



Factor VIII manufactured from plasma—the ups and downs, and the up again: a personal journey—part 2: aspects of factor VIII manufacture from plasma

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Abstract: Despite the advances in the manufacture of plasma derived FVIII (pd-FVIII), there has been practically no improvement in the yield of biological activity over the past 20 years, with 80% of the activity in the donated plasma being lost over the process. In this review, I look back on the decades of scientific progression of pd-FVIII development, including 40 years of personal involvement, with the intention of proposing routes to improved understanding and manufacture.

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The journey of Factor VIII in plasma—from donor to concentrates

Factor VIII in donors

The role of ABO blood groups

The effect of ABO blood groups on FVIII levels has been well described (1) and has been suggested to involve the secretor locus (2). The possibility of selecting plasma donors with high blood group A-associated FVIII levels was also raised in the 1980s (3,4). The practical difficulties in restricting plasma donors intended for FVIII manufacture to group A donors disallowed this approach. Concurrently, the occasional hemolytic adverse effect of contaminating isoagglutinins in early FVIII concentrates (5) led to at least one manufacturer KABI Vitrum in Sweden, now part of Octapharma supplying blood group specific plasma derived FVIII (pd-FVIII) manufactured solely from group A or group O donors). Experiments in Chris Prowse's laboratory in the early 1980s indicated that both FVIII:C and VWF, as measured through their respective antigens, were associated with group A substance (*Figure 1*).

The stimulation of donors to increase plasma FVIII levels

Mannucci's seminal work on the use of 1-Deamino-8-d-arginine vasopressin (DDAVP) for treating mild haemophilia A and von Willebrand disease (VWD) through the release of endogenous stores (6) led to the application of this agent to increase FVIII and von Willebrand factor (VWF) levels in normal individuals (7) (*Figure 2*). By increasing the FVIII level in blood donors, this approach could increase FVIII yields in cryoprecipitate using a variety of protocols for treating haemophilia A (8-10) and VWD (11). Issues around the ethics of administering DDAVP led to the discontinuation of this promising development. It is unlikely that the stimulation of donors with DDAVP has had any significant adverse effects and the use of agents to elicit hyperimmune antibodies and hematopoietic stem cells (12,13).

Factor VIII in plasma

The development of the first assays of FVIII confirmed

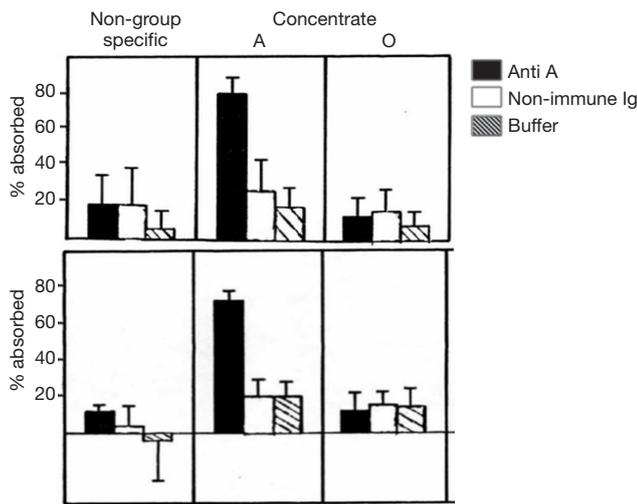


Figure 1 Absorption of FVIII and VWF from group specific concentrates on immobilized antibodies. Upper panel, FVIII Ag; lower panel, VWF Ag (Farrugia, 1984).

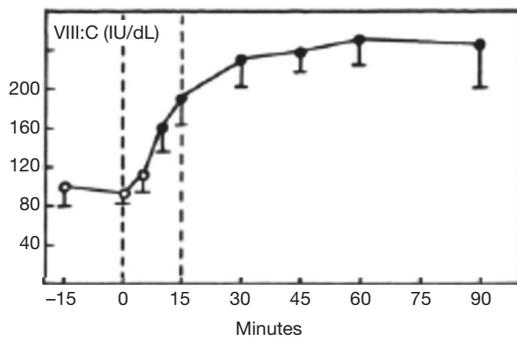


Figure 2 Changes in FVIII following 0.3 µg/kg in normal men (From Prowse *et al.*, 1984).

the early observations that the biological activity in plasma under blood bank conditions was extremely labile (14). Since the storage conditions of banked blood demanded refrigeration to preserve red cells and minimize bacterial growth, most early workers studied the stability of FVIII in banked blood at 4 °C, in order to assist the harvesting of FVIII from blood donations. This established the “bi-phasic” decay of FVIII, with a “golden window” within the first 6 hours after collection, when FVIII levels fall faster than during the subsequent period. We confirmed this in the early 1980s, but we also observed that refrigeration of whole blood resulted in losses of FVIII through cryoprecipitation, which were then lost into the red cell fraction when plasma was separated. These losses could be

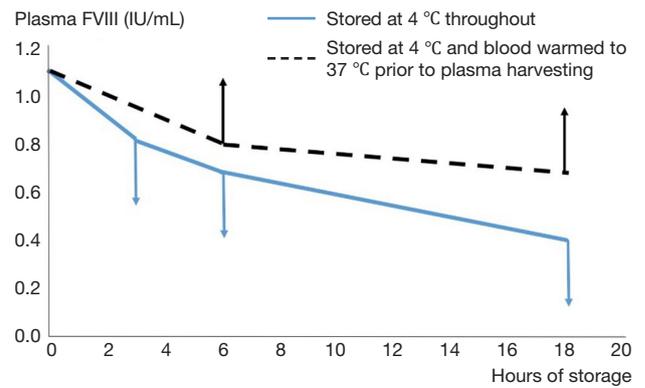


Figure 3 FVIII levels in blood collected and stored at 4 °C. 10 mL samples were centrifuged at the time points shown and assayed for FVIII. Further details in Farrugia 1984.

recovered through warming the blood prior to recovery of the plasma (Figure 3) (15).

These observations demonstrate the tension which has always existed when the raw material for pd-FVIII manufacture is plasma recovered from whole blood donations. The competing needs for red cell and FVIII preservation have continued to “favour” red cells, as is to be expected in the mainstream transfusion context. It does accentuate the advantages which are accruable if plasma destined for pd-FVIII manufacture is harvested through plasmapheresis. Another aspect studied intensely has been the role of preservative, or anticoagulant, for the initial collection. This merits a section to itself.

Factor VIII and “M²⁺”

Weiss’ work from over fifty years ago demonstrated that the stability of FVIII was dependable on divalent metal ions, and that increasing levels of chelating anticoagulant affected the FVIII in plasma substantially (16). For many years it was thought that this was due to calcium ions, but recent developments involving our current knowledge of the molecular structure of FVIII (Figure 4) suggests that the divalent metal ion mediating the association between the two chains of FVII may also include copper (17). Our own studies suggested that adding Ca²⁺ with heparin cover to prevent clotting allowed the recovery of FVIII activity in stored blood after 3 hours but not after 18 hours, when the lost activity was irrecoverable (Figure 5).

Studies by Rock *et al.* (18) attempted to exploit this dependence through collection of blood in heparin, in order

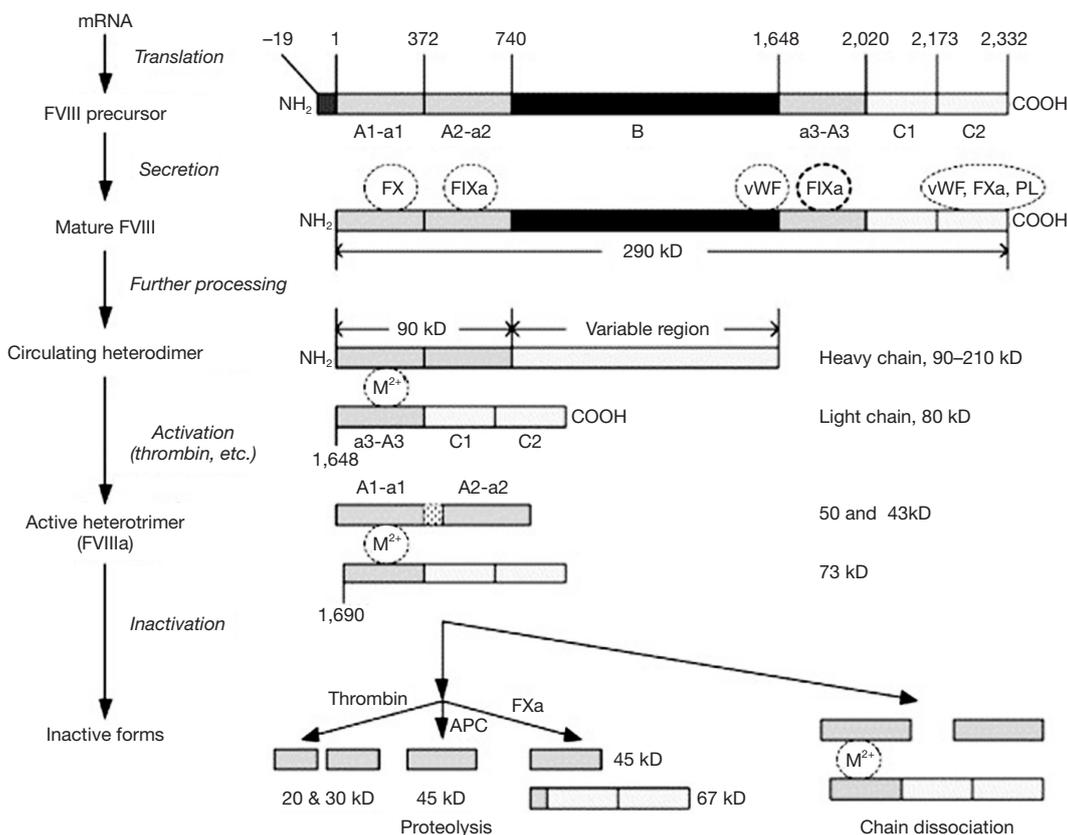


Figure 4 FVIII structure, function, and processing. The sites of FVIII interaction with other clotting factors, vWF, phospholipids (PL), and metal ions (M^{2+}) are illustrated by dotted circles (from Wang, 2003).

to retain physiological M^{2+} levels, but this would affect the capacity to harvest additional proteins. Weiss' work (16) suggested that the citrate concentrations achieved in standard anticoagulants were in excess of what was needed to prevent gross coagulation. We therefore approached this issue by a number of alternative ways [Farrugia, 1984 (15)], finally settling on the collection of blood into citrate anticoagulants with half the standard citrate concentration—half-strength citrate [$\frac{1}{2}$ citrate-phosphate-dextrose (CPD)]. This stabilized FVIII in plasma significantly (Table 1) (19).

But...does it matter really?

Preserving FVIII in plasma to ensure high levels in frozen raw material would be anticipated to result in final FVIII yields which are commensurately improved. Similarly, the modifications in formulation of plasma and intermediate fractions to preserve Ca^{2+} levels which are compatible with FVIII would have little practical use if they were not able to

achieve higher yields in FVIII purification to products. As an example, the logistical efforts (and expense) involved in ensuring rapid separation and freezing of recovered plasma within the “golden window” to ensure that the FVIII levels are high would be of little use if the higher plasma FVIII are not recovered downstream, compared to plasma frozen outside the “golden window”. Yes, there is little evidence to suggest that this may be the case; plasma frozen within the “golden window” does yield higher FVIII levels in the cryoprecipitate, but this is not always reflected into final concentrate (20-22) (Table 2).

One well known authority has addressed this issue with some well-justified skepticism (23), describing manufacturing problems encountered with the use of $\frac{1}{2}$ CPD. Experience with blood bank cryoprecipitate (Table 3) (19) and small-scale models (Figure 6) (24) indicates yield improvements which are reflected downstream of the initial plasma. In large scale manufacture, formulating in-process intermediates to higher Ca^{2+} levels resulted in improved

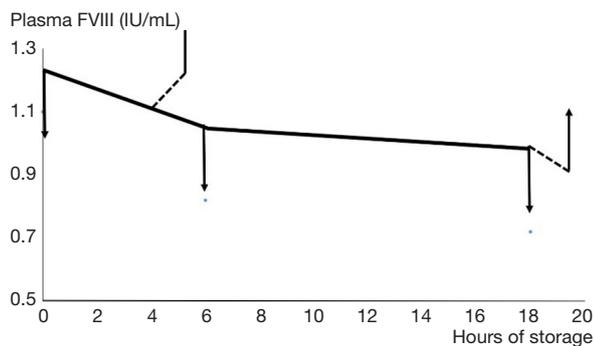


Figure 5 Recalcification of blood in donations collected in standard citrate-phosphate-dextrose (CPD) anticoagulant. A Ca^{2+} /heparin solution (10 mM/5 U/mL final concentration) was added to the blood at 3 and 18 hours post donation, and plasma was separated and assayed for FVIII at 6 and 21 hours.

Table 1 Effect of citrate concentrations on FVIII in plasma (Prowse *et al.*, 1987)

Estimated plasma citrate (mM)	Residual percentage FVIII:C		Median FpA (ng/mL)
	3 hours	23 hours	
20	100	71±9	40
16	82±10	76±9	30
12	105±13	84±9	28
10	112±22	80±13	25
8	118±15	92±18	17

FpA, fibrinopeptide A.

FVIII stability (25), but limited data using ½ CPD did not confirm higher yields further downstream (26). It is probable that the two factors assessed in this section—rapid freezing of uncooled plasma and low citrate levels in machine-delivered anticoagulants (27-29)—are converging to result in the higher FVIII yields which current era fractionators know (but seldom publish) are derived with plasmapheresis, as compared to recovered, plasma.

From plasma to cryoprecipitate

What makes cryoprecipitate?

The production of cryoprecipitate has produced a voluminous, literature, although not well maintained. An area of interest in attempting to optimize cryoprecipitate production is to understand the actual mechanism of

Table 2 FVIII production—plasma frozen 6 and 18 hours post donation (Hughes *et al.*, 1988)

Production stage	6-hour plasma (FVIII yield IU/kg plasma)	18-hour plasma (FVIII yield IU/kg plasma)
Cryoprecipitate	630±195	428±140
Intermediate purity concentrate	203±19	192±16

this phenomenon. A hypothesis based on conventional eutectics led Polson (30) to propose that a simple “salting out” of proteins through the increasing salt concentrations generated by the freezing of plasma was involved. Mackenzie’s work (31) cited by McIntosh *et al.* (32), based on electric resistivity measurements in plasma and other colloid solutions, indicated that there was no simple eutectic transition, and was confirmed by McIntosh’s measurements showing no evidence of any eutectic freezing (Figure 7) (32). Our own work increasing the protein concentration of plasma through the addition of albumin suggested that a similar concentration effect of the hydrophilic protein albumin might contribute to a differential precipitation of the least soluble plasma proteins in the cold (33) (Figure 8). Additional studies indicated the crucial role of the cold insoluble proteins themselves, in the form of fibrinogen and fibronectin, in the cryoprecipitation of FVIII (Table 4); or rather FVIII complexed to VWF, as Over’s earlier work had shown that cryoprecipitate from VWD plasma was deficient in FVIII (35).

Cryoprecipitate—the processing of plasma

Plasma destined for FVIII production needs to be frozen within a timeframe which preserves recoverable FVIII. Currently available blast freezers were not available in the early years of development, but a variety of methods were shown to be adequate as far as cryoprecipitate yields were concerned (36). In our work in the early 1980s, freezing in $-40\text{ }^{\circ}\text{C}$ cabinet freezers gave inferior results to faster freezing achieved in ethanol/dry ice baths (37) (Figure 9).

Once frozen, the storage conditions of plasma require the optimization of FVIII yield and cryoprecipitate quality, compatible with its further purification. Storage temperatures of $-20\text{ }^{\circ}\text{C}$ appear satisfactory (37), but the maintenance of steady temperatures during frozen storage are important. Temperature insults of the frozen plasma,

Table 3 Effect of plasma citrate on [Ca²⁺] and cryoprecipitate FVIII yields (Prowse *et al.*, 1987)

Production phase	Standard citrate		Half-strength citrate	
	Plasma frozen 3 hours post donation	Plasma frozen 18 hours post donation	Plasma frozen 3 hours post donation	Plasma frozen 18 hours post donation
Plasma				
FVIII:C (IU/mL)	0.86±0.06	0.56±0.11	1.01±0.13	0.89±0.05
Ca ²⁺ (µM)	50±5	48±6	95±2	95±5
Cryoprecipitate FVIII:C (IU from 100 mL of plasma)				
Fast-thaw method	54±7	44±8*	55±5	53±8
Thaw-siphon method	67±20	41±11	69±10	75±7
All methods	60±15	42±9**	62±11	64±14

*, P<0.05 for difference between 3-hour and corresponding 18-hour units; **, P<0.01 for difference between 3-hour and corresponding 18-hour units.

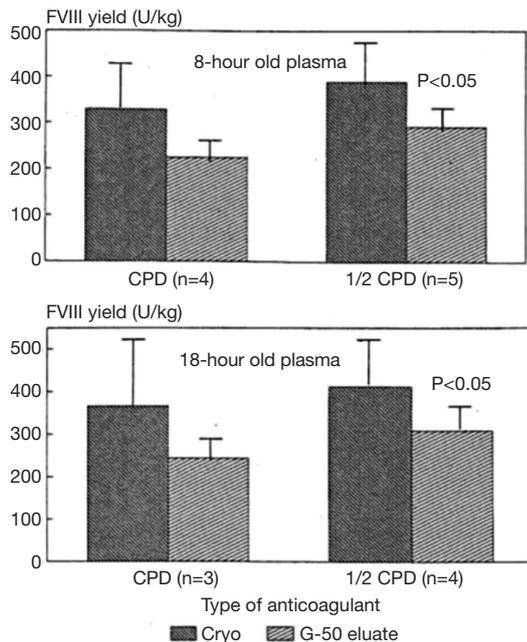


Figure 6 Effect of anticoagulant on FVIII yield in fractionation to small scale FVIII intermediate purity FVIII concentrate (from Farrugia *et al.*, 1990). CPD, citrate-phosphate-dextrose.

such as may be encountered during power cuts have some effect on cryoprecipitate FVIII yield but have a much more marked effect on the fibrinogen content of the cryoprecipitate (37) (Figure 10).

The generation of cryoprecipitate on an industrial scale requires the frozen plasma packs to be conditioned to a state which will allow the plasma to be thawed under controlled conditions. The frozen plasma has to be softened to a

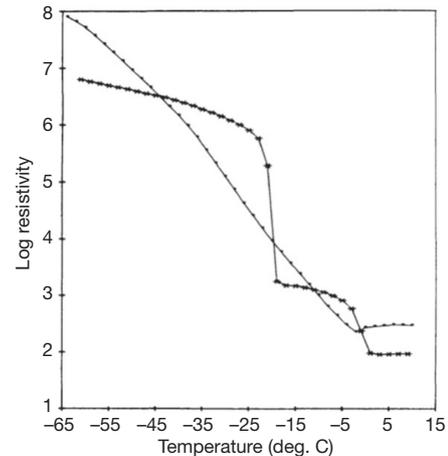


Figure 7 Resistivity of frozen plasma and normal saline. Measurements made during slow thawing (at room temperature) after fast freezing (in solid CO₂) (from McIntosh, 1990).

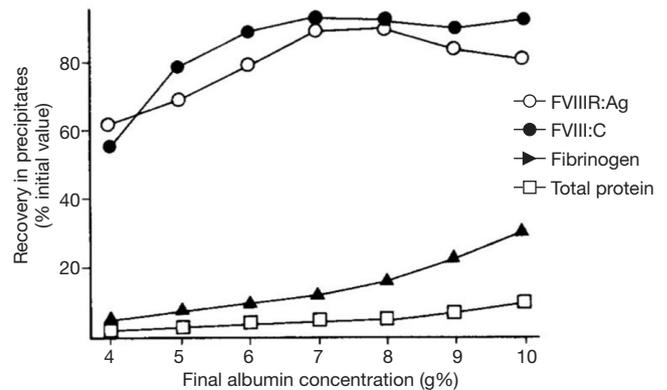


Figure 8 Precipitating effect of albumin on human plasma at 0 °C (from Farrugia, 1984).

Table 4 Effect of plasma composition on cryoprecipitation (from Farrugia, 1992) (34)

Plasma composition	Yield in cryoprecipitate (% of plasma) (mean ± SD for six experiments)			
	FVIII:C	VWF Ag	Fibrinogen	Fibronectin
Physiologic ionic strength	47±4	64±12	34±17	56±22
Low ionic strength	60±13	51±6	42±14	34±12
High ionic strength	5±2	31±19	19±7	89±47
Physiologic protein content	53	84	50	94
Low protein content	Unmeasurable	10	2	2
Physiologic fibrinogen level	47±8	73±24	55±28	68±26
Low fibrinogen	11±6	11±6	92±33	13±26
Physiologic fibronectin level	42±4	48±20	32±6	88±23
Low fibronectin	3±1	5±3	2±1	25

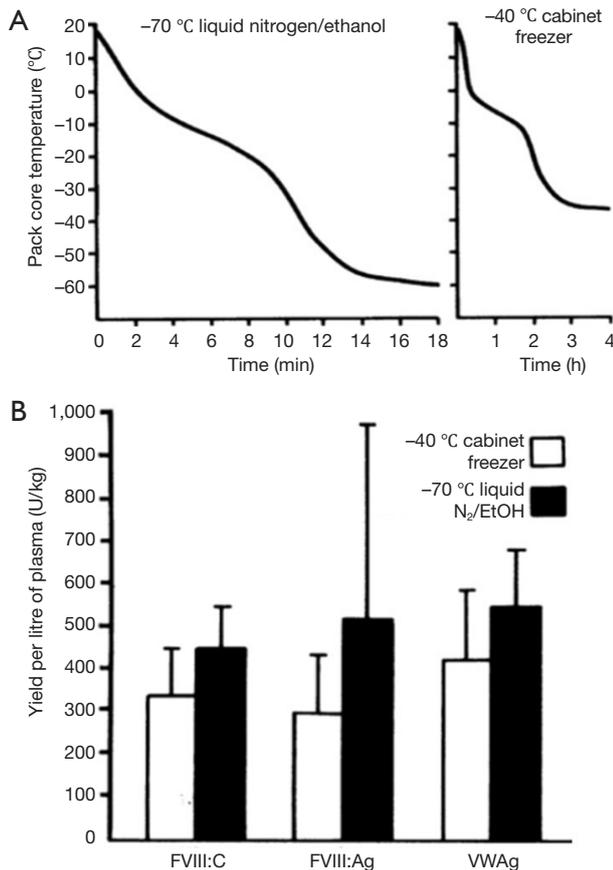


Figure 9 Effect of plasma freezing rates on FVIII yields and distribution in cryoprecipitate. (A) Temperature recorded in plasma packs frozen in different media; (B) distribution of factor VIII related activities in cryoprecipitate derived from plasma frozen at different rates. Mean ± SD of six experiments expressed as U/kg of starting plasma (from Farrugia and Prowse, 1985).

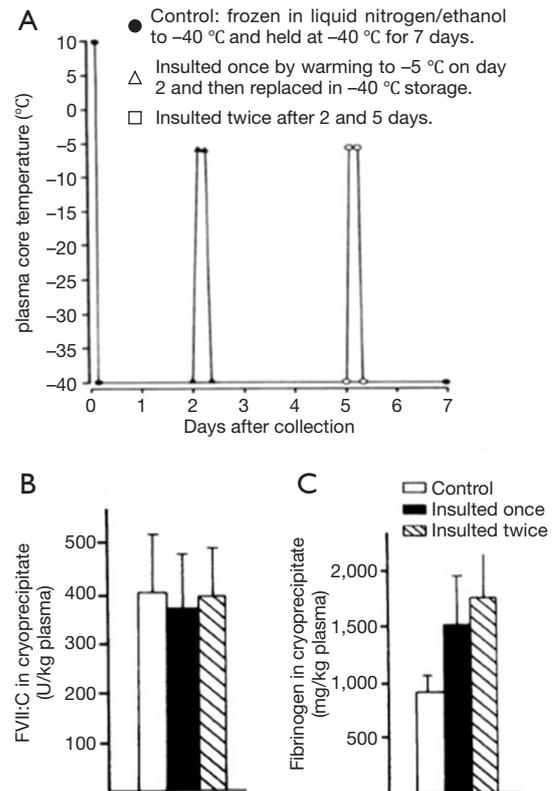
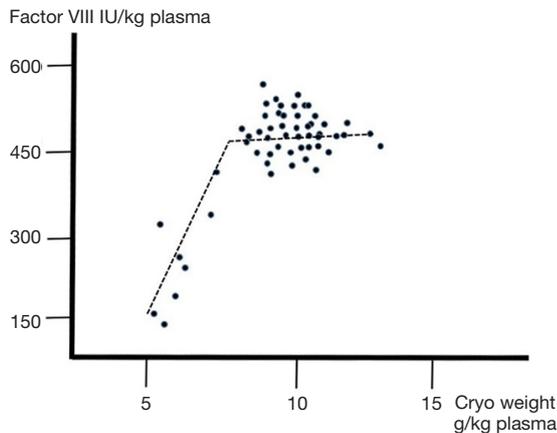


Figure 10 The effect of temperature fluctuations during plasma storage on cryoprecipitate (from Farrugia and Prowse, 1985). (A) Temperature recorded in plasma cores; (B,C) effect of temperature insult (warming and refreezing) on cryoprecipitate preparation: (B) yield of VIII:C in plasma fractions; (C) yield of fibrinogen in cryoprecipitate. Mean and SD for six experiments. All units were processed to cryoprecipitate 7 days after initial plasma freezing.

Table 5 Effect of plasma-softening on factor VIII production (Farrugia *et al.*, 1992)

Softening method [n]	Cryoprecipitate weight (g/kg plasma)	Fibrinogen (mg/kg plasma)		FVIII (IU/kg plasma)	
		Cryoprecipitate	Final eluate	Cryoprecipitate	Final eluate
Warm [10]	10.9±0.61	1,200±247	90±33	435±51	276±42
Cold [9]	8.8±0.12	800±148	30±6	411±68	264±29
None [7]	7.9±0.07	480±140	28±3	452±77	250±26

**Figure 11** The effect of cryoprecipitate weight on FVIII yield. Data from modeled bulk cryoprecipitate production from 10 kg lots of plasma.

higher temperature to allow detachment of the plasma from the plastic pack. The softened but still frozen plasma is then thawed. Extending the observations made when studying the conditions for storing frozen plasma for blood bank cryoprecipitate, we and others (38,39) developed conditions for conditioning plasma which minimized the fibrinogen content of cryoprecipitate, thus facilitating its extraction and purification to concentrate (40) (Table 5). This approach allows the fibrinogen content of cryoprecipitate to be adjusted to low levels as required in fractionation to pd-FVIII, and increased to heighten the efficacy of blood bank cryoprecipitate as a source of therapeutic fibrinogen.

The effect of plasma conditioning on cryoprecipitate weight has also been described for large-scale manufacture [table 3 in (32) confirming the independence of FVIII yield from the weight of solid cryoprecipitate]. Studying this relationship some years ago, I concluded that this independence held as long as cryoprecipitate weights did not go below 7 g/kg of plasma. Once weights became lower than this level, losses of FVIII were increasingly observed, indicating that

the structure of the solid cryoprecipitate was insufficiently robust to retain the FVIII (unpublished observations, Figure 11). It is important to note that FVIII is an incidental “contaminant” in cryoprecipitate, whose bulk composition is other proteins. I continue to be surprised at fractionation chemists in today’s modern plants who express astonishment when told that cryoprecipitate from people with haemophilia forms as easily as from normal donors!

The thawing of industrial scale lots of frozen plasma to cryoprecipitate was shown by the group under Peter Foster at the Protein Fractionation Centre to result in optimized FVIII yields when using continuous thawing (41), analogous to the “thaw-siphon” technique for blood bank cryoprecipitate developed in Brisbane and Edinburgh (42,43). I reflect that these principles, both at blood bank scale and at industrial scale, achieved FVIII yields in cryoprecipitate approaching 600 IU/kg. This indicates that, if progress had been made at commensurate levels in the further purification of FVIII, yields would exceed the apparently immutable 200 IU/kg. But, events, particularly the need to develop viral inactivation, overtook these developments, and it appears that the dominance of recombinant products has dissuaded fractionators from continuing to study the optimization of FVIII.

Final reflections

For the generation of patients born after the 1980s, pd-FVIII, at least in the developed economies, has little relevance. And for the scientists engaged in the rapidly consolidating Western plasma industry, dominated by companies which generate most of their revenue from recombinant products, pd-FVIII is rapidly assuming the status of a quaint anachronism.

Balancing this perspective is the importance of pd-FVIII in delivering care to haemophiliacs in the developing world. Efforts are continuing to attempt to divert the surplus of pd-FVIII in publicly funded blood systems to areas which



Figure 12 The author in his laboratory in the Edinburgh and South East Scotland Blood Transfusion Service in 1983.

need them and cannot afford the sophisticated, but hugely expensive, biotechnology products (44). Hence, pd-FVIII is still important and life-saving.

As discussed in the accompanying paper, the SIPPET study (ref) has only been the most recent addition to an impressive body of evidence that pd-FVIII results in a lower incidence of inhibitors in previously untreated patients (PUPs) than recombinant FVIII (rFVIII). This evidence cannot continue to be ignored. Until a final cure is achieved for haemophilia A, the problem of inhibitors demands the implementation of any measure which can lessen it. The patients deserve nothing less.

As for this author, I am happy with my career with factor VIII. The fresh-faced researcher who thawed plasma in Edinburgh 35 years ago (*Figure 12*) has seen much in the field since then. And pd-FVIII was up at that time, then it went down...and now, happily, it is up again.

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I thank Dr. Chris Prowse, in whose laboratory at the Scottish National Blood Transfusion Service in Edinburgh I performed most of the studies in the 1980s cited in this paper. Dr. Peter Foster, the then head of Research and Development at the Protein Fractionation Centre (now closed, alas!) and Dr. James Smith, then head of Research and Development in the Plasma Fractionation Laboratory in Oxford (also no longer existing) taught me most of what I know about the practical aspects of manufacturing

FVIII. All these colleagues are now enjoying a well-earned retirement, and it is perhaps time to join them. In the meantime, I hope that their wisdom is somewhat reflected in this paper, and that the new generation of fractionators may benefit from it.

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