The role of proteomics in plasma fractionation and quality control of plasma-derived therapeutic proteins

Dajana Gaso-Sokac[1], Djuro Josic[2]

[1]Department of Chemistry, J.J. Strossmayer University, Osijek, Croatia; [2]COBRE, Center for Cancer Research Development, Rhode Island Hospital and Brown University, Providence, USA.

Introduction

Human plasma is still an important row material for isolation of therapeutic proteins. Human serum albumin (HSA) was the first therapeutic protein isolated from human plasma, and plasma-derived (pd) HSA concentrates are still being used for patient treatment[1, 2]. Preparations of human immunoglobulins for intravenous use (IVIG) are currently the driving force for usage of human plasma in the fractionation industry[1]. Von Willebrand factor (VWF), protease inhibitors such as alpha-1 antitrypsin (A1-AT) and antithrombin III (AT III) and clotting factors and inhibitors are further important therapeutic proteins that are isolated form human plasma. Their concentration in this biological fluid is up to six orders of magnitude lower than HSA and IgG. Consequently, the manufacturing process for isolation of these proteins with economically sufficient yield and in form of safe and active concentrates can be a true challenge[2,3].

Virological safety of human plasma and human plasma-derived therapeutic products is still a primary concern, and high safety standards have been implemented to make these products virologically safe[2-4]. Although all plasma-derived therapeutic preparations that are on the market belong to so-called "well-characterized biologicals" [5] they still contain relatively high amount of foreign proteins. In contradiction to this name, most impurities are poorly characterized[6].

After a relatively slow start, proteomics has finally progressed into different branches of transfusion medicine[1,7,8]. As shown in figure 1, proteomic technologies can be used as an efficient tool for process development and characterization of human plasma-derived therapeutic proteins. As shown in this figure, proteomics can be used for process validation and quality control of these therapeutic proteins. It has been demonstrated that proteomics also offer fast and efficient ways for the identification of potentially harmful impurities[6]. After their identification, the time for process optimization towards their removal can be significantly shortened[6, 9]. For quality control of final products, proteomics can be used for characterization of the active component, detection of impurities and determination of batch-to-batch variations[10-12].

Human serum albumin

Human serum albumin (HSA) is present in human plasma at about 35 mg/mL, and it is the most abundant protein in this biological fluid. Essentially all therapeutic pd HSA preparations are manufactured

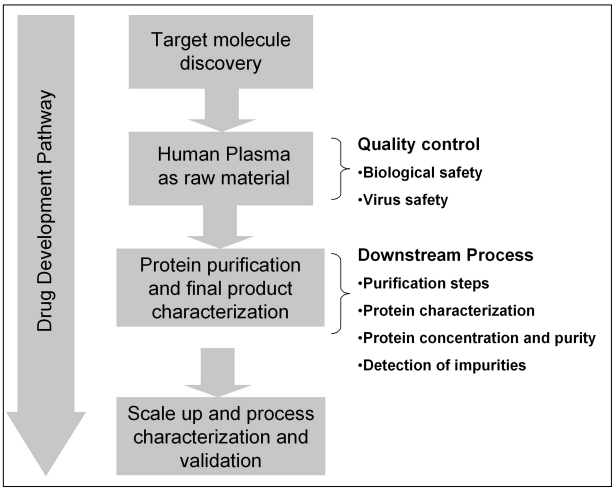

Figure 1 - Application of proteomics for validation of the production process of a therapeutic protein concentrate from human plasma and for final product characterization.

form human plasma after cryoprecipitation by ethanol fractionation[2]. These concentrates contain between 95 and 98% HSA, and it is well known that they contain some plasma proteins as impurities. To get highly pure HSA, e.g. for measurement of its potential biological activity, additional purification steps are necessary[13]. Fortis et al. [10] analyzed the 98% pure, injectable HSA solution, and after direct analysis by SDS-PAGE followed by mass spectrometry of electrophoretically separated proteins, only a single impurity, haptoglobin, was identified. However, after enrichment of trace proteins by use of hexapeptide library beads, 13 additional plasma proteins were identified as impurities in this preparation. The list of these impurities is given in table I. As shown in this table, no potentially harmful proteins such as proteases, clotting factors and inhibitors were identified, and this preparation can be considered as biologically safe. However, this work demonstrates, that proteomic investigation gives a lot of additional information about the concentrate composition, and can be very useful, especially if rare side reactions occur after use of such therapeutics. Consequently, further investigations, especially comparison between the products of different producers and batch-to-batch variations for single products are still necessary.

Table I - Proteins identified from injectable HSA concentrate after treatment with peptide ligand library. Modified from Reference[10] with permission

Identified proteins	gi	% Sequence coverage
Human serum albumin	178,344	44
Haptoglobin	386,783	26
Hemopexin	204,621	18
Alpha2-HS glycoprotein	7,106,502	2
Ferroxidase (cerruloplasmin)	116,117	7
Afamin precursor	4,501,987	9
Human beta-2-glyco-protein-I (Apolipoprot. H)	6,573,461	10
Peptidoglycan recognition protein	18,202,143	7
L precursor C1 esterase inhibitor	124,096	9
Alpha-1B-glycoprotein precursor	21,071,030	6
Transthyretin	4,572,572	41
Hemoglobin chain D	56,749,856	11
Vitamin D-binding protein	639,896	5
Immunoglobulin lambda chain	791,179	8

IVIG

Clotting factor VIII used to be the driving force for human plasma usage at the end of the last century. Now, IVIG concentrates are the leading products of the plasma fractionation industry. Recent reviews about the production and quality control of these concentrates deal mostly the aspects of yield and virus safety[14, 15]. Quality controls that are performed for release of IVIG batches are determination of subclass composition and contents of some potentially harmful proteins that can use adverse reactions (e.g. IgA and IgM), endotoxins and chemical (e.g. residues of viral inactivation treatments) impurities[15]. Recently, Buchacher et al.[16] investigated the anticomplementary activity (ACA) of IVIG concentrates caused by high concentration of large size polymers in some concentrates. They could demonstrate that conditions under which polymers are formed have an influence on the ACA outcome, but also other impurities related to the starting material and unforeseen changes of process condition might affect the ACA and lead to batch-to-batch variations. Further detection and characterization of these impurities, e.g. by above discussed hexapeptide library beads[10] could give additional information about their identity and possible side effects. In an earlier paper, Page et al. [17] demonstrate that IgG fragmentation that can cause impaired efficacy of IVIG concentrates can be caused by contamination with serum proteases such as plasmin and kallikrein. However, proteomic investigations of different IVIG preparations and batch-to-batch comparison to show potential variations of the concentrates that are on the market are still outstanding and have to be performed.

Clotting factor VIII and von Willebrand factor

Clotting factor VIII (FVIII) circulates in human plasma in a stable complex with von Willebrand factor (VWF). VWF also functions as a carrier and stabilizing protein that protects FVIII from proteolysis and clearance, and it is the reason that in some pd FVIII concentrates this clotting factor is still in complex with his carrying and protecting protein VWF[2,12]. All pd FVIII concentrates that are currently

on the market are purified from cryoprecipitate. Further purification steps are precipitation with aluminum hydroxide or glycine followed by chromatographic steps such as anion-exchange, monoclonal antibody (using immobilized anti-FVIII or anti-VWF murine antibodies) and heparin affinity chromatography . All products are, at least, double virus inactivated[2]. Plasma-derived FVIII concentrates that are on the market have a specific activity between about 80 and 250 IU/mg. Specific activity of some plasma-derived FVIII concentrates that contain human serum albumin is even lower. Theoretic specific activity of pure FVIII is one to two orders of magnitude higher[12]. That means that all plasma-derived FVIII concentrates contain a significant amount of foreign proteins. Although the purity of pd FVIII concentrates has not been convincingly demonstrated to enhance their immunological safety[2], the thorough investigation of their composition and batch-to-batch variations is necessary. FVIII molecule is very sensitive to proteolysis, activation and degradation, and the most serious immunological complication in the treatment of haemophilia A is the development of inhibitory antibodies, called inhibitors, most frequently at an early stage of therapy[18]. There have been dangerous outbreaks of inhibitors in previously treated patients in the past, and they seem to be due to creation of neoepitopes in the clotting factor molecule during the manufacturing process[19]. This possibility necessitates careful monitoring of the production process, batch-to-batch variations and additional characterization of the final product by use of proteomics[12].

In order to identify the levels of impurities in three commercial, double virus inactivated pd FVIII/VWF concentrates, we used proteomic methods for their analysis. Anion-exchange was the chromatographic method used for isolation of all three concentrates. For purification of Octanate and Haemoctin, two concentrates developed in 1990s, a low-capacity polymer-based resin was used[12]. Wilate, a recently developed FVIII/VWF concentrate, was isolated by use of anion-exchange chromatography on a newly developed, high-capacity polymer-based resin. In order to remove low-molecular weight impurities, an additional size-exclusion chromatographic step was added[12,18]. The major impurities in all three concentrates were inter-alpha inhibitor proteins, fibrinogen and fibronectin. Clotting factor II (FII, prothrombin), the protein known as a potential activator of FVIII, was also identified. In some F VIII batches that were purified by anion-exchange chromatography we also identified hyaluronan binding protein 2 (HBP2), a serum protease that also can cause unpredicted FVIII activation and fragmentation [Gaso-Sokac, Josic, unpublished results]. In further analyses, batch-to-batch variations and differences between three investigated products were analyzes using iTRAQ, an isotope labeling technique for comparative mass spectrometry[20]. As shown in table II, the amount of impurities in Wilate was significantly reduced, and HBP2 was never detected in this concentrate. The amount of potentially harmful component FII was also more than one order of magnitude lower. These investigations demonstrate the potential value of proteomic technology for quality control analysis, batch-to-batch control and comparison between different products of this clotting factor concentrate.

FVIII concentrates that contain high molecular weight VWF multimers, such as Wilate, can also be used as VWF source for treatment of von Willebrand disease (VWD)[2, 18]. The VWF concentrate Wilfactin that is used in France for exclusive treatment of VWD is double virus inactivated, and a virus removal step by nanofiltration was also added. There are functional and some biochemical studies of this concentrate[21], but a proteomic analysis of the production process and the final product is still outstanding.

Clotting factor IX

Plasma derived clotting factor IX (pd FIX) concentrates are isolated from cryopoor human plasma by a combination of anion-exchange with either immobilized heparin, metal chelate affinity or monoclonal antibody immunoaffinity chromatography. All products on the market are virus inactivated[2]. Some of pd FIX concentrates that are on the market still contain a relatively high amount of foreign proteins, especially if their isolation process does not contain the affinity step. Even if affinity chromatography step is added into the purification scheme, these processes yield FIX concentrates with a specific activity of 100-150 IU/mg[2,22]. The specific activity of pure FIX is higher that 200 IU/mg. That means that these FIX concentrates still contain a

Table II - Quantitative comparison of impurities in some pd FVIII concentrates. Reprinted from Reference[12] with permission

			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	Fibrogen, 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	Fibrogen, βchain	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^{-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.

significant amount of foreign proteins. Complement C4 and inter-alpha inhibitor have been identified as contaminating proteins in FIX concentrates that are isolated by a combination of anion-exchange and heparin affinity chromatography[23]. As shown in an early proteomic analysis of similar pd FIX concentrates, vitronectin was overseen as main contaminant in these investigations[22].

Recently, proteomics were used for validation of the production process of pd FIX and for final product characterization[24]. It was demonstrated some serine proteases such as mannan binding lectin serine protease (MBLSP) and HBP2 were not completely removed by anion-exchange chromatography. These potentially harmful components that may be responsible for some side effects if low-purity FIX concentrates were used[2] are now removed by affinity chromatography on immobilized heparin. The quantitative comparison of FIX content and impurities in two final production steps, affinity chromatography on immobilized heparin and nanofiltration was performed by use of isobaric tag for relative and absolute quantitation (iTRAQ). It was demonstrated that residual impurities that are still present after heparin affinity chromatography such as inter-alpha inhibitor proteins and C1 inhibitor are removed by nanofiltration. Final product was pure FIX with a specific activity higher than 200 IU/mg[24]. As shown in this paper, the use of proteomics can significantly extend the knowledge of this industrial process, and proteomics technology can be used for further characterization of final FIX preparations.

Prothrombin complex concentrate

Prothrombin complex concentrate (PCC) is a mixture of vitamin K dependent clotting factors II, VII, IX and X (FII, FVII, FIX and FX) and clotting inhibitors protein C, protein S and protein Z. All clotting factors and inhibitors in PCC have a low specific activity that is not higher than 0.5-2 IU/mg[2]. The production process is based on a method that involves solid-phase extraction with strong anion-exchanger Q Sephadex and subsequent anion-exchange chromatography[3].

Brigulla et al.[25] analyzed three PCC concentrates by use of one and two-dimensional electrophoresis and MALDI-TOF mass spectrometry. In all three concentrates, active components FII, FIX, FX, protein C and protein Z and 35 additional impurities were identified. Kovac et al.[6] fractionated dilute cryopoor plasma by anion-exchange chromatography. In the fraction containing highly enriched vitamin K dependent clotting factors and inhibitors (in this case, protein S was also identified), some potentially harmful proteases such as MBLSP and HBP2 were also identified[6]. These impurities that were not identified by Brigulla et al.[25] may cause thrombogenicity of some PCC concentrates and cannot be completely removed if such isolation scheme was applied. Interestingly, in both investigations FVII clotting activity was measured in an *in vitro test*, but this clotting factor was not identified by mass spectrometry. The reason may be degradation of this highly sensitive protein by serum proteases that are still present in this highly complex protein mixture[24].

Other therapeutic proteins isolated from human plasma

The pd anticoagulant antithrombin III (AT III) is usually isolated from cryopoor plasma by a combination of anion-exchange chromatography and affinity chromatography on immobilized heparin. Viral inactivation is achieved by pasteurization and/or solvent/detergent treatment. In order to remove partially denatured AT III, affinity chromatography on immobilized heparin can be added to the purification scheme[2]. AT III is a glycoprotein from human plasma for which a correlation between a certain pattern in glycosylation and anticoagulant activity is well known, and most proteomic investigations were performed in order to investigate its glycosylation pattern. Interestingly, the variability in glycosylation of this physiologically important glycoprotein was found to be relatively low[11, 26]. Unfortunately, no investigations of potential correlation between changes in quantitative glycosylation patterns and AT III activity and possible role of glycosylation of this protein in certain diseases that can be caused by AT III abnormality were performed.

The Cohn fraction IV is usually used as starting material for production of alpha-1-antitrypsin (A1-AT). Further purification steps involve PEG precipitation and anion-exchange chromatography. Two virus reduction steps, mostly solvent/detergent treatment followed by heat treatmen are now used to insure the virus safety[2]. Kolarich et al.[27] analyzed three different A1-AT commercial products were analyzed by use of different high-resolution separation methods followed by mass spectrometry, peptide mapping and investigation of the glycosylation pattern. This impressive study showed that all commercially available A1-AT products differ from A1-AT directly analyzed in human plasma. These alternations seem to occur during the production process. The authors conclude that the observed differences most likely do not play a substantial biological role, but a direct comparison of these products and their *in vivo* activity was not performed[27]. Unfortunately for both protease inhibitors, AT III and A1-AT, proteomic investigations in order to validate the production process and to detect impurities, especially possible harmful components, and batch-to-batch variations were still not performed.

Inter-alpha-inhibitor proteins (IaIp) can be isolated from the side fraction after heparin affinity chromatography during F IX isolation[28]. Recent results in animal studies IaIp suggest the potential of this protein family as a diagnostic and therapeutic agent for treatment of sepsis[29]. A proteomic validation of the isolation process and characterization of the final product was performed. Prothrombin (FII) was identified as a potentially harmful contamination in the final product, and the production process was modified in order to remove this protein[29].

Conclusions

Proteomics technology is a very useful tool for validation of existing processes for production of therapeutic proteins from human plasma, and for development and fine-tuning of new production processes. Furthermore, this technology can be used for characterization and quality control of final products, and for detection and minimizing of batch-to-batch variations.

Key words: proteomics, quality control, plasma fractionation, Factor VIII.

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Correspondence: Djuro Josic
Proteomics Core
COBRE Center for Cancer Research Development
CORO West, Suite 4.206, One Hoppkin St
Providence, RI 02903, USA
E-mail: Djuro_Josic@brown.cdu