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Journal of Chromatography B, 715 (1998) 357–367

JOURNAL OF CHROMATOGRAPHY B

Isolation of the factor VIII–von Willebrand factor complex directly from plasma by gel filtration

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Received 6 March 1998; received in revised form 15 May 1998; accepted 15 May 1998

Abstract

A high capacity gel filtration system was developed with the purpose of isolating factor VIII (FVIII) and von Willebrand factor (vWF) directly from plasma in significantly higher yields than obtained by cryoprecipitation, the technique most commonly used to recover FVIII–vWF from human plasma. After laboratory-scale gel filtration of plasma, a FVIII-containing fraction was collected containing about 90% of FVIII in the applied plasma and with almost tenfold higher purity than that obtained by cryoprecipitation. The gel filtration step has been scaled up for use as the initial step in the manufacturing process for a FVIII preparation (Nordiate).

Keywords: Factor VIII; Von Willebrand factor; Gel filtration

1. Introduction

Haemophiliacs suffering from haemophilia A and patients suffering from severe cases of von Willebrand's disease are treated with concentrates comprising factor VIII (FVIII) and/or von Willebrand factor (vWF), a treatment which has improved their quality of life considerably [1,2]. Preparations containing FVIII may be produced by recombinant technology, or FVIII may be purified from human fresh frozen plasma, either free from vWF or as a complex with vWF. Common to nearly all purification methods starting with plasma, is initial capturing of the cold-insoluble proteins including FVIII–vWF by a cryoprecipitation. By using small-pool techniques, cryoprecipitation may give recoveries of up to 70% of the factor VIII coagulation activity (FVIII:C) present in the plasma before freezing [3–5]. However, when used on a larger scale, e.g. pools of 500 kg or more, cryoprecipitation gives a FVIII:C yield of only 30–50% [6–9] and the purity is low (about 0.3 I.U. FVIII:C/mg of protein [8–10]).

Furthermore, a marked degree of vWF-fragmentation in the cryoprecipitate prepared from pooled plasmapheresis plasma is seen which makes this technique less suitable as an initial step in the production of a vWF preparation [11]. Attempts to prepare FVIII directly from plasma by chromatography have been hampered by the activation of the coagulation system leading to inactivation of FVIII:C and an initial step to remove the prothrombin complex was found necessary [8,12]. A new and gentle method for isolating the FVIII–vWF complex directly from plasma has been sought as up to 70% of the content of FVIII:C in plasma is lost as early as in, or before, the cryoprecipitation.

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0378-4347/98/$19.00
PII: S0378-4347(98)00250-3

Gel filtration is a diffusion controlled process used for separating solutes, such as proteins, according to their size and may be carried out in two ways [13]. In the group separation mode, the solutes are separated into two groups having a great difference in their molecular sizes, one group being eluted with the void volume and the other group being eluted later with a much larger elution volume, often close to the total bed volume. This procedure is primarily used for separating proteins from dissolved salts or to exchange buffer and is referred to as 'desalting' [14]. In the fractionation mode, solutes with similar molecular masses are separated; this procedure is often used for separating proteins. The substances are eluted more closely than when using group separation conditions and may overlap. High flow-rates are often not desirable because this does not allow for an effective separation of proteins and the column load must be kept low in order to obtain a reasonable separation of the individual proteins. Thus, gel filtration used in the fractionation mode has been limited to separation of proteins as a last polishing step where the volume to be fractionated is small [15]. Because FVIII–vWF is the largest protein complex known in plasma (M_r, $5 \cdot 10^5$–$20 \cdot 10^6$ [16]), it seemed possible to separate this complex from the other plasma proteins by high capacity gel filtration of the plasma in the group separation mode.

Isolation of FVIII directly from plasma by gel filtration was previously investigated by Ratnoff et al. [17,18] and by Paulssen et al. [19]. The yields were only about 40–50%, however, and the purity of the resulting FVIII-containing fraction, although high, was dependent on the starting plasma, as a high content of lipids and chylomicrons gave rise to a turbid FVIII-fraction. It was also noted that the gel filtration technique used did not allow handling of large amounts of plasma even though preliminary experiments indicated that gel filtration of the much more concentrated Cohn fraction I seemed possible. Since then gel filtration in large scale for purification of FVIII–vWF has only been used on more concentrated fractions [20,21]. However, by selecting gel filtration materials designed for high flow-rates, the capacity for isolating the FVIII–vWF complex directly from plasma can be increased considerably. This paper describes the optimization of a high capacity gel filtration technique for isolating the FVIII–vWF complex directly from plasma in high yields.

2. Experimental

2.1. Materials

Heparin was Noparin from Novo Nordisk (Bagsværd, Denmark). All other reagents were of analytical grade. Sepharose CL-6B, Sepharose CL-4B, Sepharose CL-2B, Sepharose 6FF, Sepharose 4FF, Sephacryl S-400 and Sephacryl S-500 were all from Pharmacia (Uppsala, Sweden). Fractogel TSK HW-65 (F) was from Merck (Darmstadt, Germany), and Matrex Cellufine GCL 2000 was from Amicon (Danvers, MA, USA). Laboratory columns were all from Pharmacia.

2.2. Assays

FVIII:C was measured either by a chromogenic assay in microtitre plates (Coatest Factor VIII, Chromogenix, Sweden) or by a one-stage coagulation assay using the activated partial thromboplastin time method at a Coag-A-Mate X2 (General Diagnostics, Oklahoma City, OK, USA) and FVIII-deficient plasma (General Diagnostics, Oklahoma City, OK, USA) as substrate. Internal standards had been calibrated against either the 3rd International WHO Standard of FVIII, Human Plasma, 3.9 I.U./ml or the 5th International Standard for Blood Coagulation Factor VIII (NIBSC, Potters Bar, UK).

The following specific protein analyses were performed as sandwich ELISAs using peroxidase (HRP)-conjugated antibodies and TMB (Kirkegaard & Perry, Gaithersburg, MD, USA) for visualization:
– Factor VIII antigen: FVIII antibodies from a haemophilia A inhibitor patient as catching antibody and HRP-labelled $F(ab')_2$ fragments from the same inhibitor patient as conjugate.
– Von Willebrand factor antigen (vWF:Ag): anti-human-vWF (A082, Dako, Denmark) as catching antibody and HRP–anti-human-vWF (P226, Dako) as conjugate. Standard was an in-house plasma pool calibrated against WHO 3rd International Standard

for Factor VIII and von Willebrand Factor in Plasma, code 91/666 (NIBSC, Potters Bar, UK).
– Fibrinogen: anti-human-fibrinogen (A080, Dako) as catching antibody and HRP–anti-human-fibrinogen (P445, Dako) as conjugate. Behring control plasma (Behringwerke, Marburg, Germany) was used as standard.
– Fibronectin: anti-human-fibronectin (A245, Dako) as catching antibody and HRP–anti-human-fibronectin (P246, Dako) as conjugate. Behring plasma protein standard (Behringwerke) was used as standard.
– IgM: anti-human-IgM (M702, Dako) as catching antibody and HRP–anti-human-IgM (05-4920, Zymed, San Francisco, CA, USA) as conjugate. Seronorm protein standard (Nycomed, Oslo, Norway) was used as standard.
– Protein C: anti-human-protein C (A370, Dako) as catching antibody and HRP–anti-human-protein C (P374, Dako) as conjugate. An internal plasma pool was used as standard.
– Prothrombin (FII): anti-human-prothrombin (A325, Dako) as catching antibody and HRP–anti-human-prothrombin (P446, Dako) as conjugate. An internal plasma pool was used as standard.

Albumin, α_2-macroglobulin, haptoglobin, antithrombin III, factor X, factor IX and β-lipoprotein were determined by rocket immunoelectrophoresis according to Laurell [22]. IgG was measured by radial immunodiffusion. The antibodies used were all from Dako. Standard human serum (Behringwerke) was used as standard for α_2-macroglobulin, haptoglobin, antithrombin III and albumin. An internal plasma pool was used as standard in the assay for factor IX, factor X and β-lipoprotein.

Protein concentrations were determined either by the method of Bradford [23] with human serum albumin as a standard or by a filter paper protein assay, essentially as described by Minamide [24]. In short: protein was fixed to filter paper and potential interfering substances were washed away. The fixed protein was visualized by binding of Coomassie brilliant blue G 250. Then the colour was extracted and quantitated at 600 nm. Standard was an in-house plasma pool with a protein content measured with the Kjeldahl assay.

2.3. Chromatographic procedures

If not specified otherwise in figure and table legends, the following chromatographic procedure was used: frozen plasma (Danish or US origin) was thawed to 25°C in a water bath and 1 I.U. of heparin per ml of plasma was added. The plasma was then applied at room temperature to a column packed with Sepharose 4FF using a linear flow-rate of 20 cm/h, whereafter the elution was performed at a linear flow-rate of 40 cm/h. Flow-rates were maintained using peristaltic pumps. Equilibration and elution were carried out with a 0.02 *M* citrate buffer, pH 7.0, containing 0.15 *M* NaCl and 2.55 m*M* $CaCl_2$, corresponding to a free calcium ion (Ca^{2+}) concentration of about $7 \cdot 10^{-5}$ *M* (checked by a calcium-selective electrode, Ingold, Frankfurt/Main, Germany).

After each gel filtration experiment, the packed columns were sanitized by flushing with 0.5 *M* NaOH. The columns were left with 0.5 *M* NaOH for at least 4 h.

3. Results and discussion

3.1. High capacity gel filtration of plasma

For large-scale gel filtration of plasma, high capacity is required. A high flow-rate and a high load of the column is, therefore, desirable and the gel filtration should be performed in the group separation mode. Moreover, since other plasma proteins such as albumin, IgG, antithrombin III and the prothrombin complex are often required for further purification into other medicinal preparations, high yields of these proteins are desired as well as small volumes of the collected fractions. In order not to change the anticoagulant–buffer composition of the FVIII–vWF-containing fraction and the albumin-containing fraction, a citrate buffer containing 0.15 *M* of NaCl at neutral pH was chosen for equilibration and elution of the columns. However, because a buffer exchange into a citrate buffer would decrease the level of free calcium ions below the critical level for the stability of FVIII:C [25,26], 2.55 m*M* of $CaCl_2$ was added. Addition of calcium has been found

important when using citrate buffers during purification of FVIII [27].

Fig. 1 shows the elution profile of total protein, measured by the absorbance at 280 nm, obtained by gel filtration of plasma on Sepharose 4FF. A tiny protein peak at the void volume (elution volume from 84 to 143 ml in Fig. 1) was followed by a much larger peak comprising most of the plasma proteins. The major part of the FVIII–vWF complex, measured both as activity and antigen, eluted at the void volume together with the tiny protein peak, whereas albumin and IgG eluted with the larger protein peak, clearly separated from the FVIII–vWF complex. IgM eluted between the FVIII–vWF complex and IgG/albumin. Other smaller proteins like protein C, antithrombin III, prothrombin (FII), factor X and factor IX eluted together with albumin and IgG whereas other large proteins like α_2-macroglobulin, fibrinogen and fibronectin eluted slightly later than IgM (results not shown). Almost complete separation of IgM and the FVIII main fraction (see Fig. 1) was obtained. A small part of vWF eluted later than the FVIII main fraction with an elution volume from 200 to 300 ml (see Fig. 1), showing that part of vWF may be able to penetrate the pores of the gel material. The elution volume of this vWF peak indicated that it was vWF dimer (M_r~$5 \cdot 10^5$). No FVIII:C was complexed with this low-molecular-mass vWF.

Tailings of both the FVIII main peak and the vWF main peak probably were complexes of FVIII bound to vWF multimers with low to medium size, which partly can penetrate some of the largest gel pores.

If large proteins like IgM, fibrinogen or α_2-macroglobulin are not going to be used in further purification processes, then a fraction between the FVIII main fraction and the albumin/IgG-containing fraction can be discarded, reducing the volume of the albumin/IgG-pool. The clear separation between the FVIII main fraction and the albumin/IgG-containing fraction also indicated that the load of the column

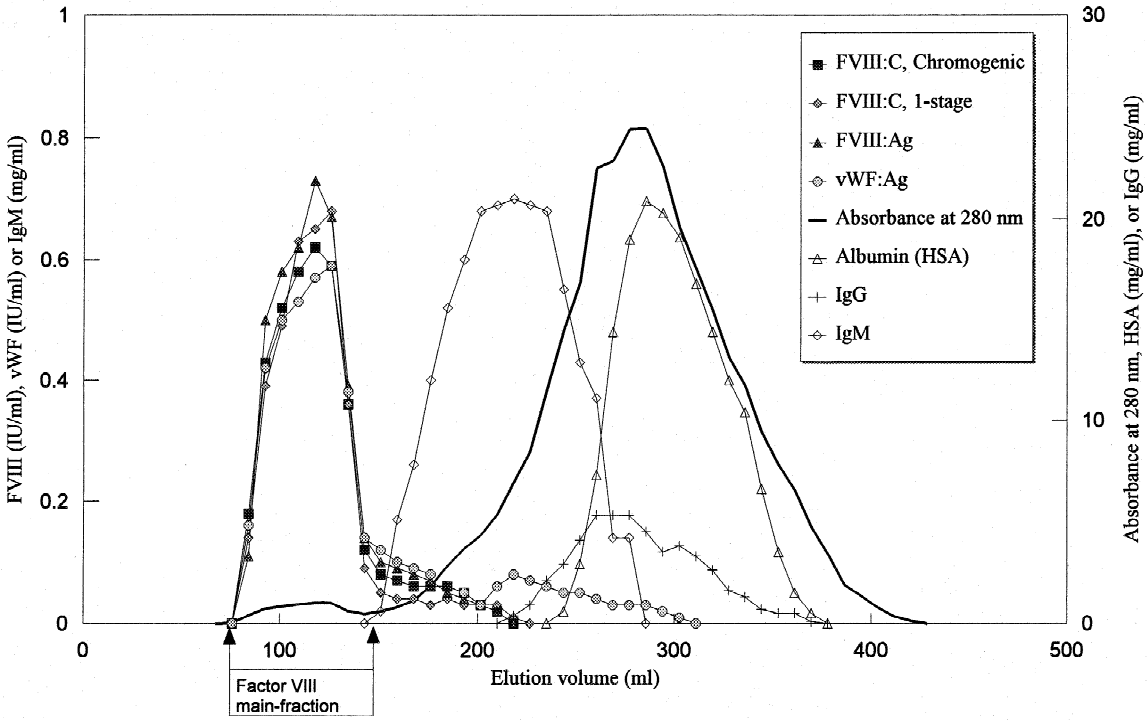

Fig. 1. Plasma (50 ml) was applied to a column (I.D. 2.6 cm, bed height 60 cm) packed with 300 ml of Sepharose 4 FF. Fractions of 8.4 ml were collected and the absorbance at 280 nm was measured. The content of various proteins in selected fractions was measured.

could be increased and/or the yield of FVIII:C could be increased slightly by collecting a larger volume, although more IgM was then also collected.

Using the same conditions as described in legend to Fig. 1, 15 experiments were performed. The FVIII main fraction was collected. Collection was started as soon as the absorbance at 280 nm indicated that protein had begun to elute and ended when the absorbance at 280 nm indicated that the local minimum in absorbance was reached. Start and end of collection are indicated by arrows on the *X*-axis in Fig. 1. The mean yield of FVIII:C (chromogenic assay) was 90% with a standard deviation of 7%.

An additional 7% of FVIII:C in the applied plasma could be recovered in the eluate immediately after the FVIII main fraction. The high recovery indicates that no inactivation of FVIII:C occurred during the fast gel filtration procedure and that there was no adsorption to the gel material. The mean volume of the FVIII main fractions was 81 ml with a standard deviation of only 3 ml, reflecting the high reproducibility. The low variation in the volume of the FVIII main fraction also means that it may be collected by volume if the local minimum in absorbance at 280 nm is difficult to observe. The specific activity of FVIII:C in the FVIII main fraction was determined as the concentration of FVIII:C divided by the protein concentration, where protein concentration was judged by absorbance at 280 nm (extinction coefficient=10). The absorbance at 280 nm was, however, very dependent on the turbidity of the FVIII main fraction. The turbidity reflected the content of lipids and lipoproteins in the applied plasma. Therefore, the separation effectiveness could only be compared in experiments performed with the same plasma pools, when judged by OD_{280}-based specific activity in individual FVIII main fractions.

The standard deviation for the yield in the 15 experiments was used for calculation of 95 % confidence limits for the mean yield in the other experiments.

3.2. Gel materials

Several gel materials were tested to find the most effective gel (Table 1). Gel filtration on gel materials with exclusion limits for globular proteins of approximately $5 \cdot 10^6$, like Sepharose CL-6B, Sepharose 6FF, Sephacryl S-400, Fractogel TSK HW-65 (F) and Matrex Cellufine GCL 2000 resulted in narrow elution peaks for FVIII, but no clear separations from the main protein peaks were obtained. Thus, the yields were high but the specific activities were mainly low. Experiments with Ultrogel A6 gave similar elution profiles to Sepharose 6FF (results not shown). Gel filtration on gel materials with very large pore sizes, like Sepharose CL-2B and Sephacryl S-500, on the other hand gave very broad FVIII:C elution peaks with elution volumes from 27

Table 1
Gel filtration of plasma using various materials

Gel material	M_r fractionation range	Number of experiments	FVIII yield (%)	FVIII mean specific activity (I.U./ml/OD_{280})
Sepharose CL-6B	$1 \cdot 10^4$–$4 \cdot 10^6$	3	95±9	0.16
Sepharose CL-4B	$6 \cdot 10^4$–$2 \cdot 10^7$	4	82±8	0.88
Sepharose CL-2B	$7 \cdot 10^5$–$4 \cdot 10^7$	3	68±9	0.24
Sepharose 6FF	$1 \cdot 10^4$–$4 \cdot 10^6$	3	96±9	0.71
Sepharose 4FF	$6 \cdot 10^4$–$2 \cdot 10^7$	3	86±9	1.35
Sephacryl S-400	$1 \cdot 10^4$–$8 \cdot 10^6$	3	101±9	0.75
Sephacryl S-500	$7 \cdot 10^5$–$4 \cdot 10^7$	3	91±9	0.28
Fractogel TSK HW-65 (F)	$5 \cdot 10^4$–$5 \cdot 10^6$	2	84±11	0.18
Matrex Cellufine GCL 2000	$1 \cdot 10^4$–$4 \cdot 10^6$	2	92±11	0.18

Plasma (50 ml, 16% of the bed volume) was applied to columns (bed height 60 cm×2.6 cm I.D.) packed with 300 ml of various gel filtration materials. Yields are given as 95% confidence intervals.

to 80% of the total bed volume. This resulted in poor separation of the FVIII–vWF complex from the other plasma proteins and thus a low specific activity in the FVIII main fraction. For Sepharose CL-2B, this also resulted in a low yield (Table 1). The best separations, and thus the highest capacity, were obtained with Sepharose CL-4B and Sepharose 4FF. With both gel materials, high specific activities were seen in the FVIII main fractions and the yields were more than 80% (Table 1). When aiming at large-scale gel filtration of plasma, Sepharose 4FF seems most suited because it is more rigid than Sepharose CL-4B and therefore allows a higher flow-rate. On a smaller laboratory scale, however, gels like Sepharose 6 FF, Sephacryl S-400 or Ultrogel A6 may be most suited if only smaller quantities of plasma are used and a high yield is needed, e.g. FVIII–vWF may be isolated in very high yield from 100–150 ml of plasma within 1 h on a column with 1 l of gel material.

Gel filtration of plasma was also attempted on softer gel materials like Biogel A-5m, Sepharose 4B and Sepharose 6B, but because of increasing back-pressure, the flow-rate had to be lowered. Experiments with loading of 20 ml of plasma (10% of the bed volume) on columns (I.D. 2.5 cm) packed with either 200 ml of Sepharose 4B or 200 ml of Sepharose 6B, at a linear flow-rate of 10 cm/h, and using the 13 m*M* citrate buffer containing 0.14 *M* NaCl at pH 7.0 that was used by Paulssen et al. [19], gave recoveries of FVIII:C below 50% in all experiments (results not shown). This may partly be due to the use of a calcium free citrate buffer, but since Ratnoff et al. [18] used a barbital-saline buffer, pH 7.5, and still obtained low recoveries, the low recoveries might as well have been due to the low flow-rates needed on softer gels, allowing time-dependent inactivation of the labile FVIII to take place.

3.3. Plasma load

To obtain high capacity gel filtration, a high load of plasma is essential. A column packed with Sepharose 4FF was loaded with increasing amounts of plasma (Table 2). With loading of plasma volumes constituting up to 25% of the bed volume, high yields were obtained. The specific activities were not significantly influenced by plasma loads up to 25% of the bed volume, but the resolution became poorer with higher loads, resulting in lower specific activities and yields. The volume of the FVIII main fraction decreased from about 230 to 125% of the applied plasma volume when the plasma load was increased from about 10 to 25% of the bed volume. Thus, the optimum plasma load of gel filtration columns packed with Sepharose 4FF seems to be 25% of the bed volume.

3.4. Column height and linear flow-rate

Gel filtration of plasma on columns packed with Sepharose 4FF to different bed heights between 40 and 60 cm were performed, but no differences in either yields or specific activities were observed (results not shown). The yield of FVIII:C was not influenced by variation in linear flow-rate between 19 and 57 cm/h. The yield was 86% at 19 cm/h, 87% at 38 cm/h, and 88% at 57 cm/h. When the

Table 2
Gel filtration of various amounts of plasma on a column packed with sepharose 4FF

Amount of plasma (% of bed volume)	Volume of FVIII-fraction (% of applied volume)	FVIII yield (%)	Specific activity (I.U./ml/OD_{280})
9.4	233	91	0.54
12.6	175	85	0.49
15.7	160	89	0.43
18.8	138	86	0.53
22.0	133	89	0.52
25.1	125	84	0.41
31.8	124	73	0.22

Different volumes of plasma were applied to a column (bed height 60 cm×2.6 cm I.D.) packed with 300 ml of Sepharose 4FF. Three experiments were performed with three different plasma pools for each amount of plasma. The 95% confidence limits for the mean yield were ±9 for each amount of plasma.

specific activities were calculated by dividing FVIII:C per millilitre by the absorbance at 280 nm no significant difference was seen. However, when the filter paper protein assay was used (which is more specific for protein) a more pronounced decrease in specific activity was seen when the linear flow-rate was increased from 20 to 60 cm/h, especially when it was increased above 30 cm/h (Fig. 2). At the same time, there seemed to be an increased amount of IgM and a steady amount of fibrinogen in the FVIII main fraction. The volume of the FVIII main fraction was not influenced by the linear flow-rate. To obtain a high specific activity in the FVIII main fraction, the linear flow-rate should not be higher than approximately 30 cm/h, but because a low linear flow-rate will lower the daily capacity in a large production scale, a compromise may have to be made. Thus, by keeping the bed height at e.g. approximately 45 cm and the linear flow-rate at 30–40 cm/h, plasma corresponding to 25% of the bed volume can easily be applied every second hour.

3.5. Temperature, conductivity and pH

Variation of the temperature between 15 and 30°C in the applied plasma and during gel filtration had no influence on either yield (78 to 88%) or separation of factor VIII:C. The specific activity decreased insignificantly from 0.43 to 0.31 when the temperature was lowered from 30 to 15°C. The volume of the FVIII main fraction was not influenced by the temperature. On one hand, a high temperature during gel filtration may be desirable as this will lower the viscosity of the plasma, giving a lower back-pressure and allowing of easier diffusion of proteins into the pores of the gel material. On the other hand, high temperatures may lead to faster inactivation of unstable proteins like FVIII. As recoveries of FVIII:C near 100% were constantly found when performing gel filtrations of plasma at room temperature (approximately 25°C), any temperature between 20 and 30°C seems useful.

The conductivity during gel filtration was varied

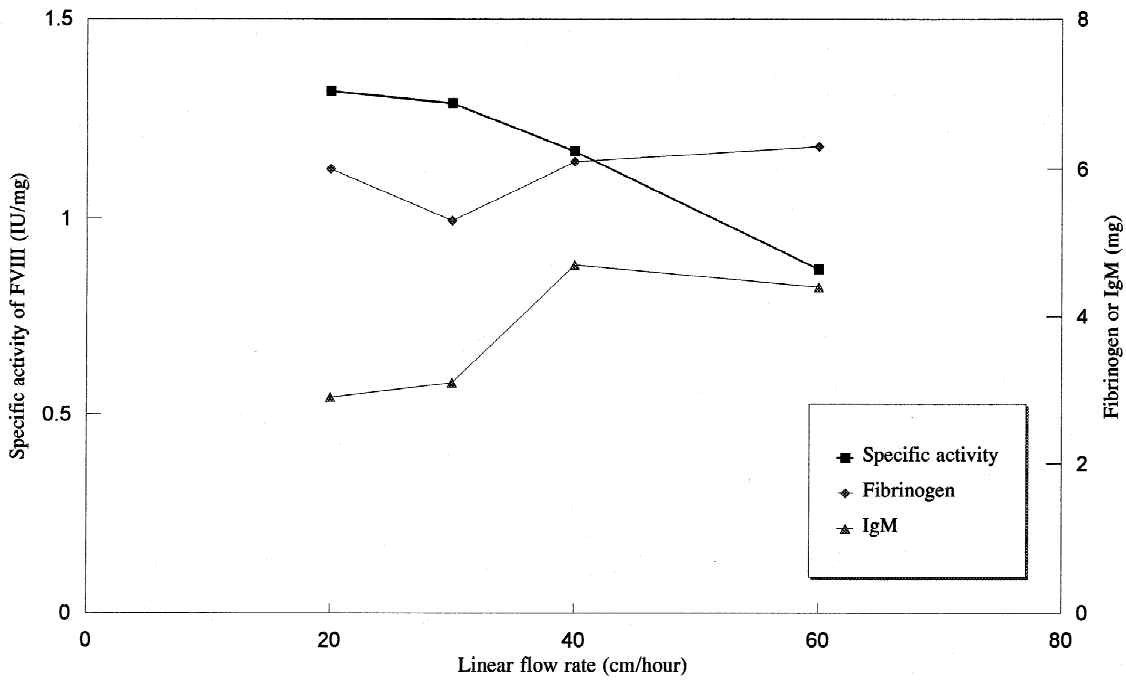

Fig. 2. Plasma (75 ml) was applied to a column (I.D. 2.6 cm, bed height 60 cm) packed with 300 ml of Sepharose 4FF. Three experiments were performed with each of the following linear flow-rates during loading of the plasma and subsequent elution with equilibration buffer: 20, 30, 40 and 60 cm/h. The FVIII main fraction was collected and the contents of IgM, fibrinogen and total protein (filter paper protein assay) were measured.

by varying the concentration of NaCl in the equilibration and elution buffer and, if necessary, adding NaCl to the plasma samples. The concentration of NaCl had no effect on the yield of FVIII:C and specific activity in the FVIII main fraction in the interval from 0.1 *M* to 0.2 *M*. When the concentration of NaCl was decreased to 0.05 *M* there seemed to be a slightly lower yield of FVIII:C (76%), but the yield was not significantly different from the results obtained with 0.1–0.2 *M* of NaCl (88–90%). Thus if desired, the conductivity in the FVIII main fraction may be lowered be using a buffer with a lower content of NaCl for equilibration and elution.

No differences were seen in yield of FVIII:C after gel filtration of plasma at either pH 7.0 (91%) or pH 7.8 (92%) nor were there any differences in the elution profiles and thus the separation of the FVIII–vWF complex is not influenced by pH in this range.

3.6. Addition of heparin

Heparin is a non-chelating anticoagulant that has been found to stabilize FVIII:C in plasma, especially when the level of calcium ions are controlled [25]. Without addition of heparin to the plasma before gel filtration, a slight but significantly ($P=0.03$, paired *t*-test) lower yield was obtained. With addition of heparin to the plasma, the mean yield (± 1 S.D.) in six experiments performed using six different plasma pools was $94\pm6\%$ whereas the mean yield in experiments with the same six plasma pools, but without addition of heparin to the plasma, was only $84\pm6\%$. The lower yield of FVIII:C without addition of heparin was apparently caused by destabilisation of FVIII:C and not by a change in the elution characteristics of the FVIII–vWF complex, as the recovery of FVIII:C decreased (from 100% to 90%) as well. By addition of heparin, all FVIII:C in the plasma was recovered after the gel filtration and the yield of FVIII:C was thus dependent only on the volume of the collected FVIII main fraction.

3.7. Characterisation of FVIII main fraction

IgM and fibrinogen were the main impurities measured in the FVIII main fraction (Table 3). Other impurities, identified by crossed immunoelectrophoresis (Fig. 3), were α_2-macroglobulin, albumin, haptoglobin and β-lipoprotein. Fibronectin was not visible in crossed immunoelectrophoresis but

Table 3
Characterisation of FVIII main fraction

Protein	FVIII main fraction
FVIII:C (I.U.)	49
vWF:Ag (I.U.)	52
IgM (mg)	2.5
Fibrinogen (mg)	6.2
Fibronectin (mg)	0.5
α_2-Macroglobulin (mg)	1.6
Albumin (mg)	1.0
Haptoglobin (mg)	<d.l.
β-Lipoprotein (I.U.)	2.0
Total protein (Bradford, mg)	16

Plasma (60 ml) was applied to a column (bed height 46 cm×2.6 cm I.D.) packed with 242 ml of Sepharose 4FF. The linear flow-rate was 47 cm/h both during loading of the plasma and during the subsequent elution with equilibration buffer. FVIII main fraction was collected and analysed for various proteins. d.l.: less than detection limit.

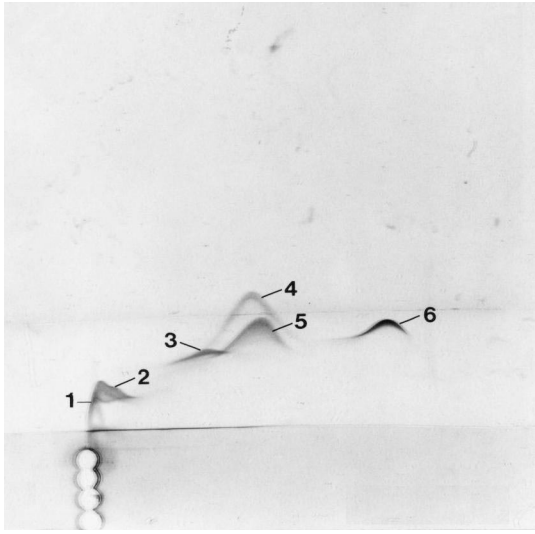

Fig. 3. Crossed immunoelectrophoresis of the FVIII main fraction. A sample of 50 μl was applied and run against 6 μl/cm^2 of anti-human-total plasma (Dako A134) in the second dimension. The proteins were identified by including specific antibodies in the intermediate gel as: vWF (1), fibrinogen (2), β-lipoprotein (3), haptoglobulin (4), α_2-macroglobulin (5) and albumin (6).

a low level could be detected by a sensitive ELISA. It was expected that large proteins like IgM, α_2-macroglobulin, fibrinogen and fibronectin would constitute most of the protein impurities in the FVIII main fraction, but it was surprising that the amount of albumin was so high, because complete separation of the FVIII–vWF complex from the albumin peak had apparently been achieved (Fig. 1). The reasons for the relatively high content of albumin may simply be that the content in plasma is very high and/or that some albumin is aggregated or complexed with other macromolecules [28]. Nevertheless, the amount of albumin was still approximately 2100 times less than in the applied plasma. The amount of β-lipoprotein was determined as 2.0 I.U., but may vary considerably in small-scale because of very different amounts of lipoproteins in the plasmas used.

The total amount of measured proteins was estimated to 14.4 mg or 90% of the total protein measured by the Bradford assay. A factor of 1.03 was used for calculating the approximate amount of protein in the 2 I.U. of β-lipoprotein present, giving about 2.1 mg of protein. One I.U. of vWF was considered to be approximately 10 μg of protein.

The specific activity of FVIII:C in the collected fraction was 3.06 I.U./mg protein, using the amount of total protein as measured by Bradford. The Bradford assay may, however, underestimate the amount of protein. Thus, from Fig. 2, where the filter paper protein assay was used for estimating the protein concentration, a specific activity of 1–1.5 was achieved. These experiments were, however, performed with plasma that had been thawed, pooled and then refrozen in portions of appropriate sizes and the concentration of FVIII:C was, therefore, low. In other experiments, where the Kjeldahl method was used for measuring the concentration of protein, specific activities from 2 to 2.5 were obtained.

Thus, gel filtration of plasma seems to give a purification factor for FVIII:C of more than 100-fold. The specific activity of FVIII:C obtained by large-scale cryoprecipitation is almost ten times lower (about 0.3 I.U./mg protein). More than 50% of the impurities in cryoprecipitates have been found to be fibrinogen and albumin [29]. The level of fibrinogen in cryoprecipitates has been found to be 1.3–2.5 mg per I.U. of FVIII:C [10,30–32] or more than ten times the level obtained in the FVIII main fraction from gel filtration (0.13 mg/I.U. FVIII:C, Table 3). Fibronectin, another major impurity in cryoprecipitates, is also more than ten times higher (0.24–0.4 mg/I.U. FVIII:C) in cryoprecipitates [31,32] than in the FVIII main fraction collected after gel filtration (0.01 mg/I.U. FVIII:C, Table 3). IgM, on the other hand, is on the same level in both cryoprecipitates (0.06 mg/I.U. FVIII:C [30]) and the FVIII main fraction obtained after gel filtration (0.05 mg/I.U. FVIII:C, Table 3).

Because the FVIII main fraction is isolated by a separation method based on size, the fraction contains all (or is even enriched in) the high-molecular-mass vWF molecules [33] which are the haemostatically most important. This is important if the technique is used as the initial step in a purification process for a vWF preparation.

Unless plasma with a very low content of lipids is used, the FVIII main fraction will be turbid. It may also contain debris from degraded blood cells as all high-molecular-mass substances that are able to enter the column will be eluted in the void volume together with FVIII–vWF. The IgG/albumin-containing fraction is, on the other hand, very clear even after gel filtration of plasma containing high amounts of lipids. Thus, the method may also be useful for removal of lipids from plasma or other protein solutions that often cause difficulties in initial chromatographic steps.

3.8. Scaling-up

The high capacity gel filtration of plasma has been introduced as the initial step in a large-scale manufacturing process for the FVIII product Nordiate [34,35], and is also used as the initial step in a purification process for a vWF preparation [33]. During development of the manufacturing process for Nordiate, the gel filtration step was first scaled-up in the laboratory to a column (bed height 65–70 cm×10 cm I.D.) containing 5.4 l of Sepharose 4FF. By loading 1350 ml of plasma with a flow of approximately 5 l/h, FVIII–vWF could be collected with a mean yield for FVIII:C of 90% (48 experiments, chromogenic assay). However, when the gel filtration step was further scaled-up in the pilot plant

to columns (bed height 63 cm×45 cm I.D.) containing 100 l of Sepharose 4FF, the mean yield of FVIII:C dropped to 65%. The reason for this is not clearly understood, but may be due to collection of a relatively smaller volume of FVIII main fraction, freezing of samples before assay and/or the transfer of the technique from laboratory equipment to manufacturing equipment. A yield of 65% is, however, much higher than the yield achieved in large-scale cryoprecipitation (30–50%), and the purity of the isolated FVIII–vWF is almost ten times higher. Gel filtration of plasma in the large-scale production of Nordiate is performed on two columns (bed height 45 cm×140 cm I.D.), each packed with 690 l of Sepharose 4FF. Within 8 h, 175 l of thawed plasma (27°C) are applied four times to each column with a linear flow-rate of 25 cm/h. The plasma is filtered through a 40 μm filter for removal of potential inorganic materials like e.g. small plastic particles from cutting of the frozen plasma portions. The collected FVIII main fractions are pooled and further purified as one batch into Nordiate. This gives a total capacity of 1400 l of plasma per batch.

4. Conclusion

A high capacity gel filtration for capturing the FVIII–vWF complex directly from plasma has been described. The isolated FVIII–vWF complex has a purity more than 100 times greater than in plasma and the yield of FVIII:C is about 90%. The high yields are obtained in laboratory scale from plasma volumes of up to 1.5 l. In large-scale, with gel filtration of up to 1400 kg of plasma on columns (bed height 45 cm×140 cm I.D.) packed with 690 l of Sepharose 4FF, the yield of FVIII:C is lower, averaging about 65%. This yield is, however, significantly higher than the yields obtained in the normally used cryoprecipitation method for isolating FVIII and vWF from plasma (typically 30–50% in large scale). The purity of the isolated FVIII–vWF is almost ten times higher than that obtained by cryoprecipitation, and the amount of the haemostatically effective high-molecular-mass vWF is also higher than obtained by cryoprecipitation.

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