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Characterization of factor VIII pharmaceutical preparations by means of MudPIT proteomic approach

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ABSTRACT

For a good clinical outcome of Haemophilia A substitutive therapy a detailed characterization of factor VIII (FVIII) concentrates is required, in order to disclose the eventual relations between differently composed concentrates and their biological effects. This preliminary work could be a first step towards a deep structural characterization of FVIII concentrates, using the fast and simply manageable MudPIT technology, which enables the identification and characterization of protein mixtures taking advantage of both the high separation capacity of two-dimensional chromatography and the powerful peptide characterization ability of tandem mass spectrometry. The aim of this study was to evaluate the suitability of for the characterization of FVIII molecule in complex mixtures such its commercial concentrates, both plasma-derived and recombinant, and for the determination of the protein composition of different FVIII preparations. By means of Multidimensional Protein Identification Technology (MudPIT) it was possible to assess the presence of factor VIII in its preparations and to identify most of the contaminant proteins without gel separation.

In particular, 125 and 42 proteins were identified in plasma-derived and recombinant concentrates, respectively. Concerning investigation of FVIII, 24 different peptides were identified in plasma-derived corresponding to 7, 29, 27, 19 and 67 of percentage coverage for A1, A2, A3, C1 and C2 domains, respectively. About its multimeric carrier von Willebrand factor (VWF), we have sequenced 42% of domain interacting with A3 and C2 domains of FVIII. Finally, it has been observed that normalized parameters, such as total peptide hits obtained by SEQUEST may be used for evaluation of the relative abundance of FVIII in different preparations.

1. Introduction

Haemophilia A is the most common severe life-long bleeding disorder. It is caused by an inherited deficiency of factor VIII (FVIII) due to mutations in the corresponding gene. Its severity correlates with the residual FVIII activity found in plasma and depends on the type of FVIII gene mutations [1]. In its most serious forms, when FVIII activity is below 1% of normal, this disorder is life-threatening [2]. In such affected individuals, haemostasis may be restored by replacing the missing or defective protein with FVIII either isolated from natural source or expressed as a recombinant analogue in mammalian cells [3].

FVIII is a large multi-domain glycoprotein with domain structure A1-α1-A2-α2-B-α3-A3-C1-C2, and is involved in the cascade of biochemical reactions leading to coagulation [4,5]. In the serum, FVIII is cleaved into heavy (A1-α1-A2-α2-B) and light chains (α3-A3-C1-C2), which are held together by a divalent metal ion [4,6]. This heterodimer circulates in association with von Willebrand factor (VWF), a large multimeric glycoprotein whose monomers are held together by disulfide bridges and non-covalent interactions [7,8]. The stoichiometry of factor VIII and VWF in plasma is approximately 1:50; therefore, VWF represents most of factor VIII–VWF complex in terms of weight [9]. Binding of factor VIII to VWF is essential for the survival, stabilization and function of FVIII in vivo [5]. The underlying mechanism probably relies on the fact that FVIII is bound to VWF and is therefore protected from phospholipid-dependent proteolysis by activated protein C and factor Xa [10,11].

Because FVIII and VWF form a tightly bound non-covalent complex in plasma [12], both proteins are co-purified when isolated from plasma, unless special measures are taken [13]. FVIII cir-

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culates in plasma at a very low concentration (0.1–0.2 μg/ml), and represents only a small proportion of plasma protein content. In plasma-derived preparations (pdFVIII), often defined as high-purity products, FVIII still represents only 1–2% of total protein content. PdFVIII concentrates are in fact complex protein mixtures, as they contain other plasma proteins such as fibrinogen and fibronectin [11–14].

Recombinant preparations (rFVIII) contain instead FVIII highly purified, but they require the addition of large amounts of stabilizers such as human serum albumin (HSA) or VWF [15].

One of the most important complications in patients treated with FVIII concentrates is the development of antibodies inhibiting FVIII activity. The latter are called inhibitors, and are directed towards the A2, C2 and A3 domains of the molecule. About 25–30% of patients develop antibodies inhibiting FVIII activity. The inhibitory effects of antibodies occur at various stages of the FVIII functional pathway, including FVIII binding to VWF, activation of FVIII by thrombin, and activated FVIII (FVIIIa) incorporation into the Xase complex [16].

The detailed analysis of FVIII preparation composition and of the structure of FVIII itself is therefore crucial in order to improve the clinical outcome of FVIII replacement therapy. Because FVIII preparations are complex protein mixtures, their analysis has represented a challenge, due to the relatively low levels of this protein in concentrates, the high abundance of VWF and the presence of other plasma proteins [13,14].

Clinical chemistry assays of FVIII preparations are based on the clotting and chromogenic methods, which determine the FVIII procoagulant activity [17], and ELISA methods and immunoblotting which allow the determination of FVIII:Ag. Molecular characterization of FVIII preparations is based on electrophoresis (mono-, 1D and bi-dimensional, 2D) [18], chromatography (like size exclusion, immunoaffinity and reversed phase) [19] and mass spectrometry using MALDI-TOF [18] or electrospray usually coupled to liquid chromatography (LC/ESI-MS) [19].

However, these methodologies give a poor characterization of the protein content of FVIII enriched preparations. A new proteomic methodology based on two-dimensional capillary chromatography coupled to tandem mass spectrometry (2DC-MS/MS), also named Multidimensional Protein Identification Technology (MudPIT), has been developed recently [20]. This approach does not require gel separation and it is a mass spectrometry-based method. It involves the generation of peptides by enzymatic digestion of a complex protein mixture, their separation by means of two micro-HPLC columns (cation exchange and reversed phase, 2DC) and direct analysis of eluted peptides by tandem mass spectrometry (MS/MS). Using an appropriate software, such as the SEQUEST algorithm, based on sequence database searching, the full and MS/MS spectra are then correlated to specific peptide sequences and the corresponding protein are identified [21]. MudPIT takes advantage of both the high separation capacity of 2DC (enabling the characterization of proteins with extreme isoelectric point, molecular weight or hydrophobicity) and peptide identification by amino acid sequencing. In addition, MudPIT technology is reported to supply quantitative data [22,23].

The aim of this study was to evaluate the suitability of a MudPIT approach for the characterization of FVIII in its commercial preparations, and determination of the protein profiles of these complex mixtures. An in-depth and rapid characterization of FVIII concentrates could disclose an eventual relation between different batches or products and their biological effects.

Besides the identification of an adequate coverage of FVIII sequence, we were able to identify most of the contaminant proteins, including those unlikely to be detected with other traditional and labour-intensive approach like the electrophoretic one.

2. Materials and methods

2.1. FVIII concentrates

Emoclot D.I. (Kedrion S.p.A., Lucca, Italy) is an high-purity, plasma-derived FVIII concentrate (pdFVIII) obtained through anion-exchange chromatography (1000 IU, 10 mg total protein content); a commercial recombinant FVIII (rFVIII; 1000 IU) (Baxter International Inc., Deerfield, IL, USA) obtained from a genetically engineered Chinese Hamster Ovary cell line, stabilized with human serum albumin (HSA 12.5 mg/ml), was used [24].

2.2. Samples preparation

Due to its high amount of albumin, the rFVIII sample was treated with Microcon YM-100 (cut-off 100 kDa) Centrifugal Filter Device (Millipore, Billerica, MA, USA) in order to separate FVIII from HSA, whose high content hampers FVIII detection. After the centrifugation (14,000 × *g* for 12 min) the filter was subjected to 4 washing steps with ammonium bicarbonate 100 mM pH 8.

Treated recombinant and plasma-derived samples were diluted (1:1) with RapiGest™ SF 1% (Waters, Milford, MA, USA). Proteins/RapiGest™ SF mixtures (both rFVIII and pdFVIII) were heated to ∼100 °C for 5 min and then samples were collected before reduction and alkylation. PdFVIII and rFVIII samples (50 μL) were reduced with 20 mM dithiothreitol (DTT, Sigma–Aldrich, Milan, Italy), at 37 °C for 4 h. The samples were then alkylated by the addition of iodoacetamide (IAA, Sigma–Aldrich, Milan, Italy) to 100 mM and incubated at the same temperature, in the dark, for approximately 1 h.

Sequencing grade modified trypsin (Promega, Madison, WI, USA) was added at enzyme–substrate ratio around 1:50 (w:w). After overnight incubation at 37 °C, the reaction was stopped by acidification with trifluoroacetic acid (Sigma, St. Louis, MO, USA) to a final concentration of 0.5%, pH ∼1.5. Trifluoroacetic acid also causes RapiGest™ SF inactivation. Samples were incubated at 37 °C for 45 min and then centrifuged at 14,000 × *g* for 10 min. Peptide mixtures were purified and concentrated using PepClean™ C-18 Spin Columns (Pierce, Rockford, IL, USA). Samples were dried in Speed-Vac at 60 °C. 20 μl of 5% ACN/0.1% formic acid solution was then added.

2.3. 2DC-MS/MS analysis

Digested pdFVIII and rFVIII samples were analyzed by means of two-dimensional microchromatography coupled to ion trap mass spectrometry (2DC-MS/MS, also referred to as MudPIT), Briefly, 5 μl of the digested peptide mixture was first separated by ion exchange chromatography (BioBasic-SCX column, 0.32 i.d. × 100 mm, 5 (m, Thermo Electron Corp., Bellofonte, PA, USA) by applying a ten-step ammonium chloride concentration gradient (5, 10, 15, 20, 30, 40, 80, 120, 400, 700 mM). Each salt step eluate was directly loaded on a C_{18} reversed-phase column (BioBasic-18, 0.180 i.d. × 100 mm, 5 (m, Thermo Electron Corp., Bellefonte, PA, USA) and separated with an acetonitrile gradient (eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile); Each of the 10 cycles had the following profile: 5 min of 5% B, a 45 min gradient from 5 to 65% B, 3 min of 65% B, a 10 min gradient from 65 to 95% B, 5 min of 95% B, a 5 min gradient from 95 to 5% B, and finally 17 min of re-equilibration with 5% B; flow rate, 2 μL/min.

Peptides eluted from the C_{18} column were analyzed directly with a linear ion trap LTQ mass spectrometer equipped with nano-ESI source. The heated capillary was held at 185 °C, ion spray 1.5 kV and capillary voltage 47 V. Spectra were acquired in positive ion mode (in the range of 400–2000 m/z) using dynamic exclusion for MS/MS analysis (collision energy 35%). The mass spectra were