Vox Sang. 55: 81–89 (1988)

0042–9007/88/0552–0081 $2.75/0

Studies on the Stability of VIII:C during the Manufacture of a Factor VIII Concentrate for Clinical Use[1]

Peter R. Foster[a], *Ida H. Dickson*[a], *Thomas A. McQuillan*[a], *Christopher V. Prowse*[b], *Frank E. Boulton*[b], *Prema Greedharry*[c], *Arthur L. Bloom*[c]

[a]Scottish National Blood Transfusion Service, Protein Fractionation Centre, Edinburgh; [b]South-East Scotland Blood Transfusion Service, Royal Infirmary, Edinburgh; [c]Department of Haematology, University Hospital of Wales, Cardiff, UK

Abstract. The stability of VIII:C was investigated by monitoring samples taken at different points from a routine process for the manufacture of factor VIII concentrate and by examining the stabilising influence of a number of product formulations. Loss of VIII:C over process-finishing procedures (formulation, 0.22 µm filtration, dispensing) was associated with a citrate-induced inactivation which could be prevented by controlling the ionised calcium concentration of the solution. These results were obtained using a one-stage clotting assay but were not observed using a two-stage assay. No evidence for activation was found in vitro (e.g. by FPA generation and VIII:C stability) and the yield increase suggested by the one-stage assay was supported by results from a controlled clinical evaluation.

Introduction

In an earlier study concerning factor VIII yield [4] we monitored a routine manufacturing process over a 2-year period, using both clotting and immunological assays, to help to define points of VIII:C loss and the mechanisms involved. While cryoprecipitation was confirmed as the principal point of loss, a further yield loss of almost 22% VIII:C was noted over the process-finishing steps (i.e. product formulation, 0.22 µm membrane filtration and aseptic dispensing) prior to freeze-drying. Although redesign of the cryoprecipitation procedure has given a substantial yield improvement [2, 5, 6], we have continued this project by examining the VIII:C loss over product finishing and it is the results of this study which are reported here.

Previously [4], we concluded that finishing losses comprised a 1% loss by adsorption onto the membrane filter, a 4% volume loss and a 17% loss due to inactivation of VIII:C. To pursue this latter finding we have examined the stability of VIII:C at key points in the manufacturing process and have used this information to assist in defining a number of potential product formulations to be evaluated for their suitability in stabilising VIII:C.

During this evaluation a discrepancy in results obtained by different clotting assays became apparent; therefore, a controlled clinical assessment of a selected formulation was undertaken to help to clarify the in vitro observations.

Materials and Methods

Preparation of FVIII Concentrate

Fresh frozen plasma was prepared at Scottish Regional Blood Transfusion Centres from whole blood donations collected into CPD anticoagulant and frozen within 8 hours of donation. The blood group of the donations was not taken into account in the construction of plasma pools for fractionation. Factor VIII concentrate was prepared (table I) from pools of plasma (range from about 300–700 kg) in a manner similar to that previously described [5] using the continuous procedure for plasma thawing. The resultant cryoprecipitate was processed (table I) [5] using the low-shear agitator (Vibromixer, model E2; Chemap, Abingdon, UK) but the final pH adjustment (step 12, table I) was to pH 6.8 using hydrochloric acid rather than citric acid.

Preliminary Stability Study

Samples were taken at 3 points during the routine production process; following extraction of the cryoprecipitate, following alu-

[1] Results from this study have been presented in preliminary form at meetings of the British Society of Haematology [1]. The World Federation of Haemophilia [2] and the International Society of Thrombosis and Haemostasis [3].

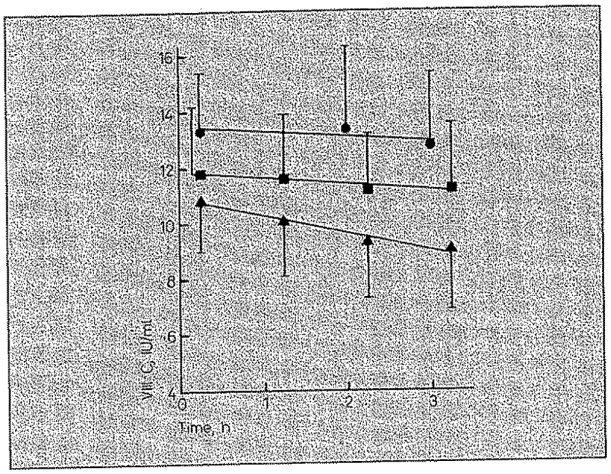

Fig. 1. Stability of VIII:C at different points in the production process (mean and SD for 10 batches). ●=Cryoprecipitate extract (step 7); ■=$Al(OH)_3$ adsorbed solution (step 10); ▲=Citrated solution (step 12).

minium hydroxide adsorption and following product formulation (i.e. after steps 7, 10 and 12; table I). The freshly taken samples were then held at 20 °C and assayed for VIII:C activity (one-stage assay) over a number of hours. Ten production batches were monitored in this manner and the results (fig. 1) suggested that VIII:C losses might be particularly associated with the addition of sodium citrate to the process solution.

Assessment of Different Formulations to Improve VIII:C Stability

The observation that the addition of sodium citrate may be implicated in the loss of yield during finishing led us to consider the development of a product formulation in which ionised calcium would be controlled at an appropriate concentration. Therefore a number of formulations were constructed involving either the exclusion of citrate, a reduced concentration of citrate or the addition of sufficient calcium chloride to return the ionised calcium concentration to that which existed in solution prior to citrate addition. Various other additives (heparin 1 U/ml; glycine 10 m*M*; glucose 1–4%; maltose 1–4%) were also incorporated into the study to establish if they could provide any beneficial effect. The principal formulations studied are listed in table II; those containing phosphate were prepared according to the method of Liu et al. [7].

To construct a set of formulated products, factor VIII solution (about 0.5–1.0 litres) was taken from a production lot following aluminium hydroxide adsorption (step 10, table I), aliquots (about 50–100 ml) were adjusted to a particular formulation and the pH adjusted to 6.8. Formulated samples, plus a pre-formulation control sample, were held for 2 h at 20 °C as a test of stability. All samples were then frozen at –40 °C with one set remaining frozen while a duplicate set was freeze-dried. The frozen samples were assayed for VIII:C activity (one-stage and two-stage assays), vWf:Ag and total protein, while the freeze-dried samples were assayed for VIII:C activity (one-stage assay), total protein and reconstitution time. A number of frozen samples were also assayed for fibrinopeptide A (FPA) content.

Table I. SNBTS process for the manufacture of factor VIII concentrate (intermediate purity)

Stage	Reagents used	Process
1		fresh-frozen plasma (–40 °C)
2		fresh-frozen plasma (–10 °C)
3		crush and thaw plasma (≤2 °C)
4		recover cryoprecipitate by centrifugation
5	Ethanol (2%), Tris (20 m*M*) →	rinse cryoprecipitate
6	Tris (20 m*M*; 30 ml/l plasma) →	extract cryoprecipitate (20 °C, 10 min)
7[1]	HCl (50 m*M*) →	adjust pH to 7.0
8	$Al(OH)_3$ (3.5 ml/l plasma) →	adsorption (20 °C, 5 min)
9		remove solids by centrifugation
10[1]		filter supernatant (to 0.45 µm)
11	Na_3 citrate (0.5 *M*) →	formulate to 20 m*M* citrate
12[1]	HCl (50 m*M*) →	adjust pH to 6.8
13		filtration (to 0.2 µm)
14		aseptic dispensing
15		freeze-drying
16		heat treatment

[1] Samples for the initial stability study were taken at the completion of each of these steps.

Full-Scale Experiments with a Selected Formulation

The effect of calcium chloride addition to a typical citrated process lot was studied in a controlled manner in two full-scale production exercises. In the first exercise a pool of plasma (530 litres) was processed to step 10 (table I). The solution was then divided into two equal portions; sodium citrate (0.5 *M*) was added to one portion in the normal manner (final concentration 20 m*M*) while the other portion was formulated by adding a mixture of sodium citrate (0.5 *M*) and calcium chloride (0.5 *M*) calculated to give a final concentration of 20 m*M* sodium citrate and 1.6 m*M* calcium chloride (the mean value derived from titrating samples from a number of factor VIII batches). Finishing of the former solution (without calcium) was completed first and the material was held while finishing operations

Table II. Details of formulations evaluated for their effects on VIII:C stability

A. Including citrate

Na_3 citrate mM	NaCl mM	$CaCl_2$ mM	Heparin U/ml	Maltose % w/v
5	30	0	0	0
5	30	0.6–1.3	0	0
5	30	0	1	0
5	30	0.6–1.3	1	0
5	30	0	0	2
5	30	0.6–1.3	0	2
5	30	0	1	2
10	10	0	0	0
10	10	0.9–1.8	0	0
10	10	0	1	0
10	10	0.9–1.8	1	0
10	10	0	0	2
10	10	0.9–1.8	0	2
10	10	0	1	2
20	0	0	0	0
20	0	1.1–2.1	0	0
20	0	0	1	0
20	0	1.1–2.1	1	0
20	0	0	0	2
20	0	1.1–2.1	0	2
20	0	0	1	2

B. Excluding citrate

NaCl mM	Na phosphate mM	Maltose % w/v
30	4	0
30	4	2

Table III. VIII:C content of products prepared for clinical evaluation

	Product without calcium adjustment	Product with calcium adjustment	Increase with calcium
Product VIII:C (IU/ml)[1] before heat treatment			
One-stage assay	7.6	11.4	50.0%
Two-stage assay	9.0	10.4	15.5%
Chromogenic assay	8.6	10.8	25.6%
Product VIII:C (IU/ml)[1] after heat treatment			
One-stage assay	7.5	9.9	32.0%
Two-stage assay	7.2	8.2	13.9%
Chromogenic assay	8.5	10.0	17.6%
Volume of concentrate used clinically, ml			
First infusion (30. 9. 85)	248		
Second infusion (7. 10. 85)		185	
Third infusion (6. 11. 85)		190	
Fourth infusion (13. 11. 85)	250		

[1] Both products dispensed at 35 ml and held for 5 h at 20 °C before freezing and freeze drying; reconstituted by adding 20 ml distilled water. Potency results are mean values from two vials of each product.

were carried out with the second solution. Both sets of vials were then frozen and freeze-dried together (i.e. on different shelves in the same freeze drier); with the total time from citrate addition to freezing being 2 h for all vials.

In a second exercise another pool of plasma (430 litres) was processed in the same manner except that the calcium chloride concentration was increased to 2.5 m*M* and the dispensed solutions were held for 5 h from citrate addition to freezing to provide a more severe test of the stabilising formulation. The freeze-dried product from the second experiment was heated in the final container at 68 °C for 2 h; this was the first factor VIII heat treatment regime used by the SNBTS and was applied to concentrates prepared from January to October 1984. (The second full-scale experiment was carried out in February 1984 using blood donations collected in late 1983.)

Clinical Evaluation of a Selected Formulation

The effect of calcium addition was studied in vivo by giving one haemophiliac four separate infusions of material from the second production experiment. The patient was a severely affected haemophiliac (64 kg) with no evidence of inhibitor who had previously received many infusions of factor VIII concentrate and was HIV antibody positive. During the study he had a pre-infusion plasma VIII:C of 0.02 IU/ml or less and had received no factor VIII therapy for at least 7 days before each treatment. He was given two infusions of the control product (without calcium) and two infusions of the experimental material (with calcium) while in a non-bleeding state (infusion details shown in table III). The protocol received ethical approval from the appropriate committee and the patient gave his informed consent to the study.

Analytical Methods

VIII:C concentration was determined by the one-stage method [8] using congenitally deficient plasma as substrate with Tris-buffered saline or imidazole buffer (patient samples) as diluent and by the two-stage method [9] using a commercial reagent kit (Diagen kit, Diagnostic Reagents) with citrate-saline as diluent. A small number of VIII:C assays were also carried out by the chromogenic substrate method (Coatest Factor VIII, Kabi Vitrum) according to the manufacturers' instructions. Plasma VIII:C assays were standardised against British Plasma Standards while results for factor VIII preparations at various stages of manufacture were determined against concentrate standards (80/556 or 83/591) provided by the National Institute for Biological Standards and Control, Hampstead, London, UK.

vWf:Ag was assayed by the standard immunoelectrophoretic method as previously described [4] and factor VIII polypeptide

distribution was determined by the method of Barrowcliffe et al. [10]. FPA measurements were carried out by radioimmunoassay [11] using the reagents and method supplied by IMCO (Sweden) with minor modification. Measurements of total protein and the reconstitution time of freeze-dried preparations were carried out as previously described [5].

Ionised Calcium

Ionised calcium determinations were made using an ion specific electrode (F2112 electrode with pHM26 or ION85 meter, Radiometer, Copenhagen) according to the manufacturers' instructions. The quantity of calcium chloride required to maintain a constant concentration of ionised calcium was determined by monitoring the fall in voltage as sodium citrate (0.5 *M*) was added (e.g. from 0 to 20 m*M* in increments of 1 m*M*) and the subsequent increase in voltage on the addition of calcium chloride (e.g. 0.055 *M* solution added to give increments of 0.1 m*M*). The concentration of apparent ionised calcium in the unformulated solution was 38 ± 5 μ*M* (n=4) and the concentration of $CaCl_2$ required to precisely compensate for the added citrate varied from batch to batch (range shown in table II). In the first full-scale exercise the quantity of calcium chloride to be added (1.6 m*M*) was selected as the mean value derived from titrations carried out with a number of factor VIII batches. This was increased to 2.5 m*M* in the second full-scale experiment to ensure that there would always be sufficient ionised calcium present (in relation to the added citrate) in any factor VIII batch.

Chemicals

Heparin (heparin sodium injection BP) was supplied by D. Flockhart & Co., London. Maltose (maltose hydrate) and calcium chloride (calcium chloride dihydrate) were obtained from Sigma Chemical Co., Poole, UK; all other chemicals (Analar grade) were obtained from BDH, Poole, UK.

Statistics

Most of the data are presented as the mean ± standard deviation from n experiments; calculations involving VIII:C concentration were carried out in the geometric rather than arithmetic form [12]. Linear correlations were obtained by the least-squares method and the significance of differences was established using Student's t test.

Results

Preliminary Stability Study

The monitoring of VIII:C activity in samples taken from the routine production process showed no significant loss of VIII:C over 3 h for both the cryoprecipitate and the $Al(0H)_3$-adsorbed solutions (fig. 1). However, compared to the initial non-citrated sample (i.e. step 10), VIII:C activity was lost progressively once citrate had been added, with a 10% loss after 1.25 h ($p>0.1$) and a 17% loss after 2.25 h ($p<0.025$). To put these results into context it is important to appreciate that the extraction and adsorption steps each required less than 30 min to carry out, whereas the finishing steps, with a citrated solution, required up to 2–3 h to complete in the full-scale process.

Comparison of Different Formulations

Results from the preliminary study suggested that the loss of VIII:C activity previously observed over product finishing [4] might be almost fully explained by a citrate-induced inactivation of VIII:C. Similar inactivation behaviour had been observed in plasma [13, 14] with one suggestion being that the presence of calcium ions is essential for VIII:C stability. Therefore a number of formulations were examined in which the concentration of ionised calcium was controlled by the addition of calcium chloride to factor VIII solutions at different citrate concentrations. The results (table IV) again showed a marked loss of VIII:C in the 20 m*M* citrate control samples (both frozen and freeze-dried), but this was largely prevented by calcium addition ($p<0.001$). There was no significant loss of VIII:C at the lower citrate concentrations in the frozen samples but the presence of calcium provided significant stabilisation at 5 m*M* citrate ($p<0.01$) and 10 m*M* citrate ($p<0.025$) in samples that were freeze-dried.

Calcium addition to citrated solutions in the presence of heparin, glycine, maltose and glucose, either individually or in combination gave similar results (results shown in table IV for heparin and 2% maltose), indicating that ionised calcium concentration was the key parameter which determined VIII:C stability. All freeze-dried samples containing glycine exhibited a rather poor solubility (reconstitution time 19.4 ± 1.3 min, n=9). VIII:C was found to be relatively stable in the phosphate-based formulations which excluded citrate (table IV), but the reconstitution time of this material (10.0 ± 6.2 min, n=9) was somewhat greater than that of the 20 m*M* citrate+calcium formulation (6.8 ± 3.3 min, n=25). This was not improved by the addition of sugars, suggesting that citrate was making an important contribution to final product solubility. Further evidence for this was seen with formulations based either on sodium chloride alone or low concentrations of citrate (e.g. 5 m*M*): Factor VIII solutions prepared in this manner were often found to be very difficult to filter, the products were poorly soluble and in some instances exhibited a tendency to clot spontaneously.

There was no significant difference in the results of vWf:Ag and total protein assays over the various formulations studied, however, in contrast to results from the one-stage assay, VIII:C values determined by the two-stage method (table V) did not show a significant

Table IV. Effect of ionised calcium on the stability of VIII:C in various formulations (one-stage assay)

Na_3 citrate m*M*	Additives	VIII:C[1], %			
		frozen sample		freeze-dried sample	
		without calcium	with calcium	without calcium	with calcium
5		94.6 ± 19.6 (23)	105.1 ± 19.2 (23)	81.4 ± 27.7 (23)	95.9 ± 16.2 (23)
10		97.1 ± 11.0 (23)	99.7 ± 14.8 (23)	85.6 ± 20.9 (23)	96.7 ± 13.2 (23)
20		83.5 ± 11.6 (23)	98.1 ± 13.1 (23)	72.6 ± 19.8 (23)	96.4 ± 19.8 (23)
5	heparin	93.8 ± 19.8 (5)	100.1 ± 13.6 (16)	86.2 ± 27.7 (5)	93.6 ± 18.0 (5)
10	heparin	94.4 ± 18.0 (5)	98.6 ± 7.2 (16)	83.4 ± 14.8 (5)	92.2 ± 18.6 (5)
20	heparin	88,1 ± 7.6 (5)	99.8 ± 12.7 (16)	74.7 ± 23.7 (5)	92.3 ± 18.2 (5)
5	maltose	101.0 ± 23.4 (8)	109.7 ± 29.3 (5)	85.4 ± 24.1 (8)	100.8 ± 13.9 (5)
10	maltose	93.3 ± 15.6 (8)	107.1 ± 13.9 (5)	73.9 ± 20.7 (8)	91.4 ± 17.7 (5)
20	maltose	86.3 ± 19.0 (8)	97.8 ± 5.8 (5)	70.9 ± 21.3 (8)	80.8 ± 17.9 (5)
0	NaCl + phosphate	105.2 ± 21.7 (9)	ND	93.8 ± 16.9 (9)	ND
0	NaCl + phosphate + maltose	109.9 ± 22.1 (8)	ND	102.7 ± 31.5 (8)	ND

All samples were held for 2 h at 20°C before freezing, as a test of stability. Figures in parentheses are numbers of samples.

[1] 100% is the VIII:C content of the frozen sample taken after removal of $Al(OH)_3$ and before the addition of any of the reagents used for product formulation (i.e. after step 10 in table I).

Table V. Effect of various formulation on the stability of VIII:C frozen sample (two-stage assay)

Na_3 citrate m*M*	Additive	VIII:C[1], %	
		without calcium adjustment	with calcium adjustment
5		96.0 ± 28.0 (14)	99.2 ± 12.7 (14)
10		96.3 ± 27.1 (14)	98.7 ± 22.3 (14)
20		90.9 ± 16.4 (14)	93.3 ± 17.8 (14)
5	heparin	101.9 ± 20.6 (4)	96.2 ± 22.0 (4)
10	heparin	85.8 ± 15.5 (4)	98.2 ± 23.6 (4)
20	heparin	99.1 ± 12.5 (4)	92.2 ± 18.1 (4)
5	maltose	107.6 ± 18.4 (4)	94.4 ± 16.7 (4)
10	maltose	104.8 ± 21.4 (4)	96.0 ± 32.1 (4)
20	maltose	93.3 ± 20.4 (4)	91.9 ± 18.1 (4)
0	NaCl + phosphate	95.4 ± 29.6 (7)	ND
0	NaCl + phosphate + maltose	105.6 ± 28.1 (7)	

Figures in parentheses are numbers of samples.

[1] See footnote to table IV.

Table VI. Effect of calcium addition on FPA content of factor VIII solutions

Na_3 citrate m*M*	FPA, ng/ml	
	without $CaCl_2$	with $CaCl_2$
0	114.8 ± 114.1 (9)	ND
5	171.5 ± 124.3 (8)	75.4 ± 41.8 (4)
10	169.1 ± 132.9 (7)	58.1 ± 12.9 (4)
20	143.2 ± 47.2 (7)	59.4 ± 37.1 (8)

All solutions held for 2 h at 20°C before freezing. Figures in parentheses are numbers of samples.

improvement in VIII:C stability with calcium addition. A repeat set of one-stage assays, using the same sub-sample provided for the two-stage assays, gave results similar to those shown in table IV, suggesting that the difference between these assays was not due to an error in sample handling. Results from the two assays were not significantly different for the unformulated control sample (designated 100%, tables IV, V), with the VIII:C content

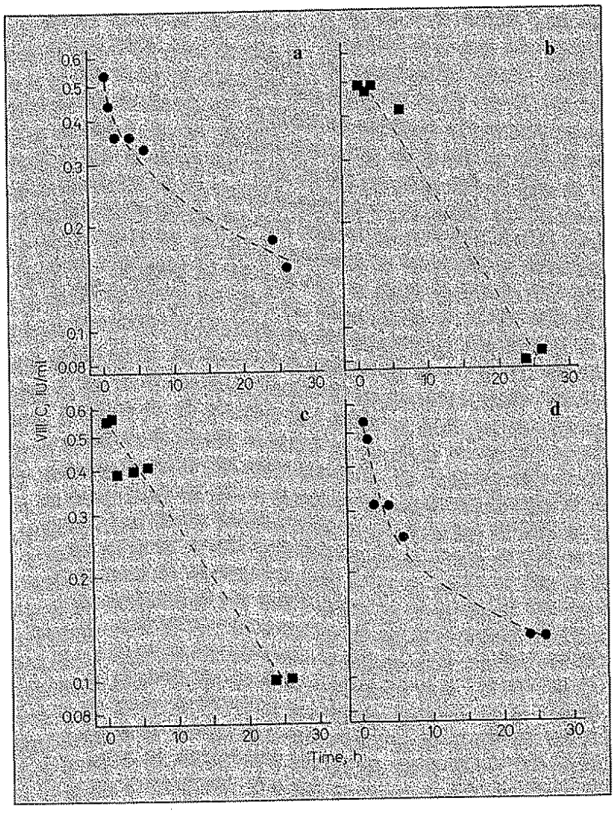

Fig. 2. VIII:C recovery in a severe haemophiliac following infusion of concentrates prepared without calcium addition (●) and with calcium addition (■). **a** First infusion (without calcium). **b** Second infusion (with calcium). **c** Third infusion (with calcium). **d** Fourth infusion (without calcium).

being 9.85 ± 1.38 IU/ml by one-stage assay and 9.36 ± 1.41 IU/ml by two-stage assay ($n=14$, $p>0.4$).

A discrepancy between one-stage and two-stage assays is often considered indicative of activation [15]; however, no evidence for activation was found from measurements of FPA, with samples containing calcium exhibiting a reduced FPA content ($p<0.005$ at 20 m*M* citrate) even though they had been held for 2 h at 20 °C as a test of stability (table VI).

Calcium Addition in Full-Scale Experiments

In the first full-scale experiment the product VIII:C potency by one-stage assay was 16.1% higher for the experimental portion (with calcium) compared to the control material. The VIII:C potency was also increased when measured by the two-stage assay, but only by 5.7%. Similar behaviour was seen in the second full-scale experiment (table III) but the magnitude of yield improvement in the presence of calcium was much greater because of the deliberately extended process time (i.e. solutions held for 5 h before freezing). The heat treatment procedure resulted in the further loss of VIII:C, particularly in the re-calcified material, but discrepancies between the different types of clotting assay were still apparent.

The stability of the heated products was assessed following reconstitution, with the re-calcified material showing no loss of activity over 3 h at 20°C (one-stage assay) but with the control material showing a corresponding loss of 11% VIII:C (one-stage assay). The factor VIII polypeptide distribution of these products was also examined, with both the re-calcified material and the control product exhibiting the same polypeptide pattern as the standard SNBTS product, shown in lane 10 in the report of Barrowcliffe et al. [10].

Clinical Evaluation

Products from the second full-scale preparation were given to one haemophiliac and the plasma VIII:C levels following each infusion are shown in figure 2. These infusions were carried out to help to determine which of the clotting assays might represent the true biological activity of the products. This was assessed by assuming a 100% recovery and calculating the potency of the products from the relationship:

$$\text{Potency (IU/ml)} = \frac{\text{Increase in plasma VIII:C (IU/ml)} \times \text{plasma volume (ml)}}{\text{Infusion volume (ml)} \times \text{recovery (\%)} \times 10^{-2}}$$

Assuming a plasma volume of 41 ml/kg, the mean potency of the control product was calculated to be 5.56 IU/ml while that of the re-calcified product was 7.26 IU/ml. Therefore, the re-calcified product had a greater in vivo effect than the control material (by 30.5%), reflecting the increase shown in vitro by the one-stage assay (32%, table III). Using the product potencies taken from the manufacturers' one-stage assay results (table III) the in vivo recoveries were calculated to be 74 and 73% for the control and re-calcified products, respectively.

The control product showed the two-phase disappearance behaviour usually seen with factor VIII infusions (infusions a, d, fig. 2) but this was not shown clearly by the re-calcified product (infusions b, c, fig. 2). Further data are required to properly assess the pharmacokinetics of this type of product but it is important to note that the more rapid first-phase disappearance associated with activated products [16] was not seen with the re-calcified material, providing further support for the validity of the one-stage assay results.

Two further sets of infusions were planned with different haemophiliacs but the clinical study was curtailed before this could be done as a potentially non-infective product [17] had become available. In these circumstances it was felt that it would no longer be ethical to continue to expose patients to the products supplied for this study as they had been prepared using a less severe heat treatment regime, introduced to destroy potential HIV contamination rather than hepatitis viruses [18].

Discussion

Even though factor VIII concentrates have been manufactured for almost 20 years their availability is still inadequate in many countries [19], with low process yields being an important component of this supply problem [20]. There have been few reports investigating the points of VIII:C loss during routine manufacturing processes: however, the fact that significant loss of VIII:C can occur over the sterile filtration or finishing procedures has been appreciated for some time [21–23]. This was believed to be largely due to the retention of factor VIII on the filter itself, but the development of immunoassays lead instead to the suggestion that some form of inactivation may provide the predominant mechanism of loss [4]. Previous studies concerning VIII:C inactivation during processing have tended to concentrate on various types of proteolytic degradation [24], whereas our preliminary stability study drew attention to the importance of product formulation and especially to the role of citrate.

Sodium citrate has a number of properties which are of benefit in the formulation of factor VIII concentrates. Studies with early preparations of fibrinogen [21, 25, 26] illustrated the value of its anticoagulant and solubilising properties which proved to be of similar value with the factor VIII concentrates that followed (23, 27, 28]. Citrate was also reported to have a marked stabilising effect on chromatographically purified factor VIII [29], a finding which helped to establish its use at a concentration of 20 m*M* in the final product formulation [30]. Virtually all manufacturers of factor VIII concentrate have adopted this concentration, exceptions being Smith et al. [31], who used 10 m*M* citrate, and Liu et al. [7], who used a phosphate buffer. Various other additives have been utilised to aid factor VIII stability or product formulation including heparin [32–34], glycine [14, 34] and sugars [26, 34, 35].

For the process described here, we found that VIII:C losses over product finishing were predominately caused by a citrate-induced inactivation and that this could be avoided by controlling the concentration of ionised calcium. This is consistent with reports that factor VIII is a calcium-linked protein complex [36, 37] and with the findings of others who have studied the stability of VIII:C in plasma [13, 14, 38, 39, 40]. While the importance of ionised calcium to VIII:C stability in plasma is well documented its relevance during the preparation of factor VIII concentrates has not been fully appreciated, possibly because the effects may not be seen with small-scale preparations or in typical bench-top experiments, where finishing procedures are completed fairly quickly or may be omitted altogether.

Our observations have been complicated by the finding that increases in VIII:C yield shown by the one-stage clotting assay were not shown with the two-stage assay. Again there may be similarities with studies of plasma VIII:C where reported yield improvements [14] were not always reproduced by other investigators [41] because of apparent discrepancies between the different clotting assays used [42]. In a clinical study undertaken to resolve this question, Rock et al. [43] concluded that the yield improvements shown in vitro by the one-stage assay were reproduced in vivo. Interference by aluminium hydroxide has been proposed [15, 44] to account for discrepancies between one-stage and two-stage assays, particularly in the presence of heparin [42]. The differences that we have observed cannot be accounted for in this way as aluminium hydroxide adsorption was not used in the assay and the discrepancy existed in the absence of heparin. A citrate-saline solution is the standard diluent used in the two-stage assay and it is conceivable that further exposure to citrate may have resulted in loss of VIII:C during the assay procedure. In a study of different diluents, Mikaelsson et al. [45] noted that VIII:C was unstable in citrate-saline and it has been suggested that citrate buffers should not be used in automated two-stage assays because of the relatively long assay time involved [46]. Further studies will be required to establish whether the use of a citrate-saline buffer, or some other factor, was responsible for the assay discrepancy observed in our results.

Most investigators have dealt with the requirement to control ionised calcium concentration by replacing citrate-based anticoagulants with heparin. However, we found citrate to be beneficial, because of its solubilising and anti-coagulant properties, and therefore examined the option of adding small quantities of calcium to the

process stream to maintain a constant concentration of ionised calcium, while retaining the standard citrate concentration used by almost all manufacturers of factor VIII concentrates. In doing this we were concerned that some form of activation might be induced and the discrepancy between one-stage and two-stage assay results gave further emphasis to this concern. No evidence for activation was found either from in vitro studies (FPA; VIII:C stability; VIII polypeptide distribution) or from in vivo studies (VIII:C recovery; VIII:C clearance rate); however, further clinical studies are required to properly establish the pharmacokinetics of factor VIII concentrates prepared this way.

Although a number of different formulations were examined, in vitro improvements in VIII:C stability were invariably associated with increases in ionised calcium concentration. None of the other additives showed any obvious beneficial effects on VIII:C stability. The addition of sugar had little effect on the solubility of freeze-dried samples, but its presence was subsequently found to be of value in retaining product solubility following heat treatment, with either 2% maltose or 2% sucrose (but not glucose) allowing heating of the freeze-dried concentrate described here to be extended from 2 to 24 h at 68 °C [Foster et al., unpubl. results].

The results of this study illustrate the importance of controlling the concentration of ionised calcium throughout VIII:C manufacturing processes and suggest that yield improvements of 20–30% may be achieved as a consequence. It is possible that even higher concentrations of ionised calcium may be of value, particularly during heat treatment [47]; however, further studies of product formulation will be required to optimise the performance of this procedure.

Acknowledgements

We are grateful to Dr. T.W. Barrowcliffe for measurements of factor VIII polypeptide distribution and to Dr. Joan Dawes for measurements of FPA. We would also like to acknowledge the contributions made by many colleagues at our respective centres, especially Dr. R.J. Perry for his help in arranging the clinical evaluation and to Susan Hardie and Petra Eaglesfield for their efforts with VIII:C assays.

References

1 Foster, P.R.; Dickson, I.H.; McQuillan, T.A.: Stability of factor VIII during the preparation of an intermediate-purity factor VIII concentrate (Abstract). Br. J. Haemat. *53:* 343 (1983).

2 Foster, P.R.; Dickson, A.J.; Dickson, I.H.: Improving yield in the manufacture of factor VIII concentrates. Scand. J. Haematol. *33:* suppl. 40, pp. 103–110 (1984).

3 Foster, P.R.; Dickson, I.H.; McQuillan, T.A.; Dawes, J.: Factor VIII stability during the manufacture of a clinical concentrate (Abstract). Thromb. Haemostasis *50:* 117 (1983).

4 Prowse, C.V.; Griffin, B.; Pepper, D.S.; Dickson, A.J.; McQuillan, T.A.; Dickson, I.H.; Foster, P.R.: Changes in factor VIII complex activities during the production of a clinical intermediate purity factor VIII concentrate. Thromb. Haemostasis *46:* 597–601 (1981).

5 Foster, P.R.; Dickson, A.J.; McQuillan, T.A.; Dickson, I.H.; Keddie, S.; Watt, J.G.: Control of large-scale plasma thawing for recovery of cryoprecipitate factor VIII. Vox Sang. *42:* 180–189 (1982).

6 Foster, P.R.; Dickson, A.J.: Control of large-scale plasma thawing. Proc. 9th Annu. Symp. Blood Transfusion, Groningen 1984, pp. 57–65 (Martinus Nijhoff, Boston 1985).

7 Liu, D.T.H.; Irwin, J.F.; Le Fave, R.O.; Grezenczyk, B.; Pai, R.C.: An improved method for the preparation of intermediate-purity antihemophilic factor concentrate for therapeutic usage. Vox Sang. *38:* 216–221 (1980).

8 Hardisty, R.M.; Macpherson, J.D.: A one-stage factor VIII assay and its use on venous and capillary plasma. Thromb. Diath. haemorrh. *7:* 215–229 (1962).

9 Denson, K.W.E.: The simplified two-stage assay for factor VIII; in Biggs, Human blood coagulation, haemostasis and thrombosis, pp. 688–692 (Blackwell, Oxford 1976).

10 Barrowcliffe, T.W.; Edwards, S.J.; Kemball-Cook, G.; Thomas, D.P.: factor VIII degradation products in heated concentrates. Lancet *i:* 1448–1449 (1986).

11 Butler, V.P.; Nossel, H.L.; Canfield, R.E.: Immunoassay of human fibrinopeptides. Meth. Enzym. *84:* 102–118 (1982).

12 Kirkwood, T.B.L.; Snape, T.J.: Biometric principles in clotting and clot lysis assays. Clin. Lab. Haemat. *2:* 155–167 (1980).

13 Stibbe, J.; Hemker, H.C.; Crevald, S. van.: The inactivation of factor VIII in vitro. Thromb. Diath. haemorrh. *27:* 43–58 (1972).

14 Rock, G.A.; Cruickshank, W.H.; Tackaberry, E.S.; Palmer, D.S.: Improved yields of factor VIII from heparinized plasma. Vox Sang. *36:* 294–300 (1979).

15 Barrowcliffe, T.W.: Comparisons of one-stage and two-stage assays of factor VIII:C. Scand. J. Haematol. *33:* suppl. 41, pp. 39–54 (1984).

16 Allain, J.P.: Principles of in vivo recovery and survival studies. Scand. J. Haematol. *33:* suppl. 41, pp. 123–130 (1984).

17 Winkelman, L.; Owen, N.E.; Haddon, M.E.; Smith, J.K.: Treatment of a new high specific activity factor VIII concentrate to inactivate viruses (Abstract). Thromb. Haemostasis *54:* 19 (1985).

18 McIntosh, R.V.; Docherty, N.; Fleming, D.; Foster, P.R.; A high-yield factor VIII concentrate suitable for advanced heat treatment (Abstract). Thromb. Haemostasis *58:* 306 (1987).

19 Britten, A.F.H.; Ala, F.; Carman, C.; Smit Sibinga, C.T.: Worldwide overview of plasma procurement and fractionation. Scand. J. Haematol. *33:* suppl. 40, pp. 479–484 (1984).

20 Hogman, C.F.: To what extent can plasma supply for factor VIII production be obtained within the frame of a blood component program? Scand. J. Haematol. *33:* suppl. 40, pp. 485–490 (1984).

21 Cohn, E.J.; Strong, L.E.; Hughes, W.L.; Mulford, D.J.; Ashworth, J.N.; Melin, M.; Taylor, H.L.: Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J. Am. chem. Soc. *68:* 459–475 (1946).
22 Blomback, B.; Blomback, M.: Purification of human and bovine fibrinogen. Ark. Kemi *10:* 415–443 (1956).
23 Hershgold, E.J.; Pool, J.G.; Pappenhagan, A.R.: The potent antihemophilic globulin concentrate derived from a cold insoluble fraction of human plasma: characterisation and further data on preparation and clinical trial. J. Lab. clin. Med. *67:* 23–32 (1966).
24 Atichartakarn, V.; Marder, V.J.; Kirby, E.P.; Budzynski, A.Z.: Effects of enzymic degradation on the subunit composition and biologic properties of human factor VIII. Blood *51:* 281–297 (1978).
25 Morrison, P.R.; Edsall, J.T.; Miller, S.G.: Preparation and properties of serum and plasma proteins. XVIII. The separation of purified fibrinogen from fraction I of human plasma. J. Am. chem. Soc. *70:* 3103–3108 (1948).
26 Fowell, A.H.: Stable fibrinogen solutions and method for producing same. US Patent 2, 826, 533 (1958).
27 Wagner, R.H.; McLester, W.D.; Smith, M.; Brinkhouse, K.M.: Purification of antihemophilic factor (factor VIII) by amino acid precipitation. Thromb. Diath. haemorrh. *11:* 64–74 (1964).
28 Johnson, A.J.; Karpatkin, M.H.; Newman, J.: Preparation and clinical experience with antihemophilic factor concentrates. Thromb. Diath. haemorrh., suppl. 35, pp. 49–59 (1969).
29 Hynes, H.E.; Owen, C.A.; Bowie, E.J.W.; Thompson, J.H.: Citrate stabilisation of chromatographically purified factor VIII. Blood *34:* 601–609 (1969).
30 Newman, J.; Johnson, A.J.; Karpatkin, M.H.; Puszkin, S.: Methods for the production of clinically effective intermediate and high-purity factor VIII concentrates. Br. J. Haemat. *21:* 1–20 (1971).
31 Smith, J.K.; Evans, D.R.; Stone, V.; Snape, T.J.: A factor VIII concentrate of intermediate purity and higher potency. Transfusion *19:* 299–306 (1979).
32 Fekete, L.F.; Holst, S.L.: Stabilisation of AHF using heparin. US Patent 3, 803, 115 (1974).
32 Rock, G.A.: Method of collecting anti-hemophilic factor VIII from blood and blood plasma using heparin or sodium heparin. US Patent 4, 203, 891 (1980).
34 Smit-Sibinga, C.T.; Welbergen, H.; Das, P.C.; Griffin, B.: High-yield method of production of freeze-dried purified factor VIII by blood banks. Lancet *ii:* 449–450 (1981).
35 Thomas, W.R.; Laguna, N.: Rapidly solubilised AHF composition and process for preparing same. US Patent 4, 089, 944 (1978).
36 Fass, D.N.; Knutson, G.J.; Katzmann, J.A.: Monoclonal antibodies to procure factor VIII coagulant and their use in the isolation of active coagulant protein. Blood *59:* 594–600 (1982).
37 Mikaelsson, M.E.; Forsman, N.; Oswaldsson, U.M.: Human factor VIII: a calcium-linked protein complex. Blood *62:* 1006–1015 (1983).
38 Rock, G.A.; Cruickshank, W.H.; Tackaberry, E.T.; Ganz, P.R.; Palmer, D.S.: Stability of VIII:C in plasma: the dependence on protease activity and calcium. Thromb. Res. *29:* 521–535 (1983).
39 Krachmalnicoff, B.; Thomas, D.P.: The stability of factor VIII in heparinised plasma. Thromb. Haemost. *49:* 224–227 (1983).
40 Prowse, C.; Waterston, Y.G.; Dawes, J.; Farrugia, A.: Studies on the procurement of blood coagulation factor VIII in vitro studies on blood components prepared in half-strength citrate anticoagulant. Vox Sang. *52:* 257–264 (1987).
41 Smith, J.K.: The assay of factor VIII in heparinized blood; in Lane, Rock, Smit-Sibinga, Development Systems for efficient separation of factor VIII from human fresh frozen plasma. Proc. Netherlands Red Cross FVIII Workshop, Groningen 1981, pp. 23–25 (1982).
42 Rock, G.; Palmer, D.S.: Disparity between one- and two-stage factor VIII assays in measuring VIII:C in heparinized plasma. Thromb. Haemostasis *54:* 780–783 (1985).
43 Rock, G.; Smiley, R.K.; Titley, P.; Palmer, D.S.: In vivo effectiveness of a high-yield factor VIII concentrate prepared in a blood bank. New Engl. J. Med. *311:* 310–313 (1984).
44 Barrowcliffe, T.W.; Kirkwood, T.B.L.; Rizza, C.R.: Aluminium hydroxide adsorption and factor VIII clotting assays. Lancet *i:* 820 (1980).
45 Mikaelsson, M.; Oswaldsson, U.: Standardisation of VIII:C assays: a manufacturer's view. Scand. J. Haematol. *33:* suppl. 41, pp. 79–86 (1984).
46 Barrowcliffe, T.W.: Methodology of the two-stage assay of factor VIII (VIII:C). Scand. J. Haematol. *33:* suppl. 41, pp. 25–38 (1984).
47 Macleod, A.J.; Welch, A.G.; Dickson, I.H.; Cuthbertson, B.; Foster, P.R.: Stabilisation of proteins to heat. Res. Disclos. *244:* 380 (1984).

Received: August 18, 1987
Accepted: December 11, 1987

Dr. P.R. Foster
Scottish National Blood Transfusion Service
Protein Fractionation Centre
21 Ellen's Glen Road
GB–Edinburgh EH17 7QT (UK)