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Recombinant Factor VIII SQ — Stability of VIII : C in Homogenates from Porcine, Monkey and Human Subcutaneous Tissue

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Abstract

The aim of this paper was to investigate whether a formulation-based approach to understanding and addressing stability could generate a subcutaneous factor VIII preparation for patients as an alternative to the existing intravenous products. The low bioavailability of subcutaneously administered factor VIII could have several causes: proteolytic degradation of the protein in the interstitium; adsorption to tissue, in particular to acidic phospholipids such as L-α phosphatidyl-L-serine (phosphatidylserine); the absence of free von Willebrand factor in the interstitum; phagocytosis by macrophages in the interstitium or in the lymph nodes; and coagulation could be initiated upon injection. This study was undertaken to investigate the first three factors in-vitro (i.e., proteolytic degradation, adsorption to tissue and the protective effect of von Willebrand factor). The influence of some other macromolecular stabilisers and protease inhibitors was also investigated. The stability of factor VIII activity (VIII : C) was investigated in homogenates from porcine, monkey and human subcutaneous tissue. Possible coagulation was prevented in these studies by the presence of both citrate and antithrombin. An exploratory in-vivo study was performed in the pig; plasma samples were assayed with a factor VIII : Ag (90 kDa) ELISA.

The decrease in VIII:C appeared to be more pronounced in homogenates from monkey and human tissues than in porcine homogenate. The results from human tissue homogenate resembled the degradation profile seen in monkey homogenate. Both the von Willebrand factor and phosphatidylserine/phosphatidylcholine (PS/PC) liposomes showed a significant stabilising effect on VIII:C in the tissue homogenates. The qualitative pattern was similar in porcine, monkey and human tissue. A combination of several protease inhibitors seemed to have a protective effect on the stability of VIII : C albeit at high concentrations of inhibitors and the effect was less than that of PS/PC. An exploratory in-vivo study was performed in the pig with phosphatidylserine in two formulations; either in the form of PS/PC liposomes or together with Polysorbate 80 in the form of mixed micelles (phosphatidylserine/P80). Including phosphatidylserine in the formulations appeared to increase the availability of subcutaneously administered r-VIII SQ in the pig. However, further studies are necessary, preferably in the monkey where in-vitro studies indicate a closer resemblance to the human.

In conclusion, a proposed inactivation mechanism for r-VIII SQ in subcutaneous tissue could be adsorption to phospholipid surfaces followed by proteolytic degradation. However, additional studies are required due to the multitude of factors influencing the subcutaneous absorption route. A combination of protease inhibitor(s) together with phosphatidylserine-containing liposomes are suggested for further investigation, preferably in a monkey animal model.

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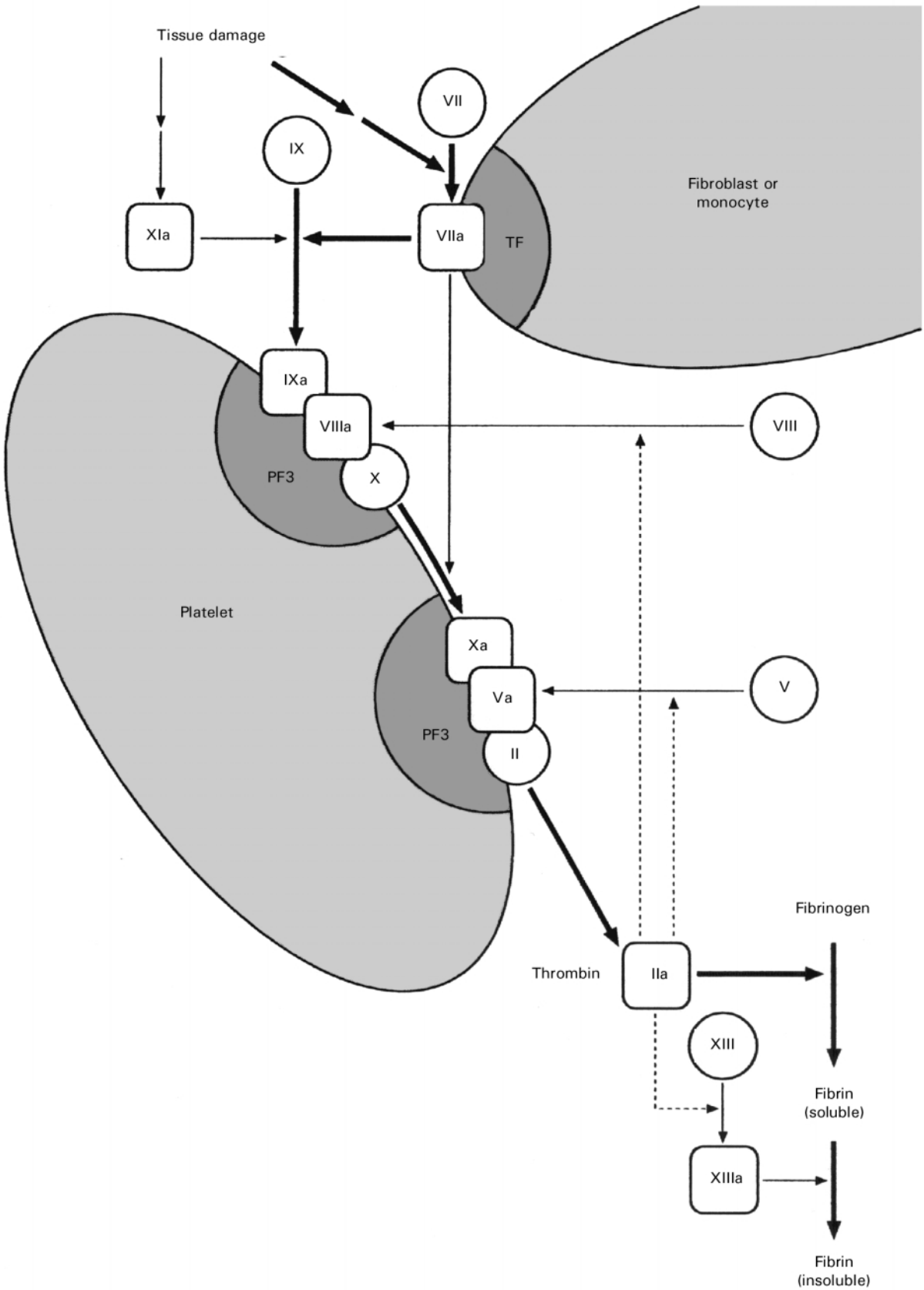

Figure 1. Current hypothesis of blood coagulation. Factor VIII is an essential cofactor to factor IXa in the activation of factor X. The requirement for calcium and possibly magnesium, as well as the regulating mechanisms of various inhibitors, have been omitted for clarity. PF3, platelet factor 3 (phospholipid); TF, tissue factor (phospholipid).

Factor VIII is a glycoprotein that acts in the coagulation pathway (Figure 1). It functions as a non-enzymatic cofactor in the activation of factor X by factor IXa in the presence of phospholipid and calcium. In plasma, it is noncovalently bound to, and stabilised by, von Willebrand factor. Factor VIII concentrates derived from human plasma contain several fully active forms of the protein with molecular masses ranging from 170 kDa to 280 kDa (Andersson et al 1986). Recombinant factor VIII SQ (r-VIII SQ) is a B-domain-deleted derivative of human factor VIII (Figure 2). It corresponds to the smallest active form, a metal ion linked 80- and 90-kDa heterodimer, present in therapeutic factor VIII concentrates (Lind et al 1995).

Factor VIII is administered by injection since the bioavailability from other routes of administration is unsatisfactory. Most patients with haemophilia administer their own treatment at home to control bleeding, and factor VIII must be given intravenously since even the subcutaneous route results in limited bioavailability (Spira et al 1995). Intravenous administration of the freeze-dried preparation requires several steps: reconstitution by transferring the solvent from its vial via a transfer needle to the dry powder, visual inspection of the reconstituted solution, drawing the solution into a plastic syringe through a filter needle, connecting the syringe to the infusion needle with tubing, finding a suitable injection site and checking that it is indeed intravenous, and slowly injecting the solution. All this has to be performed aseptically in many different situations by the patient, who may even be bleeding. Since treatment is often given several times a week, more convenient alternatives would be preferable. Subcutaneous or intramuscular injections require fewer steps and there are further injection aids available for both freeze-dried and liquid formulations (Prosser 1997). Hence, a factor VIII preparation administered subcutaneously would be desirable for both patients and physicians.

The fate of a molecule after subcutaneous injection depends, in part, on its size (Supersaxo et al 1990). Small molecules (under 16 000 Da), are able to cross the capillary wall and enter the bloodstream. Larger molecules preferentially enter the lymphatic capillaries and are transported through the lymphatic system into the bloodstream via the thoracic duct. The principal physiological role of the lymphatic system is the absorption of plasma proteins and particulate matter from the interstitial fluid and the return of these substances to the blood. In general, the chemical composition of lymph is very similar to that of plasma, but the concentrations of proteins are lower, typically 10–50% of the levels found in plasma (Stutman et al 1965; Yoffey & Courtice 1970). The uptake of interstitial fluid occurs across the thin endothelium of the initial lymphatic vessels via intercellular junctions (Hawley et al 1995). Large particles (above approximately 100 nm) are trapped in the interstitium and are not able to drain intact from the injection site.

Electron microscopy of rotary-shadowed factor VIII showed a large globular protein, 12–14 nm in diameter, with the B-domain extending as a rod-like structure upwards to 50 nm (Fowler et al 1990). The smaller size of the r-VIII SQ molecule (170 kDa, i.e. without the extending 50-nm B-domain of full-length factor VIII (280 kDa)),

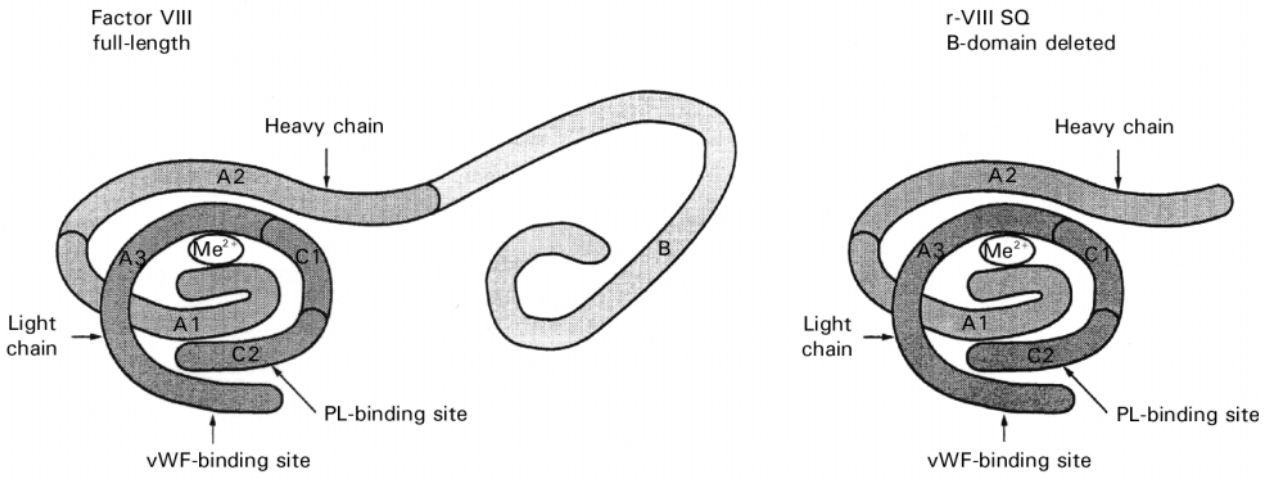

Figure 2. The relationship between recombinant factor VIII SQ (r-VIII SQ) and full-length factor VIII (adapted from Hoyer 1994). vWF, von Willerbrand factor.

should facilitate diffusion through the interstitium and absorption into the lymph capillaries (Figure 2). The size of r-VIII SQ is comparable with that of gamma globulin, 160 kDa, which has a subcutaneous bioavailability of at least 70% (Smith et al 1972). However, in several animal models a subcutaneous bioavailability of only 5–10% was noted for r-VIII SQ (Spira et al 1995).

The low bioavailability of subcutaneously administered factor VIII could have several causes: proteolytic degradation in the interstitium and in the lymphatic fluid; adsorption on tissue, in particular on acidic phospholipids such as L-α phosphatidyl-L-serine (phosphatidylserine); the absence of free von Willebrand factor in the interstitum; phagocytosis by macrophages in the interstitium or in the lymph nodes; and lastly, coagulation could be initiated upon injection. Subcutaneously administered growth hormone is, for instance, extensively degraded by proteases (Jorgensen et al 1988; Laursen et al 1996). The von Willebrand factor is known to protect factor VIII from degradation in plasma, but its size (1–12 MDa) hinders diffusion across the capillary blood vessels and its movement through the interstitium is probably restricted (Hamer 1986). This study was undertaken to investigate the first three aforementioned factors in-vitro (i.e., proteolytic degradation, adsorption on tissue, and the protective effect of von Willebrand factor). The influence of some macromolecular stabilisers and protease inhibitors on the stability of factor VIII activity (VIII : C) was assessed in tissue homogenates. Possible coagulation was prevented in these studies by the presence of both citrate and antithrombin.

Materials and Methods

Materials

Sodium chloride, calcium chloride dihydrate, L-histidine, polysorbate 80 (P80) and sucrose were of pharmacopoeial quality. Trisodium citrate dihydrate was of analytical grade. L-α Phosphatidyl-L-serine (phosphatidylserine) from bovine brain, 10 mg mL^{-1} in 95 : 5 chloroform : methanol, was purchased from Avanti Lipids and L-α phosphatidyl-L-choline (phosphatidylcholine) from egg freeze-dried powder, was purchased from SIGMA. The protease inhibitor set, containing bestatin (40 μg mL^{-1}), phosphoramidon (200 μg mL^{-1}), antipaindihydrochloride (50 μg mL^{-1}), aprotinin (2 μg mL^{-1}), pepstatin (10 μg mL^{-1}) and leupeptin (10 μg mL^{-1}), was used according to the manufacturer's instruction (Boehinger Mannheim Biochemicar, Germany). Von Willebrand factor was purified from Octonativ (Pharmacia & Upjohn AB, Sweden) to a final protein concentration of 0·51 mg mL^{-1}, corresponding to a von Willebrand factor : Ag of 142 IU mL^{-1} (Lind et al 1995). The antithrombin preparation Atenativ 500 IU (Pharmacia & Upjohn AB, Sweden) was reconstituted according to instructions. Tissue was obtained from a monkey (Cynomologus monkey) and pig (crossbred Yorkshire/Landrace/Hampshire). Human material was obtained during breast surgery and kindly donated by Karolinska Hospital, Sweden.

r-VIII SQ was produced by Chinese hamster ovary (CHO) cells cultivated in a serum-free medium. Purification was accomplished in several chromatographic steps, the final purification step being gel filtration, after which the preparation was formulated and freeze-dried. r-VIII SQ is highly purified and the specific activity is about 15 000 IU VIII : C (mg protein)$^{-1}$, $t\frac{1}{2}$ intravenously is 10–15 h.

In-vitro study

The stability of VIII : C in a tissue homogenate was investigated in-vitro. A 20% homogenate of subcutaneous tissue was prepared in physiological saline using an Ultra-Turrax homogeniser (Labassco, Sweden). To reduce the amount of intracellular content released during the homogenisation process, the material was centrifuged (3000 rev min^{-1} for 5 min) and then resuspended in a buffer containing 9 mg mL^{-1} sodium chloride, 3 mg mL^{-1} sucrose, 0·25 mg mL^{-1} calcium chloride dihydrate, 1·5 mg mL^{-1} L-histidine, 0·1 mg mL^{-1} polysorbate 80, 8 IU mL^{-1} antithrombin and 6 mM sodium citrate (buffer A).

r-VIII SQ was reconstituted according to the instructions and the reconstituted solution contained 200 IU mL^{-1}, 18 mg mL^{-1} sodium chloride, 3 mg mL^{-1} sucrose, 1·5 mg mL^{-1} L-histidine, 0·25 mg mL^{-1} calcium chloride dihydrate and 0·1 mg mL^{-1} polysorbate 80. The r-VIII SQ solution was preincubated 1 : 1 with buffer containing different stabilisers or protease inhibitors for 20 min at 37°C. Pre-incubated solution (100 μL) was then added to 0·005 mg (human) or 0·05 mg (monkey or porcine) of tissue suspended in 0·5 mL buffer A to reach a final concentration of VIII : C of 10–20 IU mL^{-1}. The samples were assayed after preparation and then incubated at 37°C. Samples (50 μL) were withdrawn after 3, 7 and 24 h.

In-vivo study

For the in-vivo study, 4 crossbred Yorkshire/Landrace/Hampshire pigs were used (7 weeks old,

approximately 30 kg). They were injected subcutaneously in the groin on days 1, 3, 5 and 7. The dose was 2000 IU kg^{-1} and plasma samples were withdrawn at 0, 1·5, 3, 6, 9, 11, 24 and 28 h. The study was terminated on day 8, before the development of anti-human factor VIII antibodies, estimated to occur after day 9.

Phospholipid vesicles

Phospholipid vesicles (phosphatidylserine/phosphatidylcholine; PS/PC) were obtained by mixing phosphatidylserine and phosphatidylcholine in a 50 : 50 (w/w) ratio. The solvent was evaporated overnight under reduced pressure and the dried lipid film was re-suspended in physiological saline to a final concentration of 10 mg mL^{-1}. The suspension was then sonicated for 5 × 2 min, on ice, with a probe sonicator (Misonix Inc., USA). The phospholipid vesicle size was determined by dynamic light scattering, also referred to as photon correlation spectroscopy (Zetamaster, Malvern Instruments, UK). The particle size by number was approximately 60 nm.

Mixed micelles consisting of phosphatidylserine and Polysorbate 80 (PS/P80) in a molar ratio of 1 : 4 were obtained by evaporating solvent from 0·2 mL phosphatidylserine (10 mg mL^{-1}) overnight and then adding 1·3 mL of a 10 mg mL^{-1} Polysorbate 80 water solution. The mixture was then sonicated (3 × 2 min) to facilitate dissolution (Misonix Inc., USA). The micelle size by number was approximately 15 nm.

Analytical methods

Factor VIII activity (VIII : C) was assayed by the chromogenic substrate method (Coatest, Chromogenix, Sweden). VIII : C refers to the biological function of the molecule, while factor VIII (or r-VIII SQ) refers to the molecule and VIII : Ag to the factor VIII antigen (Marder et al 1985). The VIII : C and VIII : Ag are expressed in international units (IU) as defined by the international concentrate standards (obtained from the National Institute for Biological Standards and Control (NIBSC), UK). The relative standard deviation of the assay is 4%. The recovery of VIII : C after storage is expressed as % recovery of the initial VIII : C value.

The amount of VIII : Ag was measured with an ELISA method specific for the heavy chain (90 kDa) of the factor VIII molecule. A microtiter plate was pre-coated with a monoclonal antibody (ESH5, American Diagnostica, USA) specific for antigenic determinants on the heavy chain of Factor VIII. Following incubation overnight, an alkaline-phosphatase-conjugated mouse monoclonal antibody (8A4-ALP, American Diagnostica, USA) was added. The enzyme activity was detected with an ELISA amplification system containing NADPH (Gibco BRL, Cat No 19589-019). The relative standard deviation of the assay was 10%.

Results and Discussion

VIII : C in tissue homogenates

The decrease of VIII:C was studied in tissue homogenates (Figure 3A). The decrease appeared to be more pronounced in monkey and human homogenates than in porcine homogenate. The kinetics also differed: pseudo-first-order kinetics were followed in the pig but a different process was observed in the monkey and human. Species-related protease populations or a varying content of phosphatidylserine in the cell membranes may have caused this. Variations in fat content of the subcutaneous tissue may also have influenced the results; the monkey tissue in particular seemed to be low in fat content. However, the decrease of VIII : C in the human tissue homogenate (Figure 3B) resembled the degradation profile seen in the monkey homogenate.

Liposomes and von Willebrand factor

The von Willebrand factor and phosphatidylserine interact with factor VIII physiologically, and therefore their effect on the stability of VIII : C in tissue homogenates was investigated.

Both von Willebrand factor and PS/PC liposomes showed a significant stabilising effect on VIII : C in tissue homogenates. The qualitative pattern was similar in porcine, monkey and human tissue (Figures 4A, B and C). The effects of PS/PC on the stability of VIII:C were particularly noteworthy. The results indicated that most of the decrease in VIII : C could be due to adsorption of r-VIII SQ on the phospholipids of cell membranes. It should be noted that no attempt was made to incorporate r-VIII SQ into the liposomes, but it cannot be ruled out that protein molecules were protected from enzymatic degradation by spontaneous diffusion into the liposome interior. The liposomes were added to the protein solution and incubated for 20 min at 37°C before mixing with the tissue homogenate.

The von Willebrand factor protects factor VIII from proteolytic degradation in plasma and also from activation by factor Xa and from inactivation

by factor C (Hamer 1986; Koedam et al 1987). Deficiency of von Willebrand factor substantially shortens the half-life of factor VIII in plasma. Von Willebrand factor is a multimeric glycoprotein with a molecular mass ranging from 1000 kDa to 12 000 kDa containing disulphide-linked subunits of about 240 kDa. Upon activation of factor VIII, thrombin cleaves the light chain, thereby releasing the A3 domain and von Willebrand factor (Figure 1). Activated factor VIII binds to phosphatidylserine on activated platelets and forms the tenase complex with factor IX and factor X (Hemker & Kahn 1967; Hemker et al 1970). The binding site for phosphatidylserine on intact factor VIII is in close proximity to the binding site for von Willebrand factor since the von Willebrand factor prevents factor VIII from binding to phospholipids. PS/PC liposomes have been used as models to simulate the phospholipid surface provided by platelets in normal haemostasis (Hemker et al 1970). The affinity of factor VIII for von Willebrand factor (Kd, the dissociation constant, = 0·2 nM) is about 10 times its affinity for synthetic phospholipid vesicles (Kd = 2 nM) or the activated platelet surface (Kd = 2·9 nM) (Fay 1993; Gilbert et al 1990).

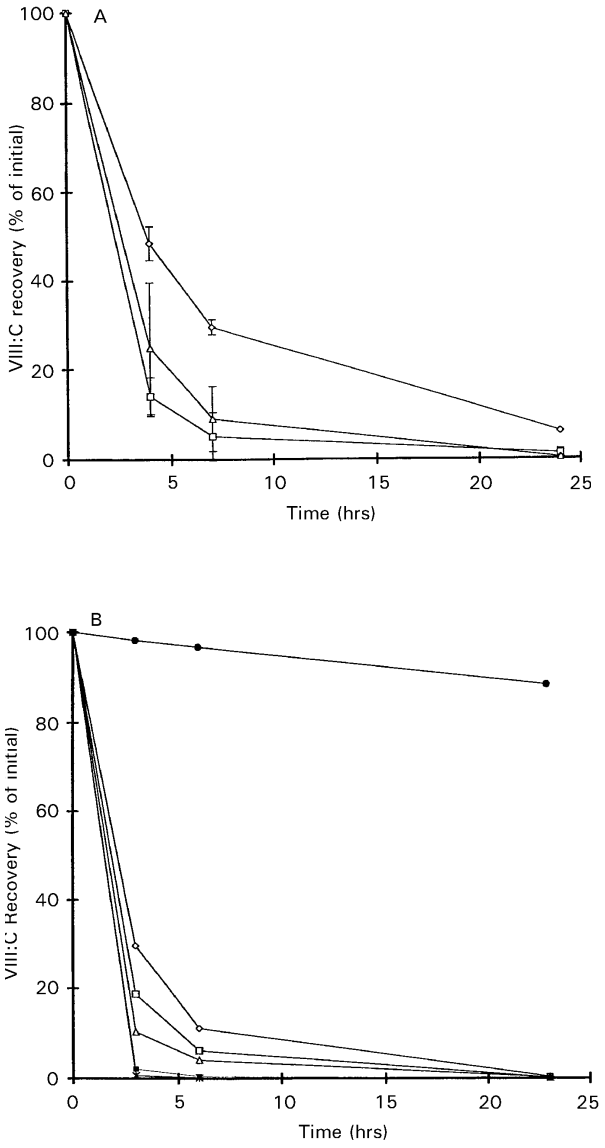

Figure 3. Recovery of VIII : C after incubation with (A) subcutaneous tissue homogenate (◇, porcine; □, monkey; and △, human) and (B) in varying amounts of human tissue homogenate (◇, 0·005 g; □, 0·02 g; △, 0·03 g; ■, 0·05 g; *, 0·1 g; and ●, buffer reference).

There is one recombinant factor VIII preparation on the market containing von Willebrand factor (Kogenate, Bayer/Miles), which could possibly facilitate subcutaneous administration. However, the factor VIII molecule in Kogenate is full-length factor VIII, so its size could restrict diffusion through the subcutaneous space.

In this study, PS/PC liposomes were comparable with von Willebrand factor, which is thought to protect factor VIII from enzymatic degradation, in stabilising VIII : C. The binding site for phosphatidylserine on the factor VIII molecule is in close proximity to the binding site for von Willebrand factor (Andersson & Brown 1981). It is therefore possible that the protective effect of von Willebrand factor also results from prevention of adsorption on phospholipid surfaces, although this will not be as complete since the phospholipid binding site on r-VIII SQ is only partly covered.

In-vivo study

An exploratory in-vivo study was made in the pig with phosphatidylserine in two formulations: either in the form of PS/PC liposomes or as PS/P80 in the form of mixed micelles. The porcine model was chosen due to the availability of a relevant analytical method. The VIII : C method could not be used since it also measures porcine endogenous factor VIII. An antigen ELISA was used containing antibodies specific for the intact r-VIII SQ molecule. The dose was 2000 IU kg^{-1}, which corresponds to 40–200 times the current intravenous dose used in the clinical setting. Since only a small number of specimens were included in this preliminary investigation, the results cannot be considered significant. However, in general, the formulations containing phosphatidylserine appear to increase the availability of subcutaneously administered r-VIII SQ in the pig (Table 1). Further studies are necessary, preferably in the monkey

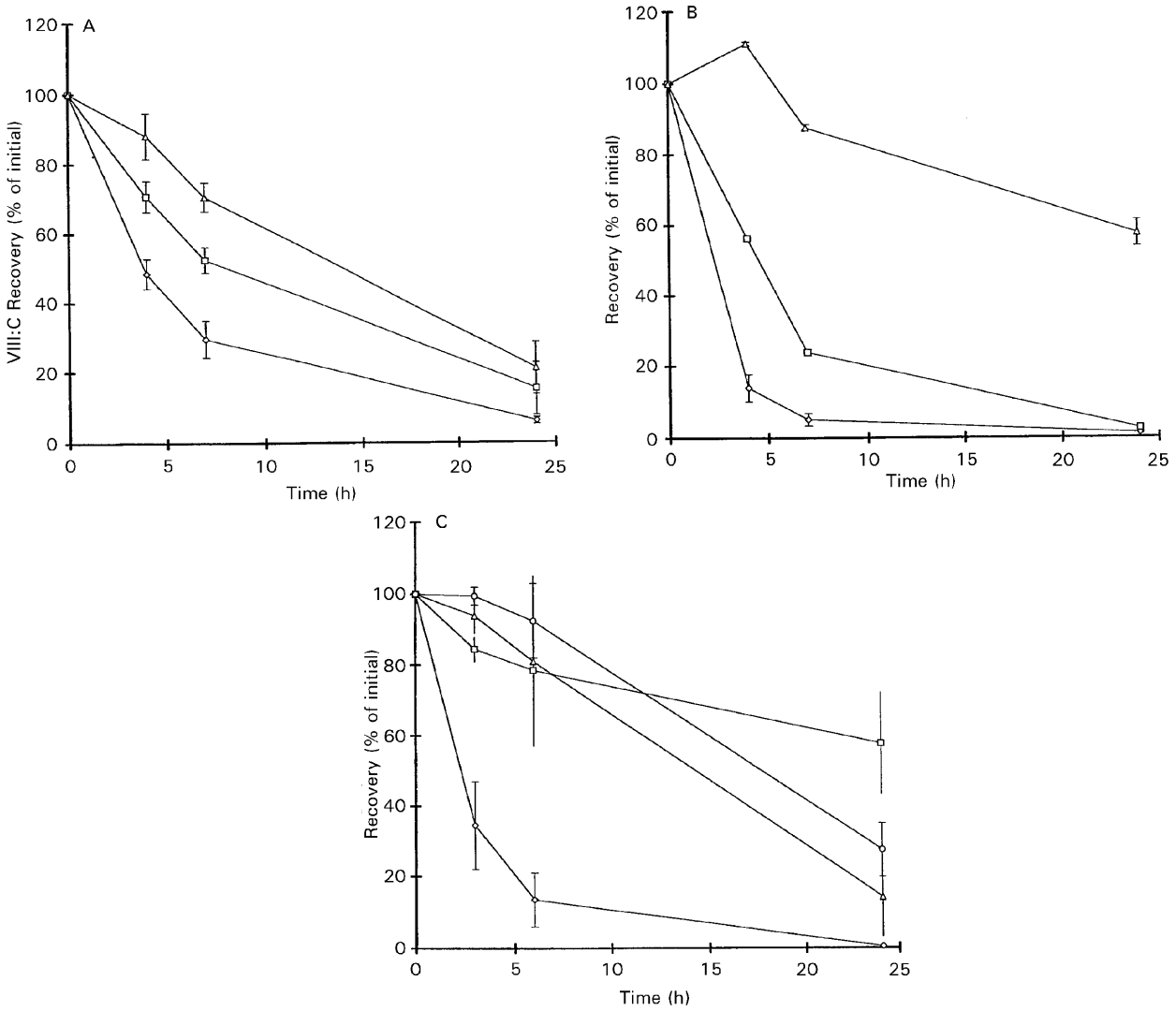

Figure 4. Recovery of VIII:C after incubation with subcutaneous tissue homogenate. A. ◇, porcine reference; □, von Willebrand factor and △, PS/PC as additives. B. ◇, monkey reference; □, von Willebrand factor; and △, PS/PC as additives. C. ◇, human reference; □, von Willebrand factor; △, PS/PC and ○, von Willebrand factor and PS/PC as additives.

where in-vitro studies indicate a closer resemblance to the human.

The average size of the liposomes used in this study was 60 nm. The size of the liposomes should be under 100 nm if they are to migrate from an injection site as intact entities (Hawley et al 1995; Patel 1988). However, it is possible that even trapped liposomes could exert an effect by protecting the protein from proteolytic attack or adsorption on tissue, while acting as a drug reservoir with an extended-release profile. Smaller liposomes will drain from the injection site and enter the lymphatic system, but most might be trapped in the lymph nodes and phagocytosed by macrophages. The negative charge on some phospholipids (e.g. phosphatidylserine) greatly enhances their binding to, and phagocytosis by, macrophages (Hawley et al 1995). If capture in the lymph nodes is to be avoided, colloids should be small or surface-modified, or both.

Table 1. In-vivo studies: area under the curve after subcutaneous injection of r-VIII SQ in the pig.

	Formulation		
	Reference	PS/PC liposomes	PS/P80 micelles
Pig 1	3·6	4·7	12·0
Pig 2	3·8	8·0	5·6
Pig 3	2·2	4·8	6·6
Pig 4	2·5	2·8	4·6
Average	3·0	5·1	7·2
s.d.	0·8	2·2	3·3

The response was measured with VIII : Ag (90 kDa) ELISA in blood plasma samples. Dose = 2000 IU kg^{-1}. Samples were withdrawn at 0, 1·5, 3, 6, 9, 11, 24 and 28 h.

Table 2. Recovery of VIII : C (% of initial) in homogenate prepared from human subcutaneous tissue with different additives.

Additives	Time (h)			
	0	3	6	23
von Willebrand factor and PS/PC	100	100	93	27
Phosphoramidon and PS/PC	100	102	90	28
PS/PC liposomes	100	94	81	14
von Willebrand factor	100	84	79	58
Phosphoramidon/bestatin/antipaindihydrochloride	100	61	43	4
Phosphoramidon/antipaindihydrochloride/leupeptin	100	46	24	2
All 6 protease inhibitors together	100	44	23	2
Antipaindihydrochloride	100	43	15	0
Leupeptin	100	42	19	0
Phosphoramidon	100	42	21	1
Aprotinin	100	36	15	0
Pepstatin	100	26	7	0
Bestatin	100	26	8	0
Leupeptin/pepstatin/aprotinin	100	24	6	0
No additive (homogenate reference)	100	30	11	0

Quantities of additives/tissue homogenate: von Willebrand factor 31 IU mL^{-1}; PS/PC 5 mg mL^{-1}; bestatin 40 μg mL^{-1}; phosphoramidon 200 μg mL^{-1}; antipaindihydrochloride 50 μg mL^{-1}; aprotinin 2 μg mL^{-1}; pepstatin 10 μg mL^{-1}; leupeptin 10 μg m^{-1}; tissue homogenate 0·005 mg.

Proteases

The influence of protease inhibitors (aprotinin, pepstatin, bestatin, leupeptin, phosphoramidon and antipaindihydrochloride) on the stability of VIII : C in human tissue homogenates was investigated. Aprotinin is specific for serine proteases, pepstatin for aspartate proteases, bestatin for amino peptidases, leupeptin for serine and cysteine proteases, antipaindihydrochloride for papain, trypsin and cathepsin A and B and phosphoramidon for metallo-endopeptidases, specifically thermolysine. A combination of several protease inhibitors seems to have a protective effect on the stability of VIII : C, especially the combination phosphoramidon/bestatin/antipaindihydrochloride (Table 2). However, this effect was not as pronounced as was the addition of von Willebrand factor and PS/PC. Furthermore, the feasiblity of using a protease inhibitor in a clinical setting is questionable, since large amounts will probably be required for an effect in-vivo.

An attempt was made to saturate proteases by addition of a surplus of proteins (albumin, collagen) and to prevent surface adsorption with some surface-active macromolecules (Polysorbate 80, sodium cholate, dextran), but no effect was seen (data not shown). The only investigated stabilisers that had a pronounced effect were von Willebrand factor and, to an even greater extent, phosphatidylserine. It is known that enzymatic stability, against both physical and proteolytic degradation, can be increased by binding to a substrate or a ligand (Shrake et al 1984; Tsai et al 1993). This could be a factor contributing to the increase in stability of VIII : C shown here.

In conclusion, a proposed inactivation mechanism for r-VIII SQ in subcutaneous tissue could be adsorption on phospholipid surfaces, followed by proteolytic degradation. However, additional studies are required due to the multitude of factors influencing the subcutaneous absorption route. Combinations of protease inhibitor(s) together with phosphatidylserine-containing liposomes are suggested for further investigation, preferably in a monkey animal model.

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