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Proteomic analysis for process development and control of therapeutic protein separation from human plasma

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Abstract

The use of proteomics technology during the development of a new process for plasma protein separation was demonstrated. In a two-step process, the two most abundant proteins, human serum albumin and IgG, were removed in a first step of anion-exchange chromatography using a gel with very high capacity. Subsequently, two fractions containing medium and low abundance proteins were re-chromatographed on a smaller column with the same type of gel. Collected fractions were separated by SDS-PAGE and 2D electrophoresis, and excised proteins were digested with trypsin and identified by LC-ESI-MS/MS. This proteomic analysis proved to be a useful method for detection of low abundance therapeutic proteins and potential harmful contaminants during process development. Based on this method low abundance therapeutic proteins, such as vitamin K dependent clotting factors and inhibitors, could be identified as present in target fractions after chromatographic separation. In addition, the tracking of potentially dangerous impurities and designing proper steps for their removal are important outcomes when developing, refining or controlling a new fractionation schema. For the purpose of in-process control, in-solution digestion of complete fractions followed by protein identification with LC-ESI-MS/MS was demonstrated as a rapid and simple alternative to the entire analysis including 1D or 2D-electrophoretic steps.

Keywords

plasma-derived therapeutic proteins; process development; proteomics; chromatographic and electrophoretic separation

1. Introduction

Human plasma is still a valuable starting material for production of therapeutic proteins [1]. Blood plasma is a fluid, and therefore it may be pooled in order to obtain a representative sample for proteomic investigations or to serve as a source for large amounts of raw material for fractionation and production of therapeutic proteins [1,2].

Proteomic analysis of plasma proteins has been discussed for more than ten years, and it continues to be a topic of intensive investigations. Large numbers of proteins and peptides are present in this most important body fluid. The dynamic range of their concentration spans at least 12 orders of magnitude [3]. Recently developed sensitive and innovative fractionation (and micro-fractionation) methods are used to identify and characterize proteins and protein fragments present in very low concentrations in human plasma [2, for review see Ref. 4]. Additionally, protein identification and detection of potential disease biomarkers in human plasma (or serum) are still big challenges for mass spectrometric and bioinformatics analyses [4–7].

Methods of plasma fractionation have been developed and used for over fifty years for the isolation of therapeutic proteins [1,8]. The development of new and safer methods for virus inactivation and removal has been for a long time the major focus in the plasma fractionation industry [9,10]. Other aspects of clinical safety were covered by extensive and very expensive clinical trials, and, except for routine procedures like protein profiling by SDS-PAGE and some immunochemical investigations, these products have never been subjected to thorough protein-chemical analyses [10,11]. Only few articles deal with both plasma fractionation and the proteomic characterization of the production process and the final product [12–16].

The use of proteomics techniques for characterization and validation of chromatographic separations has been discussed recently. In this earlier paper the separation of plasma proteins by means of a lower capacity anion-exchanger is monitored; this type of chromatographic material is routinely used in plasma fractionation for separation of clotting factors [8]. Thorough proteomic investigation was also performed during the isolation of inter alpha inhibitor proteins (IaIp), a group of protease inhibitors that has been considered as a potential new therapeutic for sepsis treatment [12]. Lion and Tissot [13] and Thiele et al. [14] also discussed the use of proteomics technology for quality assurance of the production process of blood-based therapeutic proteins. An important contribution of proteomics can be the use of this technology to reduce the inter-assay variability for the activity of these products, and for identification of potentially harmful impurities [13,15].

In this paper, we demonstrate the use of chromatographic and electrophoretic techniques, combined with LC-ESI- MS/MS, for the optimization of the separation and identification of vitamin K dependent clotting factors and inhibitors from human plasma.

2. Materials and methods

2.1. Human plasma

The starting material was cryopoor, single donor human plasma (Rhode Island Blood Center, Providence, RI). Prior to use, the cryoprecipitate was removed as previously described [17]. All plasma samples were screened to exclude the presence of blood-borne viruses (hepatitis A, B and C and HIV).

2.2. Anion-Exchange chromatography

For anion-exchange chromatography, GigaCap Q gel (Tosoh Bioseparations, Stuttgart, Germany) was used. The gel was packed in a 10 mL or a 5 mL glass column (10 mm I.D. both from Tosoh Bioseparations). After washing with HPLC water, the column was equilibrated with a low ionic strength buffer (10 mM Tris HCl, pH 7.4; BufferA). The human plasma was 4 times diluted with Buffer A and applied to the column (3 mL cryopoor plasma/mL gel, corresponding to ~180 mg protein/mL gel). Unbound proteins were collected and subsequently analyzed. After sample application, the column was washed with 5 column volumes (CV) of Buffer A, and the bound proteins were eluted with a step

gradient of Buffer B (1 M NaCl in Buffer A). The flow rates for chromatographic separation were between 1 and 5 mL/min. All chromatographic runs were performed at 4°C. For separation, a BioLogic Duo Flow chromatographic system (BioRad, Hercules, CA, USA) was used. Proteins were detected by UV absorption at 280, 260 and 210 nm.

For determination of column capacity and recovery, unbound material and eluted fractions were collected. Protein amounts were determined in all fractions and in the starting material with the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's procedure. Each experiment, namely the fractionation and subsequent identification, and determination of capacity and recovery, was performed in triplicate.

2.3. Electrophoretic separations

After total protein determination of each fraction collected during the separation by anion-exchange chromatography, about 15–20 µg protein of each sample were solubilized in NuPAGE sample buffer (Invitrogen, Carlsbad, CA, USA) and heated at 100°C for 5 min. SDS-PAGE was performed with precast 4–12% Bis-Tris gels in a Xcell Sure Lock Mini-Cell (Invitrogen), according to the manufacturer's procedure. The gels were stained with GelCode Blue (Pierce). SDS-PAGE was performed in two independent experiments.

Samples for 2-DE containing 70 µg protein were prepared using Ready Prep 2-D Cleanup kit (Bio Rad) according to manufacturer's instruction. After this preparation, samples were re-dissolved in IPG strip rehydration buffer containing 8 M urea, 2% CHAPS, 0.5% carrier ampholytes, 0.002% bromophenol blue and 20 mM DDT. The dry IPG strips were rehydrated overnight with 160 µL of the protein sample. 2-D electrophoretic separation was performed as described previously [12]. Gel scanning was performed by use of a VersaDoc Imaging System (BioRad).

2.4. “In-gel” digestion procedure

The gel bands of interest were excised by extracting six to ten gel particles with clean glass Pasteur pipettes and digested with trypsin as described previously [12,18]. The specific procedure is briefly described as follows:

After excision, the proteins present in gel particles were washed twice with analytical grade water and 1:1 v/v of 0.1 NH₄HCO₃ for 15 min with agitation. The washing solution was then removed completely and enough acetonitrile (ACN) was added to cover the gel particles. All the solvent volumes used in the washing steps should roughly equal twice the gel volume. After the gel particles shrunk and stuck together, the ACN was removed and the gel particles were rehydrated in 0.1 M NH₄HCO₃ for 10 min. An equal volume of ACN was then added to finally get 1:1 v/v of 0.1 NH₄HCO₃ / ACN. After 10 min incubation, removing all liquid, and drying down the gel particles in a vacuum centrifuge, proteins were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide in 0.1 M NH₄HCO₃. After reduction and alkylation, gel particles were washed as described above. Following tryptic digestion for 24 h at 37°C, the peptides were recovered and extracted from the gel particles by addition of a 10 µL of 25 mM NH₄HCO₃ and 5% formic acid and ACN (5 µL of each). Pooling and drying down all the extracts, the tryptic peptides were dried and redissolved in formic acid:water:acetonitrile:trifluoroacetic acid mixture (0.1:95:5:0.01) in preparation for the LC-MS/MS analysis.

2.5. “In-solution” digestion procedure

50 µg of the acetone-precipitated and denatured protein pellet was resolubilized in 100 µL of NH₄HCO₃ (pH 8.0) / 8 M 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 ammonium bicarbonate buffer 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). After incubating at 37°C overnight, the tryptic peptides were dried in a vacuum centrifuge (Vacufuge, Eppendorf, Hamburg, Germany). The material was then redissolved in a solution of 0.5% (v/v) formic acid and 20% (v/v) acetonitrile, with vacuum drying again. Subsequently, the peptides were isolated using a strong cation exchange TipTop™ (PolyLC, Inc., Columbia, MD, USA) according to the manufacturer's instructions after resuspending in the same solvent and confirming the pH value. The resulting tryptic peptides were dried once more and were subject to the LC-MS/MS analysis after being redissolved in formic acid:water:acetonitrile:trifluoroacetic acid mixture (0.1:95:5:0.01).

2.6. Identification of proteins with LC-MS/MS

Tryptic digests of whole fractions obtained by anion-exchange chromatography, or of proteins extracted from the gels after SDS-PAGE separation, were separated with an reversed-phase column (C-18 PepMap 100, LC Packings/Dionex, Sunnyvale, CA, USA) as previously described [18]. Briefly: The column eluate was introduced directly onto a QSTAR XL mass spectrometer (Applied Biosystems and Sciex, Concord, Ontario, Canada) *via* electrospray ionization (ESI). Half-second MS scans (300–1500 Thompson, Thompson(Th) = Da/z) were used to identify candidates 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 completed with ProteinPilot (Applied Biosystems and Sciex), setting with 1.5 Da mass tolerance for both MS and MS/MS and using 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 and protein scoring method. Scores above 2.0 require that at least two sequence-independent peptides will be identified [18,19].

In parallel experiments, additional LC-MS/MS system was used (Agilent Technologies, Palo Alto, CA, USA, and Thermo Electron Corporation, San Jose, CA, USA). When this system was used, tryptic peptides were separated on a 12 cm (75 µm I.D.) analytical column with 5 µm Monitor C18 resin (Column Engineering, Inc., Ontario, CA, USA) and containing an integrated ~4 µm ESI emitter tip. Solvent A was 0.1 M acetic acid in water, solvent B was 0.1 M acetic acid in acetonitrile. Peptides were eluted using a linear acetonitrile gradient (0–70% solvent B over 30 min). 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) 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.

Peptide and protein identifications were performed with software contained BioWorks version 3.2 (Thermo Electron). Peak list files were created by the program extract_msn.exe, using the following settings: The mass had to fall in the range of 600 to 4500 Daltons. The minimum total ion current for the scan had to be over 1000. The precursor tolerance for grouping was 1.5 Daltons, with no differing intermediate scans allowed and only a single scan required to create a peak file. The minimum signal-to-noise for a peak to be written to the peak file was 3, and 25 such peaks had to be found for a peak file to be created. The program calculated charge states. However, in case of ambiguity, peak files for both the +2 and +3 charge states were created.

Database searching using the peak lists was performed by the program SEQUEST [20]. 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 database contained sequences identified as human in NCBI's nr database (November, 2006), which was created using the FASTA filtering tools found in BioWorks. 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 [21]. This composite database contained approximately 490,000 entries.

3. Results

3.1. Chromatographic separation with the strong anion-exchanger Giga Cap Q

Chromatographic separation of human plasma on a 10 mL column packed with strong anion-exchanger GigaCap Q (AX-Col.1) is shown in Figure 1. Thirty mL of cryopoor plasma containing about 1900 mg protein were loaded on the column, and after washing with Buffer A, bound proteins were eluted with a step gradient containing increasing amounts of NaCl (see Figure 1). The determined column capacity was about 100 mg protein/mL gel, and the calculated recovery was higher than 95%. The SDS-PAGE analysis of collected fractions is also shown in the Figure 1.

Fractions eluted with 0.3 M (fraction 6), and 0.5 M NaCl (fraction 7), were pooled and diluted with Buffer A until the conductivity reached 20 mS/cm (about 0.16 M NaCl), and applied to a 5 mL column containing the same type of gel (AX-Col.2). Bound proteins were again eluted with a NaCl step gradient, and further analyzed with SDS-PAGE (see Figure 2).

3.2 Protein identification of collected fractions

3.2.1. Identification after in-solution digestion—Therapeutic proteins and some other proteins that were identified after re-chromatography of pooled fractions 6 and 7 from the first run and after in-solution digestion of collected fractions are listed in Table 1, right lanes (fractions labeled with "Re"). The complete list of identified proteins is given in supplemental material, Table S1.

Most therapeutic proteins identified in fraction Re₃ (re-chromatographed on AX-Col.2) are listed in Table 1. This group contains the bulk of the very important vitamin K dependent clotting factors and inhibitors. The complete list of proteins identified in the re-chromatographed fractions is given in supplemental material, Tables S2–S7. Right lanes in Table 1 (Re₂–Re₆) indicate that re-chromatography results in a much better evidence for the presence of important therapeutic proteins and undesired contaminant proteins.

3.2.2. Identification after SDS-PAGE separations and digestion of extracted proteins—The various fractions were additionally separated by SDS-PAGE (see Figure 2) and bands of interest were taken for identification by LC-ESI-MS/MS. The list of identified proteins is presented in supplemental material, Table S8. The proteins identified in the unbound fraction, were mainly IgG (see Fig. 2, bands 32–36) and Apolipoprotein B, Alpha-2-macroglobulin, Transferrin (Tf), Human serum albumin (HSA) and Hemopexin.

3.3. Identification of proteins after finally developed chromatographic separation

A chromatographic separation according to the finally developed chromatographic scheme is shown in Figure 3. In the first chromatographic run, 1900 mg protein was applied to a 10 mL column. Bound proteins were eluted in a single step with 1.0 M NaCl (chromatogram not shown). Similar to the separation shown in Fig. 1, the majority of IgG does not bind to

the column and can be collected in the front of the flow-through fraction. Under conditions employed here, a portion of HSA and some other proteins that weakly bind to the column appear later in the flow-through fraction. The eluate from the first chromatographic run was subsequently diluted with four volumes of Buffer A and applied to a 5 mL glass column containing 2 mL gel. Under these conditions, most of the residual HSA and some other proteins do not bind to the column, and are collected in the flow-through fraction, (fraction n.b.F, see Fig. 3). Bound proteins were eluted in three steps with 0.3, 0.5 and 1.0 M NaCl, respectively, giving the fractions No. 1_F, 2_F and 3_F (see Figure 3). These fractions were further separated by 2D electrophoresis (see Figures 4A and B) and proteins were identified by LC-ESI-MS/MS. Major therapeutic proteins of interest and major impurities are listed in Table 2 indicating the fractions in which they could be determined. Some of the proteins, or at least parts of their polypeptide chains, were found in more than one fraction. All proteins are listed in the supplemental material, Tables S9 and S10. In a parallel experiment, the collected fractions were directly submitted to in-solution digestion and proteins were identified with LC-ESI-MS/MS. The outcome of this shorter and thus faster procedure does not show significant differences from that given in Table 2, indicating that the faster procedure might be sufficient for a rapid in-process control. The complete list is given in the supplemental material, Tables S11 and S12.

4. Discussion

4.1. Design of a fractionation scheme for isolation of tightly bound proteins

4.1.1. First anion exchange chromatography—In the scheme presented here, the first step of plasma protein fractionation using the high-capacity anion-exchange resin (AX-Col. 1) was developed to separate the most abundant proteins, HSA and IgG. As expected, the majority of immunoglobulin G did not bind to the column and was collected in flow-through fractions (fractions 1 and 2, see Figure 1). The unbound fraction 1 contains almost pure IgG. Because of the high IgG content, proteomic analysis of this whole fraction (without subsequent SDS-PAGE separation) was not performed. The column was overloaded in order to achieve a maximal yield of strongly-binding proteins. Under these conditions, most HSA was found in late flow-through fraction (see Figure 1). Some HSA and weakly bound proteins could also be identified in the first unbound fraction, after separation with SDS-PAGE (see Figure 2, lane 7). These proteins were apolipoprotein B (band 32), alpha 2-macroglobulin (band 33), transferrin (band 35), and hemopexin (band 36, identified together with HSA). By use of the present scheme, additional HSA could be eluted in fraction 4 with 0.2 M NaCl. In order to remove residual HSA, the column was further eluted with 0.2 M NaCl (fraction 5). The next two fractions, eluted with 0.3 and 0.5 M NaCl, are the main interest of this study.

4.1.2. Rechromatography of fractions 6 and 7—After re-chromatography of pooled fractions 6 and 7 from the first run, in the first fraction (see Fig. 2, lane 1), weakly bound proteins like complement components (1, 3, 4, 4A, 4B and 9), HSA, ceruloplasmin, fibrinogen and some apolipoproteins could be removed. Further, some medium and low abundant proteins and proteoglycans such as vitronectin, lumican, paraoxonase, clusterin isoform 1, transthyretin, protein S and butyrylcholinesterase could also be detected in this fraction (see supplemental material, Table S2).

The list of active components and most important impurities is given in Table 2 (right lanes, Re₂–Re₆) and the complete list is shown in supplemental material, Tables S3–S8. Altogether, more than 140 plasma proteins were identified.

In summary, the therapeutically important low abundance proteins, vitamin K binding clotting factors and inhibitors (plasma concentration between 0.4 µg/mL for FVII and 90 µg/

mL for F II, see Ref. 17) are highly enriched after re-chromatography of pooled fractions 6 and 7 from the first run, in fraction eluted with 0.3 M NaCl (fraction Re₃). However, F VII, protein C and protein Z could not be identified in any of the fractions. A possible reason why these proteins were not detected might be proteolytic cleavage, possibly during the isolation process. This degradation may be caused by hyaluronan binding protein 2 or other proteases that are present in the sample, and this fact requires further investigation.

Mannan-binding lectin serine protease 1 could be separated from the bulk of vitamin K dependent proteins by washing the column with 0.2 M NaCl. (Band No. 9 Figure 2, see Table 2 and supplemented material, Table S8). This protease is associated with the serum mannan-binding lectin that is the part of the complement system [23]. This proteolytic enzyme can potentially cause an activation of clotting factors during the isolation process or during storage and should be completely removed from therapeutic concentrates. The bulk amount of the other protease, hyaluronan binding protein 2 [24] could also be separated from vitamin K binding proteins, but this proteomic study indicates that a part of this potentially harmful impurity is still not completely removed (see Table 1, left lane, column Re₃) by the investigated fractionation scheme. In order to remove these proteins, further optimization of the purification scheme is necessary. The fact that the main part of this protein was strongly bound to the column and can be only eluted with high salt concentration (see Figure 2 and Table 2, lane “Re₅” & “Re₆”) is an important hint in the right direction.

4.2. Finally developed fractionation scheme for isolation of vitamin K dependent factors and inhibitors

Utilizing the analytical work presented above, we developed a fractionation scheme for isolation of vitamin K dependent clotting factors and inhibitors (see Figure 3). In this second chromatographic step, residual HSA and some other proteins were removed in the unbound fraction (fraction “n.b._F”) and in the fraction eluted with 0.3 M NaCl (fraction 1_F). The major amount of vitamin K dependent plasma proteins, *i.e.*, the clotting factors F II, F IX and F X and the inhibitors protein C, protein S and protein Z (see Table 2 and supplemental material, Tables S11 and S12) were subsequently eluted with 0.5 M NaCl, (fraction 2_F). Minor amounts of FII, F X and protein Z were also found in other fractions, together with impurities (cf. Table 2). These fractions will be not utilized if this scheme is established for purification. Propitiously, F IX, the most important component in PCC [22], was detected only in the targeted fraction eluted with 0.5 M NaCl (fraction 2_F). After washing with 0.2 M NaCl, mannan-binding protease can be removed. Hyaluronan binding protein 2, together with some other tightly binding proteins, was detected only in the last fraction, eluting with 1.0 M NaCl (see Table 2). Because of their complex structure and heterogeneity, IaIp (or domains of this protein) are eluted in all three fractions, and this fractionation scheme cannot be used for their efficient isolation.

4.3. Validation and in process control by 2D electrophoresis

The proteomic data reported and discussed above were validated by means of 2D electrophoretic analyses of the re-chromatographed fractions. (see Figure 4 and supplemental material, Tables S9 and S10). The target fraction eluted with 0.5 M NaCl (fraction 2_F), and that eluted with 1.0 M M NaCl, (fraction 3_F, see Figure 3 and 4A and B) were analyzed by this method. In the target fraction 2_F, F II, F IX, F X, protein C and protein S, were identified (see supplemental material, Tables S9–S10) but not protein Z and F VII. In agreement with previous findings, no additional therapeutic proteins or potentially harmful impurities were identified [16]. One important finding is, that if 2D-electrophoresis is used for in-process control and for analysis of the final product, a relatively high number of cleavage products of different proteins can be detected.

The protein content in two fractions eluted with 0.5 (2F) and 1.0 M NaCl (3F) corresponds to the proteins in so-called prothrombin complex concentrate that contains vitamin K dependent clotting factors and inhibitors and was analyzed by Brigulla et al. [16]. These authors separated the PCC proteins by 2D-electrophoresis, and identified them by MALDI-TOF MS. Among the vitamin K dependent clotting factors and inhibitors, F II, FIX and FX were identified, together with protein C, protein S and protein Z. In addition, 44 other plasma proteins were identified and the PCC concentrate still contained a significant amount of HSA. As in this present investigation, the fourth vitamin K dependent clotting factor, F VII could not be detected. Interestingly, the hyaluronan binding protein 2 was also not detected. The reason may be the lower sensitivity of the used method. However, the content of this protease in different PCC concentrates may be also different, and also depends on the quality of starting material [24].

5. Conclusions and outlook

In this paper we demonstrated that proteomics is a very useful tool during the development and fine-tuning of a new plasma protein purification process. We simply note that this plasma fractionation scheme may have broader utility, not only for the identification of potential therapeutic proteins, but also for the tracking of cleavage products and potentially harmful impurities. One next step will be the use of proteomics methods for the characterization of final preparations of therapeutic proteins, which is a topic for future investigations.

Additionally, our investigations have shown that new techniques for human plasma fractionation, combined with proteomics methods, offer additional approaches for the identification of low and very low abundance proteins in this biological fluid. Some proteins described by others as potential biomarkers were identified here, but as this was not the main topic of our paper (see supplemented material and Reference 8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

F II, F V, F VII, F VIII, F IX, F X	clotting factors II, V, VII, VIII, IX and X
HC1, HC2, HC3	Inter alpha inhibitor protein heavy chains 1, 2 and 3
HSA	human serum albumin
IaI	inter-alpha inhibitor
IaIp	inter-alpha inhibitor proteins
PaI	pre-alpha inhibitor
PCC	prothrombin complex concentrate

References

1. Burnouf T. J Chromatogr B. 1995; 664:3–15.

2. Rose K, Bougueleret L, Baussant T, Böhm G, et al. *Proteomics*. 2004; 4:2125–2150. [PubMed: 15221774]
3. Anderson NL, Anderson NG. *Mol Cell Proteomics*. 2002; 1:845–867. [PubMed: 12488461]
4. Hu S, Loo JA, Wong DT. *Proteomics*. 2006; 6:6326–6353. [PubMed: 17083142]
5. Tiss A, Smith C, Camuzeaux S, Kabir M, et al. *Proteomics*. 2007; 7(S1):77–89. [PubMed: 17893856]
6. Anderson L, Hunter CL. *Mol Cell Proteomics*. 2006; 5:573–588. [PubMed: 16332733]
7. Gortzak-Uzan L, Ignatchenko A, Evangelou AI, Agochiya M, et al. *Mol Cell Proteomics*. 2007; 10.1021/pr0703223
8. Kovac S, Yang X, Huang F, Hixson D, Josic D. *J Chromatogr A*. 2008; 1194:38–47. [PubMed: 18486944]
9. Buchacher A, Iberer G. *Biotechnol J*. 2006; 1:148–163. [PubMed: 16892245]
10. Chtourou S, Porte P, Nogr M, Bihoreau N, et al. *Vox Sang*. 2007; 92:327–337. [PubMed: 17456157]
11. Stadler M, Gruber G, Kannicht C, Biesert L, et al. *Biologicals*. 2006; 34:281–288. [PubMed: 16500114]
12. Josic D, Brown MK, Huang F, Lim YP, et al. *Proteomics*. 2006; 6:2874–2885. [PubMed: 16596706]
13. Lion N, Tissot JD. *Exp Rev Proteomics*. 2008; 5:375–379.
14. Thiele T, Steil L, Vlker U, Greinacher A. *Biodrugs*. 2007; 21:179–193. [PubMed: 17516713]
15. Fortis F, Guerrier L, Areces LB, Antonioli P, et al. *J Proteome Res*. 2006; 5:2577–2585. [PubMed: 17022629]
16. Brigulla M, Thiele T, Scharf C, Breitner-Ruddock S, et al. *Transfusion*. 2006; 46:377–385. [PubMed: 16533279]
17. Josic, Dj; Hoffer, L.; Buchacher, A. *J Chromatogr B*. 2003; 790:183–197.
18. Lawson E, Clifton JG, Huang F, Li X, et al. *Electrophoresis*. 2006; 27:2747–2758. [PubMed: 16739230]
19. Clifton JG, Li X, Reutter W, Josic D. *J Chromatogr B*. 2007; 849:293–301.
20. Eng J, McCormack AL, Yates JR III. *J Am Soc Mass Spectrom*. 1994; 5:976–989.
21. Elias JE, Gygi SP. *Nature Methods*. 2007; 4:207–214. [PubMed: 17327847]
22. Josic D, Schwinn H, Stadler M, Strancar A. *J Chromatogr B*. 1994; 662:181–190.
23. Mller-Kristensen M, Jensenius JC, Jensen L, Thielens N, et al. *J Immunol Methods*. 2003; 282:159–167. [PubMed: 14604549]
24. Roemisch J. *Biol Chem*. 2002; 383:1119–1124. [PubMed: 12437095]

Figure 1.

Chromatographic separation of diluted human plasma (1:5 diluted with Buffer A, see Materials and Methods) on a GigaCap Q anion-exchange column (AX-Col.1), column volume 10 mL. Chromatographic conditions: flow rate 5 mL/min, pressure 0.1 mPa, temperature 4°C. The concentration of elution buffer is given in the Figure. Fractions were collected and analyzed by SDS-PAGE. For additional information, see Materials and Methods. Fractions 1, 2, and 3 were eluted without addition of NaCl, fractions 4 and 5 by the application of a 0.2 M NaCl solution, and fraction 6 and 7 by 0.3 M and 0.5 M NaCl solution, respectively.

Figure 2.

SDS-PAGE of different fractions collected for proteomic investigation

Re-chromatography of pooled fractions 6 and 7 (Re-Fr.6/7) from the first chromatographic run

Protein bands that were excised from the gel and submitted to protein identifications were labeled. Identified proteins were listed in supplemented material, Table S8

Lane 1–Re-Fr.6/7, weakly bound proteins (Re_1), NaCl concentration—about 0.16 M

Lane 2–Re-Fr.6/7, proteins eluted with 0.2 M NaCl (Re_2)

Lane 3–Re-Fr.6/7, proteins eluted with 0.3M NaCl (Re_3)

Lane 4–Re-Fr.6/7, proteins eluted with 0.4 M NaCl (Re_4)

Lane 5–Re-Fr.6/7, proteins eluted with 0.5 M NaCl (Re_5)

Lane 6–Re-Fr.6/7, proteins eluted with—1.0 M NaCl (Re_6)

Lanes 7–9–Non-bound fraction from the first chromatographic run (Figure 1, fractions 1 and 2).

Figure 3.

Chromatographic fractionation for isolation of vitamin K dependent clotting factors and inhibitors, final scheme, second anion exchange chromatography (AX-Col.2). Chromatographic separation of bound proteins eluted with 1.0 M NaCl in one step during the first chromatographic run (AX-Col.1). After 5x dilution with Buffer A, protein solution was applied on a 5 mL glass column containing 2mL gel (GigaCap Q, AX-Col.2)). Chromatographic conditions: flow rate 1 mL/min, pressure 0.1 mPa, temperature 4°C. After washing with 0.2 M NaCl, bound proteins were eluted with a step gradient as shown in the Figure. Collected fractions were analyzed by SDS-PAGE and LC-ESI-MS/MS, and additionally with 2D electrophoresis.

Figure 4A

Figure 4B

Figure 4.

2D-electrophoresis of proteins, collected after second chromatographic separation, shown in Figure 3. Hundred μ g of protein from fraction eluted with 0.5 M NaCl, (Fig. 4A) and 1.0 M NaCl, (Fig. 4B) were separated. The separated spots were excised, and digested proteins were identified with LC-ESI-MS/MS (see supplemental material, Tables S9 and S10).

Table 1

List of target proteins of therapeutic interest (upper part) and some impurities (lower part) identified in fractions 6 and 7 after first chromatographic run (see Figure 1) and fractions Re₂–Re₆ obtained after re-chromatography of these two fractions (see also Figure 2). The complete list of identified proteins is given in the supplemented material, Tables S1-S9

Column 1–Pooled fractions 6 and 7 before re-chromatography

Column 2–Re-Fr.6/7, proteins eluted with 0.2 M NaCl (Re₂)

Column 3–Re-Fr.6/7, proteins eluted with 0.3M NaCl (Re₃)

Column 4–Re-Fr.6/7, proteins eluted with 0.4 M NaCl (Re₄)

Column 5–Re-Fr.6/7, proteins eluted with 0.5 M and 1.0 M NaCl (Re₅ and Re₆)

Fraction	6 and 7	Re ₂	Re ₃	Re ₄	Re ₅ and Re ₆
Active components					
FII	++	+	+++	0	+
FVII	0	0	0	0	0
FIX	0	0	++	0	0
FX	0	0	+++	0	0
Protein C	0	0	0	0	0
Protein S	0	+	+++	+	0
Protein Z	0	0	0	0	0
IaI _p	+	+	++	+++	+++
Impurities					
HSA	++	+++	+	0	+
Compl.	++	+++	++	+	+
Kin1	++	++	++	+	0
Trth.	++	+++	++	0	0
CxPN	++	++	++	++	0
Vitronectin	+	0	++	+	+
HBP2	0	0	++	+	+++
MBLSP	0	+++	0	0	0

Compl. – Complement components; Kin1 – Kininogen 1; Trth. – Transthyretin; CxPN – Carboxypeptidase N; HBP2 – Hyaluronan binding protein 2; MBLSP – Mannan binding lectin serine protease; (+++) – Proteins identified with very high score; (++) – Proteins identified with high score; (+) – Proteins identified with low score (however, higher than 2.0)

Table 2

List of target proteins of therapeutic interest identified in fractions separated accordingly to new optimized scheme (See also Figure 3). The complete list of identified proteins is given in supplemented material, Tables S11 and S12).

Fraction	n.b. _F	1 _F	2 _F	3 _F
Active Components				
FII	+	+	+++	+
FVII	0	0	0	0
FIX	0	0	++	0
FX	+	+	+++	0
Protein C	0	+	+++	0
Protein S	0	+	+++	0
Protein Z	+	0	+++	0
IaI _p	++	++	++	+++
Impurities				
HSA	++	+++	+	+
Compl.	++	+++	+	+
Cer.	++	+++	+	+
Kin1	++	+++	+	+
Trth	++	+++	+	+
Vitronectin	0	+++	+	+
FV	0	0	+	0
HBP2	0	0	0	+++
MBLSP	+++	++	0	0

Cer. – Cerruloplasmin,

For other abbreviations see Table 1.