Recombinant Factor VIII SQ—Inactivation Kinetics in Aqueous Solution and the Influence of Disaccharides and Sugar Alcohols

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Purpose. To investigate the influence of various nonreducing disaccharides and sugar alcohols on the inactivation kinetics of recombinant factor VIII SQ (r-VIII SQ) in aqueous solution not containing albumin as a stabiliser.

Methods. The stability of r-VIII SQ was followed using measurement of activity (VIII: C) and HPLC gel filtration at different temperatures. The thermal stability was investigated using differential scanning calorimetry (DSC).

Results. The decline in VIII:C followed pseudo-first order kinetics. However, the Arrhenius plot was not linear for formulations without carbohydrate, demonstrating a distinct, reproducible curvature. The reaction rate at 5°C was faster than expected from the Arrhenius kinetics. The energy of activation (Ea) for formulations without added carbohydrates, derived from the linear part of the Arrhenius plot, varied between 77 and 86 kJ/mole in the temperature range 20–37°C. The addition of 600 mg/ml sucrose increased the Ea to 104 kJ/mole. DSC measurements showed that Tm' was 64.2 ± 0.2°C for r-VIII SQ without stabiliser. This value increased linearly with increasing concentrations of carbohydrate. This stabilising effect is most probably explained by the theory of preferential hydration.

Conclusions. The inactivation kinetics of r-VIII SQ in aqueous solution without addition of carbohydrates followed pseudo-first order kinetics but the Arrhenius plot was nonlinear. Sucrose and sorbitol both had highly stabilising effects on r-VIII SQ at concentrations above 300 mg/ml. The preparation containing 600 mg/ml sucrose was stable for at least 12 months at 5°C and 6 months at 25°C.

KEY WORDS: solution; protein stability; factor VIII; formulation; kinetics; DSC.

INTRODUCTION

Haemophilia A is a congenital bleeding disorder caused by a functional deficiency in coagulation factor VIII. Recombinant factor VIII SQ[5] (r-VIII SQ) is a new genetically engineered factor VIII. It corresponds to the smallest active form, a metal ion-linked 80 and 90 kDa heterodimer, present in all factor VIII products for therapeutic use (1,2).

In the production of factor VIII concentrates, the stability problems are usually solved by addition of albumin (human) and freeze-drying. However, the potential risk of contamination with blood-borne viruses makes albumin undesirable as an excipient. Recent studies have shown that albumin can be replaced by a combination of a non-ionic surfactant such as polysorbate 80 and non-crystallising excipients (3). Most patients with haemophilia administer their own treatment at home to control bleeding. Mastering the aseptic reconstitution of the dry powder and administration of an intravenous injection in many different situations is not ideal for the patient, and more convenient-alternatives would be preferable. In addition, freeze-drying is a costly and time-consuming process for the manufacturer. Hence, a stable ready-to-use factor VIII solution formulated without addition of albumin would be desirable for both patients and physicians as well as for the manufacturers of the product.

Hitherto, all attempts to produce stable factor VIII solutions have been unsuccessful. The r-VIII SQ molecule is large and sensitive to both chemical and physical degradation. It consists of two glycosylated chains (containing a total of 1437 amino acid residues), 7 disulphide bridges, 5 sulfhydryl groups and a metal ion bridge. Physical degradation involves changes in the higher order structure, i.e. secondary, tertiary and quaternary. These changes can include denaturation, aggregation, subunit separation, precipitation or adsorption onto surfaces.

Furthermore, factor VIII is therapeutically active at low concentrations; an average dosage of 1000 IU of r-VIII SQ is dissolved in 4 ml, to a final concentration of 17 μg/ml. This makes it very difficult to follow specific degradation pathways. The main indicator of stability used in this study was the assessment of factor VIII activity (VIII:C). Many different domains have to be intact for the molecule to show activity and therefore the results of the VIII:C assay represent the sum of the degradation events leading to inactivation.

In a previous study it was shown that the stability of r-VIII SQ in solution was improved by reducing the oxygen content of the formulation, by retaining a pH in the range of 6.5–7, and by increasing both the concentration of calcium ions and the ionic strength (4). Although the results were promising, this formulation did not demonstrate an acceptable shelf life. The purpose of the present study was, thus, to investigate the inactivation kinetics of r-VIII SQ stored in solution and the possible stabilising effects of some disaccharides and sugar alcohols. Differential scanning calorimetry (DSC) was also used to measure the thermal stability of some formulations.

MATERIALS AND METHODS

Materials

r-VIII SQ was produced at Pharmacia & Upjohn AB, Sweden. Sodium chloride, calcium chloride dihydrate, polysorbate 80, L-histidine, sucrose and mannitol were of pharmacopoeial quality. Trehalose and sorbitol were of analytical grade. Water for injection or equivalent was used for all preparations. The sterile filter used was 0.22 μm, Millex-GV Millipore (Sweden). The container was an injection vial, glass type 1 (Ph. Eur. 2nd Ed. and USP), stoppered with a bromobutyl rubber stopper (Helvoet FM 157, Belgium) and sealed with an aluminium flip-

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[5] The abbreviation SQ refers to the amino acid linkage (ser-gln) which characterizes this specific molecule.

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off seal. Nitrogen was quality 5.5 (AGA, Sweden, less than 0.5 ppm impurities).

Methods

r-VIII SQ was produced by Chinese Hamster Ovary (CHO) cells cultivated in a serum-free medium. Purification was accomplished by several chromatographic steps using ion exchange, immunoaffinity, hydrophobic interaction and molecular size exclusion as separation principles. The specific activity was about 15 000 IU VIII:C per mg protein. Bulk material of r-VIII SQ obtained after the final chromatographic step (gel filtration) was received in a formulation buffer containing 18 mg/ml sodium chloride, 0.5 mg/ml calcium chloride dihydrate, 2 mg/ml L-histidine and 0.2 mg/ml polysorbate 80. Some preparations also contained 6 mg/ml sucrose.

A range of buffers was prepared, all containing the same amounts of sodium chloride, calcium chloride dihydrate, L-histidine and polysorbate 80, but with a varying content of disaccharides or polyalcohols. Requirements for carbohydrates in a formulation such as this are that they should be nonreducing, in order to prevent Maillard reactions (where reducing carbonyl groups react with the amine groups on the protein), and acceptable as excipients in parenteral drugs used for life-long treatment. Concentrations of 10, 300 and 600 mg/ml were investigated for sucrose and sorbitol. However, the low solubility of trehalose and mannitol limited the possible range of concentrations: 10, 300 and 500 mg/ml were used for trehalose and 10 and 150 mg/ml were used for mannitol.

The factor VIII activity and the concentrations of the inactive components were adjusted by diluting with the corresponding buffer. The solution was then sterile filtered, dispensed into glass vials and deoxygenated by reducing the pressure (0.02 bar) and introducing nitrogen over several cycles. Real-time stability studies were performed at temperatures ranging from 5 to 37°C. The samples were assayed immediately after preparation and regularly during storage in controlled incubators.

The local tolerability of the formulations after a single intravenous or perivenous injection was investigated in rabbits. Two 6-month-old New Zealand White HY/Cr male rabbits weighing 3.5–3.9 kg were used per formulation. For the intravenous model, 1 ml (500 IU) was injected into the lateral veins of both ears. For the perivenous model, 0.2 ml (100 IU) was injected into the subcutaneous tissue adjacent to the marginal medial vein of both ears. Twenty-four hours after administration, the rabbits were sacrificed and the injection site examined macroscopically. Microscopic examination was carried out on sections embedded in paraffin and stained with hematoxylin and eosin.

Analytical Methods

Factor VIII activity (VIII:C) was assayed using the chromogenic substrate method (Coatest®, Chromogenix AB, Sweden). All samples were prediluted in factor VIII-deficient plasma. The activity of factor VIII is expressed in International Units (IU) as defined by the International Concentrate Standard (IS) established by WHO. The relative standard deviation of the assay was about 7% for formulation samples.

Soluble aggregates and fragments were determined using gel filtration performed on a HPLC instrumentation setup (Hewlett Packard 1090 M USA). A prepacked Superdex 200 HR 10/30 column (Pharmacia & Upjohn Sweden) was used with a fluorescence detector (Jasco or Hewlett Packard Sweden) with an excitation wavelength of 280 nm and an emission wavelength of 340 nm. The elution buffer consisted of 18 mg/ml sodium chloride, 6.05 mg/ml Tris, 0.59 mg/ml calcium chloride dihydrate, and 0.1 mg/ml polysorbate 80 at pH 7.4. The flow rate was 0.22 ml/min. The reference solutions used were BioRad No. 151-1901 gel filtration standard (Sweden) and an inhouse r-VIII SQ standard.

The thermal stability was investigated using differential scanning calorimetry (DSC) on a Micro DSC III (Setaram) at a scanning rate of 0.75°C/min from 2 to 95°C. The sample volume was 0.8 ml and the reference cell was filled with the corresponding buffer containing the same formulation but excluding the protein. To measure Tm, a preparation containing 9 500 IU/ml (approx. 0.6 mg/ml) r-VIII SQ was used. The carbohydrates were added in solid form in order to minimise dilution of the material. However, because of changes in density, the concentration of r-VIII SQ was reduced to approximately 6 000 IU/ml at a carbohydrate concentration of 600 mg/ml, thus resulting in a smaller response.

The haemolytic potential of the formulations was assessed using heparinised human and rabbit blood. The formulations were mixed 1:1 with blood and incubated at 37°C for 45 minutes. After centrifugation (220 g for 5 min), 50 µl of the supernatant was mixed with 2.5 ml Drabkins solution. Absorbance was measured at 540 nm. The positive control was 3% saponin and the negative control was saline. The haemolytic potential of a test formulation was presented as a percentage of the value for a sample that resulted in complete haemolysis with 3% saponin. The haemolytic potential was considered significant if this percentage exceeded twice that seen with untreated plasma.

RESULTS AND DISCUSSION

Arrhenius Plot Derived from the Decline of VIII:C

The stability of r-VIII SQ in solution was followed using VIII:C measurements during storage at 5, 25, 30 and 37°C in two separate studies. In a third study, VIII:C was also measured at 10, 15, and 20°C. The decline of VIII:C roughly followed pseudo-first order kinetics (Table I). Least square linear regression analysis was utilised to determine the first-order rate constants. The degree of linear association was estimated by calculation of the correlation coefficient (R) and by visual inspection of the plots. The correlation coefficients were 0.969 or above. The $T_{90\%}$ (time taken to drop to 90% of the initial value for VIII:C) at 5°C calculated from the rate equation was in the range of 2.1–2.8 months.

Studies on inactivation of enzymes indicate that the decay of activity usually follows first-order kinetics, and that the rate constants usually conform to the Arrhenius relationship (5). Although the data presented here seem to follow pseudo-first order kinetics, the Arrhenius plot was not linear (Figure 1 A). This curvature will lead to overly optimistic results if stability testing is performed at higher temperatures and then extrapolated to a storage temperature of 5°C. The calculated rate constant would be lower than that obtained experimentally. Curved Arrhenius plots can result from temperature-induced changes

Table I. VIII:C Inactivation Rate Constants, t (90 %) and Correlation Coefficients for r-VIII SQ Stored in Solution for 12 Months[a]

	Temperature (°C)	Rate constant (10^3 day^{-1}) (n = 5–6)	t (90 %) (months)	Correlation coefficient
Study 1	5	1.42	2.47	0.995
	25	2.78	1.26	0.972
	30	5.43	0.65	0.995
	37	10.81	0.32	0.997
Study 2	5	1.24	2.84	0.996
	25	2.99	1.17	0.983
	30	4.65	0.76	0.997
	37	9.85	0.36	0.995
Study 3	5	1.68	2.09	0.969
	10	1.12	3.13	0.991
	15	1.40	2.51	0.988
	20	1.99	1.77	0.995
	25	2.94	1.19	0.995
	30	5.94	0.59	0.990
	37	12.24	0.29	0.996

[a] VIII:C was 200–250 IU/ml initially, in a formulation containing 18 mg/ml sodium chloride, 0.5 mg/ml calcium chloride dihydrate, 2 mg/ml L-histidine and 0.2 mg/ml polysorbate 80.

in the reaction mechanism or the rate determining step, or because of alterations in the higher order structure (6). Therefore, real-time measurements carried out at the actual storage temperature are often necessary for proteins stored in solution. Alterations in the tertiary structure of proteins can be observed using circular dichroism (CD) spectral changes in the near-UV. However, CD spectra of r-VIII SQ in the temperature range 5–20°C did not reveal any temperature-induced conformational changes (data not shown).

The energy of activation (Ea) was obtained by linear regression of the Arrhenius plot, over a temperature range of 20–37°C. The Ea values for the three studies were 86, 77 and 83 kJ/mole, respectively. This is a rather high value considering the complexity of the r-VIII SQ molecule and the requirement for many different domains to remain intact in order to retain activity. Factor VIII must be able to interact with the von Willebrand factor, thrombin, factor IXa, factor X and phospholipids (7). Furthermore, the conformational stability, defined as the energy of activation, of many naturally occurring globular proteins is between 21 and 63 kJ/mole (8). However, the stability of r-VIII SQ had already been improved to some extent by earlier preformulation work such as providing optimum pH conditions, using nitrogen in the headspace, including a divalent cation such as calcium and increasing the ionic strength. For example, the addition of 10 mM calcium to the formulation decreased the rate constant at 37°C from 25 × 10^{-3} to 10 × 10^{-3} day^{-1}. The Arrhenius plot for a formulation with a low concentration of calcium (0.1 mM) emphasised the non-linear behaviour at low temperatures (Figure 1 A).

Influence of Disaccharides and Sugar Alcohols on the Stability of r-VIII SQ

Sucrose, sorbitol and mannitol, but not trehalose, stabilised VIII:C in a concentration-dependent manner (Figure 1 B). The stability data followed pseudo-first order kinetics relatively

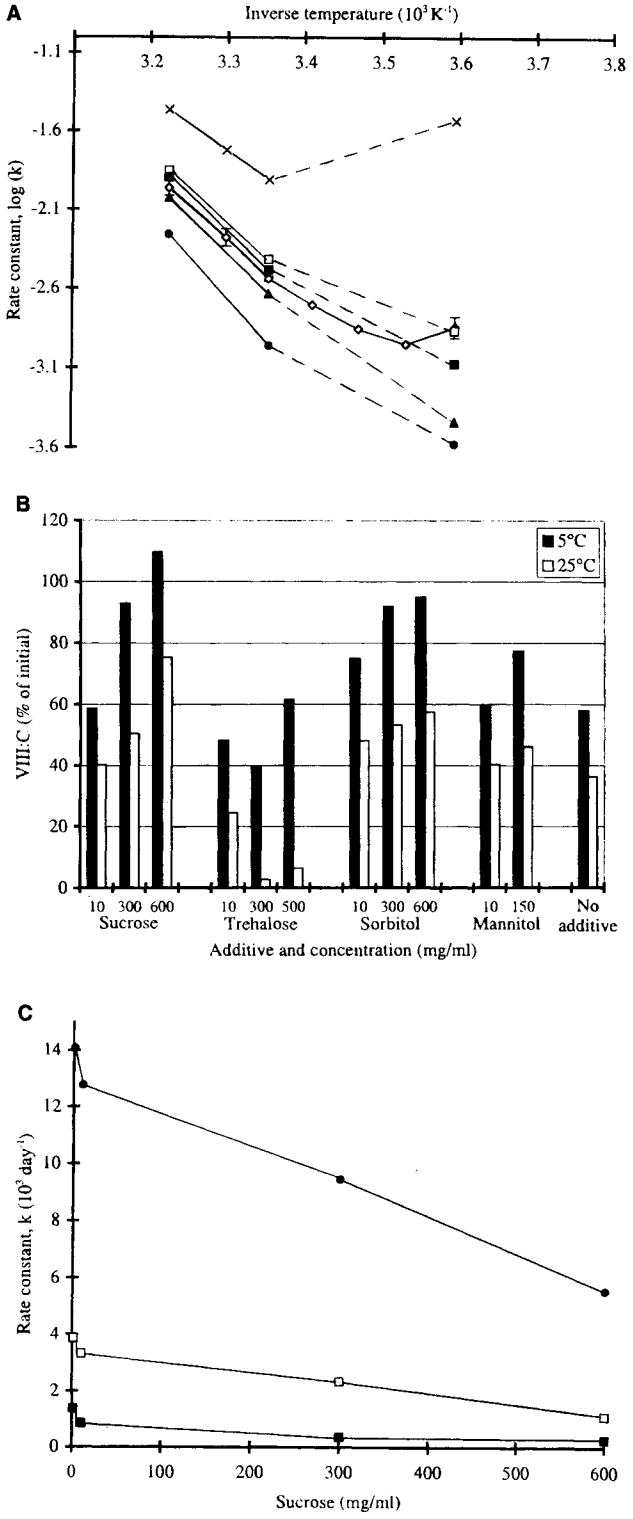

Fig. 1. A) Arrhenius plot for r-VIII SQ in solution. Without sucrose stabiliser: (◇) (average of 3 studies), with sucrose: (□) 1 mg/ml, (■) 10 mg/ml, (▲) 300 mg/ml, (●) 600 mg/ml and (X) without sucrose and a low concentration of Ca^{2+} (0.1 mM). B) Influence of disaccharides and polyalcohols on VIII:C after 9 months storage of r-VIII SQ in solution. C) Reaction rates (k) plotted as a function of sucrose concentration (■) 5°C, (□) 25°C and (●) 37°C.

well, as the correlation coefficients verify (Table II). All correlation coefficients were significant ($p < 0.05$), except for the formulation containing 600 mg/ml sucrose. The decrease in activity for 600 mg/ml sucrose was too small to give a relevant slope. For example: after 18 months 93 % and at 24 months 87 % activity remained at 5°C which is within the assay variation. At higher concentrations of carbohydrates, the rate constants were decreased and $t_{90\%}$ was markedly increased. For the formulations containing 600 mg/ml sucrose and 600 mg/ml sorbitol, the rate constant at 5°C was 0.3×10^{-3} day^{-1} and $t_{90\%}$ was at least 12 months.

The Ea calculated of the plot, in the temperature range 25–27°C, was similar to that determined in the absence of sucrose (83 and 87 kJ/mole for formulations containing 1 and 10 mg/ml sucrose, respectively). This suggests that, at low concentrations, sucrose had no noticeable effect on stability (Figure 1 A). When the concentration of sucrose was increased to 600 mg/ml, Ea rose to approximately 104 kJ/mole. The reaction rates decreased markedly with increasing sucrose (Figure 1 C), sorbitol and mannitol concentrations, but the results for trehalose were inconclusive.

Hydrophobic interactions are generally considered to be the major factor in stabilising the three-dimensional structure of proteins (9,10). The strength of the hydrophobic interaction is mainly determined by the structure of water which, in turn, is affected by the presence of co-solutes such as disaccharides and sugar alcohols (11,12). The effect of a co-solute on protein stability is defined by the preferred interaction with one of the two end states of protein unfolding: native or denatured. The strong affinity of the disaccharides and sugar alcohols for water excludes them from the domain of the protein surface. A native globular protein presents a smaller surface area to the solution than an unfolded protein. This leads to a thermodynamically more favourable state, in which contact between the co-solute (e.g. disaccharide or polyalcohol) and the protein is minimised and the protein is preferentially hydrated by water. Also, stabilisation becomes apparent only at relatively high concentrations of co-solute, i.e. above 0.3 M [which corresponds to approximately 100 mg/ml sucrose] (13). Since the concept of preferential hydration is dependent on the interactions between co-solute, water and protein, and since proteins are known to differ in their properties, the extent of stabilisation obtained with each co-solute has to be determined empirically for each protein. The inconclusive results obtained for the formulations containing trehalose as a stabiliser exemplify this.

Table II. Influence of Different Carbohydrates at Different Concentrations on the VIII:C Inactivation Rate Constant at 5°C,[a] and on the Transition Temperature (Tm') Measured by DSC[b]

Carbohydrate (mg/ml)	Rate constant (10^3 day^{-1}) (n = 6)	t (90 %) (months)	Correlation coefficient	Δ Tm' (°C)
Sucrose 10	0.85	4.1	0.962	—
Sucrose 200	—	—	—	3.8
Sucrose 300	0.37	9.6	0.992	6.3
Sucrose 600	0.27	13.2	0.643[c]	17.6
Trehalose 10	1.76	2.0	0.979	—
Trehalose 200	—	—	—	3.5
Trehalose 300	3.00	1.2	0.995	5.7
Trehalose 500	1.86	1.9	1.000	11.5
Sorbitol 10	1.07	3.3	0.993	—
Sorbitol 200	—	—	—	4.5
Sorbitol 300	0.39	9.0	0.925	6.3
Sorbitol 600	0.29	12.2	0.878	15.9
Mannitol 10	1.25	2.8	0.978	—
Mannitol 150	0.59	6.0	0.996	3.1
No additives	1.37	2.6	0.965	0

[a] VIII:C was 120 IU/ml initially.
[b] VIII:C was 9500 IU/ml.
[c] The correlation coefficient is not relevant here since values are very close to a horizontal line.

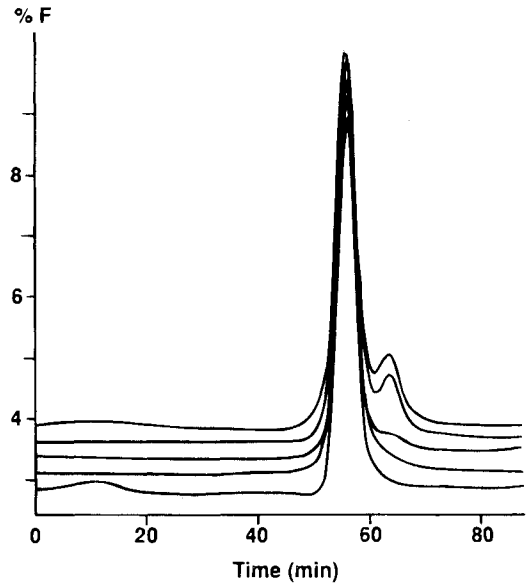

Fig. 2. HPLC gel filtration of r-VIII SQ in solution after storage for 12 months at 5°C and 25°C, from below: 1 mg/ml sucrose 25°C; 600 mg/ml sucrose 5°C, 300 mg/ml sucrose 5°C, 10 mg/ml sucrose 5°C and 1 mg/ml sucrose 5°C.

Earlier work has shown a correlation between the decline in VIII:C and the dissociation of the light (80 kDa) and heavy (90 kDa) chains of r-VIII SQ, especially at 5°C and outside the pH range of 6.5–7.0 (4). Gel filtration results in the studies reported here showed that the chain separation was proportionally reduced with increasing amounts of sucrose at pH 7 and 5°C (Figure 2). This suggests that alterations in the higher order structure of r-VIII SQ induced at low temperatures could lead to chain separation, resulting in the curved Arrhenius plot. Hydrophobic interactions are known to decrease with decreasing temperature and to increase in the presence of carbohydrates.

DSC

As r-VIII SQ without stabiliser was heated, the onset of an endotherm was first seen at approximately 56°C (Figure 4). This was because of a conformational change in the molecule, probably unfolding, which was verified by spectral changes in the near-UV CD (data not shown). The endotherm was directly followed by an exotherm corresponding to irreversible aggregation. This was directly confirmed after a scan, since the solution had turned opalescent and a precipitate had formed. A repeated scan showed the process to be irreversible. Since the onset of the endotherm is difficult to define, especially at increased concentrations of carbohydrates, and since both processes occur

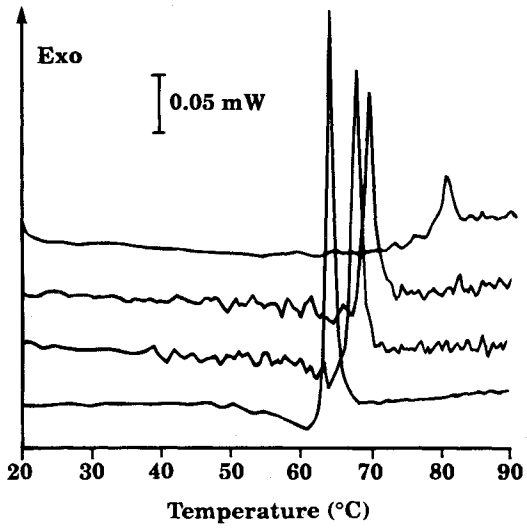

Fig. 3. DSC scans of r-VIII SQ with, from below: 0, 200, 300 and 600 mg/ml sucrose, respectively.

almost simultaneously, Tm′ is here defined at the maximum exotherm heatflow.

Tm′ was 64.2±0.2°C for r-VIII SQ without stabiliser, increasing linearly with increasing concentrations of sucrose (Figure 3, Table II). Microcalorimetric studies have shown that disaccharides and sugar alcohols can increase the transition point for heat denaturation (Tm) in a concentration-dependent fashion (14,15). Although the thermal transition point is not directly correlated with storage stability, an indication of the conformational stability of a protein in a selected formulation follows.

Sorbitol also increased Tm′ with increasing concentration, but at the highest concentration, 600 mg/ml, the solution turned opalescent before the sample was heated. This is perhaps not so surprising since preferential exclusion does decrease solubility. The stabilising effect of sorbitol confirms this, although the concentration of protein was much lower in the storage stability study. The reason for trehalose not showing the same stabilising effect during storage (as was indicated by the Tm′ data) was not elucidated in this study. Thus, as seen with other proteins, some disaccharides and sugar alcohols improved the thermal stability of r-VIII SQ. These results give support to the findings of the real-time stability investigations.

Preliminary Investigation of Tolerability

The formulations containing high concentrations of sucrose are hypertonic and could therefore cause pain and irritation when injected parenterally. However, since factor VIII preparations are administered intravenously, the increased osmolality could very well be tolerated (16,17). Immunoglobulin preparations for intravenous use are often formulated with

Table III. *In Vitro* Assessment of Haemolytic Effects and Microscopic Findings After a Local Tolerance Study Using Intravenous and Perivenous Injection in Male Rabbits

Sucrose (mg/ml)		Haemolytic score[a]			
			Dilution with saline (v.v)		
		Undiluted	1:1	1:2	1:4
6	Haemolysis	1	1.1	1.2	1.2
300	human blood	1.2	1.1	1.1	1.1
600	(baseline = 2.0)	3.9[b]	1.4	1.1	1.1
6	Haemolysis	1.2	1.2	1.2	1.4
300	rabbit blood	1.3	2.7	1.6	1.3
600	(baseline = 1.8)	2.3[b]	1.6	2.4	1.4

		Histopathological score[c]			
	Route of administration	Loss of endothelial cells	Inflammatory cell infiltrate	Oedema	Thrombus
0 (saline)	Intravenous	0	0.25	0.5	0.25
6		0.25	0.5	0.5	0.5
300		0	0.5	0.25	0
600		0.75	0.75	0.75	0.25
0 (saline)	Perivenous	0.5	0.5	0.25	0.5
6		0	0.25	0.75	0
300		0.5	0.5	0.75	0
600		0.25	1	3	0

[a] Average of 3 samples, haemolysis is calculated as % of a completely haemolysed sample.

[b] One sample showed significant hemolysis.

[c] Histopathological findings were graded 0–3 according to (18), for example: 0 no change; 1 loss of endothelial cells (or Thrombus observed) in less than 1/3 of vein in cross-section; 2 from 1/3 to 2/3; and 3 more than 2/3. Data presented as a weighted means. Four injection sites were examined (2 sites/animal/group).

high sucrose concentrations. For example, Sandoglobulin® (Sandoz) is tolerated clinically although it contains upto 200 mg/ml sucrose.

The haemolytic effects of 6, 300 and 600 mg/ml sucrose formulations were assessed *in vitro* (Table III). Low levels of haemolysis were seen with the undiluted 600 mg/ml sucrose formulation in both human and rabbit blood.

An exploratory local tolerability study of the same formulations in rabbits used doses 3–7 times the therapeutic dose intended for human use. Histopathological findings were graded 0–3 (18) (Table III). At the site of perivenous administration, the 600 mg/ml sucrose formulation caused an increase in the degree of oedema and related inflammatory cell infiltration compared with the other formulations, whereas reactions at the site of intravenous administration were minor. Since only a small number of specimens were included in this preliminary investigation, the results cannot be considered clinically significant. However, in general, the formulation containing 300 mg/ml sucrose would appear to be reasonably well tolerated, but the addition of 600 mg/ml sucrose may cause irritation upon intravenous injection.

CONCLUSION

When formulating protein drugs, it should be recognised that every protein is unique and has its special requirements. Factor VIII is known to be very labile, especially if the conditions deviate from the physiological milieu of plasma. It is remarkable that the formulation with 600 mg/ml sucrose was stable for at least 12 months of storage in solution at 5°C or 6 months of storage at 25°C. It should be emphasised that the formulations investigated included neither albumin nor the von Willebrand factor (both stabilising proteins present in the natural plasma milieu). The data presented here show that it is possible to design liquid formulations of r-VIII SQ that are stable during long-term storage.

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