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Application of compact porous tubes for preparative isolation of clotting factor VIII from human plasma

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Abstract

Membranes as well as compact porous disks are successfully used for fast analytical separations of biopolymers. So far, technical difficulties have prevented the proper scaling-up of the processes and the use of membranes and compact disks for preparative separations in a large scale. In this paper, the use of a compact porous tube for fast preparative separations of proteins is shown as a possible solution to these problems. The units have yielded good results, in terms of performance and speed of separation. The application of compact porous tubes for the preparative isolation of clotting factor VIII from human plasma shows that this method can even be used for the separation of very sensitive biopolymers. As far as yield and purity of the isolated proteins are concerned, the method was comparable to preparative column chromatography. The period of time required for separation was five times shorter than with corresponding column chromatographic methods. Compact porous disks made of the same support material can also be used for in-process analysis in order to control the separation. The quick response, which is obtained from these units within 5 to 60 s, allows close monitoring of the purification process.

Keywords: Membranes; Preparative chromatography; Compact porous tubes; Clotting factor VIII

1. Introduction

The problems that arise in down-stream processing of clotting factors from human plasma are, above all, losses caused by the purification procedures, resulting in lower yield. Apart from reduced yield, down-stream processing can cause minor modifications in the structure of the protein molecule. Usually, these modifications do not affect the activity of the product, but may change its antigenicity. Along with virus safety, the reduction of such risks is the main object in down-stream processing of clotting factors and other therapeutical proteins. It concerns proteins from human plasma as well as from cell cultures [1–3].

Chromatographic purification, especially the introduction of ion exchange chromatography (IEC) and affinity chromatography (AC), has allowed the production of highly purified concentrates of clotting factor VIII (FVIII) and factor IX (FIX) [1–5]. Improved column chromatographic methods, especially the introduction of radial columns, have reduced considerably the period of time required for separation [6]. However, even with these methods, the risk of unwelcome changes or loss of activity during purification cannot be excluded and has to be prevented in each case by careful investigation of the production process. Therefore, a fast and effective in-process analysis is of the utmost importance.

Over the last five to six years, the use of membrane chromatography (MC) for the separation of biopolymers has been discussed. The membranes as

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well as the compact, porous disks and the tubes, all of which are used in these processes, show similar performance and capacity as the separation columns. However, they allow much higher flow-rates and thereby much faster separation. This, in turn, reduces the risk of any changes in the biological macromolecules or of loss of activity, as compared with column liquid chromatography (LC) [7].

This paper shows the isolation of the clotting factors VIII and IX by means of MC. Scaling-up and scaling-down is discussed as well as in-process analysis with fast MC.

2. Experimental

2.1. Instrumentation

A gradient HPLC system built with two Pumps 64, an injection valve with a 20-μl PEEK sample loop, a variable wavelength monitor with a 10-mm optical path set to 280 nm and with a 10-μl volume flow-cell, connected by means of 0.25 mm I.D. PEEK capillary tubes and HPLC hardware/software (data acquisition and control station), all from Knauer (Berlin, Germany) was used in all preparative separations. For analytical use, some minor modifications were carried out. Knauer's mixing chamber with its relatively high dead volume was replaced by the PEEK Biocompatible Mixing Tree, with extra low dead volume (Jour Research, Uppsala, Sweden).

2.2. Chemicals

The FVIII and FIX samples (from human plasma) were obtained from Octapharma (Vienna, Austria). The production processes for these clotting factors have been described elsewhere [3,4]. All chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Sigma (Munich, Germany). Eluents were prepared at the concentrations listed in the figures and in the legends to the figures. The water used for preparing eluents was twice distilled (Octapharma).

Before being used with columns, eluents were filtered through a 0.45-μm filter (Millipore, Vienna, Austria) and degassed on an ultrasound bath (Knauer).

2.3. Separation devices

Compact porous disks and tubes were synthesized by BIA d.o.o. (Ljubljana, Slovenia) by means of radical co-polymerisation of glycidyl methacrylate and ethylene glycol dimethacrylate in the presence of pore-producing solvents, following the method of Švec et al [8]. Before the chemical modification was carried out, the disks and tubes were rinsed with methanol, a methanol–water mixture (1:1, v/v) and finally with water to remove porogenes and any residues of compounds that had failed to react. The chemical modifications of epoxy groups turned into ion-exchange groups have been described elsewhere [9].

Disks with a thickness of 3 mm were prepared with three different sizes (10, 25 and 50 mm diameter). Tubes were 53 mm long, 23 mm in diameter and had a 1-mm inner hole. They were then mounted in special cartridges with a low dead volume (developed by BIA).

2.4. Determination of clotting factor VIII, factor IX and von Willebrand factor

The one stage coagulation assay for the determination of FVIII clotting activity has been described by Barrowcliffe [10]. FVIII-deficient plasma (Behringwerke, Marburg, Germany) 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 laboratory-made standard (Octapharma) and the standard human plasma (Behringwerke). The FVIII standard solution was previously calibrated against the latest WHO standard. The measurements were carried out with a KC 4A coagulometer (Amelung, Lemgo, Germany). The concentration of von Willebrand Factor (vWF) antigen was measured as described by Cejka [11].

2.5. Electron microscopy

Disks with bound proteins were fixed and stored in 2% (v/v) glutaraldehyde and were washed extensively in PBS. Disks without bound protein were stored in 70% (v/v) ethanol. Small fragments of the support

were dehydrated through a graded series of ethanol and embedded according to the manufacturer's protocol in LR White Resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Silver to gold ultrathin sections of the embedded membranes were examined in a Philips 300 electron microscope (Philips, Eindhoven, Netherlands) with or without the benefit of heavy metal staining.

For scanning electron microscopy (SEM), disks were dehydrated in a graded series of ethanol and air dried. Then, they were fractured to expose the interior surface. Pieces mounted on aluminium stubs with silver paint were sputter-coated with gold palladium and examined at 6 kV in a Philips Model 515 SEM. SEM images were recorded on Kodak Technical Pan film (Kodak, Rochester, NY, USA).

3. Results and discussion

3.1. Application of compact porous tubes for preparative separation of clotting factor VIII from human plasma

The use of membranes or compact porous disks instead of chromatographic columns for the preparative isolation of biopolymers has been discussed several times [7,12,13].The membranes, which are made of cellulose or synthetic material, were used successfully for the purification of proteins from plasma or supernatants of cell cultures [13,14]. Both the membranes, made of the above-mentioned materials, and the compact porous disks, which are made either of poly(styrene–co-divinylbenzene) (PS-DVB) or of poly(glycidyl methacrylate–co-ethylene glycol dimethacrylate) (GMA-EDMA), allow scaling-up to some extent [7,13]. The easiest way is the construction of units that consist of several "piled-up" membranes [7,15]. The PS-DVB, GMA-EDMA and similar materials allow the formation of monolytes, consisting of a compact porous rod [16–18]. However, if the unit exceeds a certain size, polymerization and technical problems will occur, which can hardly be overcome. The main polymerization problem is connected to the homogeneity of the basic material, while technical problems concern leakage and pressure resistance, poor sample distribution and fast blockage when complex biological solutions are applied [7,19]. Some of the problems can be dealt with to some extent by applying sample distributors and appropriate filters, which are included before and after the separation unit. As a result, scaling-up is achieved with a diameter of the separation unit of up to about 5 cm [7]. Units with a larger diameter did not work satisfactorily on a technical scale (A. Štrancar, Ph.D. Thesis, in preparation).

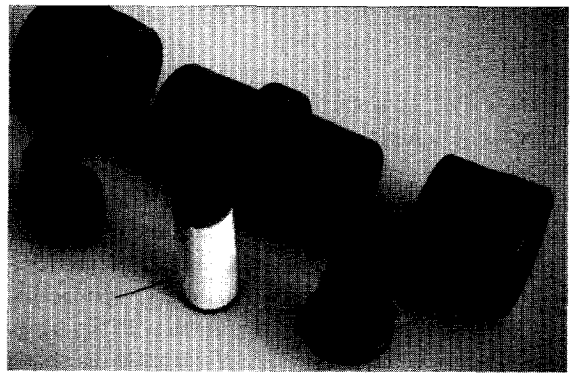

Fig. 1. Construction of the separation unit, which contains a compact porous tube (see arrow). The mobile phase or the sample is pumped from the inner side of the cylinder, flows through the wall and is collected on the other side of the unit. The separation process takes place during flow through the porous wall of the tube, as in the case of a radial column.

The axial construction of the disk does not allow further scaling-up without severely impairing its stability, capacity and resolution. Fig. 1 shows how

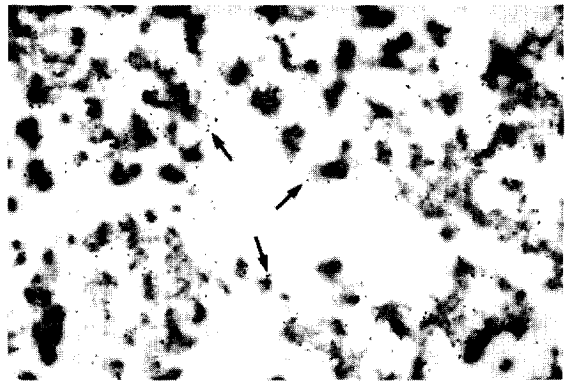

Fig. 2. Electron microscopy images of the support with immobilized antibodies. The ligand, a polyclonal rabbit IgG, was immoblilized to the epoxy-activated separation unit (cf. Ref. [7]). The immobilized ligand was subsequently made visible with gold-labelled mouse-anti-rabbit-antibodies. The gold-labelled antibodies show the position of the ligand (rabbit IgG) on the particle surface (see arrow).

the problem might be solved. The disk is replaced by a compact porous tube. Separation on such a tube is carried out in a similar way to that on a radial column. The construction minimizes the problems concerning sample distribution and pressure resistance.

SEM of such a monolyte made of GMA-EDMA shows a structure similar to perfusion chromatography materials [20]. However, instead of particles, the monolyte has a compact, porous, sponge-like structure. The interaction of the ligate with the ligand takes places on the surface of the material, as shown in Fig. 2.

Fig. 3 shows the use of a compact tube for further purification of factor VIII from human plasma. After pre-purifying the cryoprecipitate with $Al(OH)_3$, up to 2000 IU of FVIII can be bound to a tube (53 mm long, 23 mm in diameter, with an inner hole of 1 mm in diameter). The yield of the eluted FVIII–vWF complex exceeded 80%, in terms of FVIII activity. Similar to the situation of isolation on a preparative column, only a part of the vWF has bound to the support [3]. This is the part that forms a complex with FVIII. The rest of the vWF, which does not form a complex with FVIII, does not bind and is therefore separated (cf. Fig. 3).

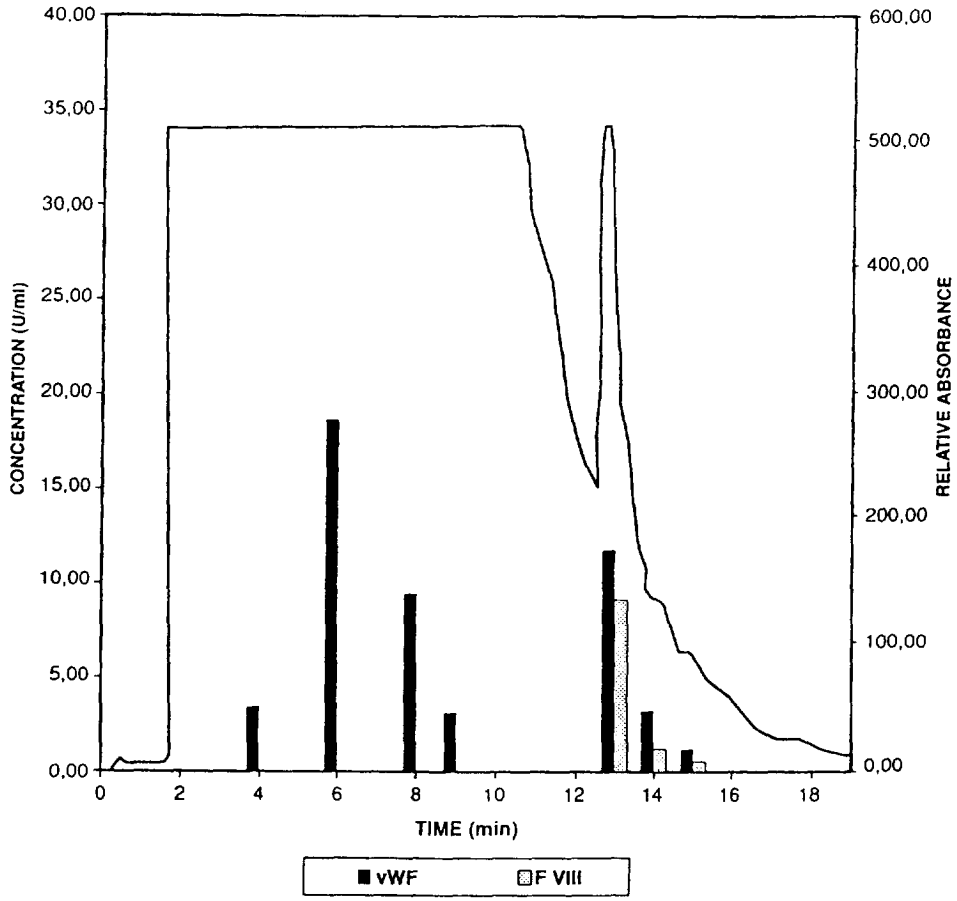

Fig. 3. Separation of sample No. 3 from FVIII production (cf. Ref. [4]), resolved cryoprecipitate after $Al(OH)_3$ precipitation and S/D virus inactivation, obtained on a QA anion-exchange compact porous tube (53 mm long, 23 mm in diameter and with a 1-mm inner hole). Conditions: Buffer A, 10 m*M* sodium citrate, 120 m*M* glycine, 1 m*M* $CaCl_2$, pH 7.0; buffer B, buffer A containing 1 *M* NaCl; injection volume, 20 ml. Preconditioning of the tube was carried out with a mixture of buffer A–buffer B (88:12, v/v). The tube was subsequently washed with 88% buffer A (2.5 min). FVIII–vWF was eluted with 50% buffer B. The flow-rate during binding was 6 ml/min and was 9 ml/min during elution; back pressure, 0.8 MPa. Relative absorbance stands for the absorbance of the UV detector set to 280 nm.

Collectively, it can be emphasized that with the unit shown in Fig. 1, several special problems, which occur in connection with the purification of highly sensitive biological materials, could be solved, along with the above-mentioned technical items. These problems are

(a) loss of biological activity, caused by adverse interaction with the support material or by decomposition during the purification process;
(b) blockage of the separation unit through non-specific interaction with components of the mixture.

In the case shown here, adverse interaction with vWF, a very sticky glycoprotein with a high molecular mass, is extremely critical. The protein often causes the clogging of the separation unit, leading to the loss of valuable sample [21].

The possibility of making analytical as well as preparative separation units out of the same support materials, allows in-process analyses under identical conditions to those used in preparative isolation. Fig. 4 shows an in-process analysis of the same sample as shown in Fig. 3. A tube that was 53 mm long and 23 mm in diameter was used. Analysis was carried out under the same conditions as used for the experiment shown in Fig. 3. A unit like this can be used for analysis of the sample before further purification and for scaling-down experiments.

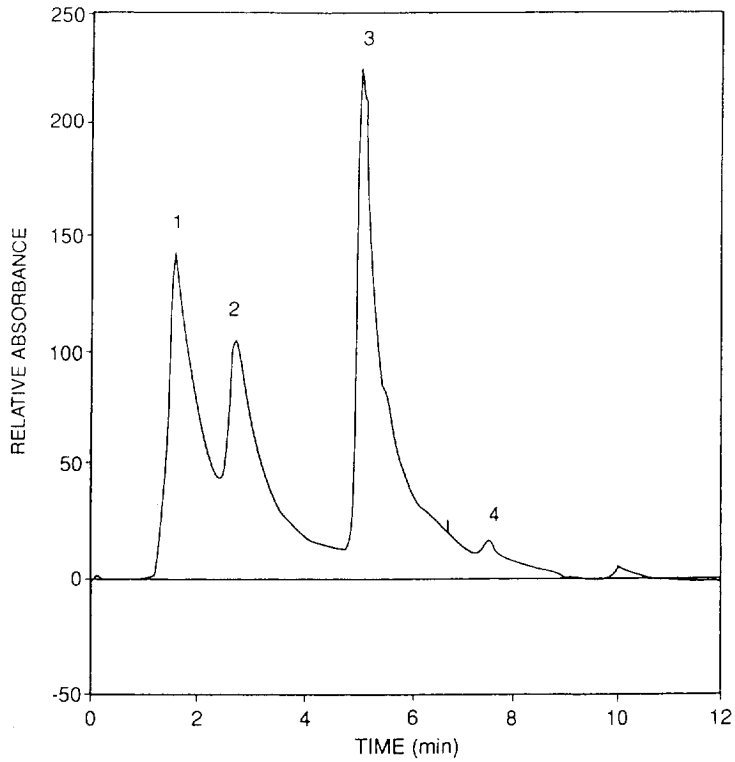

Fig. 4. Scaling-down experiment; separation of sample No. 3 (500 μl) from FVIII production, obtained on a QA anion-exchange compact porous tube (53 mm long, 23 mm in diameter and with a 1-mm inner hole). Sample no. 3 was taken from FVIII production after $Al(OH)_3$ precipitation and solvent/detergent (S/D) treatment [4]. Conditions: Buffer A, 10 m*M* sodium citrate, 120 m*M* glycine, 1 m*M* $CaCl_2$, pH 7.0. Buffer B, buffer A containing 1 *M* NaCl; injection volume, 500 μl. Preconditioning of the tube was carried out with a mixture of buffer A–buffer B (88:12, v/v). Elution conditions: 1.5 min wash with 88% buffer A; step gradients at 85% buffer A (2.5 min), 70% buffer A (2.0 min), 50% buffer A (2.0 min) and 0% buffer A (1.0 min). The flow-rate during binding was 6 ml/min and was 9 ml/min during elution; back pressure, 0.8 MPa. Peak description: Peaks 1 and 2, contaminating proteins such as transferrin and IgM; peak 3, contaminating proteins, chiefly human serum albumin (HSA); peak 4, the FVIII–vWF complex. Relative absorbance stands for the absorbance of the UV detector set to 280 nm.

3.2. On-time monitoring of factor VIII isolation by using compact, porous disks

Furthermore, the same material can be used for very fast chromatographic analyses that allow on-line monitoring of the preparative separation. Fig. 5 shows the reduction in the time required for such on-line monitoring by using a small compact disk instead of the tube. The applied unit is shown in Fig. 6. Human serum albumin and transferrin, which appear as contaminating proteins, were separated from the FVIII–vWF complex within less than 1 min. Such monitoring is required for adequate process control. The regulatory authorities demand increasingly detailed data on the production process

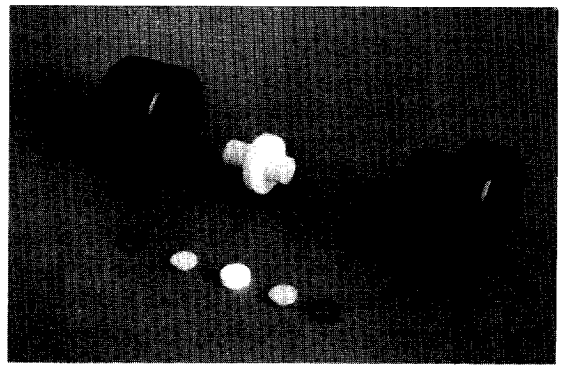

Fig. 6. Construction of the separation unit that contains the compact porous disk, which is 10 mm in diameter and 3 mm in height (see arrow).

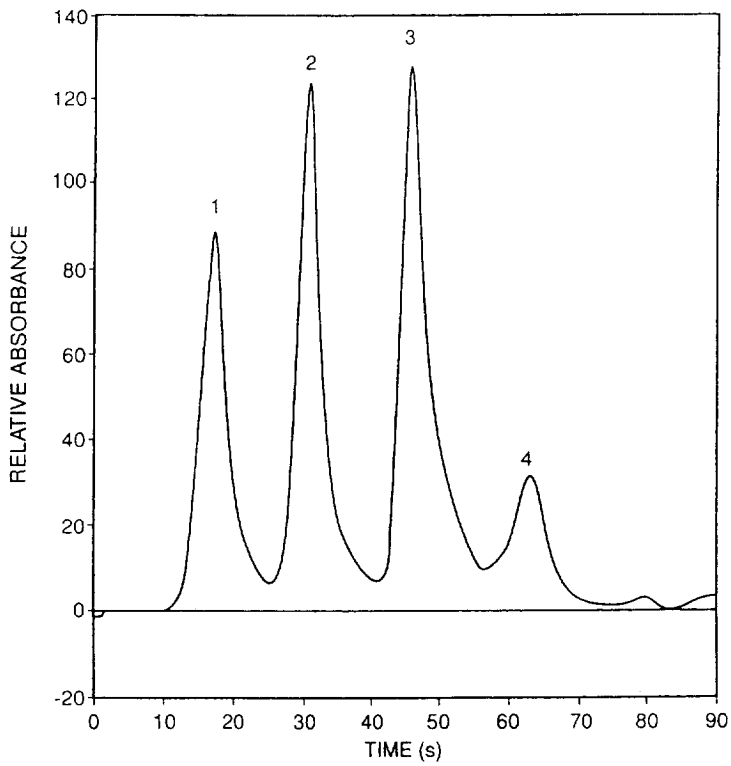

Fig. 5. Fast separation of a calibration solution obtained by a QA anion-exchange compact porous disk, 3×10 mm in diameter. Conditions: buffer A, 10 m*M* Tris–HCl, pH 7.4; buffer B, buffer A containing 1 *M* NaCl; flow-rate, 4 ml/min; back pressure, 0.8 MPa. Gradient: 1 s at 100% buffer A, subsequently step gradients at 90% buffer A (15 s), 75% buffer A (15 s), 20% buffer A (10 s) and 0% buffer A (7 s). Calibration solution, 200 μg of transferrin (peak 1), 400 μg of HSA (peak 2) and 30 units of factor VIII, bound to von Willebrand factor (peak 3) in 100 μl (volume of sample loop) of buffer A (peak 4, gradient solvent).

and the composition of the final product [22]. The analytical methods shown here allow the fast and reliable collection of such data.

4. Conclusions

Due to the lower mechanical stability, poorer sample distribution and non-homogeneity of the GMA-EDMA compact porous disks with larger diameter, scaling-up is possible only to a certain degree. In order to overcome these problems, compact porous tubes have been introduced.

These units allow fast separation of biopolymers out of complex biological materials, while the composition of the product and the yield remain the same.

Corresponding, small analytical units made of the same material allow fast scaling-down of the separation and in-process analysis within minutes, sometimes even within seconds.

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