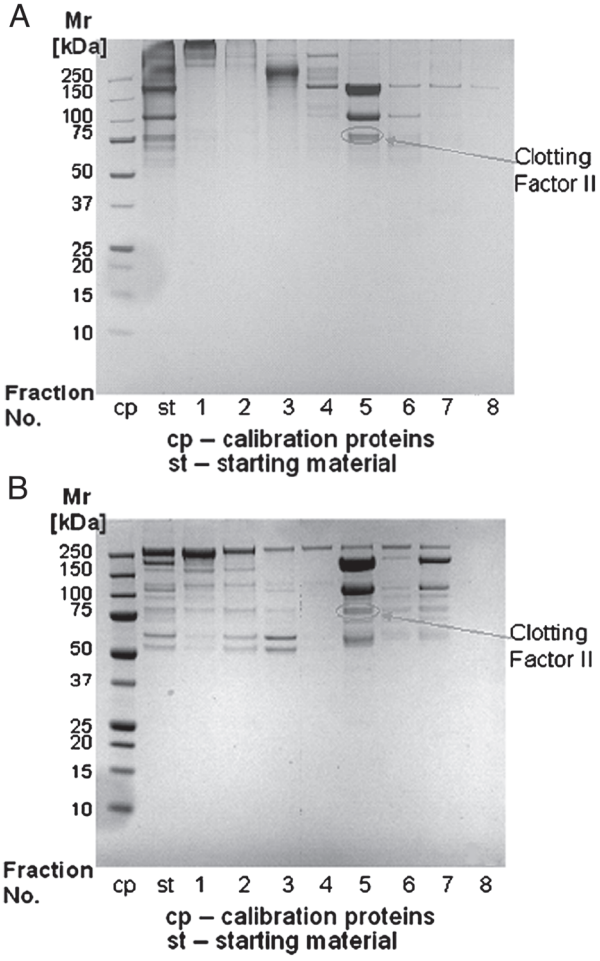

Figure 3. SDS-PAGE of collected fractions from SEC of Octanate. (A) Under reducing conditions. (B) Under non-reducing conditions. The band from fraction 5, where FII was identified by MS, is labeled.

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 FVIII concentrates by iTRAQ analysis (Table 6, for all identified proteins see also the Supporting Information Table 1) revealed both batch-to-batch and concentrate-to-concentrate variations in FVIII and VWF content and in the levels of contaminating proteins.

Table 6 shows iTRAQ comparisons of (i) two different batches of Octanate designated Octanate 1 and 2; (ii) Haemoctin and Octanate; and (iii) Wilate and Octanate. Based on ratios of the measured iTRAQ, the content of FVIII and VWF in the two Octanate batches differed by less than 15%. The content of main impurities, the IaIp (identified as subunits H1, H2, H3 and bikunin) showed much higher batch-to-batch variation. The content of clotting factor II (FII) in first Octanate (Octanate 1) batch was about two times 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 FVIII content in this sample was approximately 30% higher. The content of IaIp subunits of Octanate 1, and the amount of FII was higher than in this sample.

The content of FVIII in Wilate was at the same level as the FVIII content in Octanate 1. The content of high molecular proteins VWF (the second active component) and fibronectin and fibrinogen (both impurities) in Wilate was higher than in Octanate. The content of VWF in analyzed Wilate batch is more than two times higher than in Octanate 1, and corresponding Haemoctin batch. The level of other contaminating proteins, IaIp, kininogen and FII was significantly lower (cf. Table 6).

3.5 Determination of IaIp in FVIII concentrates by competitive ELISA

The amount of IaIp in FVIII concentrates was determined by competitive ELISA using the mAb 69.31, directed against the IaI light chain bikunin [24]. As shown in Table 7, the amount of IaIp in Octanate and one Haemoctin

Table 2. Proteins identified in excised bands after SEC of Octanate and SDS-PAGE under nonreducing conditions

Score[a)]	% coverage[b)]	Unique peptides[c)]	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, β chain
43.70	36.3	25	gi 4503689	Fibrinogen, α chain
43.22	20.2	25	gi 2506872	Fibronectin precursor
74.40	49.3	41	gi 4504781	Inter-α inhibitor H1
67.10	57.6	42	gi 125000	Inter-α-trypsin inhibitor H2 prec.
10.92	19.6	5	gi 579676	Bikunin
31.51	26.7	21	gi 54400755	Inter-α 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-α-trypsin inh., C-terminal

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

b) Percent of the protein sequence covered by sequence-unique peptide assignments.

c) Number of sequence-unique peptide assignments.

Table 3. Proteins identified in excised bands after SEC of Octanate and SDS-PAGE under reducing conditions

Score[a)]	% 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 α chain precursor
52.01	21.2	40	gi 53791223	Fibronectin 1
44.00	25.5	21	gi 4504781	Inter-α inhibitor H1
44.10	42.6	39	gi 55958062	Inter-α inhibitor H2
9.70	16.2	4	gi 4699843	Bikunin
34.95	37.2	17	gi 54400755	Inter-α 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	α-1-Antichimotrypsin

a) Score, % coverage and unique peptides are as defined in Table 2.

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 (Table 7). The protein concentrations and FVIII activity in investigated samples were comparable (between 1.3 and 2.43 mg/mL, and 180–210 IU/mL, cf. Table 7).

4 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

Table 4. Additional proteins identified in excised bands after SDS-PAGE under reducing conditions (without SEC pre-separation)

Score	% 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[a)]

Score, %coverage and unique peptides are as defined in Table 2.
a) Found in Haemoctin sample.

Table 5. Proteins identified in the FVIII/vWF concentrate Wilate[a)]

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, α 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

a) The bands after SDS-PAGE separation under reducing and non-reducing conditions were excised.

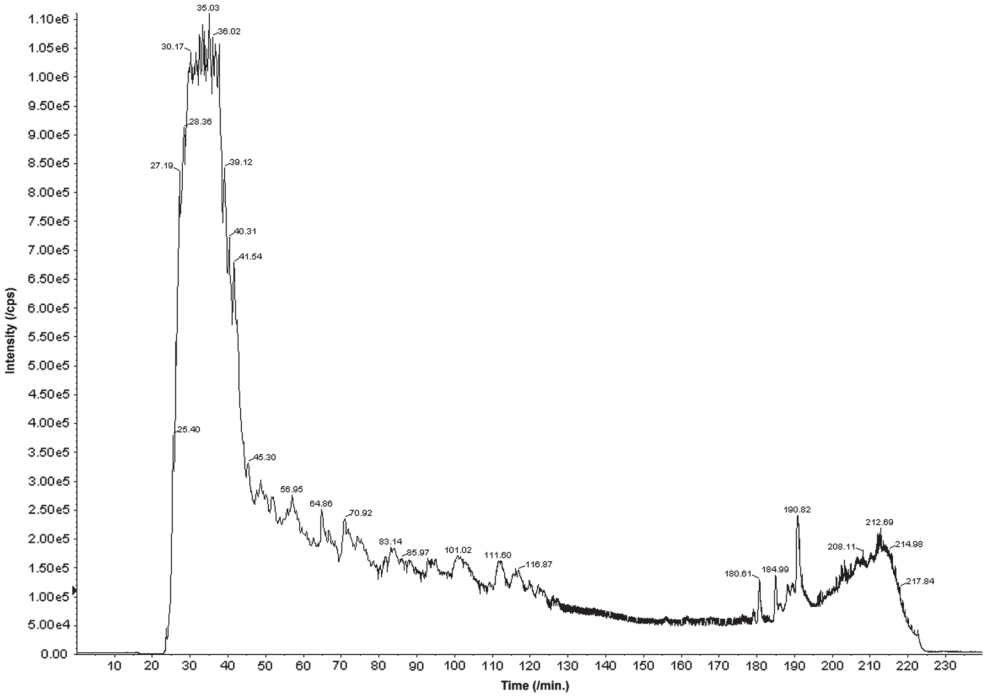

Figure 4. Total ion chromatogram of a pool of iTRAQ-labeled tryptic peptides from four different FVIII concentrates (two different Octanate batches and Haemoctin and Wilate, respectively).

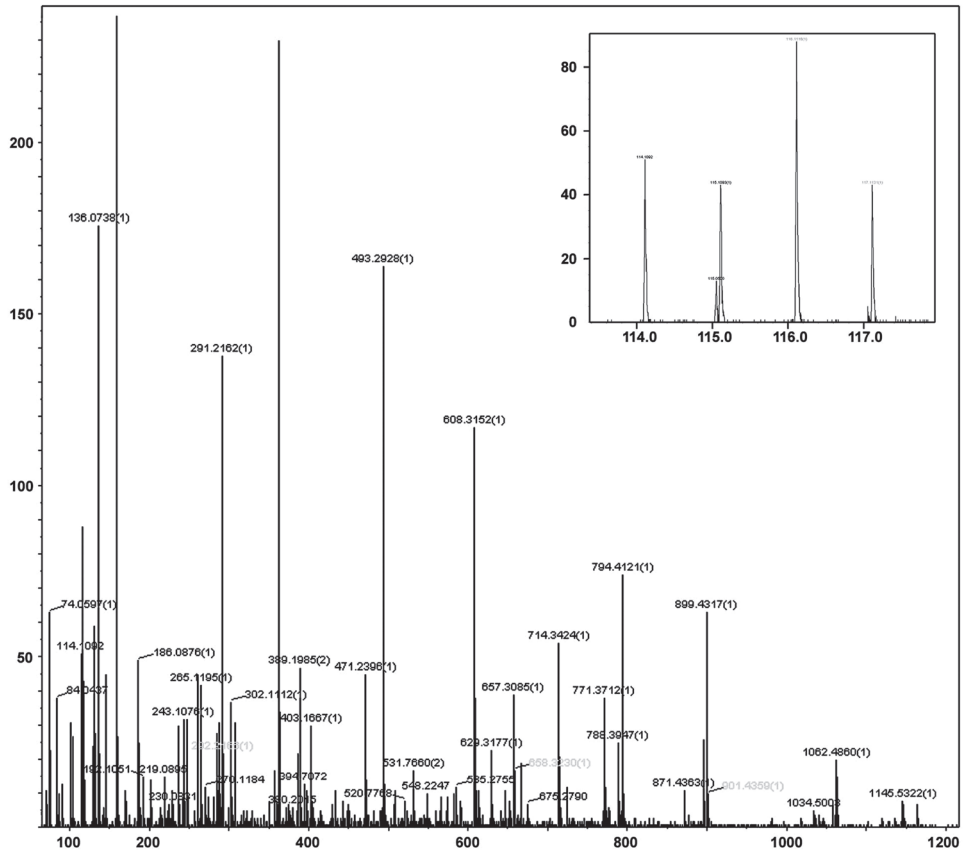

Figure 5. A representative MS/MS spectrum collected during the experiment shown in Fig. 4. This spectrum was assigned to the peptide YYWGGQYTWDMAK ($m/z = 652.98$; $z = 3$) with 99% confidence (the maximum allowed). Inset (right, upper corner): magnified view of the mass range from 113 to 118, showing 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 6. Quantitative comparison of factor VIII concentrates

			Octanate(2):Octanate(1)			Haemoctin:Octanate			Wilate:Octanate		
Score[a)]	Accession[b)]	Protein[c)]	Ratio[d)]	PVal[e)]	EF[f)]	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-α 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-α (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-α (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	α-1-microglobulin/bikunin	0.78	1.38e−08	1.08	1.15	4.34e−04	1.08	0.06	1.66e−0.6	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	Fibronectin 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, α 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, β chain	0.79	0.00e+00	1.02	1.57	0.00e+00	1.02	1.19	1.40e−45	1.04

a) The protein assignment score based on all sequence-unique peptide scores. The likelihood that the assignment is *wrong* is $10^{-\text{SCORE}}$.
b) The accession number from the searched database (DB).
c) The protein name in the DB.
d) The ratio of the measured iTRAQ levels, as determined by all contributing peptides.
e) *p*-value. Standard statistical measure of significance that the ratio deviates from unity.
f) Error factor. The multiplicative factor to determine ratio range: The true ratio should fall within ratio/EF and ratio∗EF.

unforeseen accidents after some products were introduced to the market: after the introduction of two FVIII 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

Table 7. Inter-α inhibitor proteins in FVIII samples (ELISA)

Sample No.[a)]	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.

a) Samples 4–5 – Octanate; 6–8 – Wilate.

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 FVIII [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 FVIII 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. From the analysis of potentially immunogenic, double virus-inactivated FVIII concentrates, they found that some batches showed definitive evidence of elevated FVIII hydrolysis. In surface plasmon resonance measurements, these batches showed impaired binding to phospholipid vesicles [34]. In model experiments, Smales *et al.* [35] demonstrated that heat treatment of FVIII 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 FVIII 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 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 (FII–prothrombin). Activated FII, FIIa, better known as thrombin, is potentially harmful and had to be removed from the final product. As shown in Fig. 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 FVIII concentrates, Octanate and Haemoctin. Unfortunately, the quantitative analysis of these proteins is still not a part of routine quality control of FVIII 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 FVIII concentrates (cf. Table 7). In fraction 5 (Fig. 2 and 3B), in addition to IaIp, we also detected significant amounts of FII (prothrombin; cf. Tables 2, 3 and 7). In this fraction (apparent molecular weight in SEC∼200 000, see Fig. 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 (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 FVIII concentrates of the previous generation, Octanate and Haemoctin. Similar results were obtained when biochemical and functional analyses were performed (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 FII 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 FII could be found in all three concentrates. Importantly, the content of this potentially harmful protein was almost two orders of magnitude lower in Wilate (Table 6). Besides FII, some other potentially harmful impurities, such as kininogen and clotting factor V (cf. Tables 2, 3 and Supporting Information Table 1) were also detected. Comparative analysis of FVIII 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 IaIp. As determined by ELISA with the mAb 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 mAb 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 Supporting Information Table 1). 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 1990s, 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, FII and kininogen 1 can be achieved by SEC (cf. Fig. 2, Table 6 and Ref. [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 FVIII/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 FII in these concentrates, it is very intriguing to speculate that possible FII activation during the production process, possibly during heating in combination with solvent-detergent treatment, could have caused partial degradation of FVIII. 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 FVIII/VWF concentrates [12, 17]. Unfortunately, conclusive proof of this hypothesis is no longer possible.

Most pd FVIII 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 FVIII, 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 FVIII/VWF concentrates produced from human plasma.

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