Development of a Freeze-Dried Albumin-Free Formulation of Recombinant Factor VIII SQ

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Purpose. To develop a stable freeze-dried formulation of recombinant factor VIII-SQ (r-VIII SQ) without the addition of albumin.

Methods. Different formulations were evaluated for their protective effect during sterile filtration, freeze-thawing, freeze-drying, reconstitution and long term storage. Factor VIII activity (VIII:C), visual inspection, clarity, solubility, moisture content and soluble aggregates and/or fragments were assayed.

Results. A combination of non-crystallising excipients (L-histidine and sucrose), a non-ionic surfactant (polysorbate 80) and a crystalline bulking agent (sodium chloride) was found to preserve the factor VIII activity during formulation, freeze-drying and storage. Calcium chloride was included to prevent dissociation of the heavy and light chains of r-VIII SQ. Sodium chloride was chosen as the primary bulking agent since the concentration of sodium chloride necessary for dissolution of r-VIII SQ in the buffer will inhibit the crystallization of many potential cake formers. It was found that L-histidine, besides functioning as a buffer, also protected r-VIII SQ during freeze-drying and storage. A pH close to 7 was found to be optimal. Some potential macromolecular stabilisers, PEG 4000, Haes®-steril and Haemaccel®, were evaluated but they did not improve the recovery of VIII:C. The freeze-dried formulation was stable for at least two years at 7°C and for at least one year at 25°C. The reconstituted solution was stable for at least 100 hours at 25°C.

Conclusions. The albumin-free formulation resulted in consistently high recovery of VIII:C, very low aggregate formation and good storage stability. The stability of the reconstituted solution makes the formulation suitable for continuous administration via infusion pump. The formulation strategy described here may also be useful for other proteins which require a high ionic strength.

KEY WORDS: freeze-dried; protein stability; factor VIII; formulation; surface adsorption.

INTRODUCTION

Patients with haemophilia A have been treated with factor VIII concentrates derived from human plasma since the 1960s. Although the use of plasma-derived factor VIII can be considered safe and efficient, the limited access to plasma and concern over the possible transmission of viral diseases has lead to the development of recombinant factor VIII products. Recombinant factor VIII SQ (r-VIII SQ) is a derivative of human factor VIII in which the B-domain has been deleted. It corresponds to the smallest active form of factor VIII, an 80 + 90 kDa heterodimer linked by a metal ion, present in therapeutic factor VIII concentrates (1) (Figure 1). This product is currently undergoing phase III clinical evaluation.

In circulating blood and in plasma-derived factor VIII concentrates, factor VIII is stabilised by association with a

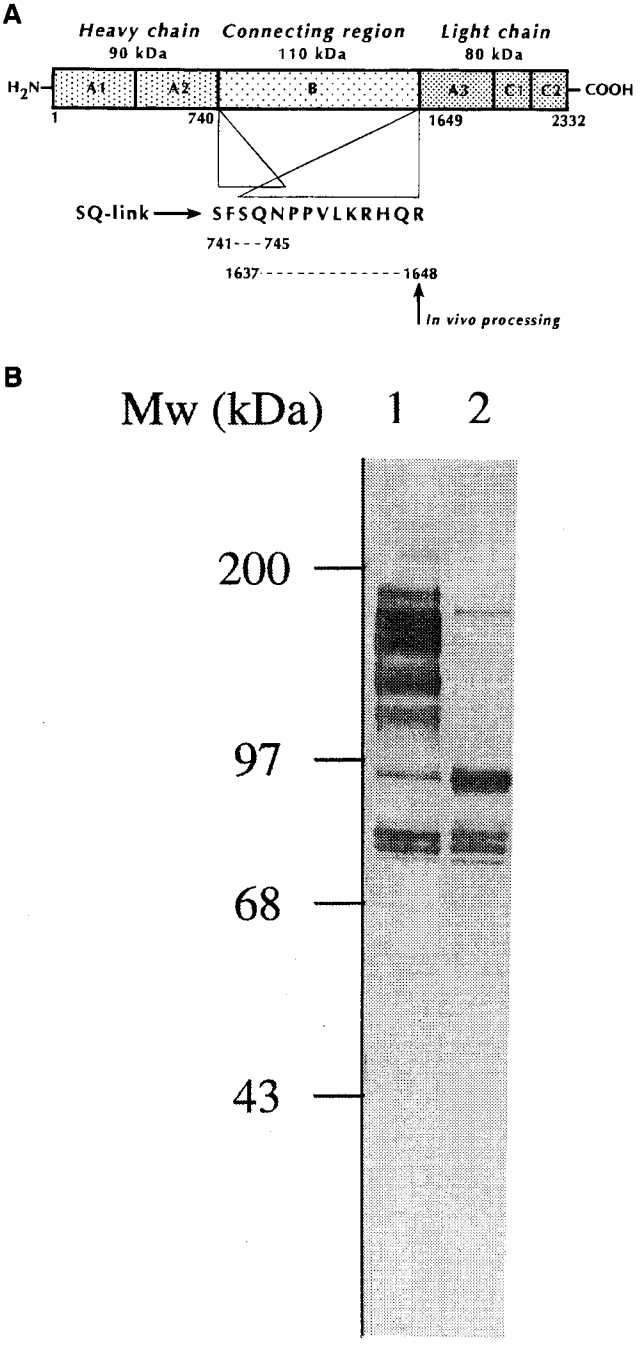

Fig. 1. (A) The relationship between recombinant factor VIII SQ (r-VIII SQ) and full-length factor VIII, (B) Western Blot profile of plasma-derived factor VIII (lane 1) and r-VIII SQ (lane 2) using anti-factor VIII polyclonal antibodies (rabbit) raised against the high molecular forms of plasma-derived factor VIII.

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carrier protein, the von Willebrand factor (vWF). In the absence of vWF, as in recombinant factor VIII products, the pure factor VIII is extremely labile. In addition, since the protein concentration after reconstitution is very low (0.02 mg protein per ml for a therapeutic dose of 1000 IU r-VIII SQ), surface adsorption becomes a major cause of loss of activity.

Surface adsorption during purification and formulation is usually prevented by adding albumin (human), while the product is stabilised by freeze drying. Albumin also functions as a stabiliser and bulking agent (cake former) in the freeze-dried cake. However, in view of the risk of viral transmission, the addition of a human-derived protein to a therapeutic protein produced by recombinant DNA technology is unattractive. The amount of albumin used to stabilise recombinant factor VIII preparations is at least 500 times the amount of factor VIII on a mass to mass basis. This means that even if the added albumin has a purity of 99%, the amount of impurities in the albumin is still higher than the amount of factor VIII itself. Furthermore, if albumin is omitted from the formulation, it becomes possible to use many powerful and sensitive analytical methods for protein characterization and quality control of the finished product. The above reasons led us to develop a freeze-dried formulation of r-VIII SQ without albumin. In this report we describe our investigations into the effects of selected excipients and the rationale for the albumin-free formulation.

MATERIALS AND METHODS

Materials

Recombinant factor VIII SQ was manufactured at Pharmacia & Upjohn AB, Sweden. Briefly, Chinese Hamster Ovary cells harbouring a modified cDNA (Glu-744 to Ser-1637 were removed), were cultivated in serum-free medium at finite passage level. The primary translation product, a 170 kDa single chain protein, was cleaved intracellularly to the desired 80 and 90 kDa heterodimer. The purification process comprised of several chromatographic steps using ion exchange, immunoaffinity, hydrophobic interaction and molecular size exclusion as separation principles. After the final purification step, the column was equilibrated and eluted with a buffer containing sodium chloride (18 mg/ml), calcium chloride dihydrate (0.5 mg/ml), L-histidine (3 mg/ml) and polysorbate 80 (0.2 mg/ml). Polysorbate 80 was omitted for selected experiments. The gel filtration eluate (hereafter denoted drug substance) was dispensed in plastic containers and used directly or stored frozen at −70°C.

Plasma volume expanders, Haemaccel® (Hoechst, Sweden), Haes®-steril (Meda, Sweden), and Human Serum Albumin (Pharmacia & Upjohn AB, Sweden) were used as received. All other chemicals were either of Ph. Eur. or USP quality. Polysorbate 80 (Tween 80® ICI, Germany) was also analysed for peroxides and aldehydes. The water used was water for injection (Pharmacia & Upjohn AB, Sweden).

Methods

Formulations containing the various excipients and the desired factor VIII activity were manufactured by mixing the drug substance with the appropriate buffers. Solid sodium chloride was added directly to the solution for some formulations. The pH was adjusted with sodium hydroxide (1 M) or hydrochloric acid (5M). The solutions were sterile filtrated (Millex®-GV 0.22 μm, Millipore, Sweden) and about 2 ml was dispensed in 10 ml moulded glass vials (Type I glass). Grey bromobutyl rubber stoppers (FM 157) were obtained from Helvoet Pharma (Belgium). Stoppers were washed, siliconized, sterilised and dried to obtain a low moisture content. Freeze-drying was carried out in a pilot freeze-drier (Edwards, Kniese & Co, Germany) with about 1 m^2 useable shelf area and 28 kg ice condensor capacity. The vials were placed on the shelves at room temperature and subsequently equilibrated at 0°C for about 1 hour. The shelves were then cooled to −60°C and held at that temperature for at least one hour. Primary drying was carried out at a product temperature of about −38°C, and secondary drying at about 35°C. Completion of the primary and secondary drying was determined by a pressure increase test. The vials were stoppered under vacuum in the freeze-drier and stored in controlled incubators for stability evaluation. Samples with nitrogen, air or oxygen in the head space were also investigated. Freeze-dried preparations were reconstituted with 4 ml sterile water for injection or with sodium chloride injection (Pharmacia & Upjohn AB).

Analytical Methods

Moisture content was determined by a coulometric Karl Fisher assay. Factor VIII activity (VIII:C) was assayed with a chromogenic substrate method (Coatest® Factor VIII, Chromogenix AB, Sweden). All samples were prediluted in factor VIII-deficient plasma. The VIII:C is expressed as international units (IU) as defined by the international concentrate standard. The relative standard deviation of the assay was about 7% for formulation samples. The specific activity was calculated as VIII:C in IU per mg protein. The protein concentration was determined by measuring absorbance at 280 nm. Visual inspection was performed in translucent light against a black background. Colour, particles, precipitation and opalescence were noted. Clarity was determined by the Ph. Eur., 2nd Ed. method (V.6.1), but the actual package was used instead of the test tubes prescribed by the pharmacopoeia. Soluble aggregates and/or fragments were determined by gel filtration on an HPLC (Hewlett Packard 1090 M, USA). A prepacked Superdex 200 HR/10/30 column (Pharmacia & Upjohn AB, Sweden) was used with a fluorescence detector (Hewlett Packard 1046 A or a Jasco 821-FP, Japan), excitation wavelength 280 nm and emission wavelength 340 nm. Chromatograms from gel filtration were evaluated by visual inspection or by integration of the peak areas if aggregates or fragments were found. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions using 7.5% slab gels. Protein bands were visualised by silver staining and samples were evaluated by comparing with a standard. Western Blot analysis was performed using anti-factor VIII polyclonal antibodies (rabbit) raised against the high molecular forms of plasma-derived factor VIII (1).

RESULTS AND DISCUSSION

The physicochemical basis for the freeze-drying process and the development of freeze-dried formulations in general have been described by MacKenzie (2,3). Freeze-drying of

biological materials in particular has been described by MacKenzie (4) and by Pikal (5, 6). Factor VIII preparations for therapeutic use, both plasma-derived and recombinant, are generally freeze-dried, and albumin is added as a stabiliser. Albumin fulfils at least three functions in this respect: (a) it acts as a bulking agent; (b) it reduces non-specific surface adsorption; and (c) it stabilises the therapeutic protein during freezing (cryoprotection) and during freeze-drying (lyoprotection). A formulation without albumin must therefore contain excipients which can fulfil all these functions.

Bulking Agent

Bulking agents such as mannitol or glycine crystallise during freezing or freeze-drying to give mechanical support to the amorphous phase containing the protein drug. Macromolecules such as albumin also function as bulking agents but in a different fashion, since they stay amorphous. Sodium chloride was chosen as the sole bulking agent in the formulation under discussion since the concentration of sodium chloride required to render r-VIII SQ soluble will actually inhibit the crystallisation of bulking agents such as mannitol or glycine. The solubility of r-VIII SQ is increased by sodium chloride and a concentration of about 6 mg/ml is necessary to dissolve moderate concentrations of r-VIII SQ in the formulation buffer. Higher concentrations of r-VIII SQ, above 1500 IU/ml, require about 18 mg/ml of sodium chloride (data not shown). A sodium chloride concentration of about 15 mg/ml produces a good freeze-dried cake. However, the use of sodium chloride as bulking agent is only successful if it crystallises out during freezing. The physico-chemical principles for crystallization of sodium chloride during freeze-drying have been described by MacKenzie (3, 7). If the content of sodium chloride is not higher than that of other excipients in the formulation, its crystallization will be retarded. A mass ratio of sodium chloride to the sum of other excipients of 2:1 was used in the final formulation of r-VIII SQ.

Physico-Chemical Characterisation

One drawback of sodium chloride as bulking agent is its rather low eutectic temperature (−21.5°C) compared to mannitol (−1°C) and glycine (−3.5°C). The eutectic temperature of the formulation determines the highest product temperature that can be used during primary drying. However, in many cases it is the amorphous phase (or phases) of the formulation that governs the maximum allowable product temperature during both primary and secondary drying.

Formulations were investigated by differential thermal analysis with simultaneous measurement of electrical resistance and with freeze-dry microscopy as described by MacKenzie (2, 8). In brief, the thermograms from the final formulation showed a glass transition (T_g') beginning at −42°C and ending at −38°C. Freeze-dry microscopy revealed freeze-drying with retention of structure up to −35°C; collapse was noted above −30°C. Based on these data, freeze-drying should preferably be carried out at a product temperature below −32°C.

Non-Specific Surface Adsorption and Macromolecular Stabilisers

Many macromolecules are known to stabilise therapeutic proteins during manufacturing. However, it is preferable to avoid macromolecular stabilisers because of the increased risk of immunogenic adverse effects, especially in drug formulations intended for lifelong treatment. Macromolecules with low potential for adverse effects, such as Haemaccel® and Haes®-steril, are clinically used as plasma volume expanders. Haemaccel® contains polypeptides (mean molecular mass about 30 kDa) derived from bovine gelatine by thermal hydrolysis and crosslinked with hexamethylene diisocyanate. Haes®-steril contains poly (O-2 hydroxyethyl) starch (mean molecular mass about 20 kDa). Macromolecules such as Haemaccel® and Haes®-steril can act as amorphous bulking agents and they also increase the collapse temperature of the formulation. They may also reduce surface adsorption and act as lyoprotectors. Polyethylene glycol 4000 (PEG 4000) was also studied since it is often used as a stabiliser in many freeze-dried protein preparations (9). When albumin, Haemaccel® and Haes®-steril formulations were prepared, the content of sodium chloride was increased from 18 mg/ml to 30 mg/ml in order to keep the same 2:1 mass ratio between sodium chloride and the amorphous excipients. Albumin, Haemaccel®, Haes®-steril (10 mg/ml) or PEG 4000 (2 mg/ml) were added to the drug substance in buffer without polysorbate 80. Non-ionic surfactants, e.g. polysorbate 80, are sometimes used to stabilise protein drugs and to reduce surface adsorption/denaturation problems (10). The effects of a surfactant on the stability of a protein are complex and require individual evaluation.

The effects of the stabilisers on the recovery of VIII:C during sterile filtration, five cycles of freeze-thawing and freeze-drying and the stability of VIII:C in reconstituted solution were studied. The different formulations freeze-dried with good cake structures and dissolved easily. Formulations with polysorbate 80, albumin and Haemaccel® gave clear solutions upon reconstitution, but formulations with PEG 4000 or Haes®-steril and those without stabiliser were opalescent even after freeze-thawing. Figure 2A shows VIII:C before and after sterile filtration, after five cycles of freeze-thawing and after freeze-drying. The formulations with polysorbate 80 or albumin protected the VIII:C to an equally high standard. The VIII:C in the formulation without stabiliser was low even before sterile filtration and was considerably decreased during sterile filtration and freeze-thawing. It is interesting to note that no further activity was lost in the freeze-drying step. The formulation containing PEG 4000 had low VIII:C values before sterile filtration but these remained essentially unchanged after sterile filtration. PEG 4000 did not stabilise VIII:C during repeated freeze-thawing, but freeze-drying was performed without further loss as occured in the formulation without stabiliser. It appears that PEG 4000 protects the protein against surface adsorption during sterile filtration, but otherwise has few stabilising effects. It has recently been shown that, while PEG 4000 is an effective cryoprotectant, it provides little or no stabilisation during dehydration; however, this is not applicable to r-VIII SQ (11). The formulation with Haemaccel® enabled good recovery of VIII:C over sterile filtration and freeze-thawing but poor recovery after freeze-drying.

Buffer and pH

The buffer must contain a divalent metal ion, e.g. calcium, since the two protein chains in r-VIII SQ are held together by a metal ion bridge. The concentration of calcium chloride

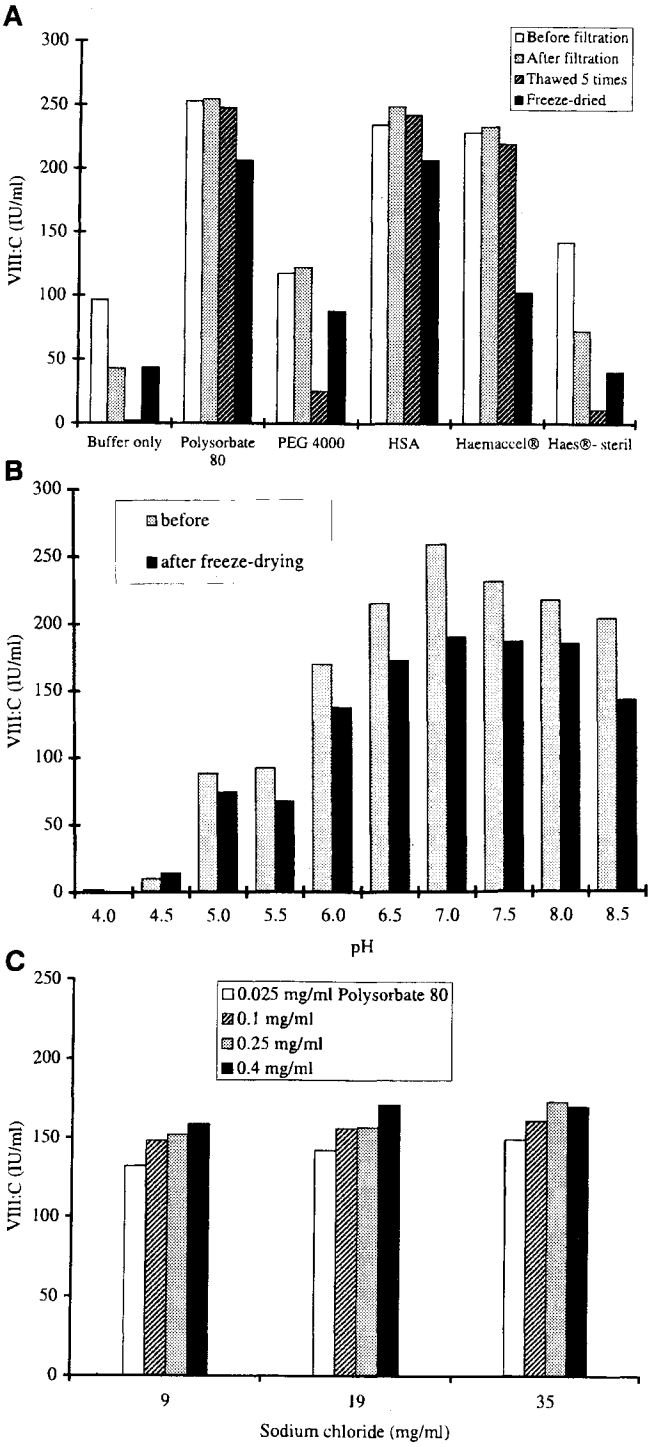

Fig. 2. (A) Factor VIII activity (VIII:C) before and after sterile filtration, freeze-thawing and freeze-drying, (B) VIII:C before and after freeze-drying at different pH, (C) VIII:C after freeze-drying at different concentrations of sodium chloride and polysorbate 80.

dihydrate was set to 0.25 mg/ml in the final formulation, since an increased concentration did not improve the stability of VIII:C (data not shown). The amount of calcium should be kept as low as possible since it decreases the T_g' of the amorphous phase. The presence of calcium ions restricts the choice of buffer substances to those that do not precipitate or form strong complexes with calcium ions. Amino acids such as histidine, lysine and arginine are conceivable buffers. L-Histidine was chosen for its buffering capacity around pH 7.

It is important to take in consideration the stability of the inactive ingredients at the selected pH. For example, it is known that acids or bases can catalyse the hydrolysis of sucrose (12). Buffer ingredients may also crystallise during freezing, causing pH shifts. Pure L-histidine does not crystallise around pH 6, which coincides with the pK of the weakly basic imidazolium function (pK = 6.0) (13). The tendency to crystallise increases as the pH moves away from the pK but the other excipients in the formulation will retard or inhibit possible crystallisation. The various formulations were freeze-dried from solutions in the pH range 4.0–8.0 and stored at 7, 25 and 37°C for up to 12 months. A pH of 6.5 to 7.5 was found to be optimum for the preservation of VIII:C (Figure 2B). Gel filtration showed no aggregation for any formulation in this pH range. It is noteworthy that the storage stability was unaffected between pH 6 and 8 (data not shown).

Cryoprotectors and Lyoprotectors

Proteins are generally stabilised and protected by excipients that stay amorphous during freezing and freeze-drying (5,14). A wide variety of excipients, including sugars, polyols, amino acids and certain salts, are effective in minimising protein denaturation during freezing and/or freeze-drying. The mechanisms by which solutes stabilise proteins during freeze-thawing and in solution seem to be similar and are described by the theory of preferential exclusion. The excipient is preferentially excluded from the protein and since the native protein conformation usually presents less surface area to the solution it is thermodynamically preferred over the denatured state (15). The means by which carbohydrates stabilise dried proteins is different from that noted in the liquid or frozen state (16,17). Carbohydrates can serve as "water substitutes", when the hydration shell of the protein is removed. However, it is likely that some proteins require a combination of these mechanisms for long-term storage stability.

Formulations with L-histidine as a lyoprotector showed good recovery of VIII:C after freeze-drying and good storage stability. L-Lysine, another basic amino acid, was therefore investigated, but the recovery and stability of VIII:C in the L-lysine formulation was considerably less than in the sucrose or L-histidine formulations (data not shown). A combination of L-histidine and sucrose was chosen as lyoprotector in the final formulation. The content of L-histidine and sucrose should be in excess compared with the content of protein (1000 IU corresponds to approximately 0.07 mg protein). Increasing the content of the amorphous phase makes the formulation more robust against possible moisture absorption from the stopper.

Optimisation of the Sodium Chloride and Polysorbate 80 Levels

Results of formulations that included sodium chloride as bulking agent and polysorbate 80 and non-crystallising excipients (L-histidine and/or sucrose) as stabilisers led to more detailed studies on the possible optimum concentrations of sodium chloride and polysorbate 80. Formulations with various concentrations of sodium chloride (9, 19 and 35 mg/ml), poly-

sorbate 80 (0.025, 0.1, 0.25 and 0.4 mg/ml) and 200 IU/ml of r-VIII SQ were freeze-dried. The results indicated that the basic formulation was robust and that increasing concentrations of sodium chloride and polysorbate 80 were increasingly favourable for the recovery of VIII:C (Figure 2C). Concentrations of 0.1 mg/ml polysorbate 80 and 18 mg/ml sodium chloride in the reconstituted solution were chosen for the final formulation.

Moisture Content

In general, proteins are more stable at lower moisture contents. However, extensive drying may lead to oxidation and denaturation of the protein (18,19). The freeze-drying process for r-VIII SQ was designed to reduce the residual moisture content below 1% w/w. The stoppers used were dried and stability studies showed no increase in moisture content in the product during storage. On the contrary, during accelerated storage, the moisture content was decreased (data not shown). This may have been caused by the uptake of moisture by the stoppers. Thus, the secondary drying process is unintentionally continued in the final package, adding complexity to the interpretation of results from accelerated stability testing.

Head Space and Stability

Factor VIII preparations should be stoppered under vacuum or inert gas according to pharmacopoeial requirements. However, oxidation may occur as a result of traces of oxygen in the nitrogen and/or diffusion of oxygen through the rubber stoppers. The impact of oxygen on the stability of VIII:C was therefore studied. Figure 3 compares the recovery of VIII:C in vials with head spaces filled with air, oxygen, nitrogen or vacuum stored at 7°C and 25°C. The stability of the formulation in vacuum and nitrogen was equally good, but air or oxygen in the head space resulted in inferior stability.

Storage Stability

The stability of VIII:C for two representative batches of the final formulation is shown in Figure 4A. The results show that this formulation is stable for at least two years at 7°C and for at least one year at 25°C. Gel filtration after 24 months at 7°C and 25°C showed no significant increase in aggregates and/or fragments (Figure 5A). Visual inspection revealed clear colourless solutions which were practically free from particles.

Figure 4B shows that VIII:C was stable for up to 100 hours at 25°C after reconstitution. Visual inspection showed clear solutions and gel filtration showed no significant changes in the molecular size distribution pattern (data not shown). The Ph. Eur. monograph for factor VIII preparations excludes the use of preservatives and prescribes that the product should be used shortly after reconstitution. However, infusion is required in some cases (e.g. surgery), and the compatibility of the reconstituted solution with some infusion pumps was therefore investigated. No decrease in VIII:C or aggregation was observed (data not shown). Stringent aseptic technique must of course be applied in these situations.

Finished Product

The finished product should be reconstituted in sodium chloride injection (9 mg/ml). The reconstituted solution contains

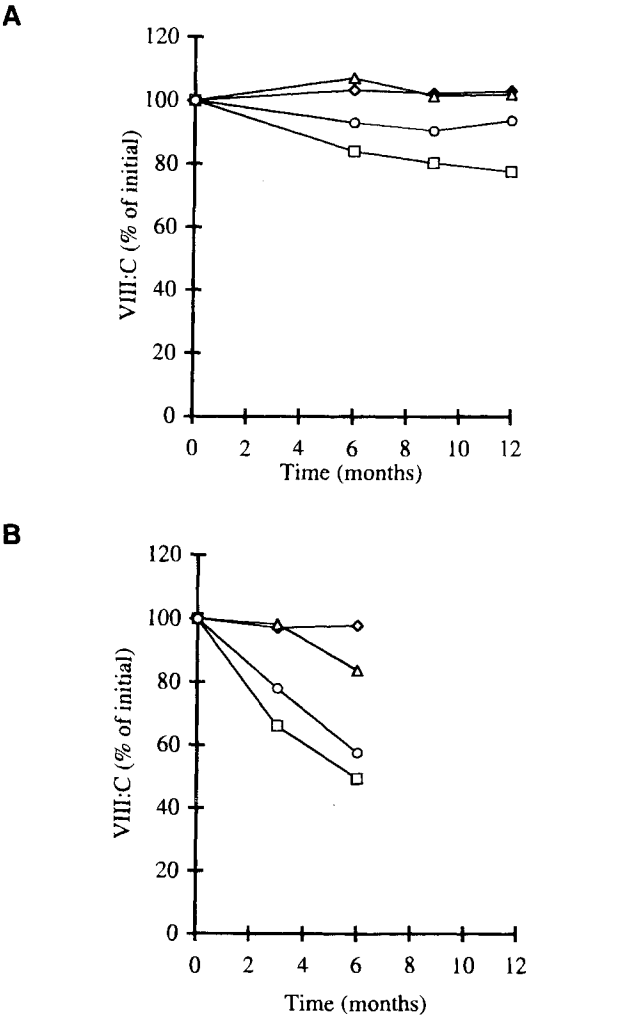

Fig. 3. Recovery of factor VIII activity (VIII:C) after storage (A) at 7°C with (◇) vacuum, (□) oxygen, (△) nitrogen and (○) air in headspace (B) at 25°C with (◇) vacuum, (□) oxygen, (△) nitrogen and (○) air in headspace.

the same concentration of excipients independent of dose: sodium chloride (18 mg/ml), sucrose (3 mg/ml), L-histidine (1.5 mg/ml), calcium chloride dihydrate (0.25 mg/ml) and polysorbate 80 (0.1 mg/ml). Considerable experience has been gained from manufacturing the finished product for clinical trials. Table 1 shows a summary of results from tests on five consecutive batches of the finished product. The recovery of VIII:C was about 90% compared to a reference sample of the solution before freeze-drying, assayed during the same run. Figure 5B shows gel filtration chromatograms of the reconstituted solutions. In all batches of r-VIII SQ, main peak was greater than 96% and there was less than 3% aggregates and less than 1% fragments. The median specific activity was about 16000 IU/mg protein. The reconstituted solutions were clear according to Ph. Eur. method and results from measurements with particle counter (HIAC/Royco) fulfilled the USP requirements. The concentration of sodium chloride in the reconstituted solution was 18 mg/ml corresponding to an osmolality of about 600 mosm/kg. Most therapeutic factor VIII concentrates have fairly high osmolality (300–900 mosm/kg) after reconstitution. The

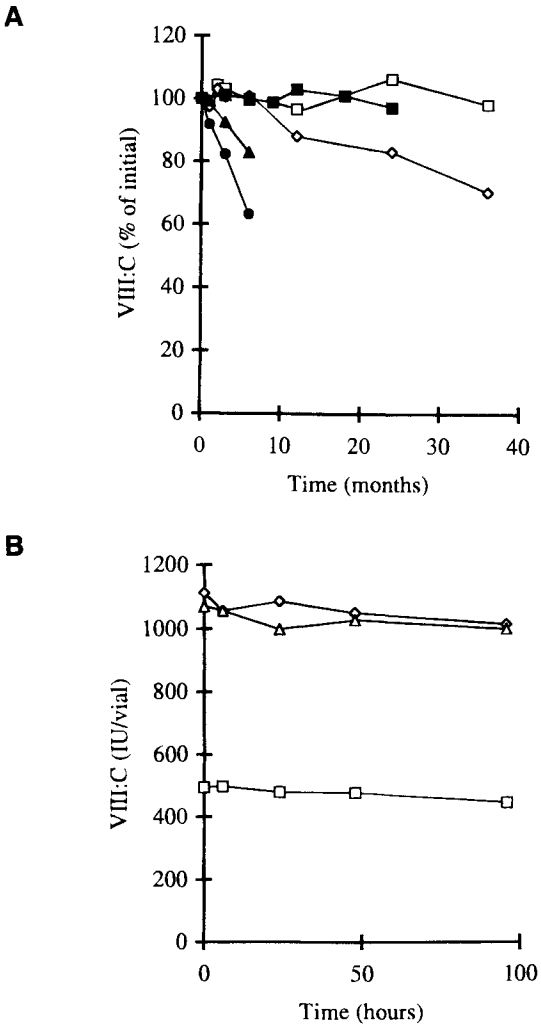

Fig. 4. (A) Recovery of factor VIII activity (VIII:C) for two representative batches of the final formulation after storage at (□) 7°C batch 1, (◇) 25°C batch 1, (■) 7°C batch 2, (▲) 30°C batch 2 and (●) 40°C batch 2, and (B) Stability of VIII:C of 3 batches after reconstitution and storage at 25°C.

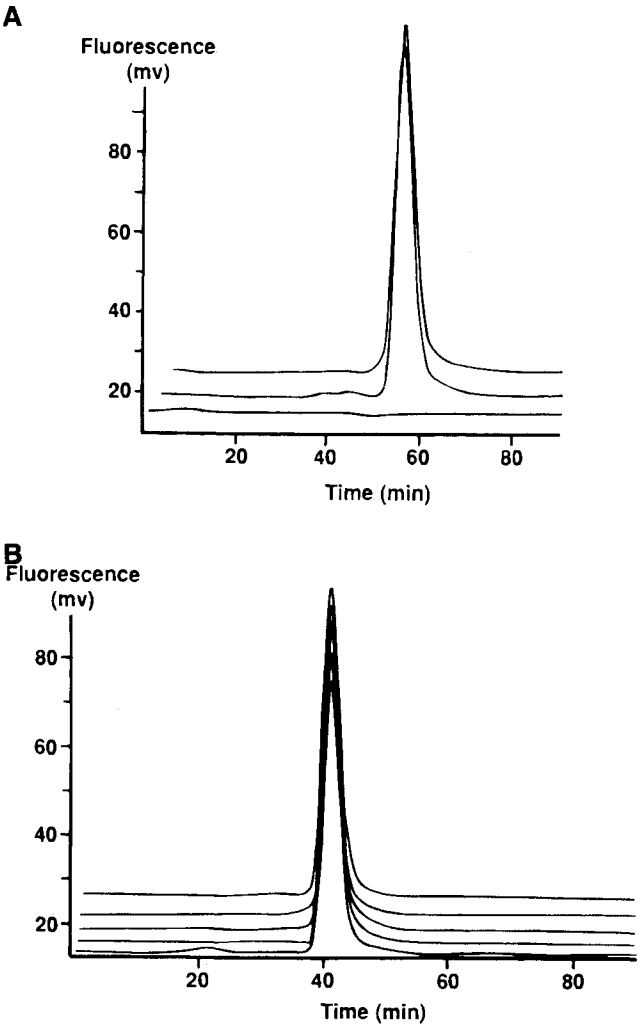

Fig. 5. HPLC gel filtration of (A), one representative batch of the final formulation and one batch placebo after storage for 24 months at 7°C (bottom) and 25°C (top) and (B) 5 consecutive batches of the r-VIII SQ finished product.

Table 1. Summary of Results from Tests on Five Consecutive Batches of Finished Product

Tests		Batch code				
		A	B	C	D	E
General appearance	White cake	A[a]	A	A	A	A
Moisture content	% w/w	0.4	0.4	0.4	0.4	0.4
Solubility	Minutes	<1	<1	<1	<1	<1
Visual inspection	Clear colourless solution	A	A	A	A	A
pH		7.0	6.9	6.9	6.9	6.9
Potency, VIII:C	IU/vial	1100	1100	1100	1100	1200
Recovery of VIII:C	% after freeze-drying	87	94	91	87	88
Specific activity	IU/mg protein	16000	15000	16000	15000	18000
SDS-PAGE	Comparison with a reference	A	A	A	A	A

[a] A = approved.

formulation presented here has been well tolerated during phase I and II clinical studies, and phase III studies are ongoing.

There are several advantages associated with an albumin-free formulation. The most essential is the elimination of the potential risk of viral transmission by a human-derived protein. This should help to allay the fears of both patients and their physicians. Furthermore, it is possible to use sensitive analytical methods such as gel filtration and SDS-PAGE for protein characterisation and quality control of the finished product. This is illustrated by the results of gel filtration of the finished product both initially and after long-term storage.

CONCLUSIONS

The experimental work presented in this report shows that r-VIII SQ can be successfully formulated for freeze-drying without the addition of albumin. Two of the necessary ingredients, sodium chloride and calcium ions, are very unfavourable in the design of a freeze-dried formulation. However, the key factor in overcoming this limitation was to use sodium chloride to form the freeze-dried cake. Polysorbate 80 in combination with amorphous excipients, L-histidine and sucrose, were used to protect r-VIII SQ from surface adsorption and to preserve the structural integrity of the molecule during sterile filtration and freeze-drying. Histidine also functions as a buffer. The albumin-free formulation is robust and results in consistently high recovery of VIII:C, very low aggregate formation and good storage stability. The shelf life of the freeze-dried product was at least two 2 years at 7°C and at least one year at 25°C. Furthermore, the reconstituted solution was stable for up to 100 hours at 25°C which would allow the product to be continuously administered via infusion pump. In conclusion, it is suggested that the formulation strategy described in this report may also be useful for other proteins which require a high ionic strength for solubility and stability.

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