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Severely Heated Therapeutic Factor VIII Concentrate of High Specific Activity

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Abstract. A new method for the manufacture of a heated factor VIII concentrate of high specific activity (2–6 IU factor VIII:C/mg protein) has been developed. Addition of heparin to cryoprecipitate extract at acid pH precipitated fibrinogen and fibronectin. Factor VIII was then recovered from the supernatant by precipitation with glycine and sodium chloride. After re-solution and desalting on Sephadex G-25, the concentrate was sterile-filtered and lyophilised. The dried product was stable to heating in the final container at 80°C for 72 h. Data from 25 consecutive batches of concentrate prepared from 1,200–1,500 kg plasma pools are presented. The mean final yield of heated product was 190 IU factor VIII:C/kg plasma. The concentrate has been found to be safe and effective in clinical use.

Introduction

One of the main hazards associated with the use of factor VIII concentrates prepared from large plasma pools is the high risk of transmission of blood-borne viruses. Studies have shown the high incidence of non-A, non-B hepatitis (NANBH) in patients receiving such concentrates for the first time [1, 2], and other reports document the transmission of hepatitis B [3] and HIV [4]. Heat treatment is an effective means of inactivation of many viruses and can be applied to the lyophilised product. However, experience with dry heated factor VIII concentrates suggests that transmission of NANBH is not eliminated by heating for 72 h at 60°C [5] and that, despite the fact that HIV has been shown to be rapidly inactivated at 60°C in some circumstances [6], patients receiving at least one factor VIII concentrate heated in the lyophilised state for 30 h at 60°C have seroconverted [7]. Thus, heating at higher temperatures or for longer periods may be required to inactivate NANBH and HIV viruses.

Heating of the Blood Products Laboratory's intermediate purity concentrates (<0.5 IU/mg [8] in the dried state at >70°C, resulted in substantial loss of factor VIII activity and unacceptable loss of solubility. This poor performance during severe heating may have been due to the presence of impurities, particularly to high concentrations of fibrinogen and fibronectin. We report here a method for substantial reduction of fibrinogen and fibronectin concentrations that allows preparation of a high-purity factor VIII concentrate in high yield. This paper describes the stability of this new concentrate to severe dry heating and the exploitation of the method for the manufacture of high purity, heat-treated factor VIII (Product Code 8Y) from 1,200–1,500 kg batches of human plasma. The initial evaluation of the safety and efficacy of the 8Y concentrate is also discussed.

Materials and Methods

Reagents

Sodium heparin with specific activity in the range 160–200 U/mg was obtained from three sources: Leo Pharmaceuticals, Ballerup, Denmark; Diosynth Inc., Chicago, Ill.; and Sigma London Chemical Co., Poole, Dorset.

'Glycine/NaCl' buffer was prepared by adding solid sodium chloride to a solution of 2.2 *M* glycine, 0.02 *M* sodium citrate, 2.4 m*M* calcium chloride, pH 7.0. Various percentages (w/v) of sodium chloride were used and the pH of the buffer readjusted to 7.0 after addition of the salt.

Buffer D was 0.10 *M* sodium chloride, 0.01 *M* Tris, 0.01 *M* sodium citrate, 1.2 m*M* calcium chloride, 1.5% (w/w) sucrose, pH 6.9.

Analytical Methods

Total protein was measured by a biuret method [9] calibrated with a human albumin standard. Clottable protein was assayed indirectly by

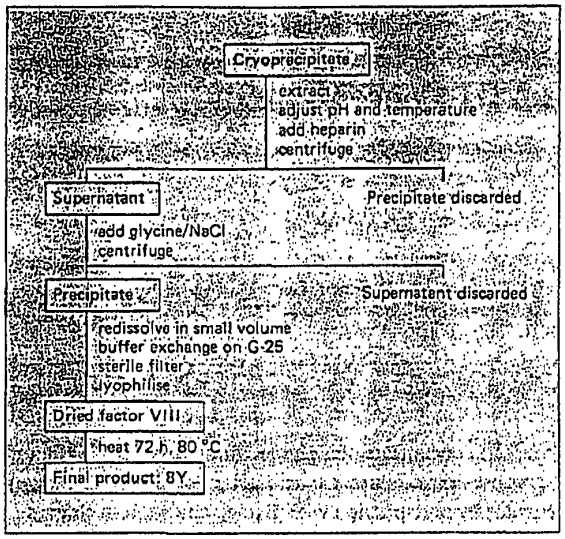

Fig. 1. 8Y processing scheme.

measuring the protein remaining in solution after the sample had been clotted by addition of thrombin. Sodium and potassium ion concentrations were measured by flame photometry; chloride [10], citrate [11], Tris [12] and glycine [13] levels were determined by colorimetric methods. Calcium ion concentration was determined with an ion-selective calcium electrode (Radiometer). Heparin was measured by an amidolytic method using chromogenic substrate S-2222 [14].

Factor VIII clotting activity was measured by a modification of the two-stage clotting assay of Biggs et al. [15]. The 4th British Working Standard (83/591) was used after calibration against the 3rd International Standard for factor VIII:C (80/556). Samples of cryoprecipitate extract and of supernatant after precipitation with heparin were absorbed with 0.02 vol aluminium hydroxide (moist gel; BDH, Poole, UK) before clotting assay, in order to eliminate interference from prothrombin complex or heparin. vWF:Ag was measured by Laurell immunoelectrophoresis using a 1% agarose gel prepared in 0.025 *M* Tris-tricine buffer, pH 8.6, containing 3% PEG 4000 and 1/2,000 sheep anti-vWF:Ag. Gels were run for 16 h at 2 V/cm. The working standard was an intermediate purity factor VIII concentrate which had been calibrated against the 1st British Standard for vWF:Ag (66/355). Fibronectin antigen and IgG were quantitated in a similar way using a 1/100 dilution of sheep anti-fibronectin or a 1/200 dilution of goat anti-IgG (Scottish Antibody Production Unit). The standard used was normal pooled plasma from 60 donors with an assigned value of 0.33 mg/ml fibronectin and 10.3 mg/ml IgG. IgM was determined by single radial immunodiffusion on Partigen plates (Behring). The standard was Standard Human Serum (Behring), 0.95 mg IgM/ml.

Anti-A haemagglutinin was measured as described by Bowell et al. [16], using a normal pooled serum standard with an assigned value of 100 U anti-A/ml.

A colorimetric Karl Fischer method [17] was used for residual water determination in the dried product before heat treatment.

Tests for pyrogens and abnormal toxicity were as prescribed in the European Pharmacopoeia using factor VIII:C doses of 20–25 IU/kg in rabbits, 40–50 IU intravenously in guinea pigs and 4–5 IU intraperitoneally in mice.

pH measurements were made at 20 °C except when titrating the Tris extract of cryoprecipitate at T >20 °C before precipitation with heparin. In these cases pH was measured using a pre-warmed electrode in a sample of the 25 or 30 °C solution but making no adjustment to the temperature compensation of the electrode which had been standardised at 20 °C.

Precipitation Experiments

Optimal conditions for the two precipitation stages were established in small-scale (1/1,000 batch scale) experiments using fractions taken from intermediate stages of production. Temperature, pH and the final concentrations of heparin and of sodium chloride were varied, and precise conditions are specified in the relevant figure legends.

Stability Studies

Vials were stored in the dark at temperatures ranging from −40 to 70 °C. At six-month intervals samples were assayed and factor VIII:C activity relative to the control sample stored at −40 °C determined. 28 batches have been entered in the study and 3 batches have completed 30 months.

Safety and Efficacy

In a preliminary study of efficacy, 17 patients at four centres received 21 infusions from 5 batches of concentrate. Doses of 28–48 IU/kg body weight were given, sufficient to raise the plasma factor VIII level to 25–100% of normal. Patients were attended by a physician for at least 1 h after infusion to observe any adverse reactions. Blood samples were taken for factor VIII determination pre-infusion; 10–30 min post-infusion and at intervals up to 24 h. Dose-response index (RI) was calculated as:

$$RI = \text{rise in plasma factor VIII, \% normal} \times \text{body weight, kg} \times (\text{IU factor VIII infused})^{-1}$$

For adults, 100% complete recovery corresponds to RI = 2.4 [18]. Half-disappearance time was estimated graphically from a plot of post-infusion factor VIII levels against time.

Production of 8Y Concentrate from 1,200–1,500 kg Plasma Batches

Cryoprecipitate Extraction. Fresh frozen plasma (in CPD or CPD-A anticoagulant) was crushed and thawed to 0–2 °C in a stirred and heated vessel and the suspension of cryoprecipitate was centrifuged in a cooled Sharples AS 16 centrifuge at flow rates of 1,300–1,500 ml/min. Recovered cryoprecipitate (≈10 g/kg plasma) was extracted with 0.02 *M* Tris buffer, pH 6.7, using 24 ml buffer per kg plasma. The extraction was performed at 20 °C using an immersed mixer/emulsifier (Silverson Machines Ltd., Chesham, UK). During the 10- to 20-min extraction, the pH was adjusted to 7.0 with 0.1 *M* HCl. The 'Tris extract' was then warmed to 25 °C and the pH adjusted to 6.55 ± 0.02 with further 0.1 *M* HCl.

The further processing of this extract, described in detail below, is illustrated schematically in figure 1.

Heparin Precipitation. Heparin solution (22 mg/ml) was added to the Tris extract with efficient mixing to give a final heparin concentra-

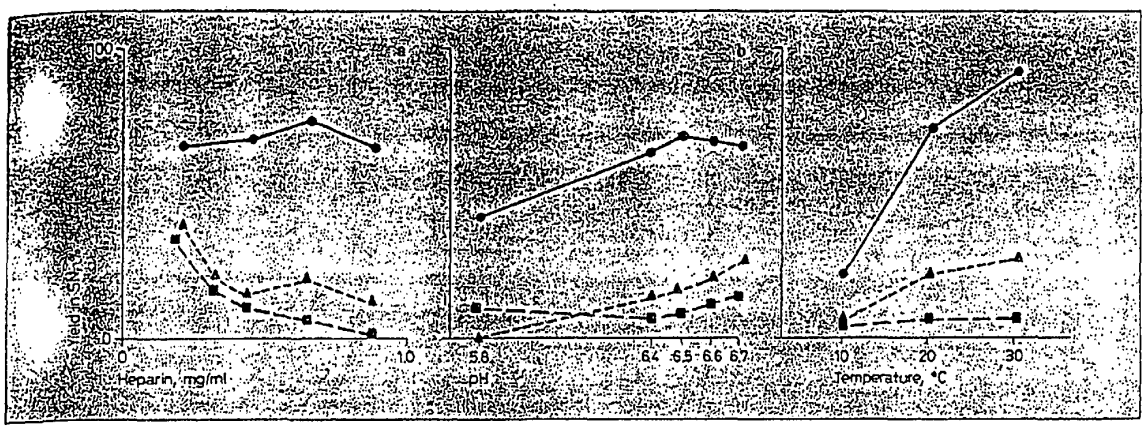

Fig. 2. Precipitation of proteins from cryoprecipitate extract using heparin. Percent recovery in the supernatant of factor VIII:C (●), fibrinogen (■) and fibronectin (▲) plotted as a function of (a) heparin concentration (at 25 °C, pH 6.55), (b) pH (0.44 mg/ml heparin, 25 °C) and (c) temperature (pH 6.55, 0.44 mg/ml heparin). Data plotted in each case are from a single experiment. SN = Supernatant.

tion of 0.88 mg/ml. The heavy precipitate was removed by centrifugation at 4,600 g, 10 min, 25 °C in Mistral 6L centrifuges (MSE, Crawley, UK).

Precipitation of factor VIII. A weight of 'glycine/NaCl' buffer (see Methods; 21% NaCl; 30°C) equal to 2.05 times the weight of the hep.. in supernatant was then added, and after stirring at 30 °C for 10 min the fine precipitate was collected by centrifugation in a cooled Sharples (30 °C) centrifuge at a flow rate of 500 ml/min. The precipitate (≈0.6 g/kg plasma) was dissolved by stirring at 30 °C in a volume of buffer D equivalent to 8% of the Tris extract volume.

Removal of Salt. Sephadex G-25 was packed in a glass-polypropylene-stainless steel chromatography column, 180 mm diameter, and equilibrated in buffer D. At a bed height of 28–30 cm a flow rate of 500 ml/min was used. The redissolved precipitate (0.1–0.2 bed volume) was loaded and the column developed with the same buffer. The absorbance of the effluent at 280 nm was monitored. Peak collection was started at the first rise in absorbance and continued until the absorbance returned to the baseline or twice the loaded sample weight had been collected, whichever happened first.

Finishing. Immediately after desalting, the solution was prefiltered through a sandwich of glass fibre discs (AP 25, AP 20, AP 15, Millipore) prior to sterilising filtration through an autoclaved 10-inch nylon cartridge 0.2 µm pore size (NRP, Pall Corp.). 10-ml doses were aseptically dispensed into 50 ml neutral glass vials, and lyophilisation was performed in a modified Lyomax 2 freeze-dryer (Edwards High Vacuum, Crawley, UK). Vials were loaded at 20 °C in direct contact with the shelves, which were then cooled rapidly to −50 °C. After 10 h at −50 °C, the chamber was evacuated and freeze-drying initiated by heating the shelves. Chamber pressure was controlled at 0.3 m bar during primary sublimation with an air bleed directly into the pumping set. A shelf temperature of 0 °C held the product at <−32 °C during sublimation. After 8 h from the start of freeze-drying, shelves were warmed (2 °C/h) to a final temperature of 25 °C for 30 h. Chamber pressure was decreased to 0.05 mbar for the final 10 h. Vials were closed under vacuum with 20-mm stoppers and overseated immediately. Sealed vials were heated in an oven at 80 °C for 72 h. The product was redissolved in the original dispensed volume before control analyses or clinical use.

Results

Heparin Precipitation

The extent of precipitation of fibrinogen and fibronectin and the recovery of factor VIII in the supernatant were dependent upon pH, temperature and the final concentration of added heparin (fig. 2) and heparin from all three sources used gave the same results. Optimal conditions for our cryoprecipitate extract (total protein 45.0±6.3 mg/ml: 66±4% clottable protein, n = 25) were found to be 0.88 mg/ml heparin, pH 6.5, 25 °C. At higher pH values, less fibrinogen and fibronectin were removed, whereas there was loss of factor VIII below pH 6.5. Temperatures <20 °C led to co-precipitation of factor VIII with fibrinogen and fibronectin.

Glycine/NaCl Precipitation

Careful adjustment of the precipitating conditions at this stage considerably reduced the amount of fibronectin co-precipitated with factor VIII. At temperatures of 25–30 °C, 75% of the factor VIII was precipitated while 90% of the fibronectin remained in the supernatant (fig. 3). At manufacturing scale, a cooled Sharples centrifuge was used for collection of this precipitate, but by keeping the suspension at 30 °C while it was pumped into the centrifuge, effluent temperatures remained above 16 °C and good separation

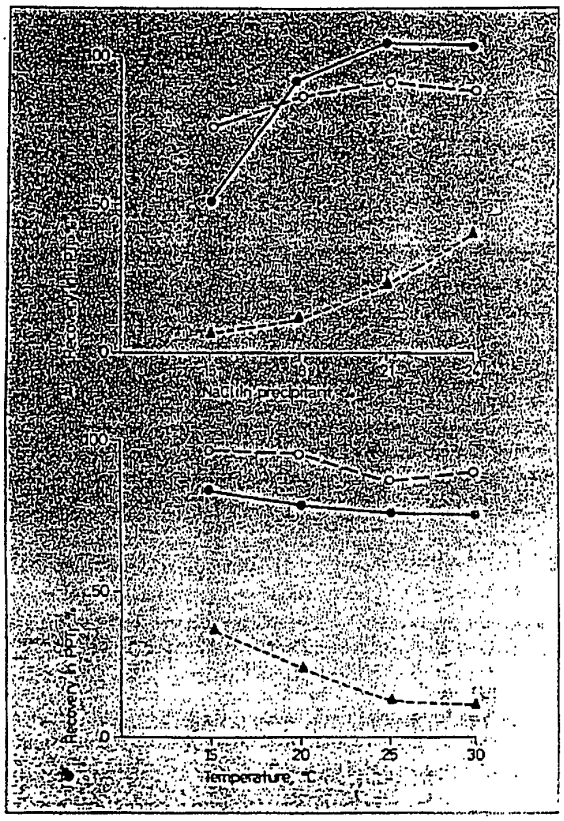

Fig. 3. Precipitation of factor VIII with 'glycine saline' buffer. The percent recovery in the redissolved precipitate of factor VIII:C (●), fibrinogen (○) and fibronectin (▲) is plotted as a function of (a) % NaCl (w/v) in the added 'glycine saline' buffer, all at 20°C, and (b) the temperature of the precipitation, all with 21% (w/v) NaCl in the added 'glycine saline' buffer. Data plotted in each case are from a single experiment. PPT = Precipitate.

was achieved. The concentration of sodium chloride in the added 'glycine/NaCl' buffer was critical; at 21% (w/v) (final 2.36 *M* sodium chloride during precipitation) complete precipitation of factor VIII was achieved and most of the fibronectin remained in solution (fig. 3a).

Heat Treatment

Recovery of factor VIII activity after heating in the lyophilised state for various times at 60–90°C is shown in figure 4. There was no change in solubility after heat treatment at 60–80°C, but after heating at 90°C samples were slower to redissolve and contained small amounts of insoluble material.

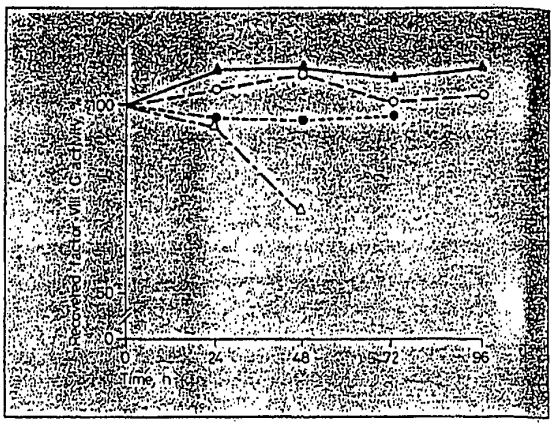

Fig. 4. Heat treatment of lyophilised 8Y concentrate. Six batches of concentrate were heated for 24–96 h at 60–90°C. Mean recoveries are expressed as a percent of the activity of an unheated control sample. ▲ = 60°C, ○ = 70°C; ● = 80°C; △ = 90°C.

8Y Production

Mean processing data for 25 consecutive plasma batches (1,200–1,500 kg) of 8Y are summarised in table 1. The loss of factor VIII during heparin precipitation was accounted for by occlusion in the massive fibrinogen precipitate. A 4-fold purification was achieved by precipitation with glycine/NaCl with a mean stage yield of 87%. The factor VIII precipitate was always easily and completely redissolved at potencies of 70–90 IU/ml. The factor VIII solution was diluted 2-fold during Sephadex G-25 desalting with nearly quantitative recoveries. Finishing processes did not cause unexpected losses. The inclusion of sucrose in the final buffer improved solubility after lyophilisation. The solubility of the dried concentrate was rapid and complete even after the final heat treatment.

Table 2 summarises final product control data for the 25 batches in table 1. In addition, all batches passed European Pharmacopoeial tests for sterility, abnormal toxicity and pyrogen. Twelve batches were subjected to complete analytical and animal testing both before and after heat treatment at 80°C for 72 h. The only significant change after heating was a mean loss of 9% factor VIII activity. Manufacture has subsequently been scaled up to 3,300 kg plasma batches; process stage yields of factor VIII and final product specific activity are comparable to those reported here for 1,200–1,500 kg batches.

Stability Studies

Interim results of ongoing stability studies of the heat-treated concentrate showed no loss of factor VIII:C activity

Table 1. Processing data and mean stage yields (±SD) for 25 batches of 8Y (1,200–1,500 kg plasma pools)

	Recovery of factor VIII: C iu/kg plasma	Potency factor VIII: C iu/ml	Specific activity iu/mg protein	Recovery of fibrinogen mg/kg plasma	Recovery of fibronectin mg/kg plasma[1]
Tris extract	364±53	10.7±1.6	0.23±0.04	1,036±99	271
Heparin supernatant	298±43	10.5±1.5	1.01±0.19	42.6±11.5	49.3
Redissolved glycine-saline precipitate	258±23	79.9±6.8	4.08±1.6	45.8±23	5.7
G-25 eluate	251±25	39.3±4.0	4.31±1.1	40.8±8.5	6.7
Sterile filtrate	237±22	29.4±2.5			
Freeze-dried product	202±23	24.9±3.3	4.3±0.8		
Heated product	190±24	23.5±3.1	4.0±0.6		

[1] n = 3.

30 months at 4 °C or at 20 °C, but further data must be accumulated before the expiry date can be extended beyond 2 months.

Safety and Efficacy

No patient in this preliminary study suffered any immediate or delayed side effect from an 8Y infusion. In the one instance where a bleeding episode was being treated, effective haemostasis was achieved. Mean RI was 1.8 (SD±0.5; n = 21; range 1.1–2.9). This corresponds approximately to 74±12% of theoretical recovery [19], assuming a normal value for haematocrit, which was not measured. The mean half-disappearance time was 10.3 h (SD± 3.8 h; n = 17; range 7–24 h). In 2 cases where data were sufficiently precise to distinguish two phases of exponential decay, the first phase (first 6 h) had half-lives of 4 and 6 h, and the second phase (6–24 h after injection) had half-lives of 11 and 9.5 h, respectively.

Table 2. Properties of 25 batches of 8Y concentrate manufactured from 1,200–1,500 kg plasma

	Mean	SD	Range
Factor VIII: C, iu/ml	23.5	3.1	17.3–31.9
Total protein, mg/ml	6.0	1.0	4.0–7.7
Clottable protein, %	77	4	67–82
vWF: Ag, u/ml	62.3	9.8	41.3–81.6
Fibronectin, mg/ml	0.31	0.09	0.20–0.60
IgG, mg/ml[1]	0.09	0.03	0.03–0.14
IgM, mg/ml[1]	0.06	0.02	0.03–0.10
Specific activity, iu/mg	4.0	0.6	2.9–5.3
Solubility time 20 °C, min	3.2	1.1	1.5–6.5
Anti-A, u/ml	165	31	81–212
Heparin, u/ml	0.23[2]	0.04[2]	<0.2–0.37
Calcium, vM	37.2	7.4	26–51
Tris	9.6	0.7	8.3–11.1
Sodium, mM	127	3.5	119–132
Chloride, mM	104	3.9	95–111
Citrate, mM	9.5	0.5	8.3–10.5
pH	6.93	0.04	6.84–7.02
Conductivity, mS	10.8	0.4	10.2–11.4
Residual water, % (w/w)	1.9	0.6	0.9–3.3

[1] Not routinely measured: n = 9 for IgG and IgM.

[2] In 13 of 25 batches, heparin was undetectable (<0.2 u/ml). For calculation of mean and SD, 0.2 u/ml was used for these batches.

Discussion

The key step in this new manufacturing process is the use of heparin at temperatures above ambient to precipitate fibrinogen and fibronectin. These two proteins are the main constituents of cryoprecipitate and substantial reduction in their concentrations is an essential part of any high-purity factor VIII preparation. Previous methods of separation have included cold precipitation [8], PEG precipitation [20], ethanol fractionation [21], glycine fractionation [22] and zinc precipitation [23]. Heparin has been used previously [24, 25] but in conjunction with cooling and at heparin concentrations lower than those which we have found optimal. For most of these methods the conditions required for maximal fibrinogen precipitation cause significant coprecipitation of factor VIII. Moreover, many are difficult to accommodate consistently in an industrial manufacturing process where many factors contribute to considerable variation in the composition of the cryoprecipitate extract. Heparin precipitation was largely independent of variable Tris extract composition because at temperatures warmer than 20 °C a wide range of heparin concentrations effectively reduced the concentrations of

fibrinogen and fibronectin to levels necessary for the solubility of the final product without causing coprecipitation of factor VIII. Although 0.44 mg/ml heparin was sufficient to precipitate more than 90% of fibrinogen in small-scale experiments (fig. 2a), 0.88 mg/ml was adopted for manufacturing to ensure maximal removal even from batches with above-average fibrinogen content. A particular advantage was that the separation could be carried out even on very concentrated Tris extract solutions (40–50 mg protein/ml), allowing large-scale processing volumes to be kept to a minimum. Careful adjustment of pH and temperature within narrow limits (pH 6.55±0.03; temperature 25±1°C) was essential to achieve maximum precipitation of fibrinogen while maintaining a high yield of factor VIII.

The second stage of the process is precipitation of the factor VIII, which removes heparin and allows re-solution at high potency. The use of sodium chloride and glycine as a factor VIII precipitant has been described, and others have also observed the importance of temperature and sodium chloride concentration on the separation of factor VIII from fibronectin [26]. Optimisation of the temperature and salt concentration of this precipitation allowed complete precipitation of factor VIII with 90% of the fibronectin remaining in solution, leading to a marked improvement in the solubility of the heated product.

The overall yield of 190 IU factor VIII:C/kg plasma (52% of the factor VIII:C in the cryoprecipitate) is very satisfactory for a severely heated, high-purity product and compares favourably with the yield previously obtained in these laboratories for an intermediate purity concentrate heated at 70°C for 24 h.

The 8Y concentrate is 5–10 times purified over most intermediate purity concentrates. This improved purity has afforded the significant benefits of increased potency and better solubility, both of which are important to manufacturer and clinician. More than 95% of the IgG and IgM are removed with fibronectin in the glycine/NaCl supernatant and the concentrations of these proteins in the final product (<0.1 mg/ml) are well below those found in intermediate purity concentrates [27]. Reduction of IgG and IgM has not resulted in decreased anti-A, the concentration of which remains similar to what it was in our earlier intermediate purity concentrate (unpubl. results).

The ability of the 8Y concentrate to withstand very severe heating in the dried state is probably a result of increased purity. Under milder heating conditions (60°C, 72 h), our dried intermediate purity factor VIII concentrates suffered loss of activity (5–35%) and solubility, pH was altered, and PKA levels rose two-fold [28]. A study of the effects of heat treatment on the 8Y concentrate has been published [29]; the only detected change after heating at 80°C for 72 h was a mean loss of 9% factor VIII activity.

Despite careful definition and control of the lyophilisation process, some variation between batches has been observed with respect to residual water content (RWC). Results of our investigations of some of the factors affecting RWC are published elsewhere [29] and suggest that much of the observed variation in RWC is due to variation in protein concentration between batches. Both the survival of factor VIII activity during heat treatment and the solubility of the concentrate are affected by RWC, with effects becoming particularly pronounced when RWC >4% [29]. Inactivation of viruses may also depend upon RWC [30]. Studies are currently in progress to assess the effects of moisture variation on virus inactivation but results from the clinical trial [31] of randomly selected batches with a range of RWC (0.13–2.10%, n = 7) suggest that inactivation of HIV-1 and NANBH at 80°C may be effective over a wide range of RWC.

The heat treatment does not appear to have had any effect on the RI or on the half-disappearance time after infusion. Although both of these parameters showed wide variation, values are compatible with recent literature values [18, 19, 32, 33]. These preliminary assessments of effectiveness should ideally have been carried out in cross-over studies with e.g. the unheated concentrate. This was not considered ethically admissible since there was no suitable 'virus-safe' concentrate available at the time. Preliminary results suggest that, despite increased purity, high molecular weight vWF multimers are present [F. G. Hill, pers. commun.] and that the concentrate is effective therapeutically in patients with von Willebrand's disease [C. R. Rizza, pers. commun.].

The 8Y concentrate has now been in use since 1984. Results of surveillance for NANBH in 33 susceptible patients showed no transmission of NANBH and are reported in detail elsewhere [31]. Recent observations indicate that the heat treatment may also inactivate human parvovirus which has been reported to contaminate some plasma pools [34].

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