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Use of proteomics for validation of the isolation process of clotting factor IX from human plasma

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Abstract

The use of proteomic techniques in the monitoring of different production steps of plasma-derived clotting factor IX (pd F IX) was demonstrated. The first step, solid-phase extraction with a weak anion-exchange resin, fractionates the bulk of human serum albumin (HSA), immunoglobulin G, and other non-binding proteins from F IX. The proteins that strongly bind to the anion-exchange resin are eluted by higher salt concentrations. In the second step, anion-exchange chromatography, residual HSA, some proteases and other contaminating proteins are separated. In the last chromatographic step, affinity chromatography with immobilized heparin, the majority of the residual impurities are removed. However, some contaminating proteins still remain in the eluate from the affinity column. The next step in the production process, virus filtration, is also an efficient step for the removal of residual impurities, mainly high molecular weight proteins, such as vitronectin and inter-alpha inhibitor proteins. In each production step, the active component, pd F IX and contaminating proteins are monitored by biochemical and immunochemical methods and by LC-MS/MS and their removal documented. Our methodology is very helpful for further process optimization, rapid identification of target proteins with relatively low abundance, and for the design of subsequent steps for their removal or purification.

Keywords

Clotting factor IX; Vitamin K dependent clotting factors; Proteomics; Human plasma; Fractionation

1. Introduction

Clotting factor IX (F IX) is a vitamin K dependent plasma glycoprotein that plays a crucial role in blood coagulation [1]. Reduced levels or a missing or dysfunctional F IX glycoprotein

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are associated with a bleeding disorder called hemophilia B [2]. In affected patients, hemostasis is maintained using replacement therapy, employing intravenous injections of F IX preparations [3]. About 20 years ago, recombinant F IX (r F IX) was developed, but these preparations only partially replace plasma-derived (pd) product. Consequently, plasma derived F IX (pd F IX) still plays an important role in hemophilia B management [4].

The concentration of F IX in human plasma is about 5 µg/mL, which is equivalent to 0.1 µmol [5,6]. Compared to the high abundance proteins human serum albumin (HSA) and IgG, concentration of F IX is approximately four orders of magnitude lower [7]. The introduction of new chromatographic resins and methods such as anion-exchange, affinity and immunoaffinity chromatography in the late 1980s and early 1990s enabled production of highly purified concentrates of this therapeutic protein [6,8,9]. The basic requirements that have to be fulfilled for the human plasma derived (pd) clotting factor concentrates are virus safety, effectiveness, and absence of side effects [10]. In the past twenty years, virus safety, of pd F IX substantially improved, and almost all pd F IX concentrates on the are at least double virus inactivated [4,10].

Together with other therapeutic proteins isolated from human plasma, pd F IX concentrates that are on the market nominally belong to the group of so-called “well-characterized biologicals”. Some of them still contain a relatively high amount of foreign proteins. In contradiction to this name, most impurities are still unidentified, and, in contrast to their name, many of these concentrates are rather poorly characterized [11]. Additionally, batch-to-batch variations during the production of these therapeutics is another important question that has to be addressed [12,13]. The use of proteomics techniques for the validation of down-stream processing and characterization of therapeutic proteins has been discussed recently [14]. Thorough proteomic investigations were performed during isolation of pd inter-alpha inhibitor proteins (IaIp) [11], as well as separation of human plasma by use of different anion-exchange resins [12,13]. The use of proteomics technology for quality assurance of the production process of blood-based therapeutic proteins and characterization of final products has also been demonstrated [15–18].

In this paper, we demonstrate the use of proteomics for the validation of the pd F IX production process and for final product characterization.

2. Materials and methods

2.1. Human plasma

Cryopoor, single donor human plasma (Rhode Island Blood Center, Providence, RI, U.S.A.) was used as starting material. All plasma samples were screened to exclude the presence of blood-borne viruses (hepatitis A, B and C and HIV). Prior to use, the cryoprecipitate was removed as described previously [6].

2.2. Isolation of F IX

The isolation protocol follows the method of Brummelhius [19], with same modifications that have been described previously [6,8]. Briefly, the initial step is a solid-phase extraction of cryopoor plasma with DEAE Sephadex A50 (GE Healthcare Bio-Sciences AB, Piscataway, NJ, U.S.A). For 3.0 L cryopoor plasma, 4.5 g Sephadex are used. The next step is further chromatographic purification on DEAE-Sepharose Fast-Flow (GE Healthcare). The eluate from the anion-exchange column is subjected to solvent/detergent (S/D) treatment with 1% (w/v) Tween 80® and 0.3% (w/v) tri-n-butyl-phosphate (TnBP) for 6h at 27°C. The S/D reagents were then removed and a final chromatographic step is performed on Heparin Sepharose Fast-Flow (FF, GE Healthcare). The eluate of the heparin affinity column undergoes

virus filtration [6,8,20]. The nanofiltration technology is based on a PVDF membrane, rendered especially hydrophilic by a patented method (Viresolve, Millipore, Billerica, MA, U.S.A.).

2.3. Determination of vitamin K dependent clotting factors and contaminating proteins

Factor IX activity was determined in a one-stage coagulation assay using an ACL 300 apparatus from Instrumentation Laboratory (Lexington, MA, USA). The assay was performed by mixing F IX-deficient plasma with diluted sample in the presence of lipid extract and caolin as activators (all reagents from Instrumentation Laboratory). Coagulation was triggered by adding CaCl_2 and the time required for a clot to form was measured. Clotting factors II, V, VII and X were determined in duplicate using commercial one-stage clotting assays from Baxter (Vienna, Austria).

Inter-alpha inhibitor proteins were determined using a competitive ELISA, as described elsewhere [18]. For determination of vitronectin, ELISA was carried out in accordance with the manufacturer's instructions (Tokara Biochemicals, Shiga, Japan).

2.3. Removal of solvent/detergent from samples prior to LC-MS/MS

Some samples, e.g. the unbound fraction from Heparin Sepharose FF chromatography, still contain significant amounts of solvent and detergent that has to be removed prior to further analysis by LC-MS/MS. To eliminate them, the samples were applied to a 1 mL column containing the strong anion-exchanger Q Sepharose FF (GE Healthcare). After washing with 5 mL application buffer (20 mM Na citrate, pH 7.2), bound proteins were eluted with 0.6 M NaCl in 20 mM NaCitrate, pH 7.2, and subjected to further analysis.

2.3. Size-Exclusion Chromatography

For size-exclusion chromatography (SEC), tandem systems containing one TSK G3000 SWXL and one TSK G4000 SWXL column (both 300×7.8 mm I.D., Tosoh Bioscience, King of Prussia, PA, USA) or a Superose 6 followed by a Superose 12 column (both 300×10 mm I.D., GE Healthcare, Piscataway, NJ, USA) were used. The mobile phase was phosphate-buffered saline, pH 7.4 (PBS). The flow rate during separation was 0.4 mL/min. All separations were performed at 4–8°C. Proteins were detected at three different wavelengths – 210 nm, 260 nm and 280 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, U.S.A.).

2.4. Protein determination

Protein amounts were determined with the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL, U.S.A) according to the manufacturer's procedure.

2.5. Electrophoretic separations

After protein determination of each *in process* fraction, collected during the isolation process, about 15–25 µg protein of each sample were solubilized in NuPAGE sample buffer (Invitrogen, Carlsbad, CA, U.S.A.), and SDS-PAGE was performed as described previously [13]. SDS-PAGE was performed in two independent experiments.

2.6. “In-gel” digestion procedure

The gel bands of interest were excised by extracting 6–10 gel particles with clean glass Pasteur pipettes and digested with trypsin as described previously [11,12].

2.7. “In-solution” digestion procedure

50 µg of the acetone-precipitated and denatured protein pellet was resolubilized in 100 µL of NH_4HCO_3 (pH 8.0)/8M urea. The resolubilized proteins were reduced with 20 mM dithiothreitol (37 °C, 45 min) and then alkylated with 50 mM iodoacetamide at room temperature for 30 min in the dark. Before tryptic digestion, 100 mM NH_4HCO_3 , pH 8.0, was added to reduce the concentration of urea. Trypsin was added to the protein mixture at an enzyme to substrate ratio of 1:60 w/w, and the digestion was performed as described previously [13]. The resulting tryptic peptides were dried and subject to the LC-MS/MS analysis after being redissolved in formic acid:water:ACN:trifluoroacetic acid mixture (0.1:95:5:0.01 v/v).

2.8. Identification of proteins with LC-MS/MS

Tryptic peptides were separated on a 12 cm (75 µm I.D.) analytical column with a 5 µm Monitor C18 resin (Column Engineering, Ontario, CA, U.S.A) and containing an integrated ~4 µm ESI emitter tip. Solvent A was 0.1 M acetic acid in water, and solvent B was 0.1 M acetic acid in ACN. Peptides were eluted using a linear ACN gradient (0–70%) solvent B over 30 min (Agilent Technologies, Paolo Alto, CA, U.S.A.). Peak parking during the time when peptides were expected to elute was accomplished by reducing the flow rate from 200 nL/min to ~20 nL/min.

Eluting peptides were introduced onto an LTQ linear ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, U.S.A.) with a 1.9 kV electrospray voltage. Full MS scans in the m/z range of 400–1800 were followed by data-dependent acquisition of MS/MS spectra for the five most abundant ions, using a 30-second dynamic exclusion time. Protein identification was performed in, at least, two independent experiments as described previously [13].

Database searching was performed using the peak lists in the SEQUEST program [21]. The precursor-ion tolerance was 2.0 Daltons and the fragment-ion tolerance was 0.8 Daltons. Enzymatic digestion was specified as trypsin, with up to 2 missed cleavages allowed. The search contained sequences identified as human in NCBI’s nr database (November, 2006), which was created using the FASTA filtering tools found in BioWorks (Thermo). A list of reversed-sequences was created from these entries and appended to them for database searching so that false positive rates could be estimated [22]. This composite database contained approximately 490,000 entries.

For parallel LC-MS/MS analysis of samples taken for the isobaric tag for relative and absolute quantification (iTRAQ) analyses (see below), a nano LC-MS/MS system was used. Tryptic digest were separated with a nano RP column (C-18 PrepMap 100, LC Packings/Dionex, Sunnyvale, CA, USA) as previously described, with the column eluate introduced directly onto QStar XL mass spectrometer (Applied Biosystems, Foster City, CA, USA and Sciex, Concord, Ontario, Canada) via electrospray ionization [23]. Half second scans (300–1500 Thompson (Th)) 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. An ion had to assigned a charge in the range +2 to +4. Dynamic exclusion was 40. Protein identifications were performed with ProteinPilot (versions 1.0 and 2.0; Applied Biosystems and Sciex) searching the human “RefSeq” databases from NCBI (<http://www.ncbi.nlm.nih.gov/Ref/Seq/>). ProteinPilot is the successor to ProID and ProGroup, and uses the same peptide/protein scoring method [23]. Briefly, a protein score, S , is 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 are identified).

2.9. Isobaric tag for relative and absolute quantitation (iTRAQ) of proteins

Isobaric tag for relative and absolute quantitation (iTRAQ) was used for comparative analysis of protein levels in *in process* after Heparin Sepharose affinity chromatography and before and after nanofiltration. This quantitative, non-gel based method is designed for comparing proteins from different sources in one single experiment [24]. After tryptic digestion and N-terminal labeling of each sample with a different tag, the sample are pooled, fractionated by RP-nano-LC, and analyzed by ESI-MS/MS in order to identify peptides 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 [24]. This method was applied to analysis of two different F IX samples before nanofiltration (F IX, HepAC#1, proteins weakly bound to HeparinSepharose column; F IX HepAC#2; eluate from the Heparin Sepharose column – main fraction that contains F IX), and two different F IX samples after nanofiltration (F IX NF#3; sample that passed the filter – main fraction that contains F IX, F IX NF#4; retentate after nanofiltration – the fraction that contains large proteins and, eventually, some F IX aggregates). The samples were precipitated with a ReadyPrep 2-D Cleanup kit (Bio-Rad), according to the manufacturer's instructions. The precipitates were dissolved with 2 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 analysis of the non-labeled tryptic digests. The remaining material was labeled with iTRAQ reagents (#1: iTRAQ 114; #2: iTRAQ 115; #3: iTRAQ 116 and #4: iTRAQ 117) according to the manufacturer's instructions.

One-tenth volume aliquots from each sample were mixed together and dried in a vacuum centrifuge. The material was twice redissolved with water and dried in a vacuum centrifuge. Subsequently, the material was twice redissolved in water and repeatedly dried, and 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 value, the peptides in the iTRAQ labeled mixture were isolated using a strong cation-exchange device TipTop™ (PolyLC, Inc, Columbia, MD, USA) according to manufacturer's instructions. The eluates were dried and redissolved in formic acid:water:acetonitrile:trifluoroacetic acid (0.1:95:5:0.1) in preparation for LC-ESI-MS/MS analysis. The experiment was performed in triplicate and LC-MS/MS data were used for quantification.

Standard information dependent acquisition (IDA) of MS and MS/MS spectra during LC separation of the peptide mixture was performed as described previously [25]. 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 with default program settings and searching a human database (see above, . *Identification of proteins with LC-MS/MS*). 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. First production step – solid phase extraction with DEAE Sephadex A50

In this production step, F IX and other vitamin K dependent plasma proteins, clotting factors II (F II) and X (F X) and clotting inhibitors protein C, protein S and protein Z together with some other strongly-interacting proteins, such as IaI_p and ceruloplasmin bind to the support, and can be eluted with 0.6 M sodium chloride [6,13,19]. The majority of HSA, IgG, transferrin and some protease inhibitors, mainly alpha-1 antitrypsin and antithrombin III do bind to the resin and can be isolated, if desired, for the next steps of Cohn fractionation [19,26]. The SDS-

PAGE of unbound proteins and proteins eluted from the support is shown in Figure 1 (lanes 1 and 2). Proteins of interest, identified in the eluate, and some proteins that do not bind to DEAE Sephadex A50 are listed in Table 1 and Table 2. The complete list of proteins identified in the eluate from DEAE Sephadex A50 is given in Supporting material, Table S1.

3.2. Anion-exchange chromatography on DEAE-Sepharose FF

Dialyzed and concentrated eluate from DEAE Sephadex A50 was applied to a DEAE Sepharose FF column. All four fractions – the unbound fraction (flow-through), proteins eluted with 0.28 M NaCl (wash), the fraction containing most of F IX which was eluted with 0.36 M NaCl and the fraction containing proteins that were subsequently eluted with 1 M NaCl – were tryptically digested, and after “in solution digestion” identified by LC-MS/MS. Additionally, the F IX containing fraction and the fraction that contains unbound proteins (flow-through) were separated by SDS-PAGE (see Figure 1, lanes 3 and 4). The separated bands were excised, and after “in-gel digestion” proteins were identified with LC-MS/MS. The proteins of interest identified in the eluate that contains F IX and was used for further purification are listed in Table 3. The list of all proteins identified in the eluate from DEAE Sepharose FF is given in Supporting material, Table S2.

3.3. Heparin affinity chromatography

Dialyzed and concentrated eluate from the DEAE Sepharose FF column (proteins eluted with 0.36 M NaCl) was virus inactivated with solvent-detergent (S/D) and applied to a Heparin Sepharose FF column. All three fractions, the unbound fraction (flow-through, see Figure 1, lane 5), the fraction eluted with 0.25 M NaCl (wash, see Figure 1, lane 6) and the fraction eluted with 0.45 M NaCl (fraction containing most of F IX, see Figure 1, lane 7) were collected. Prior to tryptic digestion and subsequent LC-MS/MS, the solvent (TnBP) and detergent (Tween 80) were removed from the flow-through fraction by chromatography on a small column containing a strong anion-exchange resin. Collected fractions were tryptically digested, and after ‘in-solution’ digestion proteins were identified with LC-MS/MS. After separation by SDS-PAGE, the bands from the fraction that contains the majority of F IX and some impurities were excised and proteins from the excised bands were identified after “in-gel digestion”. The complete list of proteins, identified in this fraction is given in Table 4. The proteins of interest that are removed in this purification step are listed in Table 5.

3.3. Virus filtration and composition of the F IX concentrate

The SDS-PAGE of the samples before and after nanofiltration with the Viresolve virus filter is shown in Figure 2 A and B. Proteins were identified in samples before and after virus filtration via tryptic digestion (both “in-solution and “in-gel”) and LC-MS/MS. In the filtrate, only F IX was identified, and all contaminating proteins listed in Table 4 were found in the retentate. Samples before and after virus filtration were also analyzed by SEC (see Figure 2).

3. 4. Quantitative comparison in process samples in two final isolation steps

Two samples after Heparin Sepharose FF affinity chromatography (samples #1 and #2) and after nanofiltration (#2 and #3) were quantitatively compared by use of iTRAQ. Sample #1 containing proteins that weakly bind to the Heparin Sepharose FF column was compared with sample #2 (eluate from Heparin Sepharose FF, containing main amount of F IX) and with sample #4 (Viresolve retentate, containing high molecular proteins, separated in this step from main amount of F IX). Samples #2 and #3, both containing F IX at different stage of purification were also compared. In order to evaluate loss of F IX during virus filtration and removal of high molecular weight proteins in this step, sample #3 (main fraction after Viresolve nanofiltration containing highly purified F IX) and #4 (Viresolve retentate containing high

molecular proteins) were also compared. Results of these quantitative comparisons are shown in Table 6.

4. Discussion

Proteomic technologies are widely used as an efficient tool for process development and characterization of recombinant therapeutic proteins, such as monoclonal antibodies, clotting factors and other physiologically active proteins [27,28]. However, there have been only a few applications of proteomics for in-process and quality control of therapeutic proteins from human plasma [11–13,17,18,29]. This fact was discussed recently, and the use of this technology has been recommended for the validation of production steps during plasma fractionation, and for characterization of final products [15,16,18,30]. It has been demonstrated that proteomics offer fast and efficient way for the identification of potentially harmful, impurities. After their identification, the time for process optimization towards their removal can be significantly shortened [12,13,29]. For the quality control of the final products, proteomics can be used for the characterization of the active component, detection of impurities and determination of batch-to-batch variations [17,18,31].

Recombinant F IX has been on the market for more than 10 years. However, especially in developing countries, pd F IX is still used for treatment of hemophilia B [4]. This vitamin K-dependent plasma glycoprotein has different PTMs. The most important post-translational modification is gamma-carboxylation (Gla). This reaction is catalyzed in the liver by gamma-glutamyl transferase, and is critical in production of this recombinant clotting factor [28]. While the PTMs of r F IX are well characterized, the structure and PTMs of pd F IX and other pd vitamin K dependent clotting factors and inhibitors are still poorly described in the literature [31,32]. As shown in Figure 3, the production of F IX from cryopoor plasma is a relatively straightforward process containing two anion-exchange chromatographic steps and one affinity step with Heparin Sepharose FF. In addition to the solvent detergent treatment for inactivation of lipid-enveloped viruses, the virus filtration was added as a second step for virus removal [20]. It was later proved that this is also a very efficient way to remove vitronectin. This protein was the main impurity in pd F IX concentrates, and it went undetected for a long time. Identification of vitronectin as a contaminating protein in pd F IX concentrates was one of the first applications of proteomics technology for the characterization of plasma-derived therapeutic proteins [32].

By use of the isolation scheme presented here (see Figure 3), highly purified and virus safe pd F IX concentrate can be produced. The lipid-enveloped viruses are first inactivated by S/D treatment. The nanofiltration is the second step for effective virus removal. This S/D treated and virus filtered concentrate has been successfully used for treatment of hemophilia B [33].

In Figure 3, the production scheme for the isolation of pd F IX is presented. The significant proteins that are removed in each isolation step are also listed. The list of quantitative changes of significant proteins in each step shown in Figure 3, determined by biochemical and immunochemical methods, is given in Table 7. The left-hand side of the schema lists the proteins that have been detected by use of immunochemical and other biochemical methods, prior to the proteomic analysis [6], and at the right-hand side, proteins detected here, by use of proteomics methods are shown. A comprehensive analysis of each isolation step is given below.

As shown in Figure 1 (lane 1), under the conditions chosen for the solid phase extraction with the weak anion-exchanger, the majority of HSA does not bind to the support. Under these conditions, immunoglobulins G also do not bind, and can be harvested in the supernatant. Together with these two highly abundant proteins, transferrin, alpha 2 macroglobulin, hemopexin, and two protease inhibitors, antithrombin III and alpha 1 trypsin inhibitor were

also identified in this fraction. In the routine Cohn fractionation, the supernatant from solid-phase extraction returns back in the fractionation process, and the therapeutic proteins of interest can be isolated in subsequent steps [6,8,19]. Alpha 2 macroglobulin, transferrin and hemopexin are also potential impurities in therapeutic IgG preparations (see Table 2), especially if chromatographic steps are used during the isolation process [12,13]. Vitamin K dependent clotting factors F II, F IX and F X, and inhibitors protein C and protein S are identified in the fraction, eluted with 0.6 M NaCl (see Table 1). Protein Z and F VII could not be identified in this step via LC-MS/MS proteomic methods. The main impurities in this fraction are ceruloplasmin, IaIp, vitronectin, complement components, transthyretin, kininogen 1 and proteases carboxypeptidase N (C_xPN), mannan binding lectin serine protease (MBLSP) and hyaluronan binding protein 2 (HBP2). The presence of these proteases poses some risk of protein degradation/activation during further processing of the material.

According to previous findings, proteases, F VII and a portion of F II should be removed from the F IX containing fraction during anion-exchange chromatography on DEAE Sepharose FF (see Reference [6] left part of Figure 3). Our proteomic investigation demonstrates that earlier conclusions are only partially true (see Table 3). For example, in the main F IX fraction, eluted from the DEAE Sepharose column, some HBP2 was still present. Other two proteases, C_xPN and MBLSP were removed below the detection limit (see Table 2 and Table 3). Residual HSA, the bulk of complement components, kininogen 1, transthyretin and ceruloplasmin were also separated from F IX. The main fraction contains vitamin K dependent clotting factors F II, F IX and F X, and inhibitors protein C, protein S and protein Z. As shown in Table 3, protein Z, the additional vitamin K dependent clotting inhibitor, could be identified in this step. However, throughout the whole process, we could not identify F VII. Brigulla *et al.* [17] had similar problems with F VII identification in prothrombin complex concentrate (PCC), when proteomic methods (2D electrophoresis followed by MS) were employed. This clotting factor can be detected with the clotting assay in both PCC and in-process samples from F IX production (not shown, cf. Reference [6]). It seems that this labile protein was degraded to below our detection limit during the sample preparation. This problem needs further investigation. Beside the above listed vitamin K dependent clotting factors and inhibitors, IaIp, C1 inhibitor, vitronectin and some residual amount of HBP2 were identified. This proteolytic enzyme which has been shown to cause thrombogenicity in some medium purity pd F IX concentrates, have to be completely removed with additional purification steps [12,13]. However, in this stage of the isolation process, the proteolytic activity of this enzyme seems to be controlled by the presence of relatively high concentration of IaIp and perhaps by other protease inhibitors such as C1 that are identified in this fraction in lower concentrations. Surprisingly, clotting factor V (F V) was also found in the major F IX fraction. This clotting factor could be identified only by use of proteomic methods, and in parallel test, using a clotting assay, no F V activity could be detected (not shown).

In the third step, heparin affinity chromatography, all contaminating proteins except vitronectin and traces of IaIp were removed (see Table 4 and Table 5, and Figure 2 and Figure 3 A and B). Because of differences in molecular size, vitronectin and IaIp are separated from F IX in the last step, virus filtration (see figure 2). IaIp and complement C4 were detected early as impurities in pd F IX concentrate that was produced by use of a similar production scheme lacking, however, a nanofiltration step [34,35]. In our earlier investigation, we identified vitronectin, as a main component that was removed during virus filtration with the Viresolve system. Complement C4 and IaIp were not detected [26]. As shown in Table 5, in present investigation, IaIp and vitronectin were detected before virus filtration, however, complement C4 was not detected in the F IX sample prior to virus filtration. Together with the majority of IaIp, F X and other components, this protein was removed during heparin affinity chromatography (see Table 4). Interestingly, in the sample before virus filtration, an additional band with low molecular weight was also separated by SDS-PAGE (see Figure 2A and Table

5). In this band, a peptide with the sequence SALVLQYLR was detected (see Figures 4 and Figure 5). This peptide belongs to the F IX heavy chain (see Figure 4 and References [36, 37]). Consequently, it appears that some proteolytic degradation has occurred during this step. This polypeptide was not detected in the final product (see Figure 2B). Consequently, it seems to be removed during virus filtration, perhaps due to interaction with some of high molecular weight proteins, possibly with vitronectin (see SDS-PAGE in Figure 2B, fraction 2).

As shown in Figure 2B in the final product, only one band, with an apparent molecular weight in SDS-PAGE of about 65,000 was detected. This product contains highly purified pd F IX. The main peak in SEC has a similar apparent molecular weight (see Figure 2B). The specific activity of this concentrate was about 240 IU F IX/mg protein (data not shown), which is close to the specific activity for pure clotting factor [6, 29]. Only peptides belonging to both F IX heavy and light chain, and no sequences from the activation peptide were detected by LC-MS/MS (see Figure 4). In our earlier investigation using 2D electrophoresis and N-terminal sequencing, we identified only peptides originating from the heavy chain. In that study, we did not identify any peptide from the light chain, and we also failed to isolate the activation peptide [38].

Isotope labeling has been used to identify quantitative differences in protein content even in very close related samples [18,24]. Results shown in Table 6 that the applied iTRAQ method enables quantitative comparison of content of F IX and impurities in two final production steps, Heparin Sepharose FF affinity chromatography and Viresolve nanofiltration. In comparison to the sample before nanofiltration (#2), F IX content in the sample after nanofiltration (#4) is 1.85x higher (see second column in Table 6). After comparison of F IX content in these samples by measuring of specific activity, the enrichment in this purification step was ~1.74x (240 IU/mg protein in sample after nanofiltration, and 138 IU/mg protein in sample before nanofiltration, see Table 7). The main impurities, F X, IaIp, vitronectin and C1 inhibitor were removed during virus filtration step. They were detected in side fractions (#1 and #4, see Table 6) and could not be detected in the final product (sample #3).

Additionally, although it was not the main topic of this paper, we have shown that this production process as a “model” example of human plasma fractionation, if combined with proteomic methods, offers additional approaches for the identification of low and very low abundance proteins in this biological fluid (see Supporting material).

5. Conclusions

In this paper, we demonstrated that proteomic technologies are very useful tools for validation of down stream processes during the production of pd F IX.

In each production step, additional proteins and potentially harmful impurities, especially serum proteases, were identified and their removal was documented. ITRAQ as an isotope labeling technique for comparative mass spectrometry demonstrated the potential value of this technology for in process control analysis. Consequently, the knowledge of this industrial process has been significantly extended.

Furthermore, proteomics can be effectively used for further characterization of final pd F IX preparations, such as detection of trace impurities and cleavage products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CxPN	carboxypeptidase N
F V, F VII, F IX, F X	clotting factors V, VII, IX and X
HBP2	hyaluronan binding protein 2
HSA	human serum albumin
IaIp	inter-alpha inhibitor proteins
MBLSP	mannan binding lectin serine protease
pd F IX	plasma-derived clotting factor IX
r F IX	recombinant clotting factor IX
SEC	size-exclusion chromatography
S/D	solvent/detergent
TnBP	tri-n-butyl phosphate

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Figure 1. SDS-PAGE under reducing conditions of in-process samples from the isolation of pd F IX. CP – calibration proteins; lane 1 – Unbound proteins after DEAE Sephadex A50 solid-phase extraction ; lane 2 – DEAE Sephadex A50 eluate (starting material for the next step – anion-exchange chromatography with DEAE Sepharose FF); lane 3 – Unbound proteins after DEAE-Sepharose FF chromatography; lane 4 – DEAE Sepharose FF, weakly bound proteins (wash with 20 mM Na-citrate buffer containing 0.28 M NaCl); lane 5 – DEAE Sepharose FF eluate (starting material for the next step – Heparin Sepharose FF affinity chromatography); lane 6 – Heparin Sepharose FF, weakly bound proteins (wash).; lane 7 – Unbound material after Heparin Sepharose FF chromatography; lane 8 – Heparin Sepharose FF eluate (fraction containing highly enriched pd F IX).

Figure 2.

Size-exclusion chromatography on a column tandem containing one TSK G3000 SW and one TSK G4000 SW column. Fractions were collected and analyzed by SDS-PAGE. Polypeptide from the proteolytic degradation containing the SALVLQYLR sequence that belongs to the F IX heavy chain is labeled (frame in lanes “co” and No. 3). **A** – sample before nanofiltration with the Viresolve virus filter (concentrated Heparin Sepharose FF eluate). SDS-PAGE under reducing conditions – Co – sample before chromatographic separation; lanes 1–3 – fractions 1, 2 and 3, after chromatographic separation. Polypeptide from the proteolytic degradation containing the SALVLQYLR sequence that belongs to the F IX heavy chain is labeled (frame in lanes “co” and No. 3). **B** – Final product, SDS-PAGE under reducing conditions – Co – sample before chromatographic separation; lanes 1 and 2 – fractions 1 and 3, after chromatographic separation.

Figure 3.

Manufacturing process for isolation of highly purified clotting factor IX concentrate from human plasma. Main impurities identified by use of biochemical and immunochemical methods, like measuring of enzymatic and clotting activity, ELISA and immunoblot are listed at the left hand part of the Figure. The proteins identified by use of proteomic methods are listed at the right side. See also Table 1–Table 5 and Supporting material, Table S1 and S2 for additional information.

Samples for further biochemical and proteomic analyses (see also Table 6)

Coagulation factor IXa (EC 3.4.21.22) precursor [validated] - human

MQRVNMIMAE **SPGLITICLL** **GYLLSAECTV** **FLDHENANKI**
LNRPKRYNSG **KLEEFVQGNL** **ERECMEEKCS** **FEEAREVFEN**
TERTTEFWKQ **YVDGDQCESN** **PCLNGGSCKD** **DINSYECWCP**
FGFEGKNCEL **DVTCNIKNGR** **CEQFCKNSAD** **NKVVCSCTEG**
YRLAENQKSC **EPAVPFPCGR** **VSVSQTSLT** **RAEAVFPD**
YVNSTEAE**TI** **LDNITOST****OS** **FNDFT****RVVGG** **EDAKPGQFPW**
QVVLNGKVDA **FCGGSIVNEK** **WIVTAAHCVE** **TGVKITVVAG**
EHNIEETEHT **EQKRN****VIRII** **PHHNYNAAIN** **KYNHDIALLE**
LDEPLVLNSY **VTPIC****ADKE** **YTNIFLKFGS** **GYVSGWGRVF**
HKGR**SALVLQ** **YLRVPLVDRA** **TCLRSTKFTI** **YNNMFCAGFH**
EGGRDSCQGD **SGGPHVTEVE** **GTSFLTGIIS** **WGEECAMKGK**
YGIYTKVSRY **VNWIKEKTKL** **T**

Figure 4.

Sequence of clotting factor IX, the peptides identified by LC-MS/MS are underlined. Gray – light chain; underlined (broken line) – activation peptide; bold – heavy chain. The peptide SALVLQYLR from the heavy chain that was identified in the cleavage product with an apparent molecular weight of about 10 kDa, separated by SDS-PAGE of the sample before nanofiltration, is framed (see also Figure 2A).

Figure 5. MS spectra collected during LC-MS/MS analysis of the band with an apparent molecular weight of 10 kDa, and the fragmentation spectrum of the peptide SALVQYLR that belongs to the heavy chain of clotting factor IX.

Table 1

List of proteins of interest, identified in DEAE Sephadex A50 eluate (the complete list of identified proteins is given in Supporting material, Table S1)

% Coverage	gi	Protein name
76.1	115298678	Complement component 3
88.2	4502027	Albumin precursor
69.4	4557485	Ceruloplasmin precursor
66.2	4504781	Inter-alpha inhibitor H1
63.8	70778918	Inter-alpha inhibitor H2
14.79	62897073	Inter-alpha (globulin) inhibitor H3 variant
12.8	4502067	Alpha-1-microglobulin/bikunin precursor
12.2	88853069	Vitronectin precursor
38.8	4507725	Transthyretin
11.6	4504893	Kininogen 1
10.8	88971372	Carboxypeptidase N
10.4	21264363	MBLSP
16.6	4758502	Hyaluronan binding protein 2
7.6	576554	Antithrombin III
5.2	151302818	Alpha-1 antitrypsin
28.6	4503635	Coagulation factor II, precursor
10.2	4506117	Protein S (alpha)
6.4	4505116	Protein C
8.1	4503649	Coagulation factor IX, precursor
5.8	4503625	Coagulation factor X, precursor

Table 2

List of proteins of interest, identified in the supernatant, after solid-phase extraction with DEAE Sephadex A50*

% Coverage	gi	Protein name
6.2	105990532	Apolipoprotein B precursor
9.5	66932947	Alpha-2-macroglobulin
23.4	4557871	Transferrin
15.8	11321561	Hemopexin
11.0	151302818	Alpha-1 antitrypsin
10.8	4502261	Antithrombin III

* Bands with apparent molecular weights in SDS-PAGE 67,000; 55,000 and ~28,000 belong to high-abundance proteins HSA and IgG and were not subject to analysis by LC-MS/MS.

Table 3

List of proteins of interest that are identified in the main fraction containing F IX after the second purification step—anion-exchange chromatography with DEAE-Sephacrose FF (the complete list of identified proteins is given in Supporting material, Table S2)

% Coverage	gi	Protein name
22.6	4503635	Coagulation factor II, precursor
14.8	36579	Protein S (alpha)
10.8	4505116	Protein C
8.2	6116779	Protein Z
24.6	4503649	Coagulation factor IX, precursor
36.2	4503625	Coagulation factor X, precursor
18.4	4503635	Coagulation factor II, precursor
1.66	163038	Coagulation factor V, precursor
32.6	88853069	Vitronectin, precursor
44.3	4504781	Inter-alpha inhibitor H1
40.8	70778918	Inter-alpha inhibitor H2
22.7	133925809	Inter-alpha inhibitor H3
10.4	4502067	Alpha-1-microglobulin/bikunin precursor
19.2	124096	C1 protease inhibitor
14.2	4758502	Hyaluronan binding protein 2

Table 4

Complete list of proteins identified in the eluate from the Heparin Sepharose FF column after “in-gel” digestion and LC-MS/MS.

% Coverage	gi	Protein name
5.81	33985	Trypsin inhibitor (inter-alpha), complete protein
7.68	33989	Inter-alpha trypsin inhibitor heavy chain 1
20.04	13477169	Vitronectin (Homo sapiens)
18.44	67596	Coagulation factor IXa precursor - human
1.95	67596	Coagulation factor IXa precursor – human *

* One peptide identified in low-molecular band-product of the proteolytic digestion during the isolation process

Table 5

List of proteins of interest, removed in the last chromatographic step–Heparin affinity chromatography

% Coverage	gi	Protein name
18.11	33989	Inter-alpha trypsin inhibitor heavy chain 1
11.31	33985	Trypsin inhibitor (inter-alpha), complete protein
5.99	288563	Inter-alpha trypsin inhibitor heavy chain 3
12.54	124096	C1 protease inhibitor
10.09	179674	Complement component C4A
9.83	36573	S-protein
12.11	68785	Kininogen, HMW precursor
8.92	4758502	Hyalouronan binding protein 2
2.38	182798	Factor V precursor
23.47	135807	Prothrombin precursor (Coagulation factor II)
13.88	67596	Coagulation factor IXa, precursor*
11.24	119761	Coagulation factor X, precursor
13.2	67599	Protein C (activated), precursor - human
4.29	36579	Preproprotein S
5.45	131092	Vitamin K-dependent protein Z precursor

* Some F IX was lost during this chromatographic step.

Table 6

Quantitative comparison of main and side fractions from 2 last production steps (after Heparin Sepharose FF chromatography and nanofiltration).

Score	Accession	Protein	#1 : #2			#3 : #2			#3 : #4			#4 : #1		
			Ratio	pVal	EF	Ratio	pVal	EF	Ratio	pVal	EF	Ratio	pVal	EF
120.5	gi 4503649	FIX	0.03	2.6e-15	1.2	1.85	8.5e-10	1.05	22.8	7.8e	1.02	2.8	1.2e-6	1.2
18.7	gi 70778918	Ialp	15.2	9.2e-10	1.04		-- *			-- *		0.12	2.4e-7	1.03
90.8	gi 88853069	VN	0.15	1.3e-15	1.08		-- *			-- *		28.2	1.2-15	1.02
6.2	gi 124096	Cl	5.8	2.2e-11	1.06		-- *			-- *		0.24	2.5e-8	1.08
9.6	gi 4503625	FX	20.2	1.8e-12	1.1		-- *			-- *			-- *	

* not detected in one of samples

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

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 value should fall within ratio/EF and ratio*EF.

Table 7

Concentration of F IX and some main impurities during the isolation process.

Sample #	Prot. [mg/ml]	FII [IU/ml]	FVII [IU/ml]	FX [IU/ml]	IaIp [mg/ml]	VN [mg/ml]	FIX [IU/ml]	FIX, spec. act. [IU/mg]	Enr. FIX
1	32.0	50.5	12.5	37.5	11.6	1.2	41.6	1.3	65x
2	6.0	5.2	0.02	4.0	5.3	0.56	27.0	4.5	225x
3	1.5	0.43	0.07	8.1	1.0*	0.05	4.5	--	--**
4	1.2	n.d.	n.d.	n.d.	0.05	0.4	115	138	6900x
5	0.8	n.d.	n.d.	n.d.	n.d.	n.d.	200	250	12500x
6	2.5	n.d.	n.d.	n.d.	0.2	2.1	26.1**	--**	--**

* Fraction containing IaIp

** Side fraction, not used for FIX isolation. For identification of fractions – see Figure 3