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Characterization of F VIII Concentrates Produced by Two Methods Incorporating Double Virus Inactivation

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ABSTRACT

The trend toward the production of high purity factor VIII concentrates for clinical use is still in progress. Although all plasma derivatives must undergo viral inactivation procedures, the possibility of transmission of viral diseases is not completely eliminated. In order to reduce such risk, we have included double virus inactivation in the procedure of factor VIII concentrate production. In a scale-up procedure for isolation of factor VIII from cryoprecipitate, two methods were used. The first is based on the chromatographic purification of factor VIII after pasteurization of cryoprecipitate solution and solvent/detergent (S/D) inactivation of viruses. The second is based on multistep precipitation of factor VIII by sodium chloride and glycine. Viral inactivation was performed by combination of S/D treatment and heating of final freeze-dried product 30 min at 100°C. The typical yield of factor VIII activity in the freeze-dried product was about 20% for the first method, and 25–30% for the second. Electrophoretic analyses of both factor VIII preparations by SDS-PAGE and IEF show very low content of contaminant proteins, in accordance with observed 400–650-fold increase of their specific activity over plasma. Both factor VIII products were stable in the liquid state for more than 24 h at room temperature. The final products, after double viral inactivation, are considered to be suitable for clinical evaluations.

Index Entries: Factor VIII manufacturing; chromatography; virus inactivation; heat treatment.

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INTRODUCTION

The administration of human coagulation factor VIII (F VIII) is necessary for patients suffering from hemophilia A. In the past 30 yr, a variety of concentrates has been used, starting with cryoprecipitate and several kinds of intermediate- and high-purity products *(1,2)*. The improved quality of the F VIII concentrates has been required to reduce the allogenic protein content, as a possible cause of immunosuppression *(3,4)*. Furthermore, in spite of sophisticated testing systems for the presence of several viruses in plasma, it is of utmost importance to expose all plasma derivatives to reliable virus inactivation procedures. Extensive virus inactivation could be promoted by an understanding of the structural similarities and differences between viruses and proteins. Different sterilizing methods have been employed based on the conditions at which the protein of interest retains its biological activity. It was shown that some often-used virus inactivation methods, such as moderate dry-heat treatment, do not fully eliminate the risk of viral infections *(5,6)*. However, pasteurization in solution *(7,8)* and solvent/detergent (S/D) treatments *(9,10)* appear to eliminate the risk of transmission of human immunodeficiency virus (HIV) and hepatitis caused by of F VIII concentrates.

In this study, we report two procedures for production of high-purity human F VIII, both of which incorporate unrelated virus inactivation steps, based on different principles. The starting material for F VIII preparation was the cryoprecipitate. The first method utilizes classical purification procedures, typical for production of intermediate-purity F VIII, followed by ion exchange chromatography *(14)*. Viral inactivation was achieved by a combination of pasteurization in solution and subsequent S/D treatment. The second method relies on the isolation of F VIII by repeated precipitation with glycine/sodium chloride mixture, according to the procedure of Myers et al. *(15)*. Virus inactivation was carried out by a combination of S/D treatment and heating of final freeze-dried product at 100°C for 30 min *(11–13)*.

MATERIALS AND METHODS

All chemicals, obtained from different sources, were of the best possible grade. Most of them fully complied with pharmacopeian requirements. A few of them, for which such requirements do not exist, were subjected, when possible, to the basic biological tests. DEAE-Fractogel TSK 650 (M) was purchased from Merck. Chromatography was performed using BioProcess system equipped with a glass column BPG 200/500 (Pharmacia, Uppsala, Sweden). Activity of F VIII was measured by automatic coagulometer (Fibrintimer, Behring, Germany). Electrophoresis was performed using PhastSystem (Pharmacia) and commercial gels and reagents Data

were analyzed using PhastImage densitometer (Pharmacia). Spectrophotometric measurements were carried out using Uvicon 930 spectrophotometer (Kontron, Switzerland), and prekallikrein activator activity was determined by iEMS MF (Labsystem, Finland) ELISA photometer.

Cryoprecipitate Collection

The starting material for the preparation of F VIII was fresh-frozen plasma collected from volunteer donors of the Zagreb region, Croatia. The plasma, collected by plasmapheresis, was rapidly frozen in liquid bath at –60°C and stored at or below –25°C until use. Each single donation of plasma was tested for hepatitis B antigen, HIV 1,2, and hepatitis C antibodies, and ALT. Individual units of plasma were removed from –25°C storage, allowed to melt at 4°C 18–20 h, and centrifuged at 4°C for 30 min at 2600*g*. The cryo-poor supernatant was transferred into a sterile container. Cryoprecipitate was resuspended in a small volume of solvent buffer, pooled, and frozen at –25°C.

Purification of Human F VIII: Method 1

The cryoprecipitate was thawed at 2–8°C, minced, and suspended in an equal volume of solvent buffer (0.08 *M* sodium chloride and 0.27 *M* glycine in water for injection). Tri(n-butyl)phosphate (TnBP) and Tween-80 were added to the suspension to the final concentrations of 0.3% and 1%, respectively. The mixture was stirred 6 h at room temperature. After that, 50 mL 2% $Al(OH)_3$ (Alhydrogel) per 1 L of solubilized cryoprecipitate was added. The suspension was gently stirred 30 min at room temperature, and centrifuged at 20°C for 30 min at 3000*g*. The precipitate was discarded. The solution was stabilized by addition of 1000 g sucrose, 0.735 g $CaCl_2 \cdot 2H_2O$, and 135 g glycine per liter of supernatant, under constant stirring. The solution was warmed up to 37°C and stirred until stabilizers were completely dissolved. The pH was adjusted to 7.0 ± 0.2 with 0.1 *M* sodium hydroxide. The temperature of the solution was increased to 60°C, and was left at that temperature for 10 h. After heat treatment (pasteurization) was completed, the solution was cooled to room temperature and diluted with 3.5 vol of starting buffer (0.01 *M* trisodium citrate, 0.003 *M* $CaCl_2 \cdot 2H_2O$, 0.016 *M* L-lysine, and 0.005 *M* sodium chloride, pH 7.0). 300 mL DEAE Fractogel TSK 650 *M* per 1 L of starting cryoprecipitate, preequilibrated in starting buffer, was added to the solution. The mixture was gently stirred and adsorption of F VIII activity on the gel was achieved during 4–6 h at room temperature. After binding of active material, the gel was left to settle at the bottom of the tank. Supernatant was carefully siphoned and discarded. The gel was washed three times in the tank by repeated resuspension in washing buffer (0.1 *M* sodium chloride in starting buffer), followed by gel setting and siphoning of the buffer. Finally, the

gel was transferred into the chromatographic column and eluted with 5–6 column vol of eluent buffer "a" (0.14 *M* sodium chloride in starting buffer). F VIII was then eluted using eluent buffer "b" (0.25 *M* sodium chloride in starting buffer), and the rest of the adsorbed material was eluted using eluent buffer "c" (1.0 *M* sodium chloride in starting buffer). The elution peak was collected and F VIII:C activity was determined by the one-stage method *(16)*. The solution containing F VIII was filtered, concentrated, and dialyzed by tangential flow ultrafiltration system (Minisette, Filtron, Northborough, MA) against sixfold volume of filling medium (0.3 *M* glycine, 0.005 *M* $CaCl_2 \cdot 2H_2O$, and 0.044 *M* sucrose, pH 7.0, adjusted by 2 *N* HCl) at room temperature. The ultrafiltrate was clarified by filtration, using a combination of 1.2/0.8 μm membranes, and sterile filtrated through 0.22 μm membrane. The F VIII activity was measured again, and material was filled into final containers and freeze dried. The flow diagram for this method is depicted in Fig. 1.

Purification of Human F VIII: Method 2

Isolation of F VIII was achieved using multistep sedimentation in NaCl/glycine, following the procedure of Myers et al. *(15)*. The final product of F VIII was dispensed into vials and freeze dried. The freeze-dried product was heated 30 min at 100°C. The flow diagram for this method is depicted in Fig. 2.

Protein Assays

F VIII activity was measured by a one-stage activated thromboplastin time test *(16)* using a F VIII-deficient human plasma (Institute of Immunology, Croatia). Fourth International Standard for blood coagulation F VIII concentrate 88/804 was used as a reference. Standard and samples were diluted 1:50, 1:100, 1:200, 1:400, and 1:800 in Owren buffer, pH 7.35, and clotting times were measured using an automatic coagulometer. Immunoglobulins G, A, and M were measured by radial immunodiffusion (Institute of Immunology). Sodium was determined by flame photometry, and aluminum was measured by atomic absorption spectrophotometry. The rabbit pyrogen test was conducted according to prescribed methods *(17)*. Isoagglutinins were determined following the recommendation of the European Pharmacopoeia, after dilution of F VIII concentrate to 3 IU/mL. Prekallikrein activator was evaluated using chromogenic substrate *N*-benzoyl- Pro-Phe-Arg *p*-nitroanilide hydrochloride. Protein concentration was determined according to the method of Bradford *(18)*, using bovine serum albumine (BSA) as a standard.

PCR Testing of Hepatitis B and C

The absence of hepatitis B and C viruses in the final products was confirmed using RIA and PCR methods, respectively.

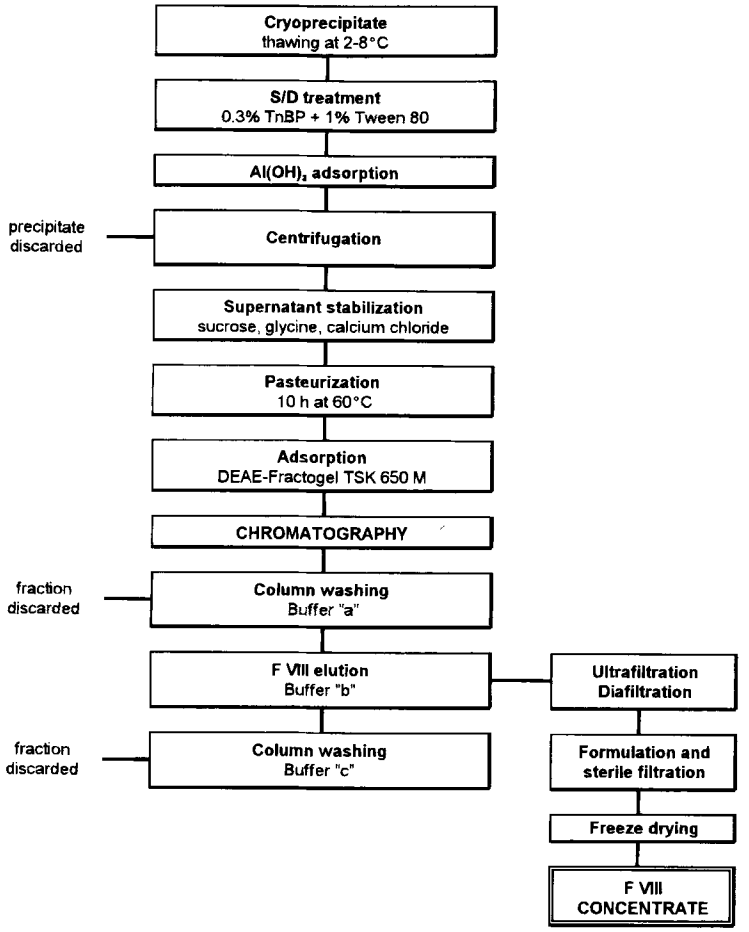

Fig. 1. Flow chart of F VIII production by Method 1 with double-step virus inactivation.

TnBP, Tween-80, and Residual Moisture Determinations

Residual TnBP and Tween-80 were determined by gas-chromatographic and spectrophotometric procedures, respectively, as previously described *(19)*. The residual moisture was determined gravimetrically.

Isoelectric Focusing and SDS-PAGE

Isoelectric focusing (IEF) was performed following the manufacturer's instructions (Pharmacia LKB Biotechnology) using PhastGel IEF 3-9. The pl of the protein was determined using pl-gradient marker proteins (Pharmacia). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions, according to Laemmli's procedure *(20)*, on semiautomatic system (PhastSystem,

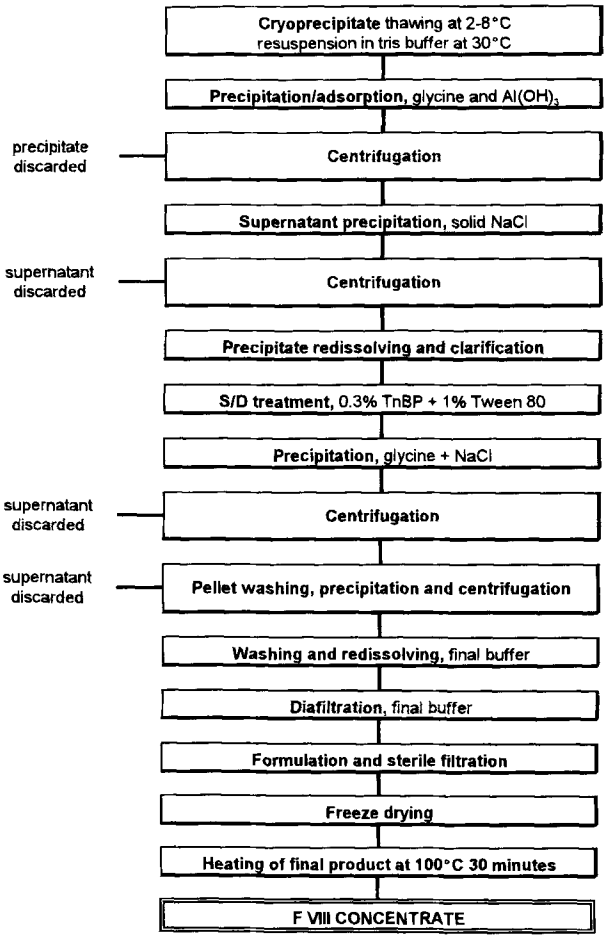

Fig. 2. Flow chart of F VIII production by Method 2 with double-step virus inactivation.

Pharmacia). The relative mol wt were estimated using Pharmacia high molecular weight (HMW) markers. The gels were stained with a Coomassie brilliant blue R-350 (Merck).

Stability Test

The F VIII activity was tested 12–24 h after reconstitution of freeze-dried material at room temperature and 4°C.

RESULTS

Chromatographic Purification of F VIII by Method 1

After S/D treatment, $Al(OH)_3$ adsorption, and pasteurization for 10 h at 60°C, the cryoprecipitate extract was loaded onto DEAE-Fractogel TSK

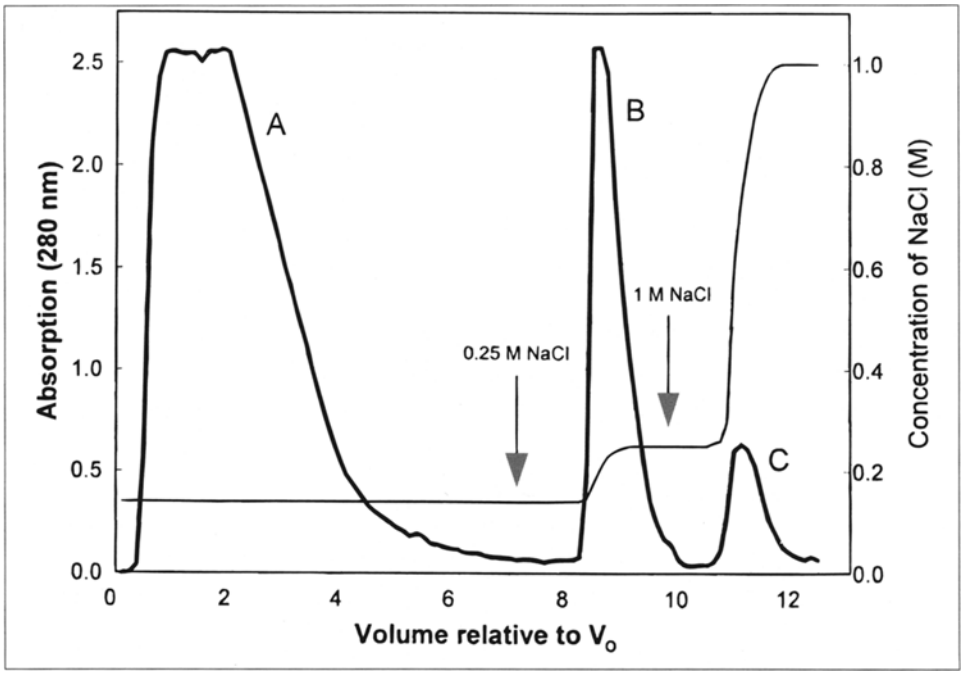

Fig. 3. Ion exchange chromatography of partially purified and virus-inactivated F VIII. **A**, fraction eluted with buffer "a"; **B**, fraction eluted with buffer "b"; and **C**, fraction eluted with buffer "c".

650 M (Fig. 3). Most proteins are eluted in the breakthrough fractions, including fibrinogen, immunoglobulins, and albumin, as well as viral sterilizing agents (*see* peak A on Fig. 3). In this step, the salt concentrations of the buffers were varied and allowed the removal of contaminant proteins, permitting the separation of the F VIII from von Willebrand factor.

Most of the F VIII activity was found in the fractions containing 0.25 *M* NaCl in the starting buffer (*see* peak B in Fig. 3). There was no F VIII activity in peak C, eluted with 1 *M* NaCl. The yield obtained with this purification method was approx 20%, based on the activity of starting cryoprecipitate. The biggest loss of F VIII activity was observed after $Al(OH)_3$ adsorption and sterile filtration (Table 1). The specific activity of the final material was increased approx 650-fold over plasma.

Purification of F VIII by Method 2

Pilot-scale experiments were conducted to examine the feasibility of the glycine procedure *(15)* with the cryoprecipitate collected in the same way as in Method 1. Mean recovery of F VIII activity for this method is shown in Table 2. The biggest loss of the F VIII activity was observed after $Al(OH)_3$ adsorption. No loss of the F VIII activity was found during the

Table 1
Recovery Balance of F VIII Purification by Method 1

Production step	Volume mL	Activity IU/mL	Total activity IU	Relative recovery %
Cryoprecipitate	9800	10	98,000	100
Supernatant after S/D and $Al(OH)_3$ treatment	13,500	4.28	57,780	59
Solution after pasteurization 10 h at 60°C	60,000	0.90	54,000	55
F VIII fraction from DEAE-Fractogel 650 (M)	2440	19.38	47,287	48
Solution after ultrafiltration/diafiltration	1200	35	42,000	43
Sterile filtration	1000	20	20,000	20
Final freeze-dried product	1000	20	20,000	20

Table 2
Recovery Balance of F VIII Purification by Method 2

Production step	Volume mL	Activity IU/mL	Total activity IU	Relative recovery %
Cryoprecipitate	1400	8.71	12,194	100
Glycine/$Al(OH)_3$ supernatant	4500	1.62	7290	60
NaCl precipitate	198	34.7	6877	56
Postviral inactivation precipitate	110	51.2	5519	45
Solution after ultrafiltration/diafiltration	100	35.5	3752	31
Sterile filtration	90	34.0	3060	25
Final freeze-dried product	90	33.7	3033	25

S/D treatment, and after heating of the final freeze-dried product for 30 min at 100°C. The overall recovery of the F VIII activity obtained from cryoprecipitate was 25–30%. The specific activity of the final material was increased approx 400-fold over plasma.

In vitro Evaluation of F VIII from Methods 1 and 2

Table 3 summarizes the major biochemical characteristics of F VIII purified according to Methods 1 and 2. Specific activity was higher by F VIII obtained according to Method 1, but content of isoaglutinins, immunoglobulins, and sodium were similar in F VIII isolated by either Methods 1 or 2. Despite the use of $Al(OH)_3$ in the beginning of both procedures, the level of aluminium in the final products were negligible. The residual levels of TnBP and Tween-80 were in the limits approved by the FDA for F VIII. All requirements from the Pharmacopeia, including pyrogen tests, were passed. No loss of F VIII activity was observed in vitro. In fact, the recon-

Table 3
Characteristics of F VIII Concentrates Isolated by Methods 1 and 2

Assay	Method 1	Method 2
Protein	2–3 g/L	5–10 g/L
F VIII activity	20–30 IU/mL	30–40 IU/mL
F VIII specific activity	≈10 IU/mg protein	5–7 IU/mg protein
Thrombin test	0	0
IgG concentration	0.06–0.1 g/L	0.1–0.14 g/L
IgA concentration	<0.03 g/L	<0.05 g/L
IgM concentration	<0.1 g/L	<0.1 g/L
Isoagglutinins: anti-A titer	1:2–1:4	1:2–1:4
anti-B titer	0–1:2	0–1:2
Prekallikrein activator	0 IU/mL	1–2 IU/mL
pH	7.1 ± 0.2	7.0 ± 0.2
TnBP concentration	0–2 µg/mL	0–2 µg/mL
Tween-80 concentration	5–10 µg/mL	5–10 µg/mL
Aluminium concentration	25–37 µg/L	40–60 µg/L
Sodium ion concentration	118–140 mmol/L	120–130 mmol/L

stituted products maintain 100% activity after 24 h at room temperature. The F VIII produced by both methods was characterized by SDS-PAGE (Fig. 4) and IEF (Fig. 5). Figure 4 shows the SDS-PAGE profiles of the starting cryoprecipitate (line 1) and final products of F VIII obtained by Method 1 (line 2) and Method 2 (line 3). Results indicate that starting cryoprecipitate is a complex mixture of proteins, especially y-proteins, β-proteins and albumin, but F VIII concentrates were free of many contaminant proteins, especially in the region of high mol wt. Using SDS-PAGE method, the molecular mass of 270 kDa for both F VIIIs were estimated. The high purity of F VIII was confirmed by PhastGel IEF (pH range 3–9), in which only one band with apparent isoelectric point of 5.9 was visible.

DISCUSSION

Much progress in the development of virus-safe, pure F VIII preparations has been achieved recently, but it seems that there is still a need for preparation of economical F VIII concentrates *(21–23)*. Both methods reported here yield a high-quality F VIII concentrate at pilot-scale batch levels of 150–200 L of plasma. These production techniques, as well as their modifications, have already been published *(14,15,24)*, so our goal was to modify them and incorporate two independent virus inactivation steps. The common inactivation step in both methods was an S/D treatment that

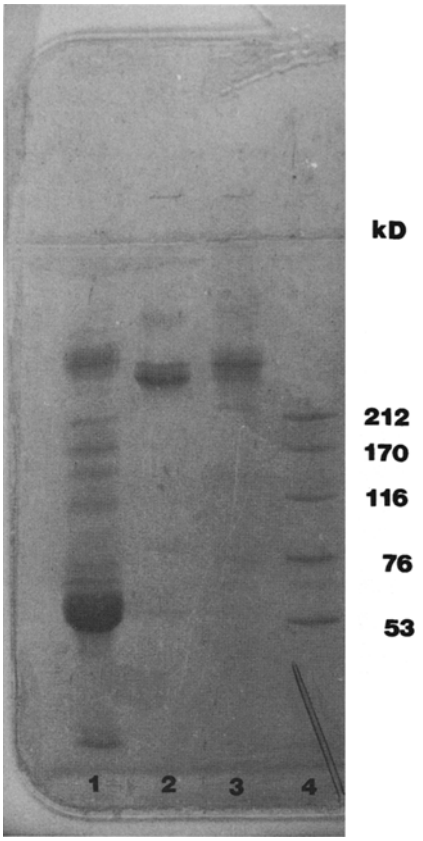

Fig. 4. SDS-PAGE analysis of purified F VIII on 4–15% gradient gel. Lane 1, starting cryoprecipitate; lane 2, F VIII concentrate produced by Method 1; lane 3, F VIII concentrate produced by Method 2; lane 4, Pharmacia HMW standards composed of glutamic dehydrogenase 53 kDa, transferin 76 kDa, -galactosidase 116 kDa, $_2$-macroglobulin 170 kDa and myosin 212 kDa.

has been shown to be effective in inactivating enveloped viruses like HIV, hepatitis B, and hepatitis C viruses. Virus sterilization by S/D has little impact on F VIII functional activity, recovery, and circulatory survival *(25,26)*. In order to inactivate nonenveloped viruses, such as hepatitis A and Parvovirus, second virus inactivation was implemented. In Method 1 that was pasteurization in solution, and in Method 2 it was dry-heat treatment at 100°C for 30 min *(11)*.

The mechanism of thermal virus inactivation is both temperature- and virus-dependent. Thermodynamic data suggest that inactivation is most probably caused by disruption of many chemical interactions, mostly hydrogen bonds *(27)*. The discrimination of thermal inactivation methods is likely to rest largely on the relative complexity of virus vs protein structures, causing their differential stabilization as a result of addition of sug-

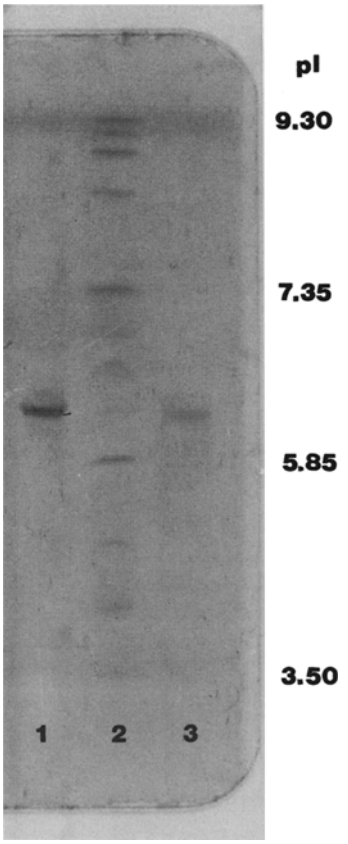

Fig. 5. pI determination of purified F VIII by IEF on 3–9 gradient gel. Lane 1, F VIII produced by Method 1; lane 2, Pharmacia broad pI calibration kit (pH 3–10) composed of amyloglucosidase, 3.50; soybean trypsin inhibitor, 4.55; β-lactoglobulin A, 5.20; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.55; horse myoglobin (acidic band), 6.85; horse myoglobin (basic band), 7.35; lentil lectin (acidic band), 8 15; lentil lectin (middle band), 8.45; lentil lectin (basic band), 8.65; trypsinogen, 9.30; lane 3, F VIII produced by Method 2.

ars and salts. Contrarily, S/D treatment would be expected to be directed toward lipid and lipophilic proteins *(28)*.

The recovery and purity of F VIII obtained by these methods was similar, but lower than was reported in the literature *(14,15,25)*. Part of the reason for this can be found in the inadequate technique of cryoprecipitate collection. It is well known that the recovery of F VIII and the level of contaminating proteins in cryoprecipitate may be significantly influenced by the methods of thawing plasma and collection of cryoprecipitate *(29)*. However, in both procedures, residual vitamin K-dependent clotting factors, such as factor VII and IX, were removed by adsorption of aluminium hydroxide, but this step also results in the biggest single F VIII activity loss

(30). Effective treatment by $Al(OH)_3$ may also explain the absence of proteolytic activity in the F VIII preparation. Despite the use of $Al(OH)_3$ in the early stage of the process, the level of aluminum in the final F VIII concentrate was between 40 and 60 μg/L. In both processes, Tween-80 and TnBP were efficiently removed, and, in the final product, were present only in trace amounts (less than 10 ppm). The stability of the product, evaluated by one-stage clotting assay, has been found to be excellent for 24 h after reconstitution.

In conclusion, F VIII obtained by Methods 1 and 2 seems to have two essential prerequisites: purity and safety. The final lyophilized products of F VIII are considered acceptable for clinical use.

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