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Purification of factor VIII and von Willebrand factor from human plasma by anion-exchange chromatography

Dj. Josic[a,*], H. Schwinn[a], M. Stadler[a], A. Strancar[b]

[a]*Octapharma Produktionsges, Oberlaaer Str. 235, A-1100 Wien, Austria*
[b]*BIA d.o.o., Adamiceva 4, SLO-61000 Ljubljana, Slovenia*

Abstract

Factor VIII (anti-hemophilia A factor) is isolated from human plasma. Purification is carried out by a combination of precipitation and chromatographic procedures. After precipitation, the first step in virus inactivation is achieved through the effect of a non-ionic detergent such as Tween 80, and a solvent, e.g. tri-*n*-butylphosphate (TnBP). By subsequent anion-exchange chromatography, a highly enriched product is isolated, consisting of a complex formed by factor VIII and von Willebrand factor (FVIII–vWF). This treatment also removes the virus-inactivating reagents to quantities in the low ppm range. The second step in virus inactivation is aimed specifically at the non-enveloped viruses and consists of pasteurization at temperatures higher than 60°C for 10 h. Through the addition of stabilizers, between 80% and 90% of the initial activity of FVIII is preserved during the modified pasteurisation. Along with the possibly denatured proteins the stabilizers, such as sugars, amino acids and bivalent cations, are subsequently removed by ion-exchange chromatography. The two-fold virus inactivation, by solvent/detergent treatment and subsequent pasteurisation, allows the destruction of both lipid-enveloped and non-enveloped viruses. During the procedure FVIII is stabilized through the high content of vWF. The complex consisting of FVIII and vWF can be dissociated by adding calcium ions. Subsequently both glycoproteins from this complex are separated from one another by further anion-exchange chromatography.

1. Introduction

Factor VIII (anti-hemophilia A factor, FVIII) is the protein which is either deficient or absent in individuals suffering from hemophilia A. This glycoprotein participates in the intrinsic pathway of blood coagulation as a co-factor together with phospholipid and factor IXa to activate factor IX [1]. In plasma, FVIII is noncovalently bound to vWF. This binding is very strong and can only be reversed for instance in the presence of highly concentrated sodium chloride solutions (>1 *M*), by chaotropic reagents such as urea and guanidine hydrochloride, or calcium ions [2]. Von Willebrand factor (vWF) is a multimeric glycoprotein with a high molecular mass, and is involved in primary hemostasis. The main role of this protein is to promote the adhesion of platelets to the subendothelium, with subsequent formation of a plug at the sites of vascular injury, as well as to serve as the transport protein for FVIII in the blood stream [1].

SDS-PAGE of purified FVIII shows a rather complicated pattern. Under non-reducing conditions several bands with apparent molecular masses between ca. 80 kDa and 220 kDa can be

* Corresponding author.

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detected [1–3]. The pattern depends also on the applied purification procedure.

The human FVIII gene has been characterized, and the glycoprotein has been synthesized by recombinant DNA techniques [4]. The first commercially available products have recently been marketed [5]. Despite the existence of recombinant products of FVIII for hemophilia A therapy, the bulk of this coagulation factor is still made from pooled human plasma. The first step in the production process of FVIII from human plasma is cryoprecipitation. This is followed by combined extraction and precipitation with aluminium hydroxide. Final purification is carried out with either anion-exchange chromatography [6] or immunoaffinity chromatography with immobilized monoclonal antibodies [2,7]. If FVIII is purified by anion-exchange chromatography, vWF is usually not removed. Consequently, such a product contains FVIII complexed with vWF [6]. In immunoaffinity chromatography the FVIII–vWF complex is usually dissociated, giving non-complexed FVIII as product. However, as FVIII alone is unstable, human serum albumin has to be added to the product to achieve the stability necessary to store the drug until used [7].

The absence of contamination with virus is a key element in the production of plasma products. Since FVIII is a protein which only occurs in traces in the plasma (ca. 0.11 mg/l), a large plasma volume is required. Especially during cryoprecipitation, the viruses that may be found in the starting material can be enriched. By appropriate treatment with a combination of a solvent such as tri-*n*-butyl phosphate (TnBP) and a non-ionic detergent, frequently Tween 80 or Triton X-100, the most serious transfusion relevant viruses can be inactivated, including HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV) [8]. When chromatographic methods are used in the subsequent purification step, the virus inactivating reagents can easily be removed, as they do not bind to the chromatographic matrices employed [6–8]. In order to increase virus safety in general and to inactivate non-enveloped viruses such as parvoviruses and the hepatitis A virus (HAV), a second step for thermal virus inactivation has recently been introduced. In this procedure, the purified FVIII product is heated at 63°C for at least 10 h with added stabilizing substances. The stabilizers are sugars and amino acids in high concentrations and bivalent cations in millimolar concentration [9].

FVIII presents quite a challenge to purification by chromatography. This glycoprotein has a complex structure and is sensitive towards proteolysis. It is therefore difficult to obtain a product with a high specific activity and high yield. The carrier protein for FVIII, the vWF, is an adhesive protein by nature [1]. Consequently, it is difficult to elute this protein quantitatively during chromatography without causing adverse non-specific interactions with the support.

Here, the behaviour of FVIII and vWF in different chromatographic systems is described. As a practical example, a newly designed production process for double virus inactivated, highly enriched FVIII is described. A key role in this process is played by anion-exchange chromatography with biocompatible synthetic resins.

2. Experimental

2.1. Plasma products and chemicals

Human plasma was obtained from Octapharma (Vienna, Austria). Detergents and other analytical grade chemicals were purchased from Merck (Darmstadt, Germany), Sigma (Munich, Germany) or Serva (Heidelberg, Germany).

2.2. Assays for factor VIII activity

The quantitative, photometric determination of FVIII activity, the so-called chromogenic assay, is based on the fact that FVIII together with factor IXa catalyzes the transformation of factor X into factor Xa, in the presence of phospholipids and calcium ions. Factor Xa is a protease which then hydrolyzes the chromogenic substrates S-2222 and I-2581 into a peptide and *p*-nitroaniline, which is then determined by measurement of the absorbance at 405 nm. The

activity of FVIII is determined on the basis of a scale which is defined by a WHO standard. A house-made standard (Octapharma) or a standard human plasma (Behringwerke, Marburg, Germany) can be used instead, provided it is calibrated against the WHO standard. The chromogenic assay has been described by Von Wagenvoord et al. [10] . The COATEST® factor VIII:c/4 testkit was purchased from Chromogenix AB (Mölndal, Sweden). It consists of the chromogenic substrates S-2222 and I-2581, factor IXa and factor X (lyophilyzed), 20 m*M* $CaCl_2$ solution, phospholipid and buffer stock solution. The measurements were carried out with an ELISA reader 340 ATTC, with automated analysis and evaluation of the readings by a personal computer and Soft 2000 software (SLT-Labinstruments, Gröding, Austria).

The one-stage coagluation assay for the determination of FVIII clotting activity has been described by Barrowcliffe [11]. FVIII deficient plasma (Behringwerke) is mixed with the sample containing FVIII, in the presence of an activator such as lipid extract and kaolin (Behringwerke). Coagulation is triggered by adding $CaCl_2$ (25 m*M*). The time required for a clot to form is measured. Each result is compared to the house-made standard and the standard human plasma above. The FVIII standard solutions were previously calibrated against the latest WHO standard. The measurements were carried out with a KC 4A coagulometer (Amelung, Lemgo, Germany).

Virus validation was performed by Dr. Lothar Biesert in Georg Speyer Haus, Frankfurt, Germany. Model enveloped viruses HIV-1, Herpes simplex virus, type 1 and Pseudorabies virus and non-enveloped HAV, Polio virus type 1 and Coxsackie virus type B6 were used as models. The methods for virus validation have been described elsewhere [12].

2.3. Analytical methods for quality control of factor VIII product

Traces of the solvent tri-*n*-butylphosphate (TnBP) and detergent Tween 80 were determined by the methods described by Horowitz et al. [13]. The protein content in the samples was determined according to the procedure of Lowry et al. [14] or according to Smith et al. [15], using the protein determination kit manufactured by Pierce (Rodgau, Germany). Bovine serum albumin was used as the protein standard.

The determination of other clotting factors and the immunochemical determination of plasma proteins have been described previously [6,11,16]. The concentration of vWF antigen was measured by ELISA as described by Cejka [17].

2.4. Purification of factor VIII

The FVIII enriched fraction was obtained from plasma through cryoprecipitation [6,18,19]. The precipitate was separated by centrifugation at 7000 *g* (Separator, Alfa Laval, Tumba, Sweden) and stored at −30°C until further use. The supernatant is used for the production of other plasma products such as the vitamin-K dependent factors and human albumin [16].

On a production scale between 15 and 40 kg of cryoprecipitate are taken for each batch; for subsequent FVIII purification on a laboratory scale, between 100 and 1000 g of cryoprecipitate were used. In both cases, the cryoprecipitate is dissolved in 10–50 m*M* sodium citrate, pH 7.2, the quantity of the sodium citrate being three times larger than that of the precipitate. It is then filtered through a filter with a 1-μm pore size (Pall, Vienna). A large part of the fibrinogen, the fibronectin and unwanted clotting factors are removed through adsorption to $Al(OH)_3$ (between 1 and 15%, w/v). Aluminium hydroxide and adsorbed proteins are removed by centrifugation at 2000 *g* with a laboratory centrifuge (Beckmann, Vienna) or at 7000 *g* using a separator (Alfa Laval).

The supernatant is used for a first anion-exchange chromatographic run. The anion-exchange supports used were Toyopearl DEAE 650 (S) (Tosohaas, Stuttgart, Germany) and tentacle support Fractogel TMAE 650 (S) (Merck). About 0.25–0.8 kg of gel was taken for each kg of cryoprecipitate. An XK 26-column with a diameter of 26 mm and a bed with a height of 100–120 mm (Pharmacia LKB, Vienna, Austria) is used on a laboratory scale. On a

production scale either a 25-l radial column (Sepragen, ict Chemikalien Vertriebs-Ges., Vienna, Austria) is used or a preparative axial column with a diameter of 250 mm and a bed height of 120 mm (Pharmacia, LKB or Merck). The sample is applied to the column in 10 m*M* citrate buffer, pH 7.5, with 110–200 m*M* NaCl. The weakly bound proteins are eluted with ca. 5 column volumes of the above NaCl solution. Osmolarity of the rinsing buffer was 400–600 mOsmol depending on the gel used (Osmomat 030 Cryosopic Osmometer, Gonotec, Berlin, Germany). The fraction which contains the enriched FVIII in a complex with vWF is subsequently eluted with a step gradient of a NaCl solution (550–900 mOsmol). The column is then rinsed with 1 *M* NaCl and the gel sanitized with 0.5 *M* NaOH.

Between 1 and 2 g/ml of a sugar such as maltose, glucose, sorbitol or trehalose, between 0.1 and 0.2 g/ml of amino acids such as alanine, arginine, glycine and histidine, heparin (0.1 IU, heparin/1 IU FVIII) and 1–5 mmol/l calcium and/or magnesium are subsequently added to the eluate containing the FVIII. The exact mixture of stabilizers used in the second virus inactivation step strongly depends on the components found in the eluate from the first anion-exchange step. After virus inactivation the stabilizers are removed by anion-exchange chromatography, using Toyopearl DEAE 650 (S) (Tosohaas) or Fractogel EMD TMAE (Merck) on a 60 × 26 mm I.D. column. The FVIII–vWF complex is eluted with a step gradient, as described above. The FVIII product is then diafiltrated (100-K membrane, Filtron, Vienna), sterile filtered (0.2-μm Sterilfilter, Millipore, Vienna), and aliquoted into vials [250, 500 or 1000 international units (IU) each] and lyophilized.

To separate FVIII from its complex with vWF anion-exchange HPLC on a tentacle support Fractogel EMD TMAE or DEAE G 650 (S) column (Merck) was used. The column dimensions were 120 × 16 mm I.D. The sample was applied in 20 m*M* Tris-HCl buffer, pH 7.4, containing 10 to 50 m*M* $CaCl_2$ and 10 to 20 m*M* lysine with a gradient of 0 to 200 m*M* NaCl. Both the application buffer and the elution buffer contained calcium chloride. Other chromatographic conditions are given in the legends to the figures.

2.5. Chromatographic equipment

The ferric-ion free HPLC system consisted of two pumps, a programmer, a spectrophotometer with a deuterium lamp and a Rheodyne loop injection valve (Knauer Gerätebau, Berlin, Germany). A BioPilot system (Pharmacia-LKB) was used for semi-preparative and preparative chromatographic runs. On a production scale, a pump and a preparative photometer with a 280-nm filter (Pharmacia-LKB) were used. The salt gradient in ion-exchange chromatography was controlled by measuring the osmotic pressure (Gonotec osmometer).

2.6. Electrophoretic techniques

For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the dialysed and freeze-dried samples were dissolved in 62.5 m*M* Tris-HCl buffer (pH 6.8) containing 3% (w/v) of SDS, 10% (v/v) of glycerol and 0.001% (w/v) of bromophenol blue. In other experiments, samples of 10–30 μl were taken from the collected fractions after chromatography and mixed with a buffer containing a five times higher concentration of the above-mentioned substances. The amount of the buffer taken was such that the original concentration (between 0.2 and 2 mg/ml protein) was obtained after dilution of the sample. SDS-PAGE of the FVIII–vWF complex was carried out according to the Laemmli method [20] under non-reducing conditions using a Hoefer system (Hoefer Scientific Instruments, Vienna, Austria); 5–15% gradient gels were used. Staining of the gels was performed with 0.1% Coomassie Brilliant Blue. The amount of protein applied was between 5 and 50 μg per each 15 cm line.

Von Willebrand factor multimers were analysed by SDS-agarose discontinuous gel electrophoresis as described by Ruggeri et al. [21] and Budde et al. [22]. Briefly, samples were diluted between 1:20 and 1:100 fold according to their

vWF-antigen content, using a buffer consisting of 10 m*M* Tris-HCl, 1 m*M* EDTA and 2% (w/v) SDS, pH 8.0, and subjected to electrophoresis overnight. The separated multimers were then transferred to nitrocellulose sheets by electroblotting with a 50 m*M* sodium phosphate buffer, pH 7.4, containing 0.04% SDS and incubated with rabbit anti-vWF antibody (Dakopatts, Copenhagen, Denmark) followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio Rad Laboratories, Vienna, Austria). Detection of the vWF multimers was achieved by luminography. The luminiscent blots were covered by a translucent polyethylene film and exposed to an X-ray film (X-omat S, Kodak, Stuttgart, Germany).

3. Results and discussion

FVIII is extracted from pooled human plasma by a combination of precipitation, ultrafiltration and chromatography. The production scheme is shown in Fig. 1. At the beginning of the purification process, the specific activity of FVIII in the plasma is 0.015 to 0.020 U/mg of protein. Between 1500 and 5000 kg of plasma are used per batch. After cryoprecipitation and centrifugation, precipitation with 1–15% $Al(OH)_3$ is carried out, with simultaneous cooling to 16°C. In this step, a large part of the fibrinogen, fibronectin, factor V and other contaminating proteins from the clotting cascade are removed. Although only traces of some of these proteins, e.g. of factor Xa, are found in the cryoprecipitate, they may still activate FVIII during the subsequent purification steps, thereby reducing the yield and shelf-life of the product [6,7]. A change in the extraction conditions, e.g. in the components of the buffer used to dissolve the cryoprecipitate, or else in the temperature or in the amount of $Al(OH)_3$ added may considerably influence both the yield of FVIII and the composition of the isolated protein fraction. The conditions used for the purification as described above, including the addition of 1–15% $Al(OH)_3$, are aimed to result in a combination of proteins which allows the best possible yield

Step No.	Process Step	Step No.	Spec.Act. of FVIII (IU/mg)	Yield (%) / Enrichment
1.	PLASMA	1.	0.015 - 0.020	100 / Start
2.	→ cryoprecipitation	2.	-	-
3	Cryoprecipitate Solution	3.	0.3	35-50 / 15-20x
	↓			
4.	$Al(OH)_3$ Adsorption and Cold Precipitation	4.	0.8	30-40
	↓			
5.	S/D-Treatment	5.	0.8	30-40 / 40-55x
	↓			
6.	1. Anion-Exchange Purification			
	↓ ↓ ↓			
	Throughput cont.proteins + solvent/detergent; Wash; 1. FVIII-Eluate Purified	6.	90	25-32
	↓			
7.	Heating in Presence of Sugars and Amino Acids 10 h 63°C	7.	-	- / 4500-6000x
	↓			
8.	Removing of stabilizers ← 2. Anion-exchange Purification	8.	125	20-26
	↓			
9.	3. FVIII-Eluate Purified and Double Virusinactivated	9.	125	20-26 / 6300-8300x
	↓			
10.	Buffer Adjustment Sterile Filtration Filling and Freeze-Drying	10.	125	18-22 / 6300-8300x

Fig. 1. Production scheme for a double-virus inactivated FVIII–vWF complex from human plasma as starting material.

and specific activity of FVIII in the subsequent purification and pasteurization steps.

After extraction, the first virus inactivation is carried out by a combination of 0.3% (w/v) TnBP and 1% (w/v) Tween 80. The subsequent purification step, anion-exchange chromatography with anion-exchange tentacle supports DEAE or TMAE Fractogel EMD, results in a product with a specific activity of FVIII of 100 U/mg of protein, representing an enrichment of at least 6000 times compared to the initial human plasma. The chromatogram and SDS-PAGE of enriched FVIII–vWF are shown in Figs. 2a and b. As the virus inactivating reagents Tween 80 and TnBP do not bind to the anion-exchanger, they are removed in this step (see Figs. 1 and 2a). Factor VIII and its carrier protein, the vWF, are glycosylated, and both contain a high amount

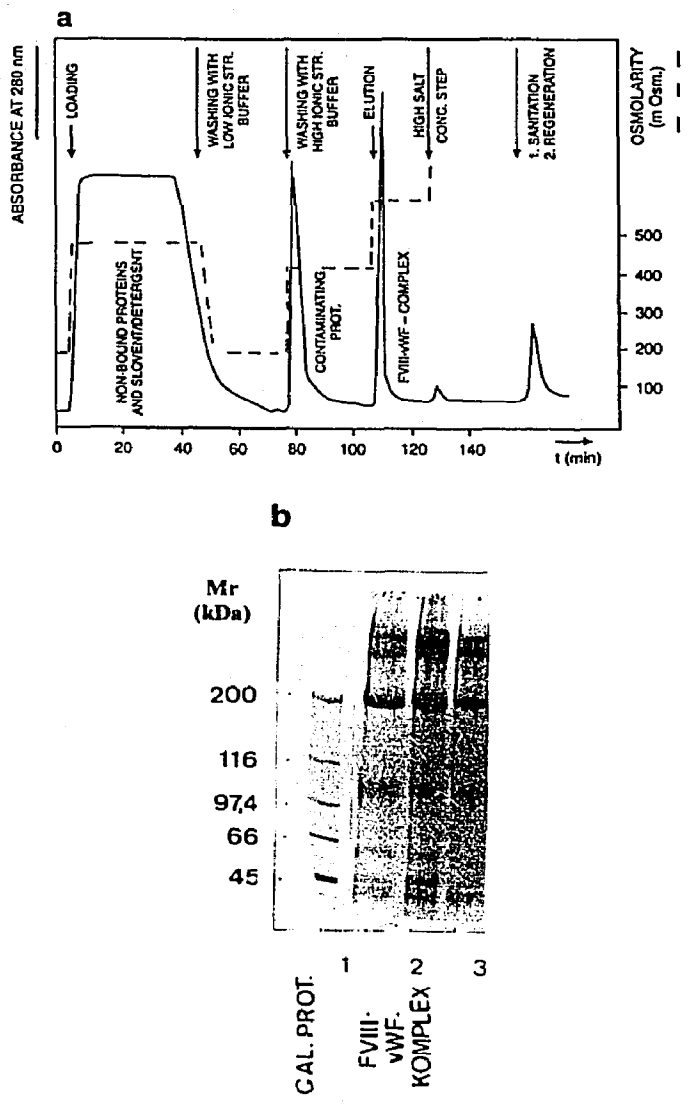

Fig 2. (a) Isolation of FVIII–vWF complex. First anion-exchange chromatography after S/D treatment. A tentacle gel Fractogel EMD-DEAE (Merck) was used. Chromatographic conditions: column, 120 × 16 mm I.D.; particle size of the support 20–40 μm (S-type); flow-rate 15 ml/min; pressure 1–3 bar ($1–3 \cdot 10^5$ Pa); room temperature. The gradient is shown in the chromatogram in part (a). (b) SDS-PAGE under non-reducing conditions of isolated FVIII–vWF complex. Three batches are analyzed (lines 1 to 3).

of sialic acid. Consequently the isoelectric points of both proteins are found in the low pH range. Both proteins bind very strongly and can be eluted only with rather high salt concentrations, even from a weak ion-exchange substance such as DEAE-Tentacle gel (see Fig. 2a). Under such conditions, the contaminating substances either do not bind at all, e.g. transferrin and immunoglobulins, or they are previously washed out by a step gradient of buffer with a low concentration (see Fig. 2a). Factor VIII and vWF are complexed by non-covalent binding. However, this binding is very strong, so that both glycoproteins can only be eluted as their FVIII–vWF complex under the mild conditions used in the present isolation scheme (Fig. 2b). The stoichiometric composition of the FVIII–vWF complex strongly depends on the extraction and chromatographic conditions applied. The relative amounts of FVIII and vWF assayed in the complex vary between 2:1 and 1:2.5. Under the conditions chosen here, one unit of FVIII is found per 0.4–0.8 units of vWF. The vWF is of utmost importance for preserving the activity of FVIII. The complex can be dissociated only under specific conditions, e.g. by adding calcium ions in relatively high concentration [2]. When FVIII is separated from the complex, another protein such as human serum albumin has to be added to protect the molecule from proteolytic degradation and loss of biological activity [7] (see also below), as well as to provide protein mass in manipulations such as freeze drying.

The chromatographic purification is shown in Fig. 2a. Only step gradients are used. The degree of purification resulting from this step is considerable, ca. 100 to 150 times compared with the original applied sample (see Fig. 1). The choice of support is important, and the same applies to the application buffer, the washing buffer and the elution buffer, in order to optimize the separation conditions. Factor VIII is a protein with a complex structure, the biological activity of which is easily lost [1–3]. The vWF functions as an adhesive protein [1] therefore readily binds irreversibly to the matrix, leading to a loss in yield or even plugging of the column. The lack of a suitable support was the reason that successful chromatographic purification of FVIII could not be carried out before the mid-80s [2,6,7,19]. Silica-based supports cannot be used for several reasons. Such supports may cause the activation of FVIII during the purification process. Also, the usual sanitation of the

column with 0.5 *M* sodium hydroxide cannot be carried out when a silica gel-based column is used because of its sensitivity to high pH.

The inadequacy of silica-based matrices is mirrored by matrices from organic polymers such as cellulose or styrene–polydivinylbenzene. The use of these supports resulted in a much lower yield, between 30% and 60% (not shown). Either partial activation of FVIII or non-specific interaction may be responsible for the losses in the purification process. The vWF has a particular tendency towards non-specific interaction, especially with slightly hydrophobic, organic supports. Other anion-exchange supports such as agarose (DEAE Sepharose or Q-Sepharose Fast Flow), and the polymer-based support Toyopearl DEAE [6,19] and tentacle supports (DEAE and TMAE) can be used successfully for the isolation of the FVIII–vWF complex.

The solvent/detergent step used in the first phase of virus inactivation is an effective tool for the destruction of lipid-enveloped viruses such as HIV, HBV and HCV [8,13]. However, non-enveloped viruses such as human parvovirus B19 and HAV remain largely unaffected by this treatment. Therefore, a second virus inactivation step is strongly recommended [9].

In the process described here, the FVIII–vWF complex is pasteurized for 10 h at 63°C. In order to protect the FVIII molecule during pasteurization, stabilizers such as sugar, amino acids, heparin and bivalent cations may be added. With 1–2 g/l of a sugar such as glucose, sorbitol, maltose or trehalose and with amino acids such as glycine, alanine, arginine or histidine, FVIII may retain up to 95% of its clotting activity. However, this kind of pasteurization will cause some loss of vWF (see Table 1). After pasteurization, the stabilizers are removed by anion-exchange chromatography. In order to lower viscosity, the sample is diluted with two volumes of a buffer with low ionic strength. Neither the sugar nor the amino acids bind to the anion-exchange column under these conditions and they can subsequently be washed out (see Fig. 3). The denatured proteins, in particular the vWF, also no longer bind to the column. Thus subsequently a FVIII–vWF complex is eluted which contains a slightly lower amount of vWF which in turn has a reduced content of vWF multimers (see Fig. 4 and Table 1). The yield of this purification step is very high, above 90%. This step is carried out for two important reasons, firstly the removal of the stabilizers as mentioned above and secondly concentration of FVIII on the column. The FVIII concentration

Table 1
Analytical characterization of FVII–vWF complex

Component	Only S/D treated	S/D treated and pasteurized
FVIII C activity	101 ± 20 IU/ml	98 ± 18 IU/ml
FVIII C antigen	110 ± 22 IU/ml	108 ± 22 IU/ml
vWF antigen	81 ± 16 IU/ml	69 ± 14 IU/ml
Total protein	1.0 mg/ml	0.8 mg/ml
Spec. act. (FVIII C)	101 ± 20 IU/mg	123 ± 20 IU/mg
vWF Ag/FIII C	0.8 ± 0.2	0.7 ± 0.2
FVIII C Ag/C	1.1 ± 0.4	1.1 ± 0.4
Fibrinogen	0.11 mg/ml	0.07 mg/ml
Fibronectin	0.10 mg/ml	≤0.05 mg/ml
Albumin	<0.03	<0.03
IgA	<0.008 mg/ml	<0.008 mg/ml
IgG	<0.008 mg/ml	<0.008 mg/ml
IgM	0.03 mg/ml	<0.01 mg/ml
TnBP	<0.5 μg/ml	<0.5 μg/ml
Tween 80	<10 μg/ml	<10 μg/ml

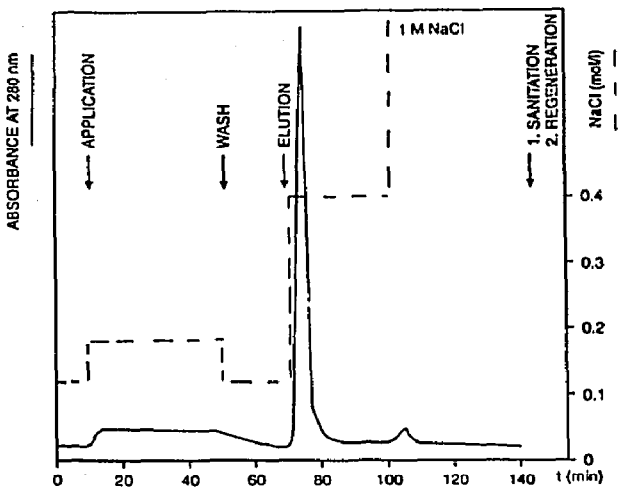

Fig. 3. Second anion-exchange chromatography as a step for removal of stabilizers after pasteurisation. For chromatographic conditions cf. Fig. 2a, except for column dimensions which are 40 × 26 mm I.D.

in the eluate is 35–60 IU/ml. This is an advantage for the following step, further concentration and diafiltration. Concentration always carries the risk of a loss of yield. As both FVIII and vWF are large proteins with a complex structure, mechanical treatment may cause a loss of activity. The specific activity of the FVIII product after subsequent lyophilization is 125 U/mg of protein, the enrichment factor compared to the original plasma being 6000–8000. The reduction of enveloped and non-enveloped viruses exceeded 5 logarithmic steps. The most harmful enveloped viruses, HIV, HBV and HCV, were reduced in all cases below detection, while HAV and Coxsackie, used as models of both non-enveloped and highly resistent viruses, are reduced to amounts lower than the detection limit [12].

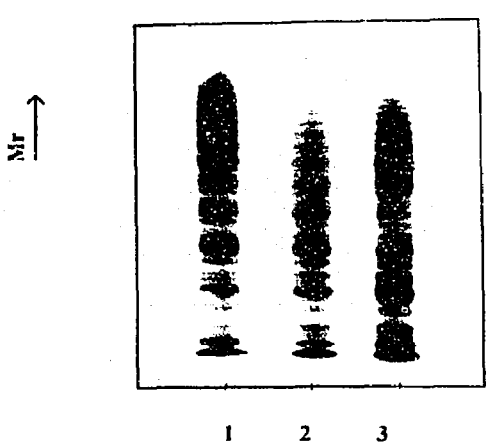

Fig. 4. Separation of vWF multimers in plasma (lane 1) and pasteurized (lane 2) and non-pasteurized (only S/D treated) (lane 3) FVIII–vWF complex, using the method according to Budde et al. [22].

3.1. Chromatographic behaviour of the factor VIII–von Willebrand Factor complex

The FVIII–vWF complex, when isolated as described above, is very stable and can hardly be dissociated at all under non-denaturing conditions. Fig. 5 shows a chromatogram obtained with a strong ion-exchange support, TMAE-tentacle gel. In these experiments, only traces of contaminants were found and the FVIII–vWF complex was eluted as one homogenic peak.

It is well known that the FVIII–vWF complex can be dissociated through the addition of calcium [2]. This method has often been used for isolating pure FVIII without vWF [2,23]. However, attempts to isolate FVIII through anion-exchange chromatography after addition of calcium failed several times. All the columns used for the experiments, packed with polymer-based supports, were plugged during elution. Fig. 6, however, shows the separation of single proteins from the FVIII–vWF complex by anion-ex-

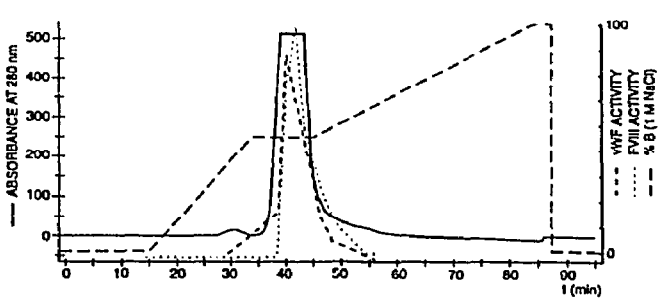

Fig. 5. Anion-exchange chromatography of FVIII–vWF complex. A strong AE-support Fractogel TMAE-tentacle (20–40 μm particle size) was used. Three hundred units of FVIII were loaded on the column. The recovery was ca. 90%. Chromatographic conditions: Column, glass, 120 × 16 mm I.D.; flow-rate 3 ml/min; pressure 1–3 bar ($1–3 \cdot 10^5$ Pa), room temperature. The gradient is shown in the figure.

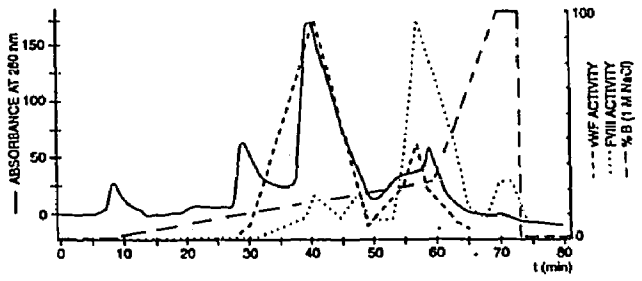

Fig. 6. Dissociation and separation of FVIII and vWF from the complex in presence of 50 m*M* calcium in the buffers. For other chromatographic conditions, cf. Fig. 5.

change HPLC, using tentacle supports. The purified FVIII is unstable and was stabilized after isolation with human serum albumin [5]. The association between FVIII and vWF in blood is very tight; dissociation can however be brought about in vitro by a high concentration of calcium ions [1]. The association and dissociation between these two glycoproteins is a reversible reaction. The heterogenity in glycosylation of FVIII and vWF as well as their possible interaction are the most likely reasons for their chromatographic behaviour (one main peak and an additional small peak for vWF and one main peak as well as three small peaks for FVIII, see Fig. 6).

The DEAE and TMAE tentacle gels were the only hydrophilic polymer supports with which elution of vWF could be carried out under the chosen conditions in the presence of calcium ions, after dissociation of the FVIII–vWF complex. Plugging of the column in the case of all the other investigated supports, such as Toyopearl DEAE, MonoQ, MonoP and TSK-DEAE 5PW (experiments not shown here), was probably due to the increased concentration of isolated vWF and its interaction with the matrix. The extended tentacle ends prevented this interaction by catching the protein before it reaches the support, thus allowing its subsequent elution. The FVIII–vWF complex has all the characteristics of a model substance suitable for the investigation of the behaviour of complex biological macromolecules during chromatographic separation:

– Factor VIII and von Willebrand factor are proteins which are found in low concrentrations in all sources.

– Purity as well as virus safety of the product are of utmost importance.

– The proteins are very sensitive, easily losing their biological activity.

– Both proteins, especially von Willebrand factor, tend towards nonspecific interactions with the support.

There may be some differences in the glycosylation pattern of proteins originating from a natural source such as plasma, and proteins produced by gene-technology; this will be a topic in further investigations.

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