

A heat-treated factor VIII concentrate prepared by controlled-pore glass adsorption chromatography

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Four years' experience with a method for preparing a high-purity, low-fibrinogen, heat-treated, factor VIII concentrate is reported. The process, batch adsorption of a cryoprecipitate extract with controlled-pore glass granules, removes 77 percent of the cryoprecipitate fibrinogen, resulting in a final concentrate-specific activity of 0.74 IU factor VIII per mg of protein at a yield of 194 IU factor VIII per kg of starting plasma. Heat treatment of the lyophilized concentrate for 72 hours at 60°C results in less than 10 percent loss of factor VIII activity. This process does not require expensive fractionation equipment, is suitable for small-to medium-scale batch concentrate production and could be adopted by moderately well-equipped regional blood processing laboratories for the decentralized production of a high-quality, heat-treated factor VIII concentrate. **TRANSFUSION** 1987;27:174-177.

A MAJOR PROBLEM facing a regional blood transfusion service wishing to become entirely self-sufficient in factor VIII components is the necessary reliance on commercial sources for the preparation of intermediate- and high-purity factor VIII concentrates. Although most regional centers can prepare cryoprecipitate, the preparation of high-purity products using traditional methods¹⁻³ is generally beyond the capability of these laboratories.

Individual laboratories can use the thaw-siphon method⁴ or cryoprecipitation in the presence of heparin⁵ to prepare single-donation or small-pool high-purity factor VIII concentrate. However, these methods are somewhat laborious, and the final products do not always have the convenience of the freeze-dried, standardized, large-pool commercial concentrates.

A new technique for preparation of a high-quality factor VIII concentrate was described by Margolis and Rhoades,⁶ who reported a factor VIII concentrate with high yield and purity, low levels of fibrinogen, good stability during heat treatment, and excellent *in vivo* recovery.⁷ Moreover, this technique did not require expensive, highly specialized fractionation equipment and could be performed by a modestly equipped regional blood processing laboratory.

We modified this method for use in our own laboratory and report on 4 years' experience in the preparation of a factor VIII concentrate.

Materials and Methods

Solutions

All buffers and electrolyte solutions were prepared by the Intravenous Solutions Unit of the Auckland Hospital Board

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from analytical-grade reagents and pyrogen-free distilled water. All solutions were autoclaved immediately after preparation.

Blood collection

Blood (450 ml) was collected from random healthy donors into anticoagulant (citrate-phosphate-double-dextrose [CP2D], 63 ml) using triple blood packs (Tuta Laboratories, Lane Cove, Australia). Other anticoagulant systems have not been evaluated for potential improvements to factor VIII recovery. The plasma was separated by centrifugation at 4000 × *g* for 7 minutes and rapidly frozen in a solid CO₂-ethanol bath at -70°C. The delay between blood collection and plasma freezing averaged 6 hours. Frozen plasma was stored at -30°C for 1 to 7 days until sufficient amounts were accumulated for further processing.

Cryoprecipitate preparation

The frozen plasma donations were thawed rapidly in a circulating 4°C waterbath.⁸ Thawing was continued with occasional manual mixing for 1 to 1.5 hours or until all of the ice-slush within the bags had disappeared. The thawed plasma was immediately centrifuged at 4°C and 3500 × *g* for 5 minutes, and all of the cryoprecipitate supernatant plasma was siphoned into a satellite bag. The cryoprecipitate was immediately frozen in a solid CO₂-ethanol bath and stored at -30°C for up to 2 weeks.

Factor VIII concentrate preparation

All subsequent procedures were carried out under clean-room conditions. Six hundred individual units of cryoprecipitate were thawed in a waterbath at 4°C and then transferred to a 70 percent (v/v) ethanol bath at 4°C for several minutes to sterilize the outside of the bags. The bags were removed and dried of excess ethanol, and the lower 40-mm section was cut with scissors, allowing the cryoprecipitate to be pooled into a fractionation vessel. After being weighed, the pooled units of cryoprecipitate were washed at 4°C with 0.02 *M* Tris-HCl, pH 7.0, at a ratio of 2.5 ml of buffer per gram of cryoprecipitate. The buffer washings were discarded.

The washed cryoprecipitate was then extracted with 0.4 ml of an intravenous synthetic amino acid solution (Synthamin 17, Travenol Laboratories, Deerfield, IL) per gram of

cryoprecipitate with continuous agitation at 25 to 30°C for 20 minutes. The extract was cooled to 20°C, the pH was adjusted to 7.0, and 0.2 ml of aluminum hydroxide gel (Alhydrogel, Superfos, Denmark) was added per gram of original washed cryoprecipitate. After being mixed for 5 minutes, the aluminum hydroxide gel and insoluble proteins were removed by centrifugation at 4000 × g for 15 minutes at 15°C. The supernatant was decanted through gauze to remove the surface residue. The citrate concentration was increased to 0.017 M by the addition of 1 M trisodium citrate, and the pH was adjusted to 7.0 with 0.1 M HCl. The cryoprecipitate extract was then ready for mixing with the controlled-pore glass (CPG) granules.

The CPG, which was stored between column runs in 0.1 M glycine, pH 1.5, was washed before use with 10 column volumes of Tris-citrate-saline buffer (0.020 M: 0.017 M: 0.026 M, pH 7.4). The moist CPG granules (mesh size, 120–200, pore size, 500 Å; Sigma Chemical Company, St. Louis, MO) were removed from the chromatography column and added to the cryoprecipitate extract in a ratio of 1.0 ml of moist beads per 50 to 60 mg of protein.

A slurry of the cryoprecipitate extract and the CPG was formed by gentle manual mixing with a wide stainless steel spatula. After being mixed, the slurry was transferred to a 140 × 500-mm acrylic chromatography column fitted with a 30 µm nylon screen supported by a stainless steel mesh. The CPG was allowed to settle in the column. The column outlet was opened and the free-running primary effluent collected into a sterile container. Factor VIII remaining in the column was recovered by eluting the column with further increments of Tris-citrate-saline buffer. These subsequent column effluents were collected and pooled with the primary effluent. The total volume of the concentrate at this stage was adjusted by the addition of further column effluent to approximately twice the cryoprecipitate extract volume.

The pH of the pooled column effluent was adjusted to 6.7 at 10°C, and the small amount of cold insoluble material that formed was removed by centrifugation at 10°C and 4000 × g for 30 minutes.

The supernatant was warmed to 25°C and the pH adjusted to 7.3. The solution was clarified through prefilter (AP 25, Millipore Corp., Bedford, MA) in a 142-mm filter holder. The pool was then sterile filtered using 0.45-µm and 0.2-µm, 0.13 m² disposable in-line filter units (Gelman Sciences, Ann Arbor, MI) connected serially. Filtration of 3500 ml of concentrate was completed within 5 minutes at a pressure of 1.5 to 2.0 psi.

The sterile filtrate was dispensed aseptically in 30-ml aliquots into 50-ml bottles. The product was frozen in liquid nitrogen and freeze-dried in a freeze-drier (Model 250 SRC, Virtis Co., Inc., Gardiner, NY) to a maximum temperature

of 25°C for at least 60 hours. The bottles were back-filled with sterile dry nitrogen and closed under a partial vacuum.

The lyophilized product was heated in a water bath at 60°C for 72 hours. When needed, the concentrate was reconstituted with 10 or 15 ml of sterile pyrogen-free distilled water.

CPG column washing

Immediately after the concentrate had been recovered from the CPG column, the CPG was removed from the column and mixed with 3 l of 2 percent (w/v) sodium dodecyl sulphate (SDS). The slurry was then returned to the column and washed with 4 column volumes of pyrogen-free water followed by 4 column volumes of 0.1 M Na₂CO₃ (pH 11.0) and 4 column volumes of 0.1 M glycine (pH 1.5). The column could be stored for long periods in the glycine if the pH was maintained below 1.5

Analytical methods

Total protein was determined by a modified biuret method.⁹ Fibrinogen was measured with a photometric modification of a standard clotting assay.¹⁰ Factor VIII procoagulant (FVIII_c) activity was assayed with an artificial substrate¹¹ with the assay calibrated against the Second International Standard for factor VIII.

Factor VIII-related antigen (FVIII_RAg) was determined by rocket immunoelectrophoresis under nonreducing conditions.¹²

Other quality control procedures were carried out according to the British Pharmacopoeia requirements for factor VIII concentrates.¹³

Results

Laboratory data

Details of the last 50 production batches of CPG factor VIII concentrate are outlined in Table 1. The washed and extracted cryoprecipitate yielded 342 ± 107 IU factor VIII per kg of plasma. Significant losses of factor VIII occurred in the aluminum hydroxide gel adsorption and in subsequent processing steps. After sterile filtration, lyophilization, and heat treatment, the average yield of factor VIII was 194 ± 31 IU per kg of plasma. Thus, 57 percent of the factor VIII in the cryoprecipitate extract was recovered in the final product. Factor VIII assays were not performed routinely on either plasma before cryoprecipitation or pooled unextracted cryoprecipitate, but experimental data indicate that 20 to 25 percent of the original factor VIII in the plasma starting material was present in the factor VIII concentrate. The

Table 1. Data on 50 production batches of CPG factor VIII concentrate.

	Factor VIII _c yield (IU/kg plasma)	Protein (mg/kg plasma)	Fibrinogen (mg/kg plasma)	Specific activity (IU FVIII/mg protein)
Cryoprecipitate extract	342 ± 107	1129 ± 361	554 ± 188	0.30
After A1 (OH) ₃ adsorption	304 ± 68	973 ± 185	385 ± 101	0.31
After CPG adsorption	275 ± 61	358 ± 110	87 ± 25	0.77
After sterile filtration	239 ± 43	305 ± 75	67 ± 18	0.78
After lyophilization, heat treatment, and reconstitution	194 ± 31	262 ± 81	55 ± 20	0.74

Table 2. Final product analyses from data on 50 production batches of heat-treated CPG factor VIII.

	Mean (\pm SD)
Factor VIII (IU/bottle)	240 \pm 30
Total protein (mg/bottle)	324 \pm 91
Fibrinogen (mg/bottle)	69 \pm 31
Specific activity (IU factor VIII/mg protein)	0.74 \pm 0.26
Yield of factor VIII (IU/kg plasma)	194 \pm 41
Factor VIII-related-antigen (IU/bottle)	685 \pm 250
pH	7.0 \pm 0.4

mean specific activity of the lyophilized, heat-treated, reconstituted factor VIII concentrate was 0.74 IU factor VIII per mg of total protein (Table 1).

The CPG adsorption step removed 63 percent of the cryoprecipitate protein and 77 percent of the fibrinogen. The final product contained less than 10 percent of the original cryoprecipitate fibrinogen. Sterile filtration and lyophilization resulted in a loss of approximately 20 percent of the factor VIII activity. The heat treatment step resulted in less than 10 percent loss of activity (range, 0–10%).

The reconstituted factor VIII concentrate lost 20 percent of activity when stored at room temperature for 24 hours. When concentrate was stored lyophilized at 4°C for 18 months, storage loss was less than 10 percent. Characteristics of the final product are outlined in Table 2.

Clinical data

Factor VIII recovery studies¹⁴ were performed on nine hemophiliac patients and showed a mean *in vivo* half-life of 11.1 hours and a mean *in vivo* recovery of 108 percent (range, 78–138%). There were no adverse clinical reactions to the concentrate.

The product has been used in hospital, clinic, and home therapy settings for the last 4 years. The concentrate has been successfully used in high doses for patients undergoing major surgery.

Discussion

Adsorption of a cryoprecipitate extract with CPG granules has proved to be a simple and useful technique for the preparation of a low-fibrinogen factor VIII concentrate. An average potency of 16 IU factor VIII per ml at a specific activity of 0.74 IU per mg protein and an overall yield of 194 IU factor VIII per kg of plasma compares favorably with reported values for factor VIII concentrates prepared by other methods.^{1–3,15}

The specific activity of the final factor VIII concentrate may be readily manipulated by changing the ratio of CPG granules to total cryoprecipitate protein. An increase in the ratio results in an increase in the specific activity of the final concentrate. Although specific activities of up to 1.9 IU per mg of protein were achieved in experimental trial batches, a considerable reduction in factor VIII stability and yield was observed in all preparations that contained more than 1.0 IU per mg of protein. This effect has not

been investigated further. An optimal balance of yield and purity was achieved when the specific activity of the final concentrate was 0.5 to 0.8 IU per mg of protein.

Incorporation of a mixture of synthetic amino acids with the buffer system used in the CPG process was based on the evidence that certain amino acids confer improved stability on the factor VIII molecule.¹⁶ Heat treatment of a CPG factor VIII concentrate prepared in the absence of amino acids result in greater than 25 percent loss of factor VIII activity and poor solubility of the heated lyophilized product.¹⁷ Synthamin 17 was chosen as a source of mixed amino acids because of its ready availability as a sterile, pyrogen-free solution suitable for intravenous use.

Current methods of cryoprecipitation result in the recovery of only about 44 percent of the plasma factor VIII. Some of the loss is due to the technical difficulties involved in manually cryoprecipitating a large number of plasma donations. It is apparent that this is the most operator-dependent step of the current technique, and a considerable improvement in final yield might be expected if a semiautomated, larger-scale cryoprecipitate batch-thawing process were adopted.¹⁸ Similarly, heparin-assisted cryoprecipitation⁵ could be considered as a means of improving the recovery of plasma factor VIII in cryoprecipitate. However, both these methods are not compatible with our laboratory facilities.

During the stages of processing, loss of factor VIII was most evident at the aluminum hydroxide adsorption, sterile filtration, and freeze-drying steps. Recent experiments have suggested that a 10-fold reduction in the amount of aluminum hydroxide added per gram of cryoprecipitate resulted in a 2 to 5 percent increase in the final yield of factor VIII, with no apparent adverse effects on the stability of the final concentrate. Mechanical losses during sterile filtration are largely unavoidable given the small total volume of the batch and the need for a rapid filtration technique.

The approximately 20 percent loss of factor VIII activity during the sterile filtration and freeze-drying steps was higher than the reported losses in similar steps of traditional factor VIII concentrate preparation methods.^{1–3} Manipulation of the CPG product temperature, ionic strength, pH, and protein concentration during processing was unsuccessful in improving the post-CPG adsorption recovery of factor VIII.

However, despite the lowered recovery of factor VIII in the steps following CPG adsorption, the final overall recovery of plasma factor VIII is acceptable in light of the advantages of the product, which include a low fibrinogen content, high factor VIII-specific activity, and excellent stability during heat treatment to inactivate the virus responsible for acquired immune

deficiency syndrome. Further advantages of the CPG method include a simple processing method, the potential for use of a small cryoprecipitate pool, and the excellent *in vivo* recovery and half-life.

The only major equipment required for the process not generally provided in a regional blood-processing laboratory are lyophilization facilities, a clean room, the CPG, and a suitable column. We used a custom-built acrylic plastic column primarily because it is significantly less expensive than the commercially available chromatography column systems. The current routine technique is not difficult for inexperienced staff to learn, but requires good aseptic technique and strict adherence to standard rules of good manufacturing practice.

In clinical use of the concentrate, there were no significant problems with reconstitution, administration, or side effects.¹⁴ The *in vivo* half-life of the factor VIII was 11.1 hours which is similar to that reported by Margolis et al.^{6,7} and comparable to values for other factor VIII concentrates.¹⁹ A wide variation of responses to infused CPG factor VIII was observed previously in the same patients who had widely varying responses to other factor VIII concentrates. To date, more than 1,500,000 IU of CPG factor VIII concentrate have been infused with no major clinical problems.

The CPG process has allowed the New Zealand Blood Transfusion Service to reduce its dependence on supplies of imported large-pool factor VIII concentrates.

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