

Immunopurification of Human Factor VIII/vWF Complex from Plasma

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Key words

Factor VIII – von Willebrand factor – Monoclonal antibodies – Immunopurification – Immunoabsorbent

Summary

In this study we describe a process for immunopurification of FVIII/vWF complex directly from plasma. A mAb against vWF has been selected that is able to bind, under physiologic conditions, the FVIII/vWF complex and to release it in slightly alkaline conditions while preserving its activity.

After investigating the influence of solid supports and of coupling methods on the recovery of active FVIII we produced an immunoabsorbent by immobilisation of the selected mAb onto a Sephacryl S-1000 support using a benzoquinone coupling method. With this immunoabsorbent we developed a purification process directly from plasma with an excellent recovery (50%) of both FVIII and vWF activities. The product obtained is very enriched (the FVIII:C specific activity is 20 IU/mg of protein) and is stable after lyophilization.

Introduction

Factor VIII (FVIII) plays a central role in intrinsic blood coagulation (1). It is the procoagulant cofactor missing or inactive in hemophilia A patients.

In the plasma FVIII is complexed with the von Willebrand factor (vWF) which has an essential role in primary hemostasis and is deficient or dysfunctional in von Willebrand's disease. These two proteins are linked by electrostatic and hydrophobic bonds (2). The physiologic importance of FVIII-vWF association is presumably due to its capacity to increase the FVIII concentration at sites of vascular damage (3) and its ability to increase the plasma half life of FVIII by protecting it from proteolytic degradation (4, 5).

The FVIII itself accounts for only 1 or 2% of the weight of the FVIII/vWF complex. It is present in plasma at extremely low concentration (100 to 200 ng/ml) and consists of a large glycoprotein (native FVIII MW = 330 kDa) (6–9) which is very susceptible to cleavage by thrombin, plasmin, activated protein C and other serine protease (10–13).

Abbreviations:

FVIII, factor VIII; FVIII:C, factor VIII procoagulant activity; FVIII:Ag, FVIII antigen; vWF, von Willebrand factor; vWF:Ag, vWF antigen; RCof, vWF ristocetin cofactor activity; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; IRMA, immunoradiometric assay.

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The vWF plasma concentration is 10 µg/ml. This molecule is composed of disulfide-linked glycoprotein subunits of approximately 220 kDa. These subunits polymerise in multimers ranging from MW 500 kDa to 20,000 kDa (14–16).

The largest multimers are of utmost importance in the intrinsic hemostatic role of vWF but the whole range of vWF multimers appears to be effective in factor VIII binding (17, 18).

Because of the low concentration of FVIII in plasma and its susceptibility to proteolytic cleavage, large scale purification is difficult. Preparations of current therapeutic FVIII concentrates include some or all of the following steps: cryoprecipitation (19); cold ethanol washing (20); glycine and polyethylene glycol precipitation (21). The limitations of these procedures are: the FVIII:C low yield (10–25%), the presence of plasma protein contaminants, the loss of vWF activity and viral contamination.

Highly purified FVIII has been obtained by gel filtration (22), anion exchange chromatography (23) and affinity chromatography on immobilized antibodies. Immunopurification of FVIII has been carried out with monoclonal antibodies toward the FVIII:Ag (24) and polyclonal (25) or monoclonal (26) antibodies directed toward vWF and dissociation of the FVIII/vWF complex by Ca²⁺ (2). These immunopurification methods lead to the recovery of FVIII deprived of vWF.

The main objective of the work presented in this article was to propose an immunopurification process able to produce a FVIII concentrate suitable for the therapy of hemophilia A. The requirements for such a process were to be as simple as possible and to allow a better FVIII:C recovery than current processes. Characteristics required for the product were to present an improvement of FVIII:C specific activity and to maintain the FVIII associated with the vWF as in the physiological form, where vWF could stabilize the FVIII:C in vitro (4) and increase the half life of the FVIII:C in vivo (5). Laboratory scale development of this process is described here.

Materials and Methods

Assays

FVIII:Ag was determined by an IRMA using the two monoclonal antibodies: CLB-CAg 27 and CLB-CAg 117 described by Stel et al. (27) and purchased from CLB, Amsterdam.

vWF:Ag was measured by using an ELISA performed with the two monoclonal antibodies: 4F91C5 and 202D3 described by Sultan et al. (28) and provided by Immunotech, Marseille. A standard curve was obtained with a vWF:Ag standard purchased by Diagnostica-Stago.

FVIII:C was determined by the one-stage assay (29) and the two-stage assay (30). The FVIII:C standard used was from Diagnostica-Stago.

RCof was quantified by platelet aggregation in the presence of the antibiotic ristocetin (31) using the Behring kit (Behringwerke AG, Marburg, F.R.G.). The relative RCof concentration of the immunopurified samples were determined by comparison with the plasma used for their preparation which was arbitrarily assumed to have a concentration of 1 unit/ml.

The mouse IgG released from immunoabsorbents were measured by an ELISA. Avidin-coated polystyrene microtiter plates (Immunotech,

patent n° 84430010.3) were coated by *n*-hydroxysuccinimidyl biotin conjugated goat anti-mouse IgG (H+L) Fab' fragments adsorbed with immobilized human proteins (1 µg/ml; 200 µl/well) provided by Immunotech. After an overnight incubation at room temperature the supernatant was aspirated and the plates dried in a dry room. ELISA was performed at room temperature. Each coated well was incubated with 200 µl of a sample in 10 mM phosphate, 100 mM NaCl, pH 7.2 (PBS) containing 0.5% bovine serum albumin. After a 3 hours incubation the wells were washed twice with 200 µl of 9% NaCl. Then, 200 µl of peroxidase conjugated goat anti-mouse IgG (H+L) Fab' fragments (Immunotech) were incubated 3 hours. The wells were then washed twice with NaCl 9% and peroxidase activity was revealed by orthophenylenediamine (Sigma) and measured at 492 nm. A standard curve was obtained with the 239 mAb. Detection sensitivity of this assay was 0.1 ng IgG/ml.

The human IgG in the fractions of FVIII/vWF immunopurified were measured by an ELISA using goat anti-human IgG (H+L) (Immunotech) as described for the mouse IgG ELISA.

Hepatitis B surface antigen (HBs) was quantified by the IRMA kit AUSRIA II purchased from S. F. Laboratoires Abbot. Plasma contaminated by HBs was obtained from CRTS, Marseille. According to the assay, the dilution of this contaminated plasma giving a c.p.m. value 2.1 fold higher than the negative control was 2.8×10^4 .

Protein concentration for calculation of the specific activity was estimated by the Bio-Rad protein assay (Bio-Rad, Richmond, CA). A standard curve was obtained with human plasma albumin obtained from CRTS Lille.

Stability of FVIII Biological Activity in Various Buffers

The effect of buffer on FVIII:C was determined by incubating 1 volume of FVIII concentrate (provided by CRTS Nancy) with 9 volumes of buffer, for 30 min at room temperature. Then the product was dialysed against PBS, for 3 hours at room temperature prior to determination of the residual activity using the one stage FVIII:C assay.

Anti-vWF Monoclonal Antibodies

Monoclonal antibodies toward vWF were produced in collaboration with the team of Dr C. Mazurier, CRTS Lille. Purified FVIII/vWF antigen (32) was injected in BALB/c mice and their spleen cells fused with mouse X63 myeloma cells (33). Hybridoma cells producing specific antibodies were identified by analysis of the cell supernatants on plates coated with purified FVIII/vWF complex (22) or FVIII molecules kindly given by Dr J. P. Allain (CNTS Les Ullys). Specificity of the mAb was assessed using the methods described by Sola et al. (34). Among the 12 anti-vWF monoclonal antibodies obtained, 4 were selected for immunopurification experiments as they were able to remove more than 50% of FVIII/vWF from plasma when bound to gel beads.

Determination of mAb Affinity for the FVIII/vWF

Polyethylene tubes were coated by an overnight incubation at room temperature each with 1 ml of PBS, containing 1 unit of immunopurified FVIII/vWF. After 3 washes with PBS-Tween 0.2%, the tubes were saturated with 1% w/v bovine serum albumin (BSA) in PBS for 3 h at room temperature. After washing with PBS-tween 0.2%, the tubes were dried in a dry room and used for radioimmunoassay. Standard curves were obtained by adding 900 µl of increasing concentrations of unlabelled mAb in 0.1 M triethanolamine, 0.1 M NaCl, 0.1% BSA, 0.2% Tween at various pH with 100 µl of ^{125}I -labelled mAb (100,000 cpm) in 0.1 M NaCl, 0.1% BSA, 0.2% Tween. After an overnight incubation at room temperature and 3 washings in PBS-tween 0.2%, bound radioactivity was counted in a gammacounter. Affinity of antibody for FVIII/vWF complex was determined by analysis of the data according to the Scatchard procedure (35).

Preparation of Immunoabsorbents

The monoclonal antibody to be coupled was purified from ascitic fluid by affinity chromatography on protein A Sepharose (36).

Ultrogel AcA 22 purchased from IBF was activated with glutaraldehyde according to Weston and Avrameas (37). Affi-gel 10 immuno-

sorbent was prepared according to the instructions of the manufacturer (Bio-Rad, Richmond, CA). Sephacryl S-1000 was purchased from Pharmacia and cyanogen bromide activation was performed according to Axen et al. (38). CH-Sephacryl S-1000 was activated by *N*'-Ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide according to Cuatrecasas (39). Epichlorohydrin activation was carried out according to Porath et al. (40). Epoxy Sephacryl affinity resin was made by Dr C. Julhin and Coll. (Pharmacia Workshop, Uppsala, Sweden). Tosyl chloride activation was carried out according to Nilsson and Mosbach (41). Trichloro-s-triazine coupling method was performed according to Finlay and Troll (42). Benzoquinone activated Sephacryl S-1000 was prepared according to the method of Brandt et al. (43). The coupling yield of the mAb on the gel beads was assessed by optical density using an extinction coefficient $E_{1\text{cm}}^{1\%} = 14$ at 280 nm for the mAb or by the mouse IgG ELISA described above.

Immunopurification

Standard immunopurification was performed on the selected immunoabsorbent (Sephacryl S-1000 benzoquinone coupled with 0.8 mg of antibody/ml of gel), as follows: all purification steps were carried out at room temperature. The starting material for the preparation of FVIII/vWF was fresh frozen plasma obtained from French C. T. S. Plasma was thawed at 37° C and adjusted to pH 7.0. Then 100 ml of plasma was loaded at a flow rate of 33 ml/h onto a 5 ml precolumn of Sephacryl S-1000 followed by the immunoabsorbent column (6.5 cm high; 5 ml of bed volume) equilibrated with solution A (0.15 M NaCl). Next, the columns were washed with 10 ml of buffer B (50 mM triethanolamine/acetic acid, 0.1 M NaCl, pH 7.0) at a 33 ml/h flow rate. Then, the precolumn was bypassed and immunoabsorbent was washed with 60 ml of buffer B at a flow rate of 60 ml/h. The FVIII/vWF was eluted with 3 column volumes of buffer C (100 mM triethanolamine, 0.1 M NaCl, pH 10.2) at 60 ml/h. Fractions of 1 ml were collected. The fractions containing at least 1 unit of FVIII:C/ml were pooled and neutralized (immediately after elution) with 2 M acetic acid. Then, the immunoabsorbent was reequilibrated in solution A for the next use or it was washed with 5 column volumes of PBS then with 5 volumes of the preserving buffer (20% ethanol, 80% PBS) at a flow rate of 60 ml/h.

Preservation of the Immunopurified FVIII/vWF

Human serum albumin at 10 mg/ml was added to the neutralized FVIII/vWF pool. This product was then lyophilized and kept at -20° C.

Gel Filtration of FVIII/vWF Complex

A 1 ml fraction of FVIII/vWF immunopurified from plasma was applied on a Sepharose CL-4B column (Pharmacia), (1 × 30 cm). The column was eluted with 0.13 M NaCl, 0.01 M sodium citrate, 0.01 M Tris-HCl, pH 7.4 as described by Perret et al. (22).

SDS-PAGE Electrophoresis

Electrophoresis was carried out using the Phast system (Pharmacia) and the 10–15% and 8–25% polyacrylamide Phast gel (Pharmacia). The sensitive silver staining method adapted by Pharmacia was used for detecting proteins in Phast gel.

Results

Stability of FVIII:C in Various Buffers

As procoagulant factor VIII is very susceptible to inactivation (8, 13), the stability of FVIII:C in different conditions allowing dissociation of antigen-antibody interactions had to be defined in order to choose the immunopurification eluting buffer.

After a short stage (30 minutes) in the studied buffer, the factor VIII solution was dialysed for 3 hours in PBS in order to assess the FVIII:C in physiological conditions and to allow completion of an eventual inactivation (Table 1).

Clearly, FVIII:C was unstable in chaotropic conditions (3 M KSCN), in acidic (pH lower than 5) and basic buffers (pH over

Table 1 Stability of FVIII:C in various buffers

Buffer	pH ¹⁾	% residual FVIII:C ²⁾
Control (0.15 M NaCl)	6.9	100
0.1 M glycine-HCl	2.2	0
0.1 M sodium-acetate	3.5	0
	4.0	0
	5.0	57 ± 2
	5.5	95 ± 5
	6.0	95 ± 5
	6.9	95 ± 5
0.1 M triethanolamine-HCl	8.1	95 ± 5
	8.8	95 ± 5
	9.4	67 ± 2
0.1 M sodium carbonate	10.1	6 ± 4
	10.9	1 ± 1
3 M potassium thiocyanate	7.2	0
50% (w/v) ethylene glycol	7.2	47 ± 2

¹⁾ final pH of the solution of FVIII concentrate in the described buffers.

²⁾ FVIII concentrate was diluted ten fold in the various buffers. After 30 minutes incubation at room temperature followed by a 3-hour dialysis in PBS FVIII:C was measured. Residual FVIII:C is expressed as the percentage of the activity of a similar sample incubated in 0.15 M NaCl. Results are the average of 2 experiments.

9.5) and partly inactivated in 50% ethylene glycol. As high ionic strength (2) and pH lower than 6.5 (44) dissociate the FVIII/vWF complex it is then clear that slightly alkaline buffers are the best eluting candidates.

Characterization of a Monoclonal Antibody Allowing Elution at Low Basic pH

Several anti-vWF and anti-FVIII monoclonal antibodies, coupled to glutaraldehyde activated Ultrogel AcA 22, were studied for their ability to specifically remove FVIII/vWF complex from plasma and to release it in low basic pH conditions. Only one of them, the anti-vWF monoclonal antibody named 239 presented the two properties.

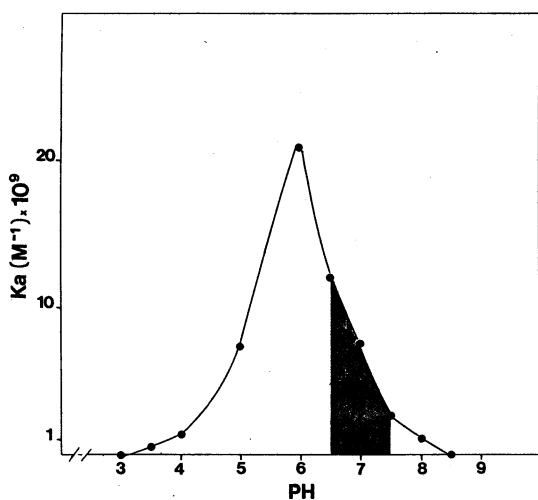


Fig. 1 Association constant (KA) of the 239 mAb as a function of pH. The range of pH allowing an efficient adsorption of FVIII/vWF complex onto an immunoadsorbent carried out with 239 mAb has been hatched (the complex is dissociated at pH lower than 6.5)

In order to determine the effect of pH on 239 mAb affinity for the FVIII/vWF complex, competition binding experiments and Scatchard analysis were performed in a pH range from 3 to 9 (Fig. 1).

The 239 mAb presents its maximum affinity at pH 6 and a high affinity constant ($\geq 3 \cdot 10^9 \text{ M}^{-1}$) from pH 4.5 to 7.5 (Fig. 1). No affinity can be detected at pH ≥ 8.5 or ≤ 3 . The antigen-antibody complex dissociation in these ranges was confirmed by adsorption experiments of FVIII/vWF complex onto immunoadsorbent followed by elution of the FVIII/vWF with pH buffers ≥ 8.5 or ≤ 3 (not shown). The results also suggest that a good elution should be obtained with a pH 8.5 buffer. In fact the elution buffer is partly neutralized during the migration on the column (Fig. 2). So a pH 10.2, 100 mM triethanolamine, 0.1 M NaCl buffer has been chosen, allowing the recovery of the FVIII/vWF complex in an elution fraction presenting a pH between 8.5 and 8.7. With a less basic elution buffer, the product is obtained in a larger fraction and is then more diluted.

Selection of a Solid Support and a Coupling Chemistry

Various coupling methods have been studied on different solid supports in order to prepare an immunoadsorbent with 239 mAb (Table 2).

Sephacryl S-1000 rapidly proved to present several advantages: its pore size allows the penetration of most of the FVIII/vWF molecules (MW 500 kDa – 20,000 kDa) into the beads, non specific binding of contaminants is low, and no activation of FVIII:C was observed (in contrast silica beads produced a FVIII:C activation).

The benzoquinone coupling method applied on Sephacryl S-1000 proved to be a very reproducible technique (constant high coupling yield). Moreover, among all the coupling methods studied, it preserved better the functions of the immobilized mAb (i.e. capacity to bind the FVIII/vWF complex). In addition, immunoadsorbent obtained with this method do not activate perceptibly the FVIII:C while with some coupling technique as the epoxy method FVIII:C activation was observed.

Nevertheless, even with the strongest coupling systems studied and after several washes and runs a 10^{-9} – 10^{-10} M release of the 239 mAb was observed in the elution fraction. When 239 mAb Fab' fragments were used, similar leakage was observed.

Attempt to remove the released antibody on a protein A Sepharose column was only partly successful (30% removal) probably because the affinity of protein A for the 239 IgG₁ mAb is relatively low. An original system, able to remove the released mAb, is under study and encouraging results have been obtained (results not shown).

Immunopurification Process

General conditions. Preliminary experiments indicated that immunoadsorption of FVIII/vWF and elution are much more efficient at room temperature than at 4 °C. The optimal loading flow rate was $42 \text{ ml} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ (for 0.8 mg mAb/ml of gel) on benzoquinone Sephacryl S-1000 immunoadsorbent. Experiments showed also that the flow rate has no effect on the elution efficiency. Therefore the highest flow rate usable with the immunoadsorbent ($76 \text{ ml} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) was applied for the elution in order to limit an eventual loss of FVIII:C in the basic buffer.

Optimal plasma pH. Previous results indicated that the 239 mAb affinity is $\geq 3 \cdot 10^9 \text{ M}^{-1}$ between pH 4.5 and 7.5 (with a maximum at pH 6). On the other hand Brocway and Fass (44) showed that FVIII/vWF dissociates in acidic pH conditions. So we

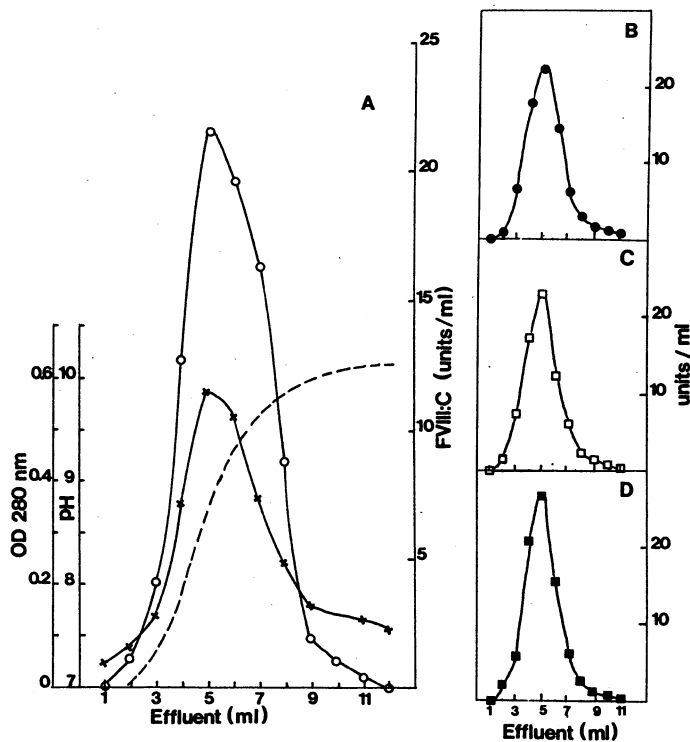


Fig. 2 Elution of the FVIII/vWF from the immunoadsorbent. Plasma (100 ml) adjusted at pH 7.0 was applied onto a 5 ml immunoadsorbent column (1 × 6.5 cm) (0.8 mg mAb/ml of Sephacryl S-1000). 2A. FVIII:C (O—O); pH — and optical density at 280 nm (x—x). 2B. FVIII:Ag (●—●). 2C. RCoF (□—□). 2D. vWF:Ag (■—■). All expressed in units per ml if we arbitrarily considered that 1 ml of the initial plasma contained 1 unit of each activity to compare the values of the different activities. FVIII:C concentration in the starting plasma was 0.6 IU/ml and vWF:Ag concentration 0.8 IU/ml

studied the loading of FVIII/vWF on the immunoadsorbent column with plasma at pH 6, 6.5, 7 and 7.4 (Table 3).

The fixation of vWF and FVIII molecules (vWF:Ag and FVIII:Ag) presents a significant discrepancy at pH 6.5 (97% and 70% respectively) and at pH 6.0 (99% and 35% respectively) suggesting that in these conditions complex dissociation partly occurs.

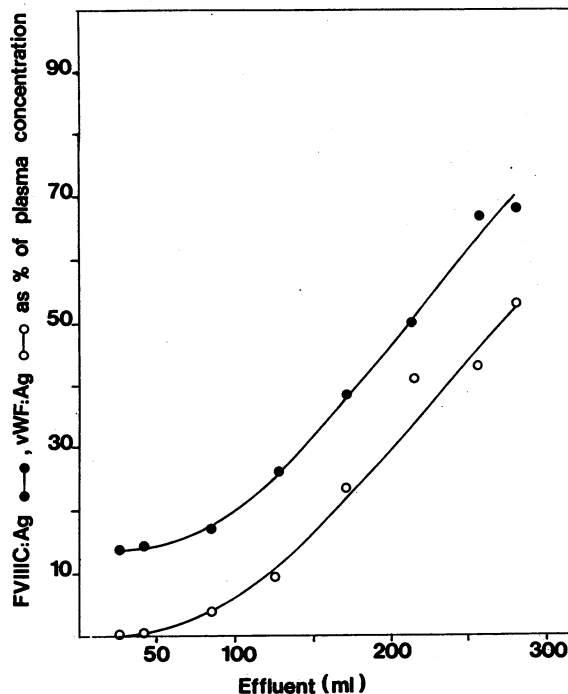


Fig. 3 Calibration of the capacity of the immunoadsorbent. Plasma (280 ml) were applied at a flow rate of 17 ml/h onto a 5 ml immunoadsorbent column as described for Fig. 2. FVIII:Ag (●—●); vWF:Ag (O—O) in unbound material expressed as % of the concentration in the applied plasma. vWF:Ag concentration in the starting plasma was 1.1 IU/ml

In addition, when plasma at pH 6 was used the elution fraction contains more FVIII:C activity (68%) than FVIII antigen (35%), clearly indicating that FVIII has been activated and will be rapidly destroyed (0% recovery after storage).

The overall results indicate that the best loading pH is 7 although satisfactory data have been obtained with pH 6.5 and pH 7.4 plasma.

Table 2 Characteristics of immunoadsorbents made with various coupling methods

Solid phase	Coupling method ¹⁾	Yield of mAb coupling (%)	Release of mAb ²⁾ (ng)	Immunoadsorbent capacity ³⁾ (vWF:Ag IU/ml of gel)	Maximum vWF:Ag recovered ⁴⁾ (IU/ml of gel)	Maximum FVIII:C recovered (IU/ml of gel)
CH Sephacryl S-1000	carbodiimide	≥80	420	30	24	15
Sephacryl S-1000	benzoquinone	≥90	90	40	32	20
	CNBr	50–80	10000	30	24	15
	epichlorohydrine	<80	1500	30	24	4
	epoxy	⁵⁾	120	30	24	<3
	tosyl chloride	40–50	100000	2	1	0
	trichloro-s-triazine	50–70	100	40	32	4
Affi-gel 10	hydroxysuccinimide ester	>90	200	5	4	<1

¹⁾ 1 mg of mAb was allowing to react with 1 ml of gel beads.

²⁾ quantity of mAb released in the elution fraction for a 1 ml immunoadsorbent column.

³⁾ maximum quantity of vWF:Ag bound per ml of gel.

⁴⁾ maximum quantity of vWF:Ag activity found in the elution fraction divided by the volume of the column.

⁵⁾ coupling performed by the Pharmacia Workshop (Uppsala, Sweden). Yield unknown.

Immunoabsorbent capacity. Several immunoabsorbents were produced with different mAb concentrations (from 0.1 to 3.2 mg/ml of gel beads). In that range the total capacity of the immunoabsorbent was directly proportional to the mAb concentration (40 IU of vWF per mg of mAb).

In the purification process currently used a 0.8 mg mAb/ml concentration have been chosen in order to limit the time of loading, i. e. the risk of FVIII:C inactivation.

On the other hand the efficiency of the binding varies during the loading of the column (Fig. 3). At the beginning all the loaded vWF:Ag is bound on the immunoabsorbent but the binding efficiency regularly decreases and when 25 IU of vWF have been bound per mg of mAb only 85% of the loaded vWF:Ag are retained on the immunoabsorbent. Thus, the usable capacity of the 0.8 mg mAb/ml of gel immunoabsorbent is 20 IU per ml for a flow rate of $42 \text{ ml} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ (3 hours loading). In addition, a 10 to 15% difference is always observed between the fixation of FVIII:Ag and vWF:Ag to the immunoabsorbent suggesting that some FVIII molecules are not firmly bound to vWF in plasma. Nevertheless this difference is not observed with freshly obtained plasma (not shown).

FVIII/vWF recovery. Elution profiles (Fig. 2) show that similar amounts of FVIII:C and RCoF activities and their associated antigens are recovered in the same elution fractions. Moreover gel filtration experiments show that FVIII:Ag and vWF:Ag are associated in the eluted product (Fig. 4).

Table 4 shows the results obtained after immunopurification performed on the same column. Differences from one cycle to another are observed, due to individual differences of the plasma used. Nevertheless a good correlation between the recovered FVIII:C and its antigenic counterpart (FVIII:Ag) is obtained, indicating that the factor VIII has not been activated nor degraded during the immunopurification process. This is confirmed by storage experiments and comparison of the FVIII activity measured by the one-stage and the two-stage assays that give consistent results (results not shown). Still, a significant discrepancy is observed between the recovery of FVIII:Ag and the recovery of vWF:Ag this is mainly due to the fact that a certain amount of the plasma FVIII does not bind to the immunoabsorbent (see Fig. 3).

On 100 experiments performed a 50% average yield was obtained on 5 ml to 500 ml columns when less than 20 cycles were performed per column. Cycle after cycle the immunoabsorbent ability to bind the FVIII/vWF is not significantly modified but the

Table 3 Influence of plasma pH on immunopurification of FVIII/vWF

pH of plasma	6.0	6.5	7.0	7.4
% adsorbed ¹⁾				
vWF:Ag	99	97	96	96
FVIII:Ag	35	70	82	85
% eluted ²⁾				
vWF:Ag	122 ⁴⁾	72	93	62
FVIII:Ag	35	58	68	49
FVIII:C	68	57	61	42
conservation of eluted FVIII:C ³⁾ %	0	100	100	100

Plasma from three different sources were pooled and divided in 100 ml fractions (FVIII:C concentration 1.0 IU/ml, vWF:Ag concentration 1.1 IU/ml) respectively adjusted with acetic acid to pH 6.0; 6.5; 7.0 or not adjusted (pH 7.4). They were loaded onto four identical 5 ml immunoabsorbent columns (see Fig. 2). Then the columns were washed with 70 ml of buffer 50 mM triethanolamine, 0.1 M NaCl) respectively at pH 6.0; 6.5; 7.0 or 7.4. Elution was carried out with buffer C in each case.

¹⁾ % adsorbed = 100% - % unbound material.

²⁾ compared to the total loaded material.

³⁾ after freezing at -20°C ; thawing at 37°C and 4 hours incubation at room temperature.

⁴⁾ this increase of vWF:Ag detection could be due to dissociation and unmasking of new antigenic sites.

recovery of the FVIII:C decreases slowly. After 20 cycles the yield is generally below that 40%. Probably the regeneration treatment used is not totally efficient and then other treatments are in study in order to improve the life of the columns.

The immunopurification process was also applied to cryoprecipitate instead of plasma. Experiments carried out with cryoprecipitates purchased from the French CTS allowed to obtain a 50% average FVIII:C recovery as from plasma.

Immunopurified FVIII/vWF

The immunopurification process presented here permitted production in one step, directly from plasma, of FVIII/vWF complex having a FVIII:C specific activity of at least 20 IU/mg (i. e. $1400 \times$ purified).

The main remaining contaminants in the immunopurified fractions were analysed by SDS-PAGE electrophoresis and seem

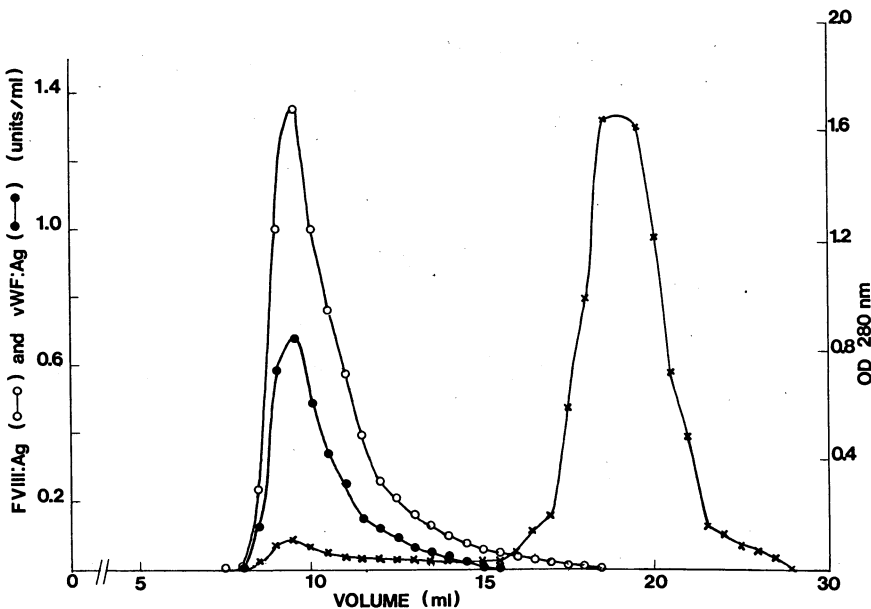


Fig. 4 Gel filtration of FVIII/vWF immunopurified on a Sepharose CL-4B column, $1 \times 30 \text{ cm}$. The 1 ml applied fraction contained 4 IU vWF:Ag, 4.1 U FVIII:Ag and 10 mg of human serum albumin as additive. FVIII:Ag (●—●); vWF:Ag (○—○) and optical density at 280 nm (×—×)

Table 4 Adsorption and elution recovery of FVIII/vWF during the immunopurification process

Cycle number	% Adsorption		% Recovery from applied plasma				Specific activity (IU of FVIII:C/mg of protein)
	FVIII:Ag	vWF:Ag	FVIII:C	FVIII:Ag	RCof	vWF:Ag	
1	77	96	56.5	61	60	75.5	20
2	75	94	82	72.5	79	81.5	45.5
3	66	96	55.5	55	40	67	35
4	71	96	43	50	57	83	not done
5	65.5	97	83	65	78	86	55
average value	71 ± 5	96 ± 1	64 ± 18	61 ± 9	63 ± 16	79 ± 8	39 ± 15

Plasma (100 ml) at pH 7.0 was loaded onto a 5 ml immunoabsorbent column (see Fig. 2). Each cycle was made with a different plasma bag. For each starting plasma, FVIII:C concentration was between 0.6 and 0.9 IU/ml and vWF:Ag concentration between 0.6 and 1.1 IU/ml.

to be human albumin (MW = 67 kDa) and human immunoglobulins (unreduced MW = 150 kDa). The presence of human immunoglobulins was confirmed with a specific immunoradiometric assay.

At the bottom of the immunoabsorbent column the pool of elutions fractions contains an average 4–5 IU of FVIII:C/ml and can be directly lyophilized as the elution buffer is volatile. The lyophilized product is stable when 10 mg/ml human serum albumin is added: 100% of its FVIII:C was recovered after 3–4 months of storage at -20°C .

Moreover, the immunopurification proved to be efficient in eliminating most of the viral contaminants. Immunopurification was performed with a plasma containing hepatitis B viruses. Hepatitis B viruses was assessed in the FVIII/vWF elution pool and the results showed that less than 0.007% of the initial virus antigen were in the purified fraction. The main part of the viral contaminants remained in the plasma and in the washing fraction.

Discussion

This work intended to develop an immunopurification process allowing the production of therapeutic purified FVIII/vWF complex directly from plasma. The difficulty of such a process lies in the characteristics of the FVIII/vWF molecule: (i) exceptional lability of the FVIII part (easily cleaved in activated, then degraded forms); (ii) reversible association of FVIII and vWF; (iii) size heterogeneity of the vWF (MW from 500 kDa to 20,000 kDa). (iv) low plasma concentration (10 $\mu\text{g/ml}$).

We have confirmed (4, 45, 46) that the FVIII:C was very unstable for pH lower than 6 and higher than 9 and in chaotropic conditions. In addition, we have substantiated that the FVIII-vWF association is reversed in acidic conditions (15% dissociation at pH 6.5 and 40% at pH 6) (44).

These characteristics require for immunopurification a monoclonal antibody having a sharp change in affinity for the FVIII/vWF complex between pH 7 to 9. Fortunately, an anti-vWF monoclonal antibody, the 239 mAb, having such properties was selected. Its maximum affinity is obtained between pH 5 to 7 and no detectable affinity was observed at pH ≥ 8.5 . Moreover, this antibody, which is easily produced (10 mg/ml of ascitic fluid) recognizes all the multimeric forms of vWF (47).

The elution buffer finally selected (100 mM triethanolamine, 0.1 M NaCl) has a pH higher than 9 (10.2) but a partial neutralization occurs on the column and the pH of the elution fraction is between 8.5 and 8.7. The buffering agent, triethanolamine, is not toxic and totally removed during the lyophilization step.

A large pore gel (Sephacryl S-1000) has been chosen to prepare the immunoabsorbent and the benzoquinone coupling method has been preferred for the following reasons: (i) good coupling

yield; (ii) better efficiency of the immobilized antibodies; (iii) low antibody release.

Nevertheless, the elution fractions were never totally devoid of released antibody and a specific process is under study to eliminate these contaminants, xenogenic for hemophilia A patients, which could induce immunological reactions.

The study of the loading step indicated that 10–15% of the plasma FVIII never binds to the anti-vWF immunoabsorbent, suggesting that this fraction of FVIII is not complexed to vWF. Nevertheless, in studies undertaken on freshly taken plasma, this phenomenon was not observed.

During the elution the FVIII and vWF activities were released in quantities proportional to the immunoabsorbed material and FVIII-vWF association remains in the product obtained. In addition, a good correlation has been observed between the FVIII:C and the FVIII:Ag recoveries strongly suggesting that the FVIII molecule is not activated, nor degraded.

Current therapeutic concentrates have a specific FVIII:C activity of 1 to 5 IU/mg and a low RCof activity with a FVIII:C recovery from plasma between 10 to 25%. The immunopurification process described here currently allowed a 50% direct recovery from plasma of FVIII/vWF complex having both FVIII:C (≥ 20 IU/mg) and RCof (≥ 20 IU/mg) activities, thus presenting both yield and purity improvements. Other FVIII immunopurification processes have been developed allowing the production of isolated FVIII (26, 48) or FVIII/vWF (46). Fass et al. (48) isolated after several steps the FVIII porcine molecule devoided of vWF having a FVIII:C activity of 6000 IU/mg suggesting that a pure FVIII/vWF complex in which FVIII represents 1% could reach a 60 IU/mg FVIII:C activity. Then, our product directly purified from plasma contains remaining contaminants that we intend to quantify.

Hornsey et al. (46) recently presented an FVIII/vWF immunopurification method using for the elution the slightly chaotropic agent KI associated with lysine which protects the FVIII:C activity. They also obtained FVIII/vWF complex from plasma. Nevertheless the FVIII:Ag recovery was 30% (74% binding and 39% release) and the FVIII:C recovery was 57% (67% binding and 85% release) suggesting that FVIII was activated during the process. In addition, KI is toxic and has to be dialysed before lyophilization.

In conclusion, the present process allows a 50% recovery of FVIII:C 1400 \times purified from plasma, associated with the vWF, directly lyophilized and presenting an excellent stability (100% preservation over 3–4 months at -20°C). The product contains all the vWF multimers (47), then it could be suitable for the therapy of both hemophilia A and von Willebrand patients. The industrial application of this process is economically feasible on the basis of 10 to 20 cycles per column (French [n $^{\circ}$ 8414488] and U.S. [n $^{\circ}$ 4670543] patents).

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References

- 1 Chavin S I. Factor VIII: structure and function in blood clotting. *Am J Hematol* 1984; 16: 297–306.
- 2 Owen W G, Wagner R H. Antihemophilic factor: separation of an active fragment following dissociation by salts or detergents. *Thromb Diath Haemorrh* 1972; 27: 502–15.
- 3 Hoyer L W. Molecular pathology and immunology of factor VIII (hemophilia A and factor VIII inhibitors). *Hum Pathol* 1987; 18: 153–61.
- 4 Weiss H J, Sussman I I, Hoyer L W. Stabilization of Factor VIII in plasma by the von Willebrand factor. *J Clin Invest* 1977; 60: 390–404.
- 5 Fay P J, Cavallaro C, Marder V J. Brief communication: comparison of the in vivo survival of human factor VIII with and without von Willebrand factor in the hemophilic dog. *Thromb Res* 1986; 37: 425–9.
- 6 Gitshier J, Wood W I, Goralka T M, Wion K L, Chen E Y, Eaton D H, Vehar G A, Capon D J, Lawn R M. Characterization of the human factor VIII gene. *Nature* 1984; 312: 326–30.
- 7 Toole J J, Knopf J L, Wozney J M, Sultzman L A, Buecker J L, Pittman D D, Kaufman R J, Brown E, Shoemaker C, Orr E C, Amphlett W G, Foster W B, Coe M L, Knutson G J, Fass D N, Hewick R M. Molecular cloning of a cDNA encoding human antihemophilic factor. *Nature* 1984; 312: 342–7.
- 8 Vehar G A, Keyt B, Eaton D, Rodriguez H, O'Brien D P, Rotblat F, Opperman H, Keck R, Wood W I, Hardins R N, Tuddenham E G D, Lawn R M, Capon D J. Structure of human factor VIII. *Nature* 1984; 312: 337–42.
- 9 Wood W I, Capon D J, Simonsen C C, Eaton D L, Gitshier J, Keyt B, Seeburg P H, Smith D H, Hollingshead P, Wion K L, Delwart E, Tuddenham E G D, Vehar G A, Lawn R M. Expression of active human factor VIII from recombinant DNA clones. *Nature* 1984; 312: 330–7.
- 10 Atichartakarn V, Marder V J, Kirby E P, Budzynski A Z. Effects of enzymatic degradation on the subunit composition and biologic properties of human factor VIII. *Blood* 1978; 51: 281–97.
- 11 Andersson L O, Forsman N, Huang K, Larsen K, Lundin A, Pavlu B, Sandberg H, Sewerin K, Smart J. Isolation and characterization of human Factor VIII: Molecular forms in commercial factor VIII concentrate, cryoprecipitate, and plasma. *Proc Natl Acad Sci USA* 1986; 83: 2979–85.
- 12 Eaton D L, Wood W I, Eaton D, Hass P E, Hollingshead P, Wion K, Mather J, Lawn R M, Vehar G A, Gorman C. Construction and characterization of an active factor VIII variant lacking the central one third of the molecule. *Biochemistry* 1986; 26: 8342–7.
- 13 Fay P J, Anderson M T, Chavin S I, Marder V J. The size of human factor VIII heterodimers and the effects produced by thrombin. *Biochim Biophys Acta* 1986; 871: 268–78.
- 14 Sadler J E, Shelton-Inloes B B, Sorace J M, Harlan J M, Titani K, Davie E W. Cloning and characterization of two cDNAs coding for human von Willebrand factor. *Proc Natl Acad Sci USA* 1985; 82: 6394–8.
- 15 Fretto L J, Fowler W E, Mc Caslin D R, Erickson H P, Mc Kee P A. Substructure of human von Willebrand Factor. *J Biol Chem* 1986; 261: 15679–89.
- 16 Furlan M. Factor VIII/von Willebrand factor: a multivalent ligand binding to platelets and collagen. *Blut* 1986; 52: 329–36.
- 17 Davies B L, Furlong R A, Peake I R. Studies on the relationship between factor VIII-related antigen (VIII:RAg) and factor VIII clotting antigen (VIII:CAG) by immunoelectrophoresis and autoradiography using ¹²⁵I anti-VIII:CAG. *Thromb Res* 1981; 22: 87–93.
- 18 Foster P A, Fulcher C A, Marti T, Titani K, Zimmerman T S. A major factor VIII binding domain resides within the amino-terminal 272 amino acid residues of von Willebrand factor. *J Biol Chem* 1987; 262: 8443–6.
- 19 Pool J G, Shannon A E. Production of high purity concentrates of anti-hemophilic globulin in a closed bag system. *N Engl J Med* 1965; 273: 1443.
- 20 Johnson A J, Karpatkin M H, Newman J. Preparation of and clinical experience with antihemophilic factor concentrates. *Thromb Diath Haemorrh* 1969; suppl 35: 49.
- 21 Brinkhous K M, Shanbrom E, Roberts H R, Webster W P, Fekete L, Wagner R H. A new high potency glycine-precipitated anti-hemophilic factor (AHF) concentrate. *J Am Med Assoc* 1968; 205: 613–7.
- 22 Perret B A, Furlan M, Beck E A. Studies on factor VIII-related protein II. Estimation of molecular size differences between factor VIII oligomers. *Biochim Biophys Acta* 1979; 578: 164.
- 23 Austen D E G. The chromatographic separation of factor VIII on aminoethyl Sepharose. *Br J Haematol* 1979; 43: 669–74.
- 24 Griffith M, Liu S, Neslund G, Tsang I, Lettelier D, Berkebile R. Preparation of high specific activity plasma AHF by anti-FVIII:C immunoaffinity chromatography. XIth International Congress on Thrombosis and Haemostasis, Bruxelles, July 4–11, 1987.
- 25 Tuddenham E G D, Trabold N C, Collins J A, Hoyer L W. The properties of factor VIII coagulant activity prepared by immunosorbent chromatography. *J Lab Clin Med* 1979; 93: 40–53.
- 26 Fulcher C A, Zimmerman T S. Characterization of the human factor VIII procoagulant protein with a heterologous precipitating antibody. *Proc Natl Acad Sci USA* 1982; 79: 1648–52.
- 27 Stel H V, Veerman E C I, Huisman J G, Janssen M C, Van Mourik J A. A rapid one-step immunoradiometric assay for factor VIII-procoagulant antigen utilizing monoclonal antibodies. *Thromb Haemostas* 1983; 50: 860–3.
- 28 Sultan Y, Avner P, Maisonneuve P, Arnaud D, Jeanneau C. An immunoradiometric assay for factor VIII related antigen (VIII:RAg) using two monoclonal antibodies. Comparison with polyclonal rabbit antibodies for use in von Willebrand's disease diagnosis. *Thromb Haemostas* 1984; 52: 250–2.
- 29 Soulier J P, Larrieu M J. Nouvelle méthode de diagnostic de l'hémophilie. Dosage des facteurs antihémophiliques A et B. *Le sang* 1953; 24: 205.
- 30 Kirwood T B L, Barrowcliffe T W. Discrepancy between one-stage and two-stage assay of factor VIII:C. *Br J Haematol* 1978; 40: 333.
- 31 Howard M A, Firkin B G. Ristocetin, a new tool in the investigation of platelet aggregation. *Thromb Diath Haemorrh* 1971; 26: 362–9.
- 32 Mazurier C, Parquet-Gernez A, Samor B, Goudemand M, Montreuil J. Etude de l'antigène lié au facteur VIII (FVIII:Ag) Purification par chromatographie d'immuno-affinité. *C R Acad Sci Paris* 1979; 296: 257.
- 33 Köhler G, Milstein C. Continuous cultured of fused cell secreting antibody of predefined specificity. *Nature* 1975; 256: 495.
- 34 Sola B, Avner P, Sultan Y, Jeanneau C, Maisonneuve P. Monoclonal antibodies against human factor FVIII molecule neutralize anti-hemophilic factor and ristocetin cofactor activities. *Proc Natl Acad Sci* 1982; 79: 183–7.
- 35 Scatchard G. The attraction of proteins to small molecules and ions. *Ann N Y Acad Sci* 1949; 51: 660.
- 36 Ey P L, Prowse S J, Jenkin C R. Isolation of pure IgG₁, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. *Immunochemistry* 1978; 15: 429–36.
- 37 Weston P D, Avrameas S. Proteins coupled to polyacrylamide beads using glutaraldehyde. *Biochem Biophys Res Commun* 1971; 45: 1574.
- 38 Axen R, Porath J, Ernback S. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature* 1967; 214: 1302.
- 39 Cuatrecasas P. Protein purification by affinity chromatography. *J Biol Chem* 1970; 245: 574.
- 40 Porath J, Fornstedt N. Group fractionation of plasma proteins on dipolar ion exchangers. *J Chromatogr* 1970; 51: 479–89.
- 41 Nilsson K, Mosbach K. Immobilization of enzymes and affinity ligands to various hydroxyl group carrying supports using highly reactive sulfonyl chlorides. *Biochem Biophys Res Commun* 1981; 102: 449.
- 42 Finlay T H, Troll V, Levy M, Johnson A, Hodgins L T. New methods for the preparation of biospecific adsorbents and immobilized enzymes utilizing trichloro-s-triazine. *Anal Biochem* 1978; 87: 77.

- 43 Brandt J, Andersson L O, Porath J. Covalent attachment of proteins to polysaccharide carriers by means of benzoquinone. *Biochim Biophys Acta* 1975; 386: 196-202.
- 44 Brocway W, Fass D N. The nature of the interaction between ristocetin-Willebrand factor and the factor VIII coagulant activity molecule. *J Lab Clin Med* 1977; 89: 1295-305.
- 45 Hoyer L W. The factor VIII complex: structure and function. *Blood* 1981; 58: 1-13.
- 46 Hornsey V S, Griffin B D, Pepper D S, Micklem L R, Prowse C V. Immunoaffinity purification of factor VIII complex. *Thromb Haemostas* 1987; 57: 102-5.
- 47 Jorieux S, Mazurier C, Deromeuf C, Goudemand M. Properties and interests of a monoclonal antibody to human von Willebrand factor, abstract 14. Bari International Conference on Factor VIII/von Willebrand Factor, June 10-15, 1985.
- 48 Fass D N, Knutson G J, Katzmann J A. Monoclonal antibodies to porcine factor VIII coagulant and their use in the isolation of active coagulant protein. *Blood* 1982; 59: 594-600.

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