Identification and differentiation of coagulation factors in Prothrombin complex concentrates (PCC's)

C. Wilhelm1, M. Mandago2, S. T. Kiessig2, S. Wittke1

[1] University of applied Sciences Bremerhaven, An der Karlstadt 8, 27568 Bremerhaven, Germany

[2] PreviPharma Consulting GmbH, CUBEX41, Universitätsmedizin Mannheim, Haus 41, Theodor-Kutzer-Ufer 1-3, D - 68165 Mannheim

E-Mail: swittke@hs-bremerhaven.de

1. Introduction:

Blood from patients receiving vitamin K antagonists has low levels of blood clotting proteins. Therefore, these patients are at increased risk of spontaneous and traumatic bleeding, leading to a progressive loss of blood clotting proteins. This creates a vicious cycle that increases the risk of disease and death [1].

Prothrombin Complex Concentrate (PCC) is a drug that contains proteins involved in the blood clotting process and is made from human plasma. It contains the blood coagulation factors II, VII, IX, X, protein C and protein S. These coagulation factors are vitamin K-dependent and in the absence of any of these factors, the blood does not clot as quickly as usual, leading to increased bleeding tendency. PCCs are therefore used to treat [2]:

- treatment of bleeding
- prevent bleeding immediately before or after surgery
- treatment of acquired or congenital deficiency of coagulation factors

In order to avoid unwanted side effects, the PCCs usually only contain the inactive coagulation factors. Greater "impurities" with the active forms should be avoided as they can cause serious fatal complications such as disseminated intravascular coagulation (DIC) or involve the risk of a reduced durability of the product, since the active forms of the coagulation factors can activate further coagulation factors from the coagulation cascade[3]. These would therefore no longer be available in the drug. This activation may occur during manufacture, during bottling, storage, reconstitution (e.g., lyophilized products) or even after reconstitution of lyophilized products with e.g. water.

2. Material and methods:

To date, direct detection of the clotting factors is difficult to achieve since factor evidence is based on PTT (prothrombin time), TPZ (thromboplasmin time), clotting and chromogenic assays ignoring the activation state of the serine proteases or just hinting at it. Factors such as F.VII, F.X, F.V, prothrombin (F.II) and fibrinogen are measured. Prolonged clotting times are an indication of a factor deficiency, which is corrected by the addition of normal plasma [4]. This approach illustrates the need for a fast, direct and reliable method for detecting and quantifying coagulation factors (active / inactive) in body fluids, plasma fractions and drugs.

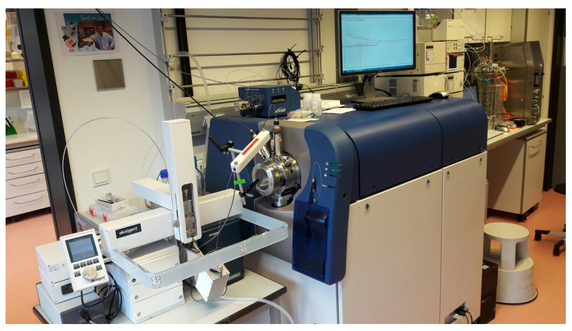

Figure 1:LC-MS-System (TripleToF 4600) used for MRM-Investigations of PCCs and coagulation factors

For identification and differentiation of the inactive and active forms of four pairs of coagulation factors F.II, F.VII, F.IX and F.X a bottom-up liquid chromatography-mass spectrometry (LC-MS/MS; Triple-Tof 4600 ABSciex, figure 1; protease digest; figure 2) assay was established.

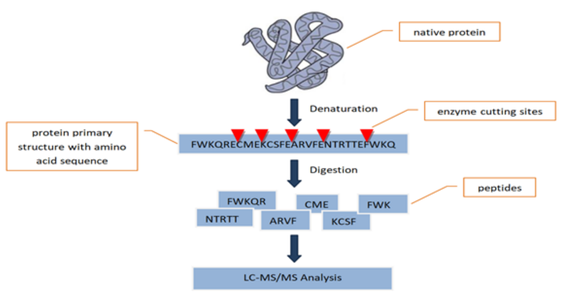

Figure 2: Bottom-up proteomics approach for the identification, differentiation and quantification of active / inactive forms of different coagulation factors

3. Results

For all coagulation factors investigated, specific peptides suitable for identification and differentiation could be identified after proteolytic digestion, as exemplarily shown for F.VII (Figure 3a,b).

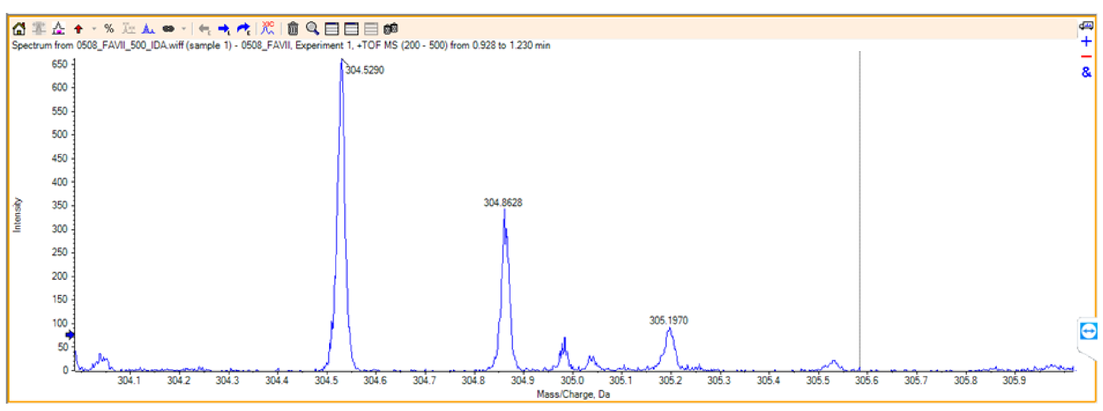

Figure 3a: Extracted Ion chromatogramm (range 304 - 306 m / z) for the monoisotopic peptide signal (indicative for F.VII; m / z = 304.5290; z = 3; M_r = 910.56 g/mol) in F.VII

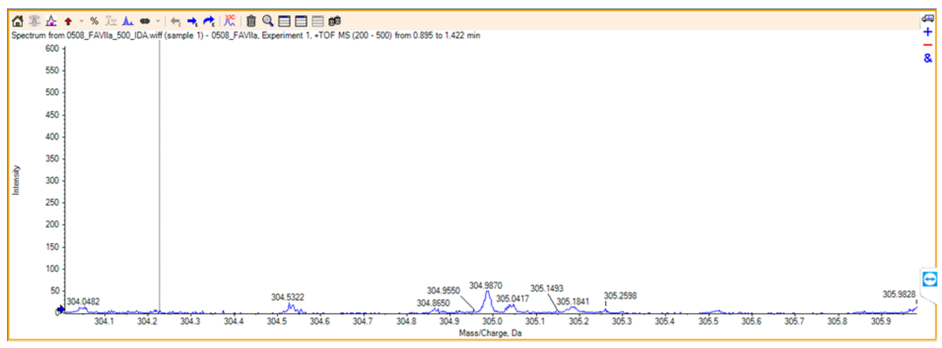

Figure 3b: Extracted Ion chromatogramm (range 304 - 306 m / z) for the monoisotopic peptide signal (indicative for F.VII; m / z = 304.5290; z = 3; M_r = 910.56 g/mol) in F.VIIa

While the indicative peptide can be detected with high intensity in F.VII, the discriminatory peptide (mass 910.53 Da; m / z: 304.53, z = 3) is only detectable in small amounts as an impurity in pure F.VIIa. Similar investigation for F.VII, F.IX, F.X indicate that the assay is suitable for all coagulation factors investigated.

Subsequently the standardized workflow (Figure 4) was adapted to PCCs to determine the purity and stability of the coagulation factors.

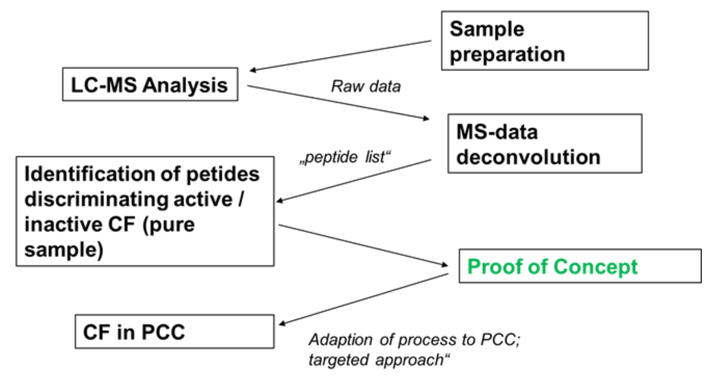

Figure 4: Final workflow for bottom-up proteomics analysis for the identification (detection), differentiation and quantification of active / inactive forms of various coagulation factors in body fluids, plasma fractions and pharmaceuticals

By the use of two discriminative peptides (Figure 5) prothrombin (F.II) can be detected in a commercially available PCC (Beriplex; upper MS-spectrum; blue) and the reference material (F.II; red) whereas the activated form (lower MS-spectrum; orange) only occurs in the reference material (F.IIa).

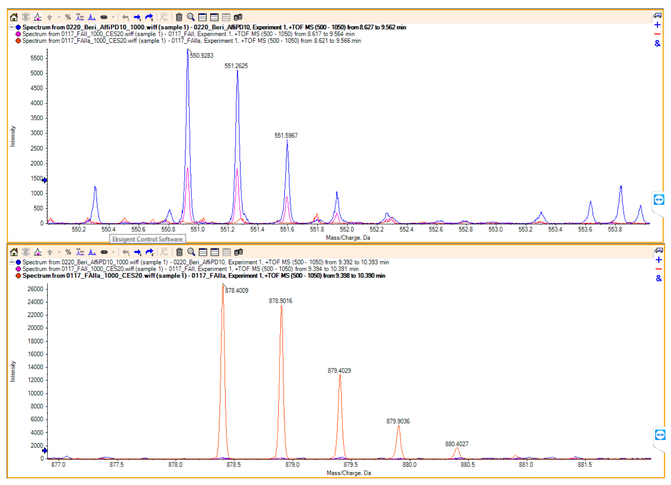

Figure 5: Differentiation of F.II and F.IIa by LC-MS/MS in PCCs. The peptide with the m/z-ratio of 550,9283 (3-fold charged) occurs only in prothrombin and the peptide with the m/z-ratio 878,4009 (2-fold charged) can only be detected in α-thrombin

5. Conclusion:

- the established assay can be used for all coagulation factors investigated,
- the assay developed, reliably discriminates between active and inactive forms of the respective coagulation factors,
- all coagulation factors can be identified and discriminated in PCCs like Beriplex and similar products supplied by Previpharma,
- recent results indicate that quantification of active and inactive factors is possible,
- assay validation confirmed that sample preparation did not influence the composition of PCCs, since spiking the original PCC with the respective coagulation factors basically only influnced the signal intensity of the added compound,

In conclusion this assay might be used in release analytics of the respective products by the pharmaceutical industry to determine the content of the respective coagulation factors as well as to determine the active - / inactive - CF-ratio.

6. Literature:

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