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Journal of Chromatography A, 796 (1998) 289–298

JOURNAL OF CHROMATOGRAPHY A

Size-exclusion chromatography of plasma proteins with high molecular masses

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Received 23 April 1997; received in revised form 17 September 1997; accepted 1 October 1997

Abstract

Two different hydrophilic materials with large pores, Superose 6 and Fractogel EMD BioSec (S), which are designed for size-exclusion chromatography (SEC) of plasma proteins with high molecular masses, are tested for their performance on a preparative scale. The model mixtures are preparations of the clotting factors VIII (FVIII) and IX (FIX). A combination of a Fractogel EMD BioSec (S) column and a Superose 6 column has proved to be particularly effective for separations in a wide molecular size range, from several millions down to about 20 000. Superose 6 showed good results on a small scale as well as on a large scale, even in the molecular mass range over 1 000 000. However, recovery of FVIII clotting activity was less than 70% with this material and therefore not satisfactory. Fractogel did not perform well in terms of separation on a small scale. However, in the case of biopolymers with high molecular masses, separation was improved by using larger columns. With Fractogel, recovery of activity of the two clotting factors FVIII and FIX was satisfactory, above 80%. On a large scale, the active fraction in the clotting factor concentrate was successfully separated from the non-active fraction with either size exclusion (SE) material. In the preparation under investigation, the clotting factor VIII is found in a complex with the von Willebrand factor (vWF). The FVIII–vWF complex has a molecular mass of several millions. It dissociates in the presence of high concentrations of Ca^{2+} ions. Under such conditions FVIII and vWF were successfully separated with both SEC columns.

Keywords: Stationary phases, LC; Preparative chromatography; Proteins; Clotting factor XIII; Clotting factor IX

1. Introduction

Size-exclusion chromatography (SEC) of proteins is a method which can provide important information about the state of the protein molecules and other biopolymers in a solution. The obtained data concern the size of the molecule or its hydrodynamic diameter and the interaction of identical molecules with one another or with other molecules.

Using SEC, information is obtained about the physical state of proteins in solution. Separation can be carried out under denaturing or non-denaturing conditions [1–3]. Reagents such as some detergents, urea or guanidinium hydrochloride are used in order to reduce to a minimum the undesirable non-specific interactions between components of the sample and the matrix used for separation. However, the reagents can also modify the original state of the components in the sample, and thereby obscure the obtained data [4]. Further data can be obtained by a combination of SEC with other methods such as sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–

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0021-9673/98/$19.00
PII S0021-9673(97)01018-2

PAGE), chemical cross-linking and immunoprecipitation.

The above-mentioned applications of SEC are usually carried out on a small scale and for analytical purposes only. For preparative isolation of biopolymers, SEC is best suited for the last phase of the process, the so-called polishing step. At this stage the sample volume is already reduced, and the sample has been pre-purified. Therefore the rather low volume capacity of the SEC columns is no longer a limiting factor [5]. If the component to be separated is physiologically active and subsequently to be used as a drug, the addition of unphysiological substances such as detergents or chaotropic reagents to the mobile phase of SEC has adverse effects. Their removal in the polishing step, at a very late stage of production, presents considerable difficulties. Besides, additional analytical and toxicological investigations are necessary about any residues of these substances in the product and their possible side-effects [6]. This production step therefore calls for another strategy, namely to find an SEC support that will not interact with the sample component, rendering the addition of the above-mentioned substances superfluous. Of course this support will have to perform best in the range of molecular masses to which the target substance belongs.

Surprisingly few supports exist for preparative chromatography of biopolymers with high molecular masses, that is above one million. This lack of material is felt in particular, if the chromatographic separation has to be carried out under non-denaturing conditions. SEC media with large pores on a silica gel basis can cause the activation of clotting factors, followed by their degradation, especially in the case of the highly sensitive clotting factor VIII (FVIII) [7]. The rather high hydrophobicity of synthetic materials with large pores is another reason why they cannot be used for such separations. The only bulk support available for preparative SEC of plasma proteins with high molecular masses has been Superose 6. This support has many advantageous characteristics including a hydrophilic surface which reduces to a minimum non-specific interactions with components of the sample. Consequently the risk of any denaturing occurring during chromatographic separation is rather low, and the components almost always retain their biological activity to a very large extent. The support also allows the separation of biopolymers with apparent molecular masses of several millions [8]. However, the low pressure resistance of this support is a severe disadvantage, as it rules out high flow rates. Sanitation, regeneration and equilibration are significantly slowed down. Other supports for SEC of biopolymers have considerable disadvantages in preparative separations including a hydrophobic surface or a lack of stability at higher pH. This will lead at least to some loss of biological activity in the substances and may therefore prevent their use altogether [2].

The support Fractogel EMD BioSec (S) has recently been developed. To the rather hydrophobic surface of the starting material on a polymethacrylate basis with large pores (1000 Å), a hydrophilic tentacle coating has been covalently bound [5,9,10]. In this paper the behavior of the support in separations of biopolymers with high molecular masses is investigated. It is also used for the isolation of highly sensitive proteins, FVIII and clotting factor IX (FIX), which retain their biological activity.

2. Experimental

2.1. Chemicals

Chemicals of analytical-reagent grade were purchased from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) or Sigma (Deisenhofen, Germany).

2.2. Chromatographic system

The unit for preparative SEC consists of a pump P-50, with a flow-rate of 0.1 to 49.9 ml/min, a photometer UV-1 (detection at 280 nm), a Rec 101 recorder and a Frac 100 fraction collector (all from Pharmacia, Vienna, Austria).

For analytical high-performance size-exclusion chromatography (HPSEC) a unit from Knauer Gerätebau (Berlin, Germany) was used. It consists of two pumps No. 64, a variable-wavelength detector and a Knauer loop injection valve. The fraction collector was from Bio-Rad (Munich, Germany). The salt concentration was controlled by measuring

the osmotic pressure (Halbmikro-Osmometer, Typ Dig. L., Knauer Gerätebau).

2.3. Columns and separation media

For analytical HPSEC a Superose 6 column (300×10 mm I.D., Pharmacia) and the TSK G 4000 PWXL and TSK G 5000 PW columns, 300×7.5 mm I.D. (both from Tosohaas, Stuttgart, Germany) were used.

For scaling-up experiments the following materials were used: Superose 6 Prep Grade (Pharmacia), packed in XK 16/60 and XK 26/60 glass columns or Superformance 1000-100, and Fractogel EMD Bio SEC (S), packed either in a semi-preparative glass column Superformance 26, 600×26 mm I.D., or a preparative glass column Superformance 1000-100, 900×100 mm I.D. (all from Merck).

2.4. Proteins

The standard protein mixture for HPSEC, consisting of thyroglobin (Thyr) (M_r 640 000), immunoglobulin G (IgG) (158 000), ovalbumin (OA) (44 000), myoglobulin (Mgb) (17 000) and vitamin B12 (1300), was purchased from Bio-Rad (Vienna, Austria). To this mixture immunoglobulin M (IgM), a protein with a high molecular mass (about 900 000) was added. It was purchased from Sigma.

The FVIII concentrate Octavi SDPlus, consisting of a complex of FVIII and its carrier protein von Willebrand factor (vWF), as well as the FIX concentrate were obtained from Octapharma (Vienna, Austria).

2.5. Determination of biological activity of plasma proteins

2.5.1. Assays for factor VIII activity

The quantitative, photometric determination of FVIII activity, the so-called chromogenic assay [11], is based on the fact that FVIII together with clotting factor IXa (FIXa) catalyses the transformation of clotting factor X (FX) into clotting factor Xa (FXa), in the presence of phospholipids and calcium ions. FXa is a protease which then hydrolyzes the chromogenic substrates S-2222 and I-2581 into a peptide and *p*-nitroaniline, which is determined by measurement of the adsorbance at 405 nm. The activity of FVIII is determined on the basis of a scale which is defined by a World Health Organization (WHO) standard. A laboratory-made standard or a standard human plasma (Behringwerke, Marburg, Germany) calibrated against the WHO standard was used. The COATEST factor VIII:c testkit was purchased from Chromogenix (Mölndal, Sweden). The measurements were carried out with an enzyme-linked immunosorbent assay (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 coagulation assay for the determination of FVIII clotting activity has been described by Barrowcliffe [12]. 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 a laboratory-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).

2.5.2. Factor IX clotting assay

A one-stage coagulation assay for FIX was performed by mixing the corresponding deficient plasma with the diluted sample and incubating it in an activator (lipid extract and kaolin). After incubation, coagulation was started by adding calcium chloride solution. The time required for the clot to form was measured. Each test was calibrated against the FIX concentrate (Octapharma). FIX deficient plasma and all other reagents were purchased from Behringwerke. Coagulation tests were carried out with a KC 4A coagulometer (Amelung).

2.5.3. Determination of other plasma proteins

The determination of other clotting factors and the immunochemical determination of plasma proteins have been described previously [13]. The concentration of von Willebrand factor antigen (vWF Ag) was measured by ELISA as described by Cejka [14].

2.6. Purification of factor VIII

The FVIII enriched fraction was obtained from plasma through cryoprecipitation [15]. The precipitate was separated by centrifugation at 7000 *g* (Separator, Alfa Laval, Tumba, Sweden) and stored at −30°C until further use. The supernatant was used for the production of other plasma products such as the vitamin K-dependent factors and human albumin [16]. The further procedure was performed as described by Josić et al. [17].

2.7. Purification of factor IX

FIX is routinely produced from human cryopoor plasma at the Octapharma production facility (Vienna, Austria). The batch size was 1300 kg. The production roughly follows the method of Brummelhuis [18]. The modifications of the process and the introduction of an additional step for the removal of viruses have been described before [13,19].

2.8. Electrophoretic techniques

For SDS–PAGE the dialysed and freeze–dried samples were dissolved in 62.5 m*M* tris(hydroxymethyl)aminomethane (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 buffer used was such that after dilution of the sample the original concentration (between 0.2 and 2 mg of protein/ml) was obtained. 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 (Sigma). The amount of protein applied was between 5 and 50 μg per lane.

The vWF multimers were analysed by SDS–agarose discontinuous gel electrophoresis as described by Budde et al. [21].

3. Results

The separation of standard proteins with molecular masses between 18 000 and 900 000 is achieved both with Superose 6 and Fractogel EMD Bio SEC (S). The material of Superose 6 yields good results in the analytical range down to a column size of 300×10 mm I.D. (cf. the separation in Fig. 1), whereas Fractogel Bio SEC (S) with its larger particles performs satisfactorily only from the semi-preparative range onwards (cf. Figs. 2 and 3).

The separation of a FVIII product on an analytical scale with a Superose 6 high-performance column (300×10 mm I.D.) is shown in Fig. 1A. The main components, all of them in the range of molecular masses between 150 000 and several millions, were well separated in four peaks. The separation according to molecular masses agrees with the corresponding SDS–PAGE under non-reducing conditions (Fig. 1B). The entire FVIII activity was measured in the first peak with an apparent molecular mass of several millions (Fig. 1A, dashed line). This is not surprising, as FVIII in this product forms a complex with the vWF multimers, whose molecular mass is at least 4 million [17,22]. Corresponding SDS–PAGE under non-denaturing conditions confirms that separation occurs according to the sizes of the molecules (Fig. 1B). However, the FVIII–vWF complex which appears in the first peak (see Fig. 1A) has such a large molecular size that it cannot even enter the separating gel (lanes 3 and 4 in Fig. 1B). The separation of vWF multimers from peak 1 in Fig. 1A was carried out with the corresponding agarose gel. Fig. 1C shows vWF multimers in different fractions from HPSEC. The separation is well reproducible, provided that the column is rinsed with 0.5 *M* NaOH after each separation in order to remove any residues of the previous sample. This means that under the given conditions a part of the sample does after all form non-specific bindings with the column. Therefore the yield did not exceed 50 to 70%.

No satisfactory separation of such a FVIII product was achieved with a Fractogel EMD BioSec (S) column, neither on an analytical nor on a semi-preparative scale (not shown here). However, a combination of a Fractogel EMD BioSec (S) column with a Superose 6 column for semi-preparative separations showed good results (Fig. 2). A semi-

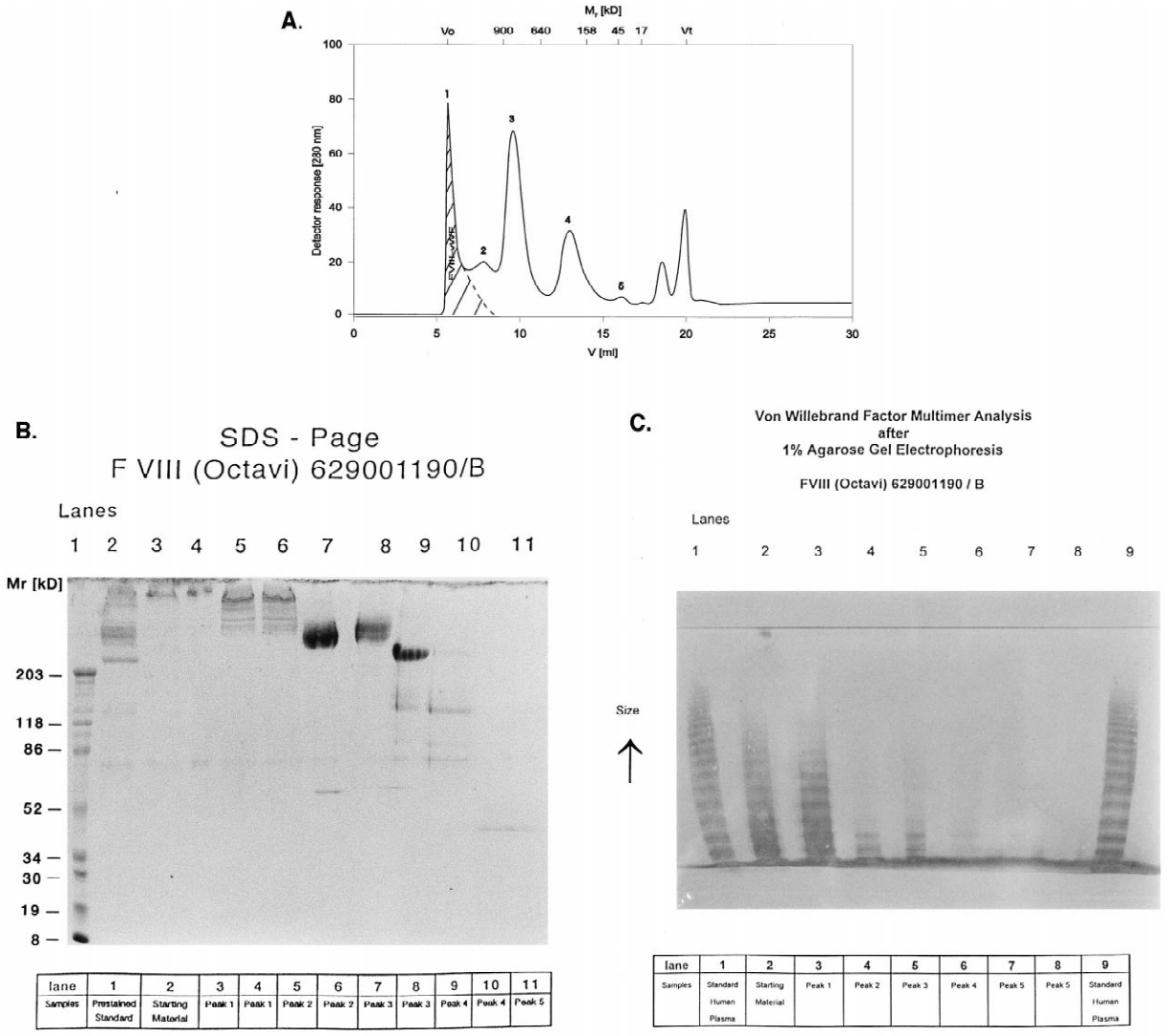

Fig. 1. (A) HPSEC of a preparation containing the FVIII–vWF complex. SEC chromatogram under non-denaturing conditions. A Superose 6 high-performance column, 300×10 mm I.D. (analytical) was used. Chromatographic conditions: 0.9 mg of protein (100 IU of FVIII and 65 IU of vWF Ag) in 0.5 ml; flow-rate 0.5 ml/min, pressure 9 bar, room temperature. Standard proteins as molecular size markers (250 μg each): IgM (M_r) about 900 000; Thyr. (640 000); IgG (158 000), OA (45 000); Mgb (17 000) and vitamin B12 (1300) (see also Section 2.4). Mobile phase: 200 m*M* NaCl, 15 m*M* Tris–HCl, pH 7.4, osmolarity: 440–460 mOsmol. After each run the column was regenerated with 0.5 *M* NaOH for 2 h (flow-rate 0.5 ml/min) and subsequently equilibrated with two column volumes of 0.2 *M* Tris–HCl, pH 8.0, and four column volumes of mobile phase (flow-rate at equilibration: 1 ml/min). The fractions were collected, and the FVIII activity and the vWF Ag were determined. Recovery, 56% FVIII and 62% vWF Ag. (B) SDS–PAGE under non-reducing conditions of collected fractions from SEC in part (A) of this Figure. (C) Multimers of vWF in fractions from SEC. kD=kilodaltons.

preparative Superose 6 column shows good performance only in the range of high molecular masses, whereas components with molecular masses below 100 000 are not sufficiently separated (not shown here). They occur in very small quantities only, but their existence can be due to cleavage of the product during the production process [23]. By applying a column tandem, which consists of one

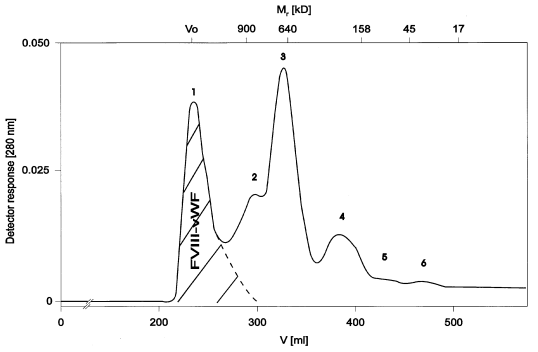

Fig. 2. Semi-preparative SEC of a preparation containing the FVIII–vWF complex. SEC chromatogram under non-denaturing conditions. A column tandem was used, consisting of a semi-preparative Fractogel EMD BioSEC (S) column and a Superose 6 Prep Grade column. The dimensions of each column were 600× 26 mm I.D. Chromatographic conditions: sample 12.5 mg of protein (1500 IU of FVIII and 1000 IU of vWF Ag) in 7 ml, flow-rate 1 ml/min, pressure 1–2 bar, room temperature. For other conditions see Fig. 1A. The fractions were collected, the activity of FVIII and of vWF Ag were determined. Recovery was 680 IU of FVIII (45%) and 700 IU of vWF (70%).

semi-preparative Fractogel and one Superose 6 column, the polypeptides with apparent molecular masses below 100 000 were well separated (Fig. 2, peaks 5 and 6).

Fig. 3a shows the separation of a FVIII concentrate, which in its original state contains a FVIII–vWF complex. This time it is carried out in the presence of 250 m*M* $CaCl_2$. The high concentration of Ca^{2+} ions causes the complex to dissociate [17]. Therefore the vWF multimers with molecular masses of several millions were easily separated from the much smaller FVIII polypeptides with their apparent molecular masses between 100 000 and 300 000. The same column tandem as in Fig. 2 was used for this separation. The separation of the proteins was excellent; however, recovery of FVIII activity was only around 40%.

If only a Fractogel EMD Bio SEC (S) column was used for the same task, separation was poor in the range of high molecular masses, even in the presence of $CaCl_2$. As is seen in Fig. 3b, FVIII and vWF were well separated. However, a baseline separation was not achieved. The recovery of FVIII in this experiment was 80%.

Separations of a FVIII concentrate consisting of a FVIII–vWF complex were unsatisfactory with Fractogel Bio SEC, both on an analytical and on a semi-preparative scale. However, a good separation was achieved with a preparative column with dimensions of 900×100 mm I.D. The separation was carried out at a flow-rate of 6.3 ml/min (Fig. 4a). When even higher flow-rates were applied, the separation was impaired (not shown here). The

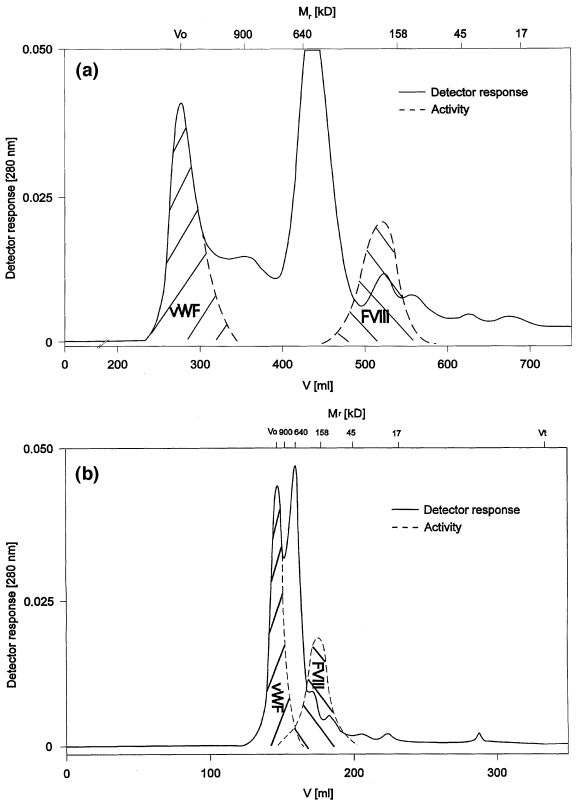

Fig. 3. Semi-preparative SEC of the FVIII–vWF complex in the presence of 250 m*M* $CaCl_2$. (a) A column tandem as in Fig. 2 was used. The mobile phase was 250 m*M* $CaCl_2$, buffered with 10 m*M* Tris–HCl, pH 7.2; 1500 IU of FVIII and 1000 IU of vWF Ag (125 mg protein) in 7 ml were applied; other conditions as in Fig. 2. The fractions were collected, the clotting activity of FVIII was measured (chromogenic test) and the vWF Ag was determined. Recovery was 600 IU of FVIII (40%) and 420 IU of vWF (42%). (b) Only a semi-preparative Fractogel EMD Bio SEC column (600×26 mm I.D.) was used. Mobile phase, see part (a) sample, 9 mg of protein, containing 1000 IU of FVIII and 680 IU of vWF Ag in 4.5 ml; flow-rate, 1.0 ml/min, practically without back pressure, room temperature. The fractions were collected. The clotting activity of FVIII (chromogenic test) and the vWF Ag were determined. Recovery: 800 IU of FVIII (80%) and 600 IU of vWF Ag (88%).

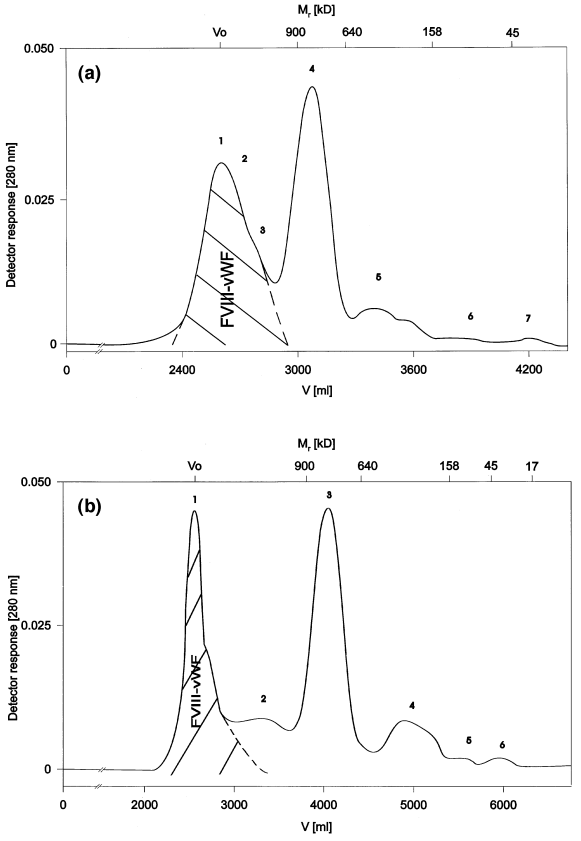

Fig. 4. Preparative separation of a FVIII concentrate: (a) on a Fractogel EMD Bio SEC column (900×100 mm I.D.). A total of 30 000 IU of FVIII and 17 000 IU of vWF Ag were dissolved in 120 ml of mobile phase and applied to the column. Chromatographic conditions: flow-rate 6.3 ml/min. Standard proteins, see Fig. 1A. For mobile phase, NaOH solution and buffers for regeneration and equilibration see Fig. 1A. Regeneration, 1 h at a flow-rate of 30 ml/min, equilibration with two column volumes of Tris buffer and subsequently with four column volumes of mobile phase at a flow-rate of 50 ml/min. The fractions were collected, and FVIII and vWF were determined. Recovery was 25 000 IU of FVIII (83%), and 14 000 IU of vWF Ag (82%). The specific activity of FVIII before separation was 110 IU/mg of protein. In the separated peak 1 the specific activity was 320 IU/mg of protein. (b) On a Superose 6 Prep Grade column (900×100 mm I.D.). A total of 30 000 IU of FVIII and 21 000 IU of vWF were dissolved in 120 ml of mobile phase and applied to the column. Chromatographic conditions: flow-rate 6.3 ml/min. For mobile phase, NaOH solution and buffers for regeneration and equilibration see part (a). Regeneration: 2 h at a flow-rate of 15 ml/min, equilibration with two column volumes of Tris buffer and subsequently with four column volumes of mobile phase at a flow-rate of 20 ml/min. The fractions were collected, FVIII and vWF were determined. Recovery was 12 400 IU of FVIII (41%) and 11 000 IU of vWF (52%). The specific activity of FVIII before separation was 90 IU/mg of protein, and about 300 IU/mg of protein in the separated peak 1.

capacity of the column was about 30 000 international units (IUs) of FVIII (about 300 mg of sample). The protein has to be dissolved in a volume not exceeding 150 ml, otherwise the quality of the separation is much poorer.

As shown in Fig. 4b, a better separation of single peaks in the range of high molecular masses was achieved with the preparative column Superose 6 (Prep Grade) (cf. the small-scale experiment in Fig. 1). However, recovery of FVIII clotting activity was only about 50% and therefore much lower. Additionally, sanitation and equilibration of a Fractogel Bio SEC column was carried out at a flow-rate of 100 ml/min, consequently lasting only 3 h. With a Superose 6 column the same steps can be carried out at a maximum flow-rate of 15–20 ml/min only.

The polishing step of a highly concentrated FIX product on a large scale with a Fractogel EMD Bio SEC (S) is shown in Fig. 5. With the Superose 6 column a separation at the baseline was not achieved (not shown here), whereas all contaminations, chiefly proteins with high molecular masses, were separated from the active FIX, when the Fractogel column was used. The specific activity of the FIX was above 200 IU/mg and therefore comparable to the specific activity of the product that had been obtained by

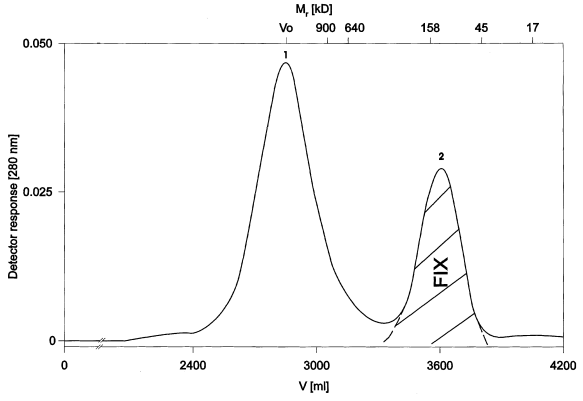

Fig. 5. Preparative SEC as the polishing step in the production of a highly purified FIX product. A total of 30 000 IU of FIX (500 mg of protein) in 100 ml of mobile phase were applied to a Fractogel EMD Bio SEC column (900×100 mm I.D.). For chromatographic conditions, regeneration and equilibration, see Fig. 4a. The fractions were collected, the activity of FIX and the protein content were determined. Recovery of FIX was 28 500 IU (95%). Its specific activity before separation was 60 IU/mg of protein, and about 280 IU/mg of protein in the separated peak 2.

immunoaffinity chromatography with monoclonal antibodies [24]. The yield was more than 90%.

4. Discussion

The separation by HPSEC of biopolymers with apparent molecular masses of over 300 000 still requires time-consuming optimization [25]. The commercially available supports with sufficiently large pores, usually >50 nm, are frequently used for separations under denaturing conditions [1–4]. Separations under non-denaturing conditions were carried out with less sensitive proteins such as bacterial enzymes or antibodies of the IgM type. Both size and solubility of these proteins are in a suitable range. Therefore their separation was possible with silica-based as well as with polymer-based SEC supports [8,26–29].

Clotting factors from human plasma are glycoproteins with molecular mass between 50 000 and 70 000 in the case of vitamin K-dependent clotting factors, and over 200 000 in the case of FVIII. These proteins are very sensitive, often losing their activity in the separation process [17,19,22,23,30].

Human plasma derived clotting factor VIII (pd FVIII) products also contain vWF. One of the physiological functions of vWF is to be a carrier for FVIII in the human plasma [16,22]. On the average, concentrates of pd FVIII contain between 0.05 and about 2 IU vWF per unit of FVIII [16,17]. The molecular masses of such FVIII–vWF complexes are usually several millions. Since vWF is also an adhesion protein, non-specific interaction with the carrier during separation is likely to occur [31]. Factor VIII products containing the FVIII–vWF complex are purified by anion-exchange chromatography. Anion-exchange material on a poly(glycidyl methacrylate) basis has been shown to be an effective tool [16]. Although this basic material, marketed under the trade name of Toyopearl, is rather hydrophobic, it has no adverse effect on the recovery of the protein or the clotting activity of FVIII [17]. However, if the same material is used for SEC of FVIII–vWF complexes, only about 10 to 20% of the initial FVIII activity are recovered [32]. Frequent plugging of the column and poor recovery are probably due to adverse interaction of the vWF with the matrix. When Toyopearl diethylaminoethyl (DEAE), an anion-exchange support is used, FVIII is applied with high concentrations of the detergent Tween 80. At this stage of the production process the detergent is used for the inactivation of lipid-enveloped viruses [16,17]. When the FVIII sample containing the detergent is separated, the non-specific interactions with the support are also reduced, allowing high recovery. In SEC, the last separation step, the detergent cannot be added, as it cannot be reliably removed in the remaining production process. Therefore other supports such as Superose 6 and Fractogel Bio SEC (S) are used for SEC of FVIII products.

An analytical separation of a FVIII product which contains the FVIII–vWF complex, can easily be carried out on a Superose 6 column (cf. Fig. 1 and Ref. [8]). The rather poor recovery of only 40 to 70% of FVIII activity has again to be attributed to certain remaining non-specific interaction of the sample with the column material. This assumption is supported by the fact that the column requires sanitation with 0.5 *M* NaOH after each run. Upscaling from the analytical Superose 6 (high-performance) material to a preparative scale (Superose 6 Prep Grade) is possible (cf. Figs. 1 and 4b).

Fractogel Bio SEC material is produced by modifying the surface of the material on a poly(glycidyl methacrylate) basis with tentacle residues [5,10]. This has made the material much more hydrophilic, reducing non-specific interaction during separation according to the size of the molecules. This also allows the observation of the dissociation of FVIII–vWF complexes, e.g., under the influence of high concentrations of $CaCl_2$ (see Fig. 3b). High recovery of activity of both FVIII and vWF also allows the determination of the degree of dissociation in this experiment. When column materials with slightly higher hydrophobicity are used, this observation is impossible under non-denaturing conditions. One reason is the non-specific adhesion of the vWF to the column material, leading to poor rates of recovery of this protein and possibly to the plugging of the column [32]. Under such conditions a loss of FVIII activity during separation is also likely to occur. The introduction of hydrophilic tentacle residues inhibits such interaction. Consequently the recovery of both proteins was high, about 80% (cf. Fig. 3b).

The material is chemically stable, allowing sanita-

tion with 0.5 *M* NaOH. When used on a preparative scale, the application of high flow rates is possible. Sanitation and regeneration of this support are therefore carried out much faster.

Successful up-scaling of the separation is carried out with Superose 6 (Prep Grade) as well as with Fractogel Bio SEC (Fig. 4a, Fig. 4b). A column of 900×100 mm I.D. is used. The best separation is achieved with a load of about 30 000 IU FVIII (300 mg of protein) and a volume of 150 ml. The solubility of the protein is a limiting factor at this point. When larger protein quantities or larger volumes are applied, poorer separations will occur in the case of both column materials.

The performance of the Superose 6 column is the same on a large scale as on a small scale (cf. Figs. 1 and 4b), both in terms of separation and recovery of FVIII activity. Recovery in the preparative experiment is less than 50% and therefore equally low.

In the case of Fractogel Bio SEC the separation on a preparative scale is much better than the results obtained with semi-preparative columns (cf. Fig. 3b, Fig. 4a), where the FVIII–vWF complex appears in the void volume of the column. This may be due to the introduction of rather large tentacle residues into the pores, reducing their size and accessibility for larger protein complexes. However, the separation of such substances with a preparative column (900× 100 mm I.D.) does not support this assumption. This phenomenon as well as the rather surprising improvement of separation behavior when flow rates are decreased, will be the object of future, more extensive research.

Preparative separation of FIX is achieved on a Fractogel Bio SEC column in an almost ideal fashion. The clotting factor is isolated from human plasma by a combination of precipitation and chromatographic methods [13,19]. If monoclonal antibodies are used for its isolation, a protein of high purity is obtained, practically without contaminations. The final product has a specific activity of more than 200 IU/mg of protein, close to the theoretical maximum [24]. However, the purification process requires additional steps to ensure that no contaminants, e.g., hydrolysed antibodies from the column, enter the product [33]. If a combination of anion-exchange chromatography and heparin affinity chromatography is used for production, co-purification of some contaminating proteins such as the complement factors C3 and C4, or protein S, cannot be completely avoided [13]. The specific activity of such a product is usually between 60 and 120 IU/mg of protein. When such a FIX concentrate is subjected to nanofiltration in order to remove any viruses from the product, additional purification is achieved through the removal of some components with high molecular masses [19]. Consequently the specific activity of a virus-filtrated product is above 140 IU/mg. However, this filtration does not remove all contaminating proteins. So far, HPSEC has been used for purity control of a FIX product only. A complete separation between the FIX and its components with high molecular masses is carried out on a column tandem, consisting of a Zorbax GF-250 column and a Zorbax GF-450 column [19]. This silica-based column material is not used for preparative purposes, as it does not allow sanitation with sodium hydroxide. The separation by SEC of FIX and its components with high molecular masses on a preparative Fractogel Bio SEC column is shown in Fig. 5. Recovery of FIX activity is over 90%. According to the results of SDS–PAGE and in terms of specific activity (over 200 IU/mg of protein), the purified FIX is comparable to the product that has been obtained with immobilized monoclonal antibodies [24].

References

[1] K. Löster, O. Baum, W. Hofmann, W. Reutter, J. Chromatogr. A 711 (1995) 187.
[2] Dj. Josič, W. Reutter, in: C.T. Mane, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991, p. 231.
[3] K. Löster, Dj. Josič, J. Chromatogr. B 699 (1997) 439.
[4] Dj. Josić, W. Schütt, R. Neumeier, W. Reutter, FEBS Lett. 185 (1985) 182.
[5] L.R. Jacob, A. Heinen, L. Britsch, Int. Lab. May (1996) 10H.
[6] Dj. Josić, P. Schulz, L. Biesert, L. Hoffer, H. Schwinn, M. Kordis-Krapez, A. Strancar, J. Chromatogr. B 694 (1997) 253.
[7] H. Saito, in: A.L. Bloom, G.L. Forbes, D.P. Thomas, E.G.D. Tuddenham (Eds.), Haemostasis and Thrombosis, Vol. 1, Churchill Livingstone, 1994, p. 289.
[8] S. Arrighi, R. Rossi, M.G. Barri, V. Lesnikov, M. Lesnikova, E. Franco, M. Divizia, M.E. DeSautis, E. Bucci, Thromb. Haemost. 74 (1995) 868.
[9] W. Müller, J. Chromatogr. 510 (1990) 133.

[10] W. Brümmer, L. Britsch, Proc. VIII Conference on Recovery of Biolog. Products, Tuscon, AR, Oct. 21–25, 1996.
[11] R. Wagenvoord, A. Hendrix, H.C. Hemker, Haemostasis 19 (1989) 196.
[12] T. Barrowcliffe, Res. Clin. Lab. 20 (1990) 155.
[13] T. Burnouf, C. Michalski, M. Goudemand, J.J. Huart, Vox Sang. 57 (1989) 225.
[14] J. Cejka, Clin. Chem. 28 (1982) 1356.
[15] J.G. Pool, E.J. Hershgold, A.R. Pappenhagen, Nature (London) 203 (1964) 312.
[16] H. Schwinn, A. Smith, D. Wolter, Arzneim.-Forsch./Drug Res. 39 (1989) 1302.
[17] Dj. Josić, H. Schwinn, M. Stadler, A. Strancar, J. Chromatogr. B 662 (1994) 181.
[18] H.G.J. Brummelhuis, in: J.M. Curling (Ed.), Methods of Plasma Protein Fractionation, Academic Press, London, Orlando, 1980, p. 117.
[19] L. Hoffer, H. Schwinn, L. Biesert, Dj. Josić, J. Chromatogr. B 669 (1995) 187.
[20] U.K. Laemmli, Nature (London) 227 (1970) 680.
[21] U. Budde, R.E. Scharf, P. Franke, K. Hartmann-Budde, J. Dent, Z.M. Ruggeri, Blood 82 (1993) 1749.
[22] D.P. O'Brien, E.G.D. Tuddenham, in: A.L. Bloom, G.L. Forbes, D.P. Thomas, E.G.D. Tuddenham (Eds.), Haemostasis and Thrombosis, Vol. 1, Churchill Livingstone, 1994, p. 333.
[23] T.W. Barrowcliffe, S.J. Edwards, G. Kemball-Cook, D.P. Thomas, Lancet June (1986) 1448.
[24] K.J. Smith, Blood 72 (1988) 1269.
[25] W.K.R. Barnikol, H. Pötzschke, J. Chromatogr. A 685 (1994) 221.
[26] Dj. Josić, K. Löster, R. Kuhl, F. Noll, J. Reusch, Biol. Chem. Hoppe-Seyler 372 (1991) 149.
[27] R.M. Carolla, L.L. Rudel, J. Lip. Res. 24 (1983) 200.
[28] N.H. Werner, G.M. Clore, A.M. Gronenborn, A. Kondoh, R.J. Fisher, FEBS Lett. 345 (1994) 125.
[29] U.H. Hole, Arch. Microbiol. 165 (1996) 55.
[30] A.P. Reiner, E.W. Davie, in: Ch.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, Vol. III, McGraw-Hill, 1995, p. 3181.
[31] K. Titani, K.A. Walsh, Trends Biochem. Sci. 13 (1988) 94.
[32] Ch. Kannicht, C. Bauer, R. Tauber, W. Reutter, Thromb. Haemost. 73(Abstr. 1196) (1995) 1212.
[33] C. Lutsch, P. Gattel, B. Franget, J.L. Veron, K. Smith, J. Armand, M. Grandgeorge, Biotechnol. Blood Prot. 227 (1993) 75.