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Recombinant factor VIII SQ—influence of oxygen, metal ions, pH and ionic strength on its stability in aqueous solution

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Abstract

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, a metal ion-linked 80 + 90 kDa heterodimer, present in therapeutic factor VIII concentrates. The stability of r-VIII SQ was investigated in aqueous solution, without albumin (human) as a stabiliser. Activity assay (VIII:C), visual inspection and gel filtration were performed after storage at different temperatures. The influence of oxygen, metal ions, pH and ionic strength was studied. The thermal stability was investigated using differential scanning calorimetry (DSC). There was a rapid loss of activity when r-VIII SQ was stored in solution in vials containing air in the headspace. The stability was markedly improved by reducing the oxygen content. pH 6.5–7.0 was optimal for stability at both low and high ionic strengths. The best results were obtained at high ionic strengths, since r-VIII SQ precipitated at sodium chloride concentrations below 5 mg/ml. The loss of VIII:C correlated with dissociation of the light (80 kDa) and heavy (90 kDa) chain complex. The separation of these chains was partly prevented by addition of calcium or strontium ions in the concentration range 1–10 mM. The transition point for heat denaturation (T'_m) was 64 ± 0.2°C in a formulation containing 9500 IU r-VIII SQ/ml, 18 mg/ml sodium chloride, 0.5 mg/ml calcium chloride dihydrate, 3 mg/ml L-histidine and 0.2 mg/ml polysorbate 80. The stability results presented here show promise and prompt further investigations into the development of a stable solution of r-VIII SQ. © 1997 Elsevier Science B.V.

Keywords: Solution; Protein stability; Factor VIII; Metal ions; pH; Differential scanning calorimetry; Oxygen

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1. Introduction

Factor VIII is a glycoprotein present in human blood in which it functions as a cofactor in the

intrinsic coagulation pathway. Functional deficiencies in this protein cause haemophilia A, an inherited X-linked recessive bleeding disorder. There are several therapeutic factor VIII concentrates presently available for replacement therapy. These products contain differing fully active forms of the protein, ranging from 170 to 280 kDa in molecular mass (Andersson et al., 1986). The smallest active form consists of two polypeptide chains of 90 kDa and 80 kDa held together by a metal ion bridge. It lacks the B-domain present in full length factor VIII. Recently a new recombinant product, termed r-VIII SQ and corresponding to the 170 kDa form of factor VIII, has been developed (Lind et al., 1995) (Fig. 1). This product is currently being evaluated in clinical trials formulated as a freeze-dried preparation.

The factor VIII molecule is very large and is one of the most complex therapeutic proteins ever developed by recombinant technology. The activity of the molecule is reliant on the presence of several different domains and it is sensitive to both chemical and physical degradation. Furthermore, factor VIII is therapeutically active at extremely low concentrations. For example, an average dose of 1000 IU of r-VIII SQ reconstituted in 4 ml amounts to only 17 μg/ml. This can lead to losses of r-VIII SQ due to non-specific surface adsorption both during manufacturing and in the final container. In addition, the low active concentrations and the large size and complexity of the molecule make it very difficult to follow specific degradation pathways.

In the natural environment, human blood, factor VIII circulates in association with the von Willebrand factor (vWF), a stabilising carrier protein also involved in primary haemostasis. The problems associated with stability and surface adsorption during the manufacturing of pure factor VIII products lacking vWF are usually solved by the addition of albumin (human) and freeze drying. This is currently done for all licensed highly purified factor VIII products, recombinant or plasma derived. However, considering the risks of viral transmission, the addition of a human blood product to a therapeutic protein manufactured by recombinant DNA technology is inadvisable. An albumin-free formulation for freeze-dried r-VIII SQ has been developed (Österberg et al., 1997). In that formulation, r-VIII SQ remained well preserved during freeze-drying and storage when polysorbate 80 in combination with amorphous excipients was used as stabilisers.

Patients with haemophilia often administer factor VIII themselves without medical supervision. A freeze-dried preparation requires reconstitution and must be handled aseptically before use. This involves several steps which the patient has to master. Furthermore, the freeze-drying process in itself is a costly and time consuming process. Therefore, a factor VIII preparation in a ready-to-use solution without albumin would be an advantage for both the haemophilia community and the industry.

In this study the properties of r-VIII SQ have been investigated on storage in solution without albumin. Stability was analysed by assessing the activity of factor VIII, visual inspection and analytical gel filtration. The influences of oxygen, different divalent metal ions, pH and ionic strength on stability were also investigated. Moreover differential scanning calorimetry (DSC) was used to measure the thermal stability of different formulations.

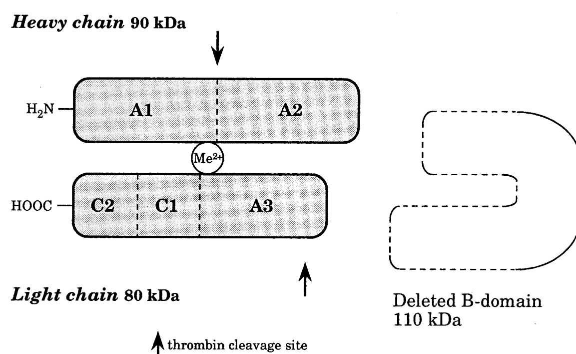

Fig. 1. The relationship between r-VIII SQ and the full-length factor VIII molecule.

Table 1
Influence of oxygen on the stability of r-VIII SQ

Temperature (°C)	Headspace	VIII:C % of initial activity					
		Storage time (months)					
		0.5	1	2	3	6	12
7	Nitrogen	—	—	—	88/88	81/82	62/66
	Air	—	—	—	—	8/61	3/46
30	Nitrogen	—	83/86	72/72	59/59	37	—
	Air	—	3/57	1/32	<1/11	—	—
37	Nitrogen	82/85	71/68	55/48	41/38	13	—
	Air	7/69	1/32	—	—	—	—

VIII:C was 250 IU/ml initially. Two separate studies are shown. All formulations contained sodium chloride 18 mg/ml, L-histidine 2 mg/ml, calcium chloride dihydrate 0.5 mg/ml, polysorbate 80 0.2 mg/ml, pH 7.

2. Materials and methods

2.1. Materials

r-VIII SQ was produced by Pharmacia & Upjohn AB (Sweden) as described below. Sodium chloride, calcium chloride dihydrate, hydrochloric acid, sodium hydroxide and polysorbate 80 used in the investigation conformed to the requirements of Ph. Eur. 2nd Ed. and USP. L-Histidine conformed to the requirements of DAB and USP. Divalent metal ions of analytical grade were in the form of chloride salts. 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-off seal. Nitrogen was of quality 5.5 (AGA, Sweden, less than 0.5 ppm impurities).

2.2. Methods

r-VIII SQ was produced in 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. r-VIII SQ is highly purified with a specific activity of about 15 000 IU VIII:C per mg protein. Bulk material of r-VIII SQ, with a factor VIII activity of 2500–6000 IU/ml, was obtained from the final purification step.

The elution buffer contained 18 mg/ml sodium chloride, 0.5 mg/ml calcium chloride dihydrate, 2 mg/ml L-histidine and 0.2 mg/ml polysorbate 80. Other buffers were also prepared, all containing L-histidine and polysorbate 80, but differing in the concentrations of sodium chloride or metal ion content. The factor VIII activity and the concentrations of the inactive components were adjusted by diluting with the corresponding buffer. pH was adjusted with hydrochloric acid (5 M) or sodium hydroxide (2 M). The solution was then sterile filtered, dispensed into glass vials and deoxygenated by subjecting the solution to reduced pressure (0.02 bar) and then introducing nitrogen in several cycles. Some samples were also prepared with air in the headspace. Real time stability studies were performed at 7, 25, 30 and 37°C. Samples were assayed immediately after preparation and during storage for up to 12 months.

The thermal stability was also investigated using calorimetry on a Micro DSC III (Setaram France) 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.

2.3. Analytical methods

The factor VIII activity (VIII:C) was assessed using a chromogenic substrate assay based on the Coatest Factor VIII kit from 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 the formulation samples. The recovery of VIII:C after storage is expressed as percentage recovery of the initial VIII:C value.

Soluble aggregates and fragments were determined by gel filtration performed on a HPLC instrumentation setup (Hewlett Packard 1090 M, USA). A prepacked Superdex 200 HR 10/30 column (Pharmacia & Upjohn AB, Sweden) was used with a fluorescence detector (Jasco or Hewlett Packard 1046 A, Sweden): excitation wavelength 280 nm, emission wavelength 340 nm. The gel filtration 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 Bio-Rad No. 151-1901 gel filtration standard (Sweden) and an in-house r-VIII SQ standard. Evaluation of results was performed by integration of the peak areas. Aggregates, fragments and the main r-VIII SQ peak are presented as percentage of the total integrated area.

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 (Lind et al., 1995).

Visual inspection was performed in translucent light against a black background. Colour, particles, precipitation and opalescence were noted.

3. Results and discussion

In a previous study a combination of polysorbate 80, L-histidine, sodium chloride and calcium chloride was found to preserve r-VIII SQ well during sterile filtration, freeze-thawing and freeze-drying (Österberg et al., 1997). The same excipients were chosen for the current study on r-VIII SQ stability in aqueous solution. The rationale behind the choice of polysorbate 80 was the prevention of surface adsorption. L-Histidine was primarily chosen for its buffering capacity at pH 7 and calcium chloride was included to prevent dissociation of the metal ion linked r-VIII SQ heterodimer. If not otherwise stated, visual inspection revealed clear, coloured solutions.

3.1. Influence of oxygen on the stability of VIII:C

The influence of oxygen was studied by comparing the use of air or nitrogen in the headspace when storing r-VIII SQ in solution for up to 12 months at 7, 30 and 37°C. As shown in Table 1, r-VIII SQ is susceptible to oxidation and a sustained improvement in stability was attained by reducing the oxygen content. The r-VIII SQ molecule is very large (170 kDa) and theoretically contains seven disulphide bridges, five sulfhydryl groups and 43 methionine residues, all of which are potential oxidation sites (McMullen et al., 1995). Furthermore, the presence of trace metals could catalyze the oxidation processes, e.g. divalent cations such as copper and iron are known to accelerate the oxidation of proteins (Lamfrom and Nielsen, 1956; Shihong et al., 1993). The addition of some antioxidants (acetylcysteine, glutathione, L-methionine) and chelators (EDTA, citrate) was therefore investigated but the stability of VIII:C was not further improved by these agents (data not shown). Mechanisms other than oxidation probably prevail once the oxygen level is reduced. In addition, L-histidine, which was included in the formulation for its buffering capacity at pH 7, may also act as a chelator and block the effects of trace metals (Sundberg and Martin, 1974). Since reduced levels of oxygen were found to be crucial, all formulations in the following studies were stored with nitrogen in the headspace.

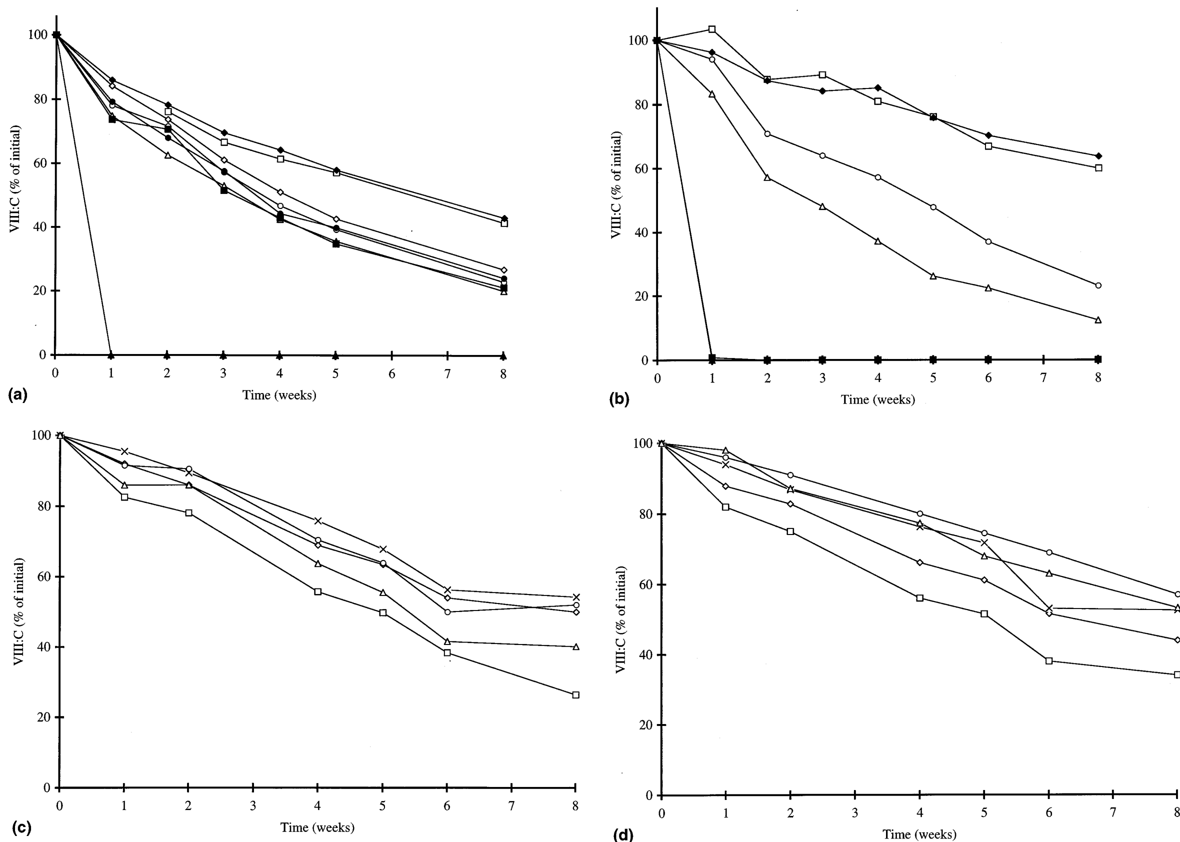

Fig. 2. Influence of divalent metal ions on the recovery of VIII:C after storage at 37°C in formulations containing 200 IU/ml, 18 mg/ml sodium chloride, 2 mg/ml L-histidine and 0.2 mg/ml polysorbate 80 at pH 7 plus; (a) 1 mM (□) Ca^{2+}, (◇) Cu^{2+}, (△) Mn^{2+}, (○) Mg^{2+}, (■) Zn^{2+}, (◆) Sr^{2+}, (▲) Fe^{2+} and (●) control (0.1 mM Ca^{2+}); (b) 10 mM (□) Ca^{2+}, (◇) Cu^{2+}, (△) Mn^{2+}, (○) Mg^{2+}, (■) Zn^{2+}, (◆) Sr^{2+} and (▲) Fe^{2+}; (c) Ca^{2+}, (□) 0.2 mM, (◇) 1 mM, (×) 10 mM, (○) 50 mM and (△) 100 mM; (d) Sr^{2+}, (□) 0.2 mM, (◇) 1 mM, (×) 10 mM, (○) 50 mM and (△) 100 mM.

3.2. Influence of divalent metal ions on the stability of r-VIII SQ stored at 37°C

Ca^{2+}, or another divalent metal ion such as Mn^{2+} or Cu^{2+}, is necessary for the preservation of factor VIII activity (Weiss, 1965; Mikaelsson et al., 1983; Nordfang and Ezban, 1988). The addition of chelating agents has been demonstrated to result in the separation of the factor VIII light and heavy chains (Andersson et al., 1986). Bihoreau et al. (1994) have shown that factor VIII preparations contain copper but their studies did not exclude the presence of other metal ions. It is possible that an increase in Ca^{2+} concentration or the addition of another metal ion could favor association of the chains. The stabilising effects of several divalent metal ions (Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Sr^{2+} and Fe^{2+}; at 1 and 10 mM) were therefore investigated.

Since the bulk material contained Ca^{2+}, all formulations included this ion at a concentration of at least 0.1 mM, but this was not sufficient for the preservation of VIII:C (Fig. 2A,B). At a concentration of 1 mM, only Ca^{2+} and Sr^{2+} increased the stability of the formulation compared with the control. Fe^{2+} was detrimental to VIII:C, probably by accelerating oxidation. At 10 mM, the stabilisation provided by Ca^{2+} and Sr^{2+} was enhanced: after 4 weeks of storage at 37°C, the residual VIII:C was more than 80%. Mn^{2+} and Mg^{2+} produced much the same result at 10 mM as at 1 mM. Cu^{2+}, Zn^{2+} and Fe^{2+} at 10 mM all

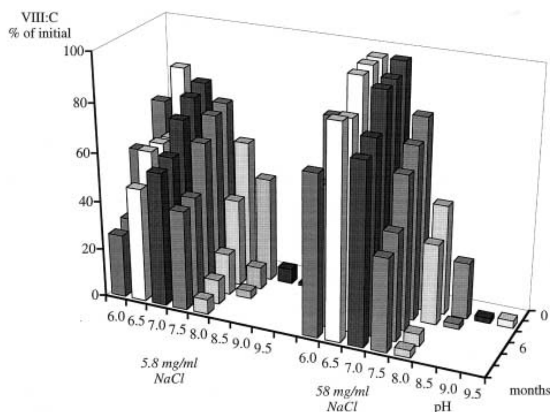

Fig. 3. Influence of pH and ionic strength on the stability of VIII:C stored at 7°C.

reduced the stability of VIII:C. All activity was lost within a week in solutions containing these concentrations.

The stability of VIII:C was further investigated using increasing concentrations of Ca^{2+} and Sr^{2+} (Fig. 2C and Fig. 2D). Both ions showed comparable results and the stability of VIII:C was improved in the concentration range 1–10 mM. Additional hydrophobic and electrostatic interactions are required besides divalent ion binding for the integrity of the factor VIII molecule. For example, reconstitution of factor VIII activity

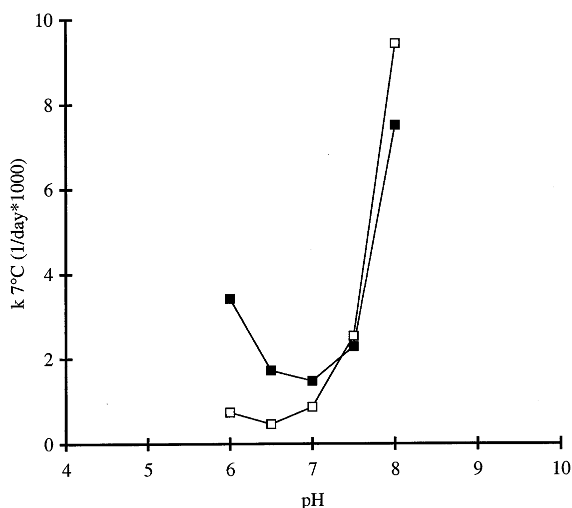

Fig. 4. Reaction rates at 7°C plotted as a function of pH: (■) 5.8 mg/ml sodium chloride, (□) 58 mg/ml sodium chloride.

from isolated (plasma derived) subunits was shown to be dependent upon ionic strength (Fay, 1988). Due to its beneficial effect on the stability of VIII:C, Ca^{2+} (as 0.5 mg/ml (4 mM) calcium chloride dihydrate) was included in the following studies.

3.3. Influence of ionic strength on the solubility of r-VIII SQ

Visual inspection of solutions with varying ionic strength revealed that r-VIII SQ (125 IU/ml) precipitated at sodium chloride concentrations below 5 mg/ml (0.1 M) at pH 7. First the protein solution turned opalescent, and after approximately 1 h a precipitate with a clear supernatant was formed. No further changes were observed after 24 h at room temperature. When the supernatant after centrifugation (3000 rpm, 10 min) was assayed for VIII:C, only 17% of the activity remained. The precipitate was dissolved by increasing the concentration of sodium chloride and almost all activity was recovered. Higher concentrations of r-VIII SQ, above 1500 IU/ml, were found to precipitate even at 9 mg/ml sodium chloride (see below).

The effect of increased ionic strength on the solubility of r-VIII SQ may be explained by the Debye–Hückel theory (Middaugh and Volkin, 1992). This theory assumes that proteins are surrounded by a counter-ion atmosphere that decreases their electrostatic free energy, producing a decrease in solution activity with a concomitant increase in protein solubility. Usually, this phenomenon is referred to as salting in. It is interesting that the full activity of a labile molecule such as r-VIII SQ can be recovered after precipitation if the precipitate is redissolved in a solution of increased ionic strength.

3.4. Influence of pH and ionic strength on the stability of r-VIII SQ stored at 7°C

The stability of r-VIII SQ in solutions with varying pH and ionic strength was evaluated using VIII:C assays and gel filtration. Earlier studies have revealed that r-VIII SQ is almost completely

Table 2
Influence of pH and ionic strength on the aggregation of r-VIII SQ stored at 7°C

Sodium chloride (mg/ml)	pH	Aggregates (% of the total integrated area)				
		Storage time (months)				
		1	3	6	9	12
5.8	6.0	0	<3	?	27	6
	6.5	0	<3	3	0	1
	7.0	3	12	<3	3	<3
	7.5	3	13	<3	5	5
	8.0	4	21	nd	14	12
	8.5	4	22	nd	nd	nd
	9.0	7	nd	nd	nd	nd
	9.5	26	nd	nd	nd	nd
58	6.0	0	<3	3	3	6
	6.5	0	<3	1	0	<3
	7.0	0	<3	<3	1	<3
	7.5	0	<3	<3	3	<3
	8.0	0	10	nd	13	15
	8.5	5	17	nd	nd	nd
	9.0	10	nd	nd	nd	nd
	9.5	11	nd	nd	nd	nd

All formulations contained L-histidine 3 mg/ml, calcium chloride dihydrate 0.5 mg/ml, polysorbate 80 0.2 mg/ml.
nd, not determined.

denatured below pH 6 (Österberg et al., 1997). Therefore the pH interval 6–9.5 was investigated here. Some caution should be exercised in the interpretation of the pH profile since different buffer species can have differing effects on stability. The effects of low ionic strength 5.8 mg/ml (0.1 M) sodium chloride) and high ionic strength (58 mg/ml (1 M) sodium chloride) were primarily investigated, but the results were also confirmed at physiological concentrations (9 mg/ml (0.15 M) sodium chloride).

The recovery of VIII:C after storage at 7°C was optimal at pH 7 at both low and high ionic strengths (Fig. 3). The best results were obtained at high ionic strength in the investigated pH interval, i.e. 6–9.5. As expected, the decline of VIII:C was faster at 25°C than at 7°C (data not shown). Otherwise the results corresponded closely. The reaction rate constants at 7°C plotted against pH show a minimum at pH 6.5–7 (Fig. 4).

The mechanisms by which a salt influences the physical properties of a molecule (such as conformation and solubility) are not yet completely understood. At least two mechanisms, preferential hydration and specific ion binding, are involved. Interactions that involve specific ion binding are sensitive to solution environmental factors such as pH. The stabilisation of proteins by the addition of co-solutes (e.g. certain salts, sugars and amino acids) is usually dependent on the concentration of the additives. In the present case the stability was markedly improved by an increase in sodium chloride from 0.1 to 1 M. This effect is in agreement with the general concept that stabilization becomes apparent only at relatively high concentrations of co-solutes, namely above 0.3 M (Arakawa et al., 1992). The co-solute molecules are preferentially excluded from contact with the protein, and the protein is preferentially hydrated.

Proteins are often (but not always) most stable at their isoelectric point. For factor VIII, however, the isoelectric point has not yet been determined. The 80 kDa subunit has been resolved in a cluster of bands with a range of isoelectric points

Table 3
Influence of pH and ionic strength on the fragmentation of r-VIII SQ stored at 7°C

Sodium chloride (mg/ml)	pH	Fragments (% of the total integrated area)				
		Storage time (months)				
		1	3	6	9	12
5.8	6.0	11	<1	?	5	41
	6.5	4	<1	15	5	18
	7.0	3	2	5	5	8
	7.5	4	4	11	15	19
	8.0	12	17	nd	36	38
	8.5	28	34	nd	nd	nd
	9.0	46	nd	nd	nd	nd
	9.5	57	nd	nd	nd	nd
58	6	6	<1	3	3	4
	6.5	4	<1	9	4	6
	7.0	3	3	8	10	12
	7.5	7	12	18	22	26
	8.0	28	28	nd	38	38
	8.5	42	42	nd	nd	nd
	9.0	49	nd	nd	nd	nd
	9.5	50	nd	nd	nd	nd

All formulations contained L-histidine 3 mg/ml, calcium chloride dihydrate 0.5 mg/ml, polysorbate 80 0.2 mg/ml.
nd, not determined.

between 6.5 and 7.2, but the larger chain has still to be focused (Eaton et al., 1987). The surface of a large protein can be regarded as a mosaic of charges. Parts of the surface may exhibit high charge densities, even though the net charge may be close to zero. Some variation in surface charge can be ascribed to heterogeneous glycosylation (Damm, 1995). The concept of maximum stability at the isoelectric point is therefore difficult to apply to large proteins such as factor VIII, especially if they consist of different subunits. Furthermore, proteins are usually most easily precipitated at their isoelectric point and this can result in solubility problems.

Besides activity measurements, the stability studies were monitored by gel filtration in order to detect fragmentation or aggregation. The results are shown in Tables 2 and 3 and in Fig. 5A. During storage, increasing amounts of both fragments and soluble aggregates appeared in the r-VIII SQ solution, especially at pH outside the range 6.5–7.0. The degree of fragmentation and aggregation was slightly higher at low ionic strength.

Loss of activity as well as occurrence of fragments and soluble aggregates were at a minimum at high ionic strength and pH 6.5–7.0, whereas alterations in pH outside this narrow range had a dramatic effect on the stability of r-VIII SQ. During storage the changes seemed to occur in the following order: activity loss, fragmentation and then aggregation. To further elucidate the cause of these reactions, the fragments detected by gel filtration were isolated and subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 5B the only findings were bands corresponding to the light (80 kDa) and heavy (90 kDa) chains of r-VIII SQ. The amount of the light chain appeared to be much greater than that of the heavy chain. There were no bands corresponding to proteolytic fragments, e.g. 45, 50 or 70 kDa forms of factor VIII. These results may indicate that r-VIII SQ dissociates into separate chains during storage in solution and that the heavy chain then aggregates.

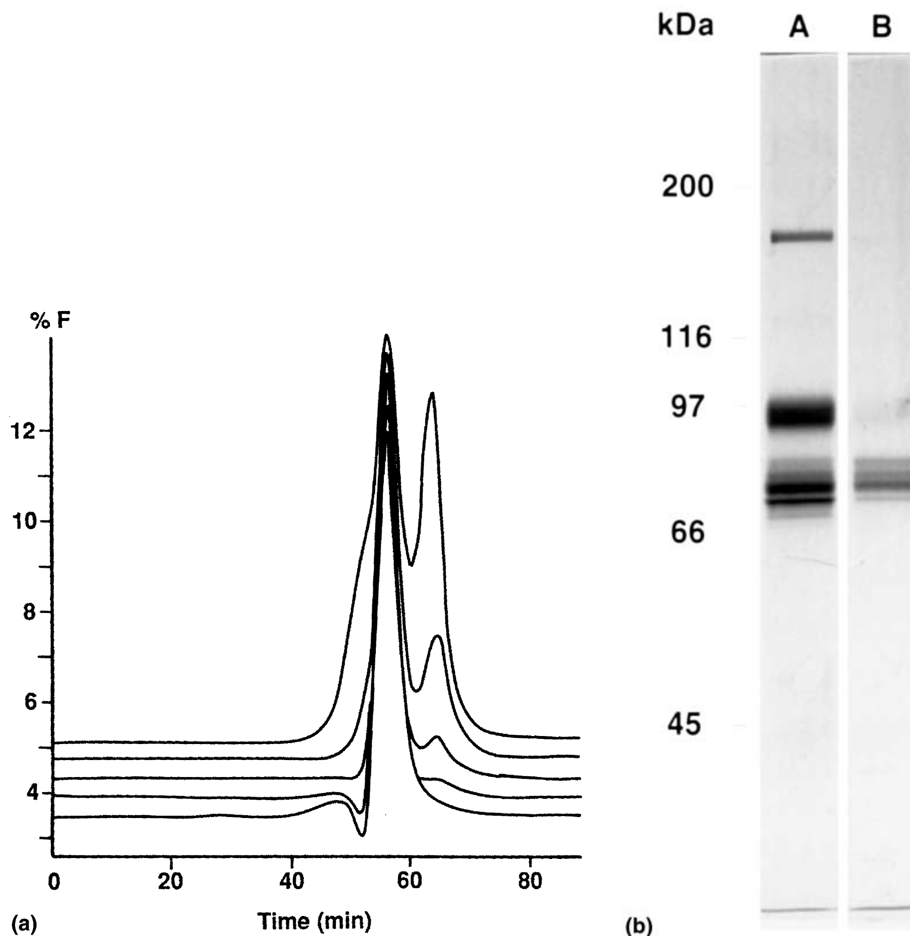

Fig. 5. (a) HPLC gel filtration of r-VIII SQ in 58 mg/ml sodium chloride after storage for 12 months at 7°C. Depicted in order from below: pH 6.0; pH 6.5; pH 7.0; pH 7.5 and pH 8.0; (b) SDS-PAGE profile of r-VIII SQ after gel filtration and fractionation, (lane A) fraction containing the main peak, (lane B) fraction containing the fragment.

For a protein like factor VIII which circulates and exerts its function in blood plasma, optimum stability is expected at physiological pH (Schein, 1990). In the absence of the B-domain and moreover the von Willebrand factor, optimum pH may deviate from that of blood. Taken together the results of the present investigation suggest that even fairly small alterations in pH may result in loss of activity, conceivably caused by small changes in the conformation induced by alterations in charge and ion interactions.

Factor VIII is a complex molecule in which many different domains have to be intact and correctly positioned for optimum interactions with the von Willebrand factor, thrombin, factor IXa, factor X, activated protein C and phospholipids (Foster and Zimmermann, 1989). With increasing changes in pH, it is conceivable that local changes in charge become more pronounced which may lead to weakening of the metal ion bridge and/or further conformational changes. Either event could induce chain separation with concomitant activity losses. It must be kept in mind however, that an apparent reduction in the activity may reflect either an almost complete loss of activity affecting part of the molecules or a conformational alteration associated with less functional activity in most of the molecules.

3.5. Differential scanning calorimetry (DSC)

The thermal stability was investigated using DSC. Although the transition point for heat denaturation (T_m) is not directly correlated with storage stability, an indication of the conformational stability of a protein in a selected formulation is provided by DSC (Boctor and Mehta, 1992; Gekko, 1982). In order to obtain T_m for r-VIII SQ, a more concentrated preparation [9500 IU/ml (approximately 0.6 mg/ml)] was used. At this high concentration, the protein was found to precipitate even at 9 mg/ml sodium chloride. A concentration of 18 mg/ml sodium chloride was therefore used to dissolve r-VIII SQ during these studies.

The onset of an endotherm on heating r-VIII SQ was first seen at approximately 56°C. This was due to a conformational change of the molecule such as unfolding, or perhaps a dissociation of the heterodimer as described above. It was directly followed by an exotherm, corresponding to an irreversible aggregation. This was confirmed directly after the scan since the solution had turned opalescent and a precipitate had formed. A repeated scan showed the process to be irreversible. Since the time of onset of the endotherm is difficult to define, and since the aggregation process occurs almost simultaneously, T'_m is here defined at the maximum exotherm heat flow.

T'_m was 64.2 ± 0.2°C for r-VIII SQ at 18 mg/ml sodium chloride concentration at pH values of both 6.5 and 7.0. At pH 5, 6, 7.5 and 8, r-VIII SQ precipitated before a scan could be made. At 58 mg/ml sodium chloride concentration and pH 7, T'_m was increased to 67°C (Fig. 6). This confirms that increasing the ionic strength at pH 7 is favourable for the stability of r-VIII SQ. A scan was also performed on a formulation without polysorbate 80 but no influence on T'_m was detected. This indicates that no direct interaction takes place between r-VIII SQ and the nonionic surfactant.

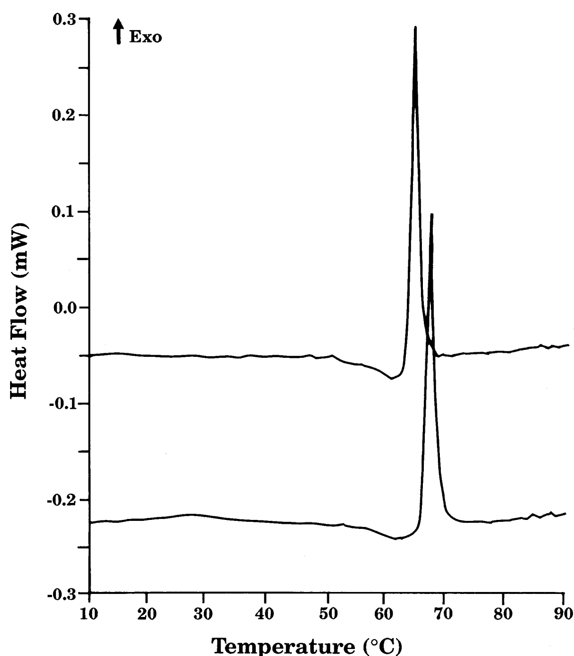

Fig. 6. DSC scans of r-VIII SQ in a formulation containing 9500 IU/ml, 0.5 mg/ml calcium chloride dihydrate, 3 mg/ml L-histidine and 0.2 mg/ml polysorbate 80 at pH 7 plus 58 mg/ml sodium chloride (curve below) and 18 mg/ml sodium chloride (curve on top).

4. Conclusions

Factor VIII is known to be very labile in solution. By elucidating the underlying mechanism, however, it is possible to enhance the stability of the solution and facilitate the design of formulations suitable for long-term storage. In this study the stability of r-VIII SQ was strongly improved by decreasing the oxygen content and increasing the ionic strength. A pH at 6.5–7.0 in an L-histidine buffer was optimal for stability. Dissociation of the light (80 kDa) and heavy (90 kDa) chains of r-VIII SQ correlated well with the loss of VIII:C and seemed to be the major cause of degradation. Addition of divalent ions like Ca^{2+} and Sr^{2+} markedly improved the stability of r-VIII SQ. To maintain the solubility of r-VIII SQ at high concentrations, it was necessary to increase the ionic strength above that normally found in physiological solutions. Since the formulation did not contain the von Willebrand factor, albumin or any other protein stabilizer it is remarkable that up to 80% of the initial activity remained after 1 year of storage at 7°C. Thus, the

stability results presented here show promise and prompt further investigations into the development of a stable, ready-to-use solution of r-VIII SQ.

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