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Review

Use of high-resolution techniques for the characterization of clotting factor VIII

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Abstract

Dealing with the structural characterization of clotting factor VIII (FVIII) requires the application of several high-resolution analytical techniques. Besides the analytical point of view, a detailed knowledge of FVIII structure, production and therapeutic application is necessary. This review gives an overview of most of the currently applied analytical methods and how they deal with the complex analytical problem, investigating FVIII in a sample matrix containing large amounts of accompanying plasma proteins.

Keywords: Reviews; Factor VIII; Proteins; Glycoproteins; Von Willebrand factor

Contents

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1. Introduction

Clotting factor VIII (FVIII) plays an important role in the intrinsic pathway of the blood coagulation cascade. It is a glycoprotein of complex structure, consisting of a heavy and a light chain and circulates in plasma in a complex with von Willebrand Factor (vWF) as a stabilizer [1]. During activation, FVIII is cleaved at defined sites, resulting in a heterotrimer that is assumed to be the active principle [2]. During inactivation, this trimer dissociates and is further cleaved [3]. Reduced levels and a missing or dysfunctional FVIII glycoprotein are associated with the disease known as hemophilia A [4]. Treatment of hemophilia A is accomplished by infusions of FVIII concentrates [5,6]. They are prepared either from human plasma or by recombinant technology. As the concentration of this glycoprotein in plasma and in the cell culture supernatant is considerably low, FVIII has to be enriched several orders of magnitude and in parallel to be purified and virus-inactivated [7,8]. FVIII is sensitive to proteolysis, activation and degradation and has therefore to be stabilized during the production process and in the final formulation. Virus safety, effectiveness and the absence of side-effects of the product have to be fulfilled as the basic requirements [9]. The most serious complication in therapy is the development of FVIII procoagulant activity-inhibiting antibodies, so-called inhibitors [10]. Inhibitors are almost always developed by patients at an early stage of therapy and seem to be inherent to the protein structure. The outbreak of inhibitors in multitransfused patients on the other hand is speculated to be due to neoantigen formation, an undesirable structural change of the protein during the manufacturing process [11,12]. Therefore, the production process must be evaluated by suitable methods and the final product thoroughly characterized in order to guarantee a highly purified, biologically active FVIII concentrate [13]. High-resolution analytical methods are necessary for enrichment from plasma and characterization of human-plasma-derived FVIII (pdFVIII) in the presence of large amounts of stabilizer, such as human serum albumin and accompanying plasma proteins. Concerning recombinant FVIII (rFVIII), structural analysis of FVIII is closely related to analyzing post-translational modifications [14–17]. They are dependent on the cell type chosen for FVIII expression [18] as well as the conditions during cell growth [19], and have to be as similar as possible to human pdFVIII in order to avoid side effects of therapy. In both cases, some known post-translational modifications on FVIII are required for its secretion (glycosylation of the B-domain [20]) and function (sulfation at Tyr1680 for binding to vWF [21]). The roles of post-translational modifications for secretion and activity of coagulation factors were recently reviewed by Kaufman [22]. Therefore, post-translational modifications have to be monitored carefully and the batch-to-batch characteristics have to be compared. Detailed knowledge of the structure of rFVIII requires the same high resolution methods for pdFVIII for comparison reasons and to fulfill the demand for a well-characterized biological pharmaceutical [23].

The methods described in this review cover those that are widely used for enrichment and purification of FVIII, in process control and product characterization of concentrates and in detailed structural analysis of the FVIII protein. They comprise immunochemical methods, chromatography, electrophoresis, mass spectrometry and biosensors. Their advantages, capabilities and drawbacks will be discussed in terms of the information gained and the limits of characterization.

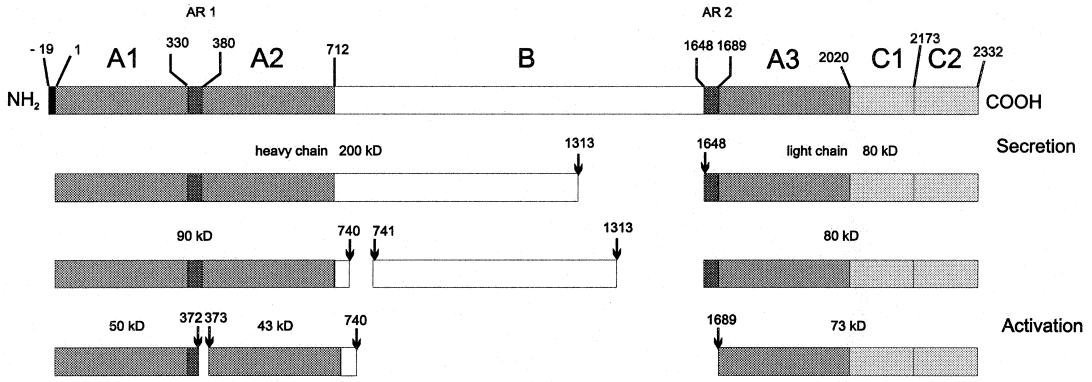

Fig. 1. Schematic representation of the structure and domain assignments of blood coagulation factor VIII after secretion and thrombin activation according to Kaufman ([3], reprinted with permission). The cleavage sites are indicated by arrows and numbers of amino acids. Numbering does not include the propeptide shown at the N-terminus, from amino acids −19 to −1.

2. Structure of factor VIII

Mature FVIII protein consists of 2332 amino acids with a calculated molecular mass on the basis of the amino acid sequence, without considering the post-translational modifications, of M_r 264 000 [2]. Due to amino acid homologies, the sequence of FVIII is divided into domains: three A domains, two C domains and one B domain [2], arranged from the N- to the C-terminus as A1–A2–B–A3–C1–C2 (Fig. 1). Furthermore, there are two acidic regions within the molecule, which are rich in aspartic and glutamic acid residues [acidic region 1, AR1 (amino acids 330–379 in the heavy chain) and acidic region 2, AR2 (amino acids 1649–1689 in the light chain)]. By cleavage at amino acids 1313 [2] and 1647, the FVIII heavy and light chain are generated. This heterodimer stays associated by a metal ion bridge and is stabilized by non-covalent binding to its carrier protein, von Willebrand Factor. The light chain has a molecular mass of about 80 000. Due to proteolysis from the C-terminus within the B-domain, the molecular mass of the heavy chain is between 90 000 and 200 000 [24].

3. Factor VIII in the blood coagulation cascade

3.1. Activation and inactivation

For activation and inactivation, the FVIII amino acid backbone is cleaved at definite sites. FVIII is activated by proteolytic cleavage by thrombin (activated FII, FIIa) or activated factor X (FXa) in the presence of phospholipids [2]. Thrombin cleaves the heavy chain between amino acids 740 and 741, resulting in the formation of a M_r 90 000 polypeptide. By further cleavage between amino acids 372 and 373, two polypeptides with molecular masses of 50 000 and 43 000 are produced. The FVIII light chain is cleaved between amino acids 1689 and 1690. The molecular mass of the remaining light chain is about 73 000. The molecular mass of the B domain is about 103 000, with fragments of lower molecular mass. Activated FVIII (FVIIIa) is a heterotrimer consisting of the A1 domain, the A2 domain and the shortened light chain. The B domain, however, does not seem to be necessary for the coagulation activity of FVIII [25]. FVIIIa does not contain the region AR2, which is assumed to be the main binding region for vWF; FVIIIa is not further associated with vWF. Inactivation occurs due to the dissociation of the three polypeptide chains and by the proteolytic action of FXa and activated protein C (APC). FXa cleaves at the same sites as thrombin and between amino acids 336–337 and 1721–1722 [26].

3.2. Binding to von Willebrand factor and in the factor Xase complex

vWF is important for stabilizing and regulating

FVIII. The biosynthesis, structure and function of FVIII and vWF and their interaction, as well as those of FVIII in the FXase complex [FVIIIa and activated FIX (FIXa) assembled on a phospholipid surface] have been reviewed by Vlot et al. [27]. It is the light chain that binds to vWF, negatively charged phospholipid [28], platelets [29], FIXa [30] and APC [31,32].

4. Factor VIII concentrates

Commercially available preparations of FVIII concentrates needed for the treatment of hemophilia A are either isolated from human plasma or from cell culture supernatants of genetically engineered cell lines. They differ in the manufacturing process, virus inactivation steps, purity and in the stabilizer used. The use of transgenic animal expression systems might be a future source for large amounts of FVIII [33].

4.1. Plasma-derived FVIII

Production of pdFVIII starts from blood plasma of healthy persons. The concentration of FVIII in plasma is very low, i.e., about 100–200 ng/ml, corresponding to one unit of FVIII per ml [34]. The production of pdFVIII concentrates thus requires large plasma volumes and includes several consecutive concentration and purification steps using cryoprecipitation techniques and chromatographic steps. Some examples of the procedures used to obtain pdFVIII products can be found in Refs. [5,7,13,35]. The main analytical problem associated with FVIII characterization and structural analysis is that, in the plasma-derived preparations, FVIII is associated with von Willebrand Factor, which has a molecular mass in the range of 250 000 for the monomer and multimers of increasing size up to $20 \cdot 10^6$ for the largest one. The percentage excess of vWF over FVIII is more than 90 % in pdFVIII preparations.

4.2. Recombinant FVIII

For the production of rFVIII, genetically engineered mammalian cells are employed, which are able to produce recombinant proteins with post-translational modifications similar to those of the natural human product [18]. The cell lines that are used frequently for this purpose are Chinese hamster ovary (CHO) cells and baby hamster kidney (BHK) cells. The rFVIII preparations on the market differ in the expression system, culture medium, stabilizer added, growth factors and in the continuity of the fermentation process. In all cases, isolation and purification of rFVIII from the raw product of fermentation is achieved by a multistep purification process, including several chromatographic steps, such as ion exchange, immunoadsorption and size-exclusion. Attempts have been made to produce B-domain-deleted FVIII from CHO cells, as the B domain is not necessary for the procoagulant function of FVIII [36]. rFVIII is available in a more concentrated and pure form, making subsequent characterization easier, as is reflected by the number of papers on rFVIII analysis compared to those on pdFVIII. Moreover, rFVIII expression offers additional features like [^{35}S]sulfate labeling, the inhibition of sulfate incorporation or the mutation of Tyr to Phe residues to study the influence of such modifications on FVIII synthesis, secretion and functionality [14,37–39].

4.3. Virus inactivation

FVIII concentrates are derived from complex biological materials and have to meet special requirements concerning product safety. In order to eliminate the risk of virus transmission with plasma or plasma products, methods have been developed that allow the inactivation of transfusion-relevant viruses [40]. Besides screening the single blood donations, virus-inactivation steps during the production process are introduced, not only for the plasma-derived products but also for the recombinant ones, because animal materials are used during the manufacturing process. The International Association of Biological Standardization (IABS) recommends that at least two independent steps for virus inactivation are carried out [41]. In general, they comprise solvent/detergent treatment and thermal inactivation. The latter can be done by heating the product with steam and plunging the final container into a water bath or by pasteurization, a heat treatment after the addition of stabilizers to the FVIII solution [42].

4.4. Stabilizer

As FVIII is very sensitive to proteolysis and degradation, the FVIII preparations need to be stabilized. Plasma-derived FVIII can be purified and enriched in the presence of its natural stabilizer, vWF [7]. If the production process includes the depletion of vWF from FVIII by affinity chromatography (with monoclonal antibodies against vWF), further stabilization is achieved with human pasteurized albumin [13]. For recombinant preparations, stabilization is achieved by coexpression of vWF and/or the addition of albumin.

4.5. Purity

The total protein content in preparations of FVIII concentrates is measured and related to the FVIII activity in international units per milliliter (IU FVIII/ml) of the final containers and is measured using a chromogenic assay (cf. Section 5.1). Depending on the specific activity (IU FVIII/mg protein), the concentrates can be classified according to purity [43,44]. The total protein content depends mainly on the stabilizers, vWF or albumin. Apart from the FVIII.vWF complex, which forms the active part of the concentrate, plasma-derived concentrates consist of a protein portion that may contain fragments of FVIII and vWF as well as other plasma proteins, such as fibrinogen, fibronectin, IgA, IgG, IgM and isoagglutinins [7]. They represent a protein burden to the patient and, in improved FVIII preparations, protein contaminations should be reduced as they may cause an immunogenic response [12]. Recombinant FVIII preparations are tested for recombinant vWF, host cell-derived impurities, such as DNA and protein, and production-process-derived impurities, such as monoclonal antibodies, microorganisms and pyrogens, to fulfill the specifications [45].

4.6. Inhibiting antibodies

A major complication in FVIII therapy results from FVIII-inhibiting antibodies, developed by the patients [2,46]. This problem is not diminished when high-purity FVIII concentrates or recombinant preparations are used [46,47]. The pathogenesis of inhibitor development is still not fully understood. Most of the inhibitors have been shown to be directed against the A2 and/or C2 domain [48–50], but it is likely that the antibody specificity may change during therapy [51]. Inhibitors against the A3 domain are developed, but less frequently, and other inhibitor-binding regions have also been reported, e.g., Lys338–Asp362 (AR1 in the M_r 50 000 fragment) [52], Val1670–Glu1684 (AR2 in the M_r 80 000 but not in the M_r 72 000 fragment) [53], 1674–1684 [54] and 2170–2332 (C1 and C2) [55]. The inhibiting effect of the antibodies may result by hindering the cleavage of FVIII to the active heterotrimer or the binding of the light chain to vWF or to phospholipid [56]. However, the mechanism of action of some antibodies is still not known. A further outbreak of inhibitors in multitransfused previously treated patients (PTPs) is rare, but was the case after the introduction of double virus-inactivated FVIII preparations that were submitted to a solvent/detergent treatment followed by pasteurization [11,12]. The increase in inhibitor formation was significant and batch-related and could be correlated with the plasma source. The existence of neoantigens in other FVIII preparations cannot be excluded at the moment and may be responsible for inhibitor formation in some cases. A neoantigen, defined as an antigenic site that is not present on the native form of a molecule, may be introduced during the production process, especially during virus-inactivation steps. The reasons why FVIII can become more immunogenic have been reviewed in detail [57]. A marker for problematic batches of cryoprecipitates containing FVIII is the presence of a fragment of approximately M_r 40 000 (originating from the heavy chain) that can be detected by using size-exclusion chromatography (SEC) and immunoblotting [58]. However, there is no evidence that this particular heavy chain fragment is responsible for the immunological response of PTPs. Raut et al. [59] described slower thrombin proteolysis of the light chain, more rapid FXa generation and a higher binding affinity to phospholipid in some double virus-inactivated FVIII batches, using surface plasmon resonance measurements. Whereas the changes in FVIII that are responsible for these findings remain unknown, the described tests may serve to screen new FVIII preparations.

5. Methods for in vitro characterization of factor VIII concentrates

5.1. Tests for the determination of clotting activity, antibody binding and clinical parameters

Besides quantitative tests to monitor possible impurities, preparations of FVIII and vWF are characterized by their clotting activity as well as by antigen tests. The biological activity of FVIII (FVIII:C) is measured by two different methods. In the so-called one-stage method, samples are added to plasma that is deficient in FVIII. Correction of the clotting time of the FVIII-deficient plasma is proportional to the activity of the FVIII concentration in the sample, which can therefore be calculated [60]. The other method is based upon the activation of clotting factor X to factor Xa by FVIII. FXa in turn cleaves a chromogenic substrate. The intensity of the color is proportional to the FVIII concentration of the sample [61]. The biological activity of vWF is expressed as ristocetin cofactor activity (RiCof). The vWF-induced aggregation of thrombocytes is mediated by ristocetin. The tests are based on macroscopic platelet agglutination [62] and, more recently, on recognition of the functional epitope of the vWF molecule by an enzyme-linked immunosorbent assay (ELISA). Besides the determination of activities, the content of antigen, FVIII:Ag and vWF:Ag can be determined [63,64]. To check the native form of the proteins, the ratio of activity to antigen is calculated. FXa generation times can be compared using a modified chromogenic assay [59,65]. For the therapeutic effectiveness of the FVIII concentrates as pharmaceuticals, clinical testing of in vitro recovery, half-life, efficacy and correction of bleeding time is done [66]. These tests are used to determine the in vitro and in vivo properties of FVIII. The ratio of the activities and antigen levels of FVIII and vWF for the preparations are monitored and activity and antigen levels are compared to prove the integrity of FVIII. The in vivo properties are necessary for dosage and control of therapy.

5.2. Slab-gel electrophoresis

This method allows the separation of complex mixtures and aggregates of proteins according to their apparent molecular mass under non-reducing or reducing conditions. Proteins with a molecular mass in the range 5000 to 300 000 can be well separated on polyacrylamide gradient gels. This is, in principle, a sufficient range for the separation of intact FVIII and FVIII fragments, as these proteins are not associated into vWF multimers under electrophoretic conditions. Electrophoresis is widely applied to FVIII samples, purified FVIII [26] and thrombin-activated FVIII [26] to monitor FVIII degradation [67], check the consistency of recombinant FVIII [45], to compare rFVIII and pdFVIII chains [20] and to study the influence of heat treatment on neoantigen formation, as reflected by band shifts [68]. Due to its size, vWF only partly enters polyacrylamide gels under non-reducing conditions. Information about the presence of small, medium and large multimers can be obtained with agarose gels, as described by Budde et al. [69]. Protein stains show the molecular-mass distributions of the proteins within the samples. Western blot and incubation with antibodies gives more detailed structural information [70]. The assignment of bands to proteins can be done by immunoblotting with specific antibodies. On choosing an appropriate antibody, the FVIII heavy and light chains can be detected, intact as well as after thrombin activation. With highly purified FVIII, the thrombin activation can also be followed by silver-staining [71]. Moreover, vWF and concomitant plasma proteins like fibrinogen can be detected. Fluorophore-assisted carbohydrate electrophoresis was used for elucidation of N-linked oligosaccharide structures and their consistency in rFVIII, as reported by Kumar et al. [17]. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of FVIII preparations was described by Pock et al. [72]. 2D-PAGE allows the further separation of proteins with similar molecular masses but a difference in their isoelectric point(s). Besides the proposed protocol from the Swiss 2D-PAGE group [73], some variations have proven useful for the isoelectric focusing (IEF) of plasma proteins, such as the use of thiourea instead of urea [74,75] or the use of the recently introduced immobilized gradient (IPG) buffers as ampholytes. The 2D spot pattern varies depending on the conditions for isoelectric focusing and sample preparation and need to be optimized for individual samples. A comparison of the gels with the standard

human plasma reference map [76,77] gives hints regarding the presence of plasma proteins other than FVIII. Further identification, as demonstrated with fibrinogen, can be done by N-terminal sequencing [78] or by peptide-mass mapping [79]. From preparations without vWF, the separate FVIII light and heavy chain isoforms could be visualized on 2D blots. Some major problems that still remain are the size of FVIII and the question of whether or not it can be separated from vWF, so that it can be moved into the first dimension gel for isoelectric focusing and so that FVIII spots can be monitored by 2D-polyacrylamide gel electrophoresis. Compared to other methods, electrophoresis is the most powerful method for the separation of proteins. They can be monitored using protein stains and the more sensitive technique of immunoblotting. Molecular masses can be estimated. Purification, activation and degradation can be monitored and batches and different products can be compared. N-terminal sequencing from the immunoblot membrane can be used to confirm the components of a product or to identify contaminant bands, provided that the protein is not blocked. The advantages of 2D-PAGE in terms of proteins penetrating the two-dimensional gels are high spot capacity, good resolution and the high loadability, making subsequent characterization of well separated spots possible.

5.3. Chromatography

Several chromatographic steps are part of the manufacturing processes of FVIII preparations, regardless of their origin. The purification process itself is a multistep procedure in which the primary principles of protein separation, i.e., according to differences in charge, size and affinity, are used for effective removal of impurities and concentration of FVIII. The principles involved comprise ion-exchange chromatography, for concentration and removal of the majority of protein contaminants, immunoadsorption chromatography, for further purification and enrichment, and size-exclusion chromatography, which is usually the last phase of the process, the so-called polishing step. Besides being useful in the production process, chromatography is a major tool for characterization of FVIII.

5.3.1. Size-exclusion chromatography

Size-exclusion chromatography can provide important information about the physical state of FVIII and concomitant proteins in solution. The data obtained provide information about the size of the molecules or their hydrodynamic diameters, and the aggregation (interaction) of identical molecules with one another or with other molecules. Separation can be carried out on an analytical as well as a preparative scale under denaturing and non-denaturing conditions [80]. FVIII complexed with vWF is eluted with the void volume of the column, as determined by activity or antigen tests of both proteins. Due to the size of the complex under non-denaturing conditions, its adhesive behavior and the sensitivity of FVIII to activation and degradation, only a few size-exclusion media can be used to obtain good resolution and product recovery. Some SEC materials have been shown to be suited for analytical-scale characterization of pdFVIII [81] and rFVIII [82]. 'One-peak products' can be distinguished from products that reveal several peaks provided that the appropriate support is chosen, i.e., one that is capable of separating biopolymers with M_r of several million down to about 20 000 [83] (Fig. 2). Degradation products can be observed [58] and the presence of aggregates in rFVIII can be detected. Moreover, by using at least 250 m*M* calcium in the running buffer, FVIII and vWF can be almost quantitatively separated from each other (on an analytical as well as a preparative scale) [81]. Fractions obtained after SEC can be further separated using sodium dodecylsulfate SDS–PAGE, to prove that the peaks are homogeneous. However, preparative SEC results in product dilution, making a subsequent concentration step necessary. Protein complexes that are stable under the chromatographic conditions used will not be separated from each other and represent some protein burden within the concentrate, although the majority of the non-clottable protein was removed. Besides being one of the last steps in FVIII manufacturing, a high-capacity gel filtration system was developed for the separation of FVIII.vWF directly from plasma instead of having to use cryoprecipitation [84].

5.3.2. Ion-exchange chromatography

Ion-exchange chromatography (IEC) is an essential part of the purification protocol for the prepara-

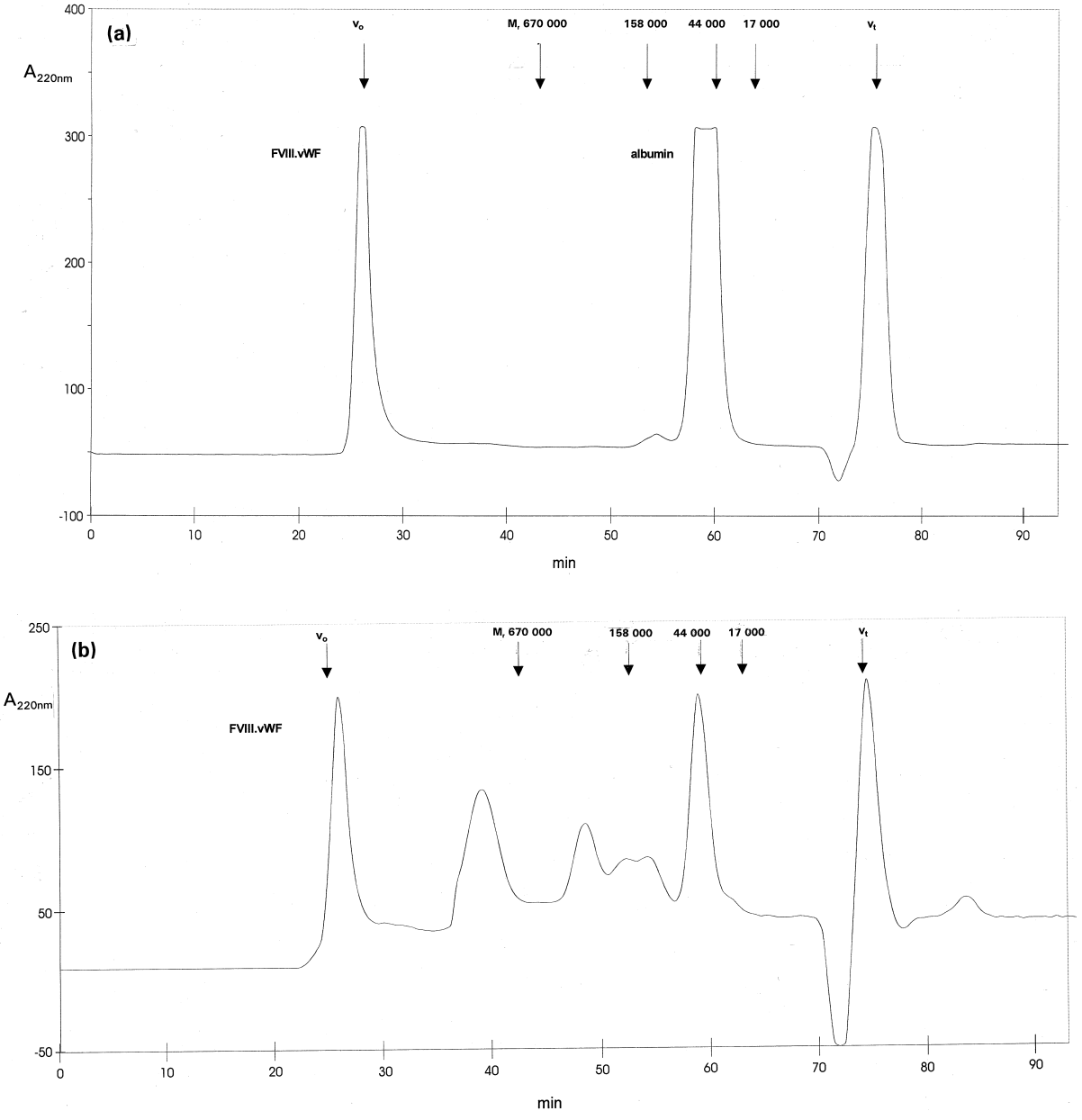

Fig. 2. Size-exclusion chromatograms of (a) a pdFVIII preparation that was highly purified by immunoaffinity chromatography and predominantly contains FVIII.vWF and albumin, and (b) a less pure pdFVIII preparation than that shown in (a). Besides FVIII.vWF, several peaks are separated that contain almost no clottable protein, as determined by Josic et al. [117]. The peak at V_t results from salt in the samples. The gel filtration standard was from Bio-Rad (Hercules, CA, USA).

tion of pdFVIII [85] and rFVIII [8,45] concentrates. This method was used for structural and functional characterization of FVIII, to investigate different activated forms of FVIII. It was also used to compare rFVIII with pdFVIII [86], rFVIII with the porcine homolog [87] and as a purification step for further identification of copper as the metal-ion bridge connecting FVIII's heavy and light chains

[88]. rFVIII with different degrees of sulfation could be separated by IEC, indicating the importance of the sulfate groups on the charge and/or conformation of FVIII [89].

5.3.3. Immunoaffinity chromatography

Immunoaffinity chromatography (IAC) with monoclonal antibodies against FVIII or vWF is incorporated in some FVIII manufacturing processes, e.g., in the production of Monoclate (a pdFVIII preparation) and Kogenate (recombinant FVIII produced from BHK cells) [8]. Affinity chromatography is combined with ion-exchange chromatography for the preparation of highly purified FVIII from pdFVIII concentrates [90]. Peptides having a sequence similar to the FVIII binding epitope on vWF are reported as ligands suitable for FVIII affinity chromatography [91].

5.3.4. Reversed-phase chromatography

rFVIII polypeptides can be separated using reversed-phase high-performance liquid chromatography (RP-HPLC) after thrombin activation. The defined cleavage products are smaller in size than the FVIII heterodimer. They are well separated and can be used for further characterization by electrophoresis and mass spectrometry [15,16,92]. RP-HPLC is restricted to FVIII that has been depleted of vWF, as vWF will block the column due to its size and its adhesive behavior. Due to the composition of the eluent, FVIII will be denatured.

5.3.5. High-performance membrane chromatography

In high-performance membrane chromatography (HPMC), the chromatographic column is replaced by either a single membrane or several membranes made of cellulose or synthetic material, such as poly(glycidyl methacrylate–co-ethylene glycol dimethacrylate) (GMAEDMA) sheets. Another option is to use compact disks made of hollow fibers or a synthetic material, such as poly(styrene–co-divinylbenzene) or poly-GMAEDMA. Both membranes and compact disks carrying functional groups allow fast separations and are thus particularly suited for in-process analysis of biopolymers [93]. Using compact porous tubes, FVIII can be isolated from human plasma [94]. In principle, this convective interaction technology allows the combination of different chromatographic modes in a single run by inserting disks of different chemistries in the same housing. This operational mode is named conjoined liquid chromatography [95].

5.4. Mass spectrometry (MS)

Mass spectrometric analysis of FVIII chains and fragments has been reported using electrospray ionization MS (ESI–MS) or matrix-assisted laser desorption ionization MS (MALDI–MS) in an off-line mode after chromatographic or electrophoretic isolation of FVIII fragments. The technique allows the determination of mass, for verifying theoretical masses or for deriving modifications due to mass differences [15,16], and detailed structural investigations of post-translational modifications, such as glycosylation and sulfation [96]. The masses of a single chain rFVIII, the heavy and light chains as well as polypeptides after thrombin activation and RP-HPLC separation have been determined and compared to the theoretical masses derived from the FVIII amino-acid sequence [92]. Peptide mapping of the A2 domain verified the sequence of the A2 domain and its modifications. Fibrinogen could be identified after 2D separation of a FVIII concentrate, followed by tryptic digestion of the spots, peptide mass mapping [79] and comparison with the masses derived from the amino acid sequences collected in databases like SwissProt [97]. This result was verified by N-terminal sequencing of spots from 2D blots [78]. The latest development in MS instrumentation is a good example of the progress in analytical results running in parallel with technology. Before MS, e.g., sugar chains of pdFVIII and rFVIII were compared after their release, radioactive labeling and separation by paper electrophoresis in 1992 [98], but no assessment of the occupied sites was possible. In 1994, glycane fingerprinting (enzymatic cleavage of sugars, their purification and characterization with high-performance anion-exchange chromatography with pulsed amperometric detection) for rFVIII was published [99]. The method is used for batch control but no information about the occupied sites and the glycane type is available. In 1996, the glycosylation of a rFVIII was investigated in detail, using electrophoresis, HPLC and ESI–MS [16]. In

1997, the site occupancy and the site-specific carbohydrate microheterogeneity of N-linked oligosaccharides in rFVIII from CHO cells was investigated using RP-HPLC and ESI–MS [15]. A map of all carbohydrate structures of the *N*-glycanes present in a rFVIII was established. Oligomannose and complex carbohydrates were detected at the glycosylation sites of the M_r 50 000 and 73 000 polypeptides originating from the FVIII heavy and light chains, respectively, while the oligosaccharides identified on the B domain were complex-type structures. The M_r 43 000 polypeptide from the FVIII heavy chain was found not to be glycosylated, in agreement with other publications [92,100]. A biantennary core-fucosylated carbohydrate was identified as the major substituent of the heavy chain M_r 50 000 polypeptide, the B domain and the light chain, consistent with the results of Kumar et al. [17]. In addition, the presence of a complex nonfucosylated oligosaccharide that had not been reported previously for this glycoprotein was revealed. The investigation of O-glycosylation is more complicated than that of the N-glycosylation and, therefore, published data on that topic are very scarce. Maszaroff et al. [16] have reported mass spectrometric approaches for the determination of O-glycosylation and its presence on the FVIII B domain. With mass spectra, the molecular mass can be determined much more precisely than from electrophoresis in a mass range of several hundred thousand when using MALDI–MS. After enzymatic cleavage of a protein of known sequence, the peptides can be monitored. The peptides carrying no modification can be used for protein verification. In cases where post-translational modifications are present on the protein, the difference between the mass of the modified peptides and the same peptides without modifications is provided. Correlation of the mass differences with modifications needs further analysis, because of possible isomeric structures and very similar or identical residue masses that can only be differentiated, e.g., by collision-induced MS, to produce diagnostic fragment ions, or by complementary analysis. The combination of MS with HPLC and/or electrophoresis allows multidimensional separation and prepurification of samples. More detailed results on FVIII glycosylation are the basis of a further comparison between pdFVIII and rFVIII from CHO and BHK cells and are examples of the increasing importance of mass spectrometric methods.

5.5. Determination of factor VIII interaction with polypeptide ligands

FVIII under investigation may be the plasma-derived protein in a concentrate containing vWF and other proteins or after further high purification as well as the recombinant one, after or without the addition of albumin. In any case, the samples are used in a native state or after thrombin activation. Expressed FVIII sequences, domains or chains from *Escherichia coli* can be used for epitope binding studies [48]. The expression of deletion fragments or site-directed mutagenesis may help to determine the influence of single amino acids or sequences for the procoagulant activity of FVIII as well as for its binding to other proteins [101]. The most restrictive methods use synthetic peptides that are designed to mimic epitopes on FVIII or on substrates that react with FVIII [53,102,103]. The interaction of FVIII with other proteins and with phospholipid can be determined by immunological approaches and by using sensor chips.

5.5.1. Immunochemical investigations

Protein–protein interactions, in particular, the reactivity of FVIII with antibodies, are mainly determined using ELISA systems [104,105] and immunoblotting [49,70]. Major requirements for the antibodies are high specificity, no cross-reactivity, e.g. with vWF or other plasma proteins, and unequivocal reactivity with single FVIII domains. The more that is known about the binding region on FVIII, the more information about FVIII can be gained. Activation and degradation products can be detected if the antibody still reacts with the products. Potential methods to detect neoantigen formation and increased immunogenicity are ELISA and neonatal mouse immune-tolerance methods [106–108]. If neoantigens could be identified, antibodies could be raised against them and used for screening different batches as well as new preparations [109]. Determination of binding epitopes is commonly done using competitive assays in solutions, using synthetic peptides [102]. If FVIII binding to an antibody is blocked by synthetic peptides, it is concluded that

the corresponding peptide sequence represents the sequence of the binding epitope on FVIII or the antibody. Structural information is gained using these methods, e.g., the band pattern in Western blots and epitope reactivities in ELISA and identification of binding epitopes by mimicking them using synthetic peptides.

5.5.2. Sensor chips

With mass-sensitive chips, binding events and subsequent dissociation can be monitored in real time and the kinetic constants of association and dissociation can be determined [110]. In the BIACore instrument, detection is based on the surface plasmon resonance phenomenon, which measures the change in the refractive index upon association of the analyte with the immobilized ligand or upon the dissociation of a formed complex. The resulting signal is proportional to the amount of protein bound [111]. The binding constant can be evaluated from the ratio of the kinetic constants of the association and dissociation processes as well as from the binding isotherm obtained when measurement are performed at different protein concentrations. When interpreting kinetic data, any influence of the transfer kinetic in solution has to be carefully excluded [112]. The BIACore instrument was used to study the kinetics of interaction between the human FVIIIa subunits, which varied depending on the pH, ionic strength, calcium concentration, heparin and activated protein C-catalyzed proteolysis [111]. Other applications of sensor chips are in following the interaction between FVIII and vWF [113] or between FVIII and phospholipid after thrombin activation [114] or using non-activated FVIII [59,115]. A mixture of dioleoylphosphatidylcholine and dioleoylphosphatidylserine can be used to simulate FVIII–phospholipid binding under non-physiological conditions [116]. The sensor chip has also been used to measure the interactions of FVIII with antibodies. Interactions with antibodies, vWF and phospholipid mimic the in vivo situation by in vitro investigations. Reactivities and reaction constants can be determined and the reactivity of different preparations can be compared. The experimental set-up is reduced to some parameters that can be experimentally controlled but may not reflect the actual complex in vivo situation. Interactions of FVIII can be measured but no information can be obtained about the structural prerequisites for its reactivity on the molecular level.

6. Conclusion

The characterization of FVIII and FVIII concentrates involves a wide field of analytical techniques, most of which are complementary to each other. The analytical methods for characterization can be separated roughly into analysis of binding functionality, protein pattern and the structure of FVIII. All therapeutic FVIII concentrates contain high amounts of stabilizing protein. Therefore, the contribution of FVIII to the total protein is only about 1–2% and FVIII characterization becomes very difficult. Investigation of the affinity and binding sites, using mainly ELISA and sensor chips, addresses the reactivity of FVIII with antibodies and the proteins involved in the processing of FVIII during blood coagulation. Many binding sites on FVIII could be determined until now. This is helpful for a better understanding of function and inhibition of FVIII procoagulant activity. Protein patterns are obtained using electrophoretic methods, such as one- and two-dimensional electrophoresis, isoelectric focusing and Western blotting, resulting in bands and spots. The molecular mass of single chains of activated and non-activated FVIII can be estimated and preparations can be compared. Chromatographic methods, regardless of the principle of separation, reveal records of the absorbance, reflecting protein concentrations. Protein assessments can be done by activity or antigen tests of the individual fractions. Compared to the vWF–free recombinant counterpart, pdFVIII is attached to vWF during the chromatographic steps; otherwise, measures are taken to separate them. In ion-exchange and affinity chromatography, impurities are in the flow-through whereas, in size-exclusion chromatography, the whole preparation is reflected by a characteristic pattern. The purity of preparations can be estimated and compared. Primary structure analysis is performed mainly by N-terminal sequencing and mass spectrometric methods. However, several methods are necessary to characterize therapeutic FVIII concentrates with respect to FVIII and the whole preparation. At present, the sensitivity of mass spectrometric meth-

ods is required to prove the neoantigen hypothesis. This hypothesis states that a considerable small alteration of the FVIII molecule makes some preparations more immunogenic.

7. Abbreviations

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
APC	activated protein C
AR1	acidic region 1
AR2	acidic region 2
BHK	baby hamster kidney
CHO	Chinese hamster ovary
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
FIIa	activated factor II
FVIII	factor VIII
FVIII.vWF	complex of FVIII and vWF
FVIII:Ag	FVIII antigen
FVIII:C	factor VIII activity
FVIIIa	activated factor VIII
FIXa	activated factor IX
FXa	activated factor X
FXase	factor Xase complex
GMA–EDMA	poly(glycidyl methacrylate–co-ethylene glycol dimethacrylate)
HPAEC–PAD	high-performance anion-exchange chromatography–pulsed amperometric detection
HPMC	high-performance membrane chromatography
IAC	immunoaffinity chromatography
IEC	ion-exchange chromatography
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IEF	isoelectric focusing
IU	international units
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
pdFVIII	plasma-derived FVIII
pdFVIII	plasma-derived FVIII
PTP	previously treated patients
rFVIII	recombinant FVIII
RiCof	ristocetin cofactor activity
RP-HPLC	reversed-phase high-performance liquid chromatography
SDS–PAGE	sodium dodecylsulfate–polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
vWF	von Willebrand factor
vWF:Ag	antigen

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References

[1] G.A. Vehar, B. Keyt, D. Eaton, H. Rodriguez, D.P. O'Brien, F. Rotblat, H. Oppermann, R. Keck, W.I. Wood, R.N. Harkins, E.G.D. Tuddenham, R.M. Lawn, D.J. Capon, Nature 312 (1984) 337.
[2] H.H.J. Kazazian, E.G.D. Tuddenham, S.E. Antonarakis, in: C.R. Scriver, A.L. Blandet, W.S. Sly, D. Valle (Eds.), Hemophilia A and Parahemophilia: Deficiencies of Coagulation Factors VIII and V, Vol. III, McGraw Hill, New York, 1995, p. 3241.
[3] R.J. Kaufman, Annu. Rev. Med. 43 (1992) 325.
[4] G. Kemball-Cook, J.E. Tubbs, N.J. Dawson, T.W. Barrowcliffe, Br. J. Haematol. 84 (1993) 273.
[5] C.K. Kasper, J.M. Lusher, Transfusion 33 (1993) 422.
[6] K. Schimpf, Hämostaseologie 14 (1994) 44.
[7] D. Josic, H. Schwinn, M. Stadler, A. Strancar, J. Chromatogr. B 662 (1994) 181.
[8] B.G.D. Boedeker, Transfus. Med. Rev. 6 (1992) 256.
[9] D. Josic, P. Schulz, L. Biesert, L. Hoffer, H. Schwinn, M. Kordis-Krapez, A. Strancar, J. Chromatogr. B 694 (1997) 253.
[10] L.W. Hoyer, D. Scandella, Semin. Hematol. 31 (1994) 1.
[11] K. Peerlinck, J. Arnout, M.D. Giambattista, J.G. Gilles, R. Laub, M. Jacqquemin, J.M.R. Saint-Remy, J. Vermylen, Thromb. Haemost. 77 (1997) 80.
[12] Y. Sawamoto, R. Prescott, D. Zhong, E.L. Saenko, E. Mauser-Bunschoten, K. Peerlinck, M.v.d. Berg, D. Scandella, Thromb. Haemost. 79 (1998) 62.
[13] E. Berntorp, I.M. Nilsson, Eur. J. Haematol. 40 (1988) 205.
[14] J. Mikkelsen, J. Thomsen, M. Ezban, Biochemistry 30 (1991) 1533.

[15] K.F. Medzihradszky, M.J. Besman, A.L. Burlingame, Anal. Chem. 69 (1997) 3986.
[16] I. Mazsaroff, W. Yu, B.D. Kelley, J.E. Vath, Anal. Chem. 69 (1997) 2517.
[17] H.P.M. Kumar, C. Hague, T. Haley, C.M. Starr, M.J. Besman, R.L. Lundblad, D. Baker, Biotechnol. Appl. Biochem. 24 (1996) 207.
[18] D.A. Cumming, Glycobiology 1 (1991) 115.
[19] D.C. Andersen, C.F. Goochee, Curr. Opin. Biotechnol. 5 (1994) 546.
[20] R.J. Kaufman, Ann. Hematol. 63 (1991) 155.
[21] A. Leyte, H.B.v. Schijndel, C. Niehrs, W.B. Huttner, M.P. Verbeet, K. Mertens, J.A.v. Mourik, J. Biol. Chem. 266 (1991) 740.
[22] R.J. Kaufman, Thromb. Haemost. 79 (1998) 1068.
[23] K. Löster, C. Kannicht, P. Schulz, A. Buchacher, K. Pock, H. Schwinn, D. Josic, in: Proceedings of the 3rd Symposium on the Analysis of Well Characterized Biotechnology Pharmaceuticals, Washington, DC, January 1999, p. 78.
[24] L.-O. Andersson, J.E. Brown, Biochem. J. 200 (1981) 161.
[25] D.D. Pittman, E.M. Alderman, K.N. Tomkinson, J.H. Wang, A.R. Giles, R.J. Kaufman, Blood 81 (1993) 2925.
[26] D. Eaton, H. Rodriguez, G.A. Vehar, Biochemistry 25 (1986) 505.
[27] A.J. Vlot, S.J. Koppelman, B.N. Bouma, J.J. Sixma, Thromb. Haemost. 79 (1998) 456.
[28] G.E. Gilbert, B.C. Furie, B. Furie, J. Biol. Chem. 265 (1990) 815.
[29] M.E. Nesheim, D.D. Pittman, J.H. Wang, D. Slonosky, A.R. Giles, R.J. Kaufman, J. Biol. Chem. 263 (1988) 16467.
[30] P.J. Lenting, J.W. van de Loo, M.J. Donath, J.A. van Mourik, K. Mertens, J. Biol. Chem. 271 (1996) 1935.
[31] P.J. Fay, F.J. Walker, Biochim. Biophys. Acta 994 (1989) 142.
[32] F.J. Walker, D. Scandella, P.J. Fay, J. Biol. Chem. 265 (1990) 1484.
[33] R.K. Paleyanda, W.H. Velander, T.K. Lee, D.H. Scandella, F.C. Gwazdauskas, J.W. Knight, L.W. Hoyer, W.N. Drohan, H. Lubon, Nat. Biotechnol. 15 (1997) 971.
[34] A.J. Vlot, S.J. Koppelman, M.H.v.d. Berg, B.N. Bouma, J.J. Sixma, Blood 85 (1995) 3150.
[35] M.-C. Poon, Ann. Hematol. 68 (1994) 83.
[36] P. Meulien, T. Faure, F. Mischler, H. Harrer, P. Ulrich, B. Bouderbla, K. Dott, M.S. Marie, C. Mazurier, M.-L. Wiesel, H.V.d. Pol, J.-P. Cazenave, M. Courtney, A. Pavirani, Protein Eng. 2 (1988) 301.
[37] D.A. Micknick, D.D. Pittman, R.J. Wise, R.J. Kaufman, J. Biol. Chem. 269 (1994) 20095.
[38] D.D. Pittman, J.H. Wang, R.J. Kaufman, Biochemistry 31 (1992) 3315.
[39] M. Kjalke, A. Heding, G. Talbo, E. Persson, J. Thomsen, M. Ezban, Eur. J. Biochem. 234 (1995) 773.
[40] B. Horowitz, R. Bonomo, A.M. Prince, S.N. Chin, B. Brotman, R.W. Schulman, Blood 79 (1992) 826.
[41] H. Willkommen, J. Löwer, in: F. Brown (Ed.), Virological Safety Aspects of Plasma Derivatives. Development in Biological Standardization, Vol. 81, Karger, Basel, 1993, p. 109.
[42] P.M. Mannucci, Hämostaseologie 14 (1994) 60.
[43] T.W. Barrowcliffe, J. Watton, A. Harman, J.E. Tubbs, G. Kemball-Cook, Lancet 336 (1990) 124.
[44] A. Schoppmann, A. Weber, F. Hondl, Y. Linnau, Thromb. Haemost. 72 (1994) 483, letters to the editor.
[45] J. Lawrence, Ann. Hematol. 68 (1994) 21.
[46] Y. Sultan, Blood Coagul. Fibrinolysis 6 (1995) 55.
[47] J.-M.R. Saint-Remy, J.G. Gilles, Vox Sang. 70 (suppl 1) (1996) 15.
[48] D. Scandella, S. Mahoney, M. Mattingly, D. Roeder, L. Timmons, C.A. Fulcher, Proc. Natl. Acad. Sci. USA 85 (1988) 6152.
[49] D. Scandella, M. Mattingly, S.D. Graaf, C.A. Fulcher, Blood 74 (1989) 1618.
[50] C.A. Fulcher, S.d. Graaf-Mahoney, J.R. Roberts, C.K. Kasper, T.S. Zimmerman, Proc. Natl. Acad. Sci. USA 82 (1985) 7728.
[51] J.G.G. Gilles, J. Arnout, J. Vermylen, J.-M.R. Saint-Remy, Blood 82 (1993) 2452.
[52] B.C. Lubahn, J. Ware, D.W. Stafford, H.M. Reisner, Blood 2 (1989) 497.
[53] P.A. Foster, C.A. Fulcher, R.A. Houghten, T.S. Zimmerman, J. Biol. Chem. 263 (1988) 5230.
[54] J.W. Precup, B.C. Kline, D.N. Fass, Blood 77 (1991) 1929.
[55] K. Amano, M. Arai, K. Koshihara, T. Suzuki, K. Kagawa, Y. Nishida, K. Fukutake, Am. J. Hematol. 49 (1995) 310.
[56] M. Arai, D. Scandella, L.W. Hoyer, J. Clin. Invest. 83 (1989) 1978.
[57] J.G. Gilles, M.d. Giambattista, R. Laub, J.-M.R. Saint-Remy, Vox Sang. 73 (1996) 16.
[58] D. Josic, M. Stadler, A. Buchacher, K. Pock, S. Robinson, H. Schwinn, Thromb. Haemost. 78 (suppl) (1997) 576.
[59] S. Raut, M.D. Giambattista, S.A. Bevan, A.R. Hubbard, T.W. Barrowcliffe, R. Laub, Thromb. Haemost. 80 (1998) 624.
[60] W. Barrowcliffe, Res. Clin. Lab. 20 (1990) 155.
[61] R. Wagenvoord, A. Hendrix, H.C. Hemker, Haemostasis 19 (1989) 196.
[62] K.M. Brinkhous, Thromb. Res. 6 (1975) 267.
[63] J.P. Girma, E. Fressinaud, A. Houklar, Y. Laurian, J. Amiral, D. Mayer, Thromb. Haemost. 78 (suppl) (1997) 30.
[64] J. Cejka, Clin. Chem. 30 (1984) 814.
[65] G. Kemball-Cook, J.E. Tubbs, N.J. Dawson, T.W. Barrowcliffe, Br. J. Haematol. 84 (1993) 273.
[66] I. Scharrer, Hämostaseologie 14 (1994) 69.
[67] T.W. Barrowcliffe, S.J. Edwards, G. Kemball-Cook, D.P. Thomas, Lancet (1986) 1448.
[68] S. Arrighi, R. Rossi, M.G. Borri, V. Lesnikov, M. Lesnikov, E. Franco, M. Divizia, M.E. Santis, E. Bucci, Thromb. Haemost. 73 (1995) 868.
[69] U. Budde, R.E. Scharf, P. Franke, L. Holmberg, J. Dent, Z.M. Ruggeri, Blood 82 (1993) 1749.
[70] F. Rotblat, D.P. O'Brien, F.J. O'Brien, A.H. Goodall, E.G.D. Tuddenham, Biochemistry 24 (1985) 4292.
[71] C. Fulcher, J.R. Roberts, T.S. Zimmerman, Blood 61 (1983) 807.
[72] K. Pock, A. Jungbauer, C. Kannicht, K. Löster, A. Buchacher, D. Josic, Anal. Chim. Acta 372 (1998) 219.

[73] B. Bjellqvist, J.-C. Sanchez, C. Pasquali, F. Ravier, N. Paquet, S. Frutiger, G.J. Hughes, D. Hochstrasser, Electrophoresis 14 (1993) 1375.
[74] T. Rabilloud, C. Adessi, A. Giraudel, J. Lunardi, Electrophoresis 18 (1997) 307.
[75] L. Musante, G. Candiano, G.M. Ghiggeri, J. Chromatogr. B 705 (1998) 351.
[76] G.J. Hughes, S. Frutiger, N. Paquet, F. Ravier, C. Pasquali, J.-C. Sanchez, R. James, J.-D. Tissot, B. Bjellqvist, D.F. Hochstrasser, Electrophoresis 13 (1992) 707.
[77] O. Golaz, G.J. Hughes, S. Frutiger, N. Paquet, A. Bairoch, C. Pasquali, J.-C. Sanchez, J.-D. Tissot, R.D. Appel, C. Walzer, L. Balant, D.F. Hochstrasser, Electrophoresis 14 (1993) 1223.
[78] K. Pock, K. Linnemayr, A. Rizzi, K. Löster, C. Kannicht, R.
Necina, A. Buchacher, H. Schwinn, H. Lindner, A. Jungbauer, D. Josic, Anal. Hematol. 76 (Suppl. 1) (1998) 64.
[79] K. Löster, C. Kannicht, H. Schwinn, D. Josic, in: Proceedings of the 18th International Symposium on Separation of Proteins, Peptides and Polynucleotides, Vienna, Austria, November 1998, p. 44.
[80] K. Löster, D. Josic, J. Chromatogr. B 699 (1997) 439.
[81] D. Josic, H. Horn, P. Schulz, H. Schwinn, L. Britsch, J. Chromatogr. A 796 (1998) 289.
[82] Y. Cho, C. Garanchon, R. Kashi, C. Wong, M.J. Besman, Biotechnol. Appl. Biochem. 24 (1996) 55.
[83] M. Stadler, K. Löster, C. Kannicht, G. Gruber, H. Schwinn, D. Josic, Anal. Hematol. 76 (Suppl. 1) (1998) 64.
[84] P. Kaersgaard, K.A. Bariington, J. Chromatogr. B 715 (1998) 357.
[85] S. Robinson, H. Schwinn, D. Josic, M. Stadler, F. Bal, W. Gehringer, R. Schütz, Blood Coagul. Fibrinolysis 6 (1995) 40.
[86] N. Bihoreau, P. Paolantonacci, C. Bardelle, M.-P. Fontaine-Aupart, S. Krishnan, J. Yon, J.-L. Romet-Lemonne, Biochem. J. 277 (1991) 23.
[87] P. Lollar, E.T. Parker, J. Biol. Chem. 266 (1991) 12481.
[88] N. Bihoreau, S. Pin, A.-M.D. Kersabiec, F. Vidot, M.-P. Fontaine-Aupart, Eur. J. Biochem. 220 (1994) 41.
[89] J. Mikkelsen, J. Thomsen, M. Ezban, Biochemistry 30 (1991) 1533.
[90] P.J. Fay, T.M. Smudzin, J. Biol. Chem. 264 (1989) 14005.
[91] R. Necina, K. Amatschek, E. Schallaun, H. Schwinn, D. Josic, A. Jungbauer, J. Chromatogr. B 715 (1998) 191.
[92] N. Bihoreau, J.F. Veillon, C. Ramon, J.M. Scohyers, J.M. Schmitter, Rapid Commun. Mass Spectrom. 9 (1995) 1584.
[93] A. Strancar, P. Koselj, Anal. Chem. 68 (1996) 3483.
[94] A. Strancar, M. Barut, A. Podgornik, P. Koselj, H. Schwinn, P. Raspor, D. Josic, J. Chromatogr. A 760 (1997) 117.
[95] A. Strancar, M. Barut, A. Podgornik, P. Koselj, D. Josic, A. Buchacher, LC·GC Int. October (1998) 660.
[96] G. Talbo, P. Roepstorff, Rapid Commun. Mass Spectrom. 7 (1993) 201.
[97] A. Bairoch, R. Apweiler, Nucleic Acids Res. 25 (1997) 31.
[98] T. Hironaka, K. Furukawa, P.C. Esmon, M.A. Fournel, S. Sawada, M. Kato, T. Minaga, A. Kobata, J. Biol. Chem. 267 (1992) 8012.
[99] R. Adamson, Ann. Hematol. 68 (1994) 9.
[100] R.J. Kaufman, L.C. Wasley, A.J. Dorner, J. Biol. Chem. 263 (1988) 6352.
[101] D. Pittman, R. Kaufman, Methods Enzymol. 222 (1993) 236.
[102] P.A. Foster, C.A. Fulcher, R.A. Houghten, T.S. Zimmerman, Blood 75 (1990) 1999.
[103] D. Scandella, G.E. Gilbert, M. Shima, H. Nakai, C. Eagleson, M. Felch, R. Prescott, K.j. Rajalakshmi, L.W. Hoyer, E. Saenko, Blood 86 (1995) 1811.
[104] F. Verdier, S. Duthel, P. Paulmier, J.L. Poplavsky, J. Descotes, Blood Coagul. Fibrinolysis 6 (1995) 62.
[105] F. Rotblat, A.H. Goodall, P. O'Brien, E. Rawlings, S. Middleton, E.G.D. Tuddenham, J. Lab. Clin. Med. 101 (1983) 736.
[106] P.C. Esmon, H.S. Kuo, M.A. Fournel, Blood 76 (1990) 1593.
[107] I.R. MacGregor, L.F. McLaughlin, M.C. MacGregor, C.V. Prowse, D.S. Pepper, Vox Sang. 69 (1995) 319.
[108] J.G. Gilles, M. di Giambattista, R. Laub, J.-M.R. Saint-Remy, Vox Sang. 73 (1997) 16.
[109] Y.P. Lim, Presented at the 18th International Symposium on Separation of Proteins, Peptides and Polynucleotides, Vienna, November 1998, paper 23.
[110] D.J. O'Shannessy, M. Bringham-Burke, K.K. Soneson, P. Hensley, Anal. Biochem. 212 (1993) 457.
[111] E. Persson, M. Ezban, R.M. Shymko, Biochemistry 34 (1995) 12775.
[112] M. Fivash, E.M. Towler, R.J. Fisher, Curr. Opin. Biotechnol. 9 (1998) 97.
[113] B.E. Fischer, G. Kramer, A. Mitterer, L. Grillberger, M. Reiter, W. Mundt, F. Dorner, J. Eibl, Thromb. Res. 84 (1996) 55.
[114] E. Saenko, D. Scandella, A. Yakhyaev, Anal. Hematol 76 (Suppl. 1) (1998) 29.
[115] R. Necina, A. Jungbauer, A. Buchacher, H. Schwinn, D. Josic, Anal. Hematol. 76 (Suppl. I) (1998) 29.
[116] S. Raut, T. Barrowcliffe, Thromb. Haemost. 78 (Suppl.) (1997) 629.
[117] D. Josic, H. Schwinn, M. Stadler, G. Gruber, L. Biesert, Ann. Hematol. 77 (Suppl. 1) (1999) 100.