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Control of Large-Scale Plasma Thawing for Recovery of Cryoprecipitate Factor VIII[1]

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Abstract. Cryoprecipitation is commonly used as the primary step in the preparation of clinical factor VIII concentrates; yet recovery is usually very low. Much of this loss is due to poor temperature control and a process of continuous plasma thawing has been designed to overcome this. A substantial improvement has resulted, with an increase in both yield and purity of factor VIII:C of over 50% in comparison to a conventional batch thaw process.

Introduction

A notable problem in the production of factor VIII concentrates for clinical use is the low yield of procoagulant activity (VIII:C) commonly achieved [1]. A number of processing methods can be identified for the preparation of either intermediate-purity or high-purity concentrates, however, they all utilise cryoprecipitation as the initial fractionation stage [2] and it is at this point that major loss of factor VIII can be located [3, 4].

Factor VIII is at risk during the process of cryoprecipitation from both its solubility behaviour and labile nature [5]. Rapid processing is desirable to minimise inactivation, but the solution temperature must also be held below the factor VIII solubility limit, otherwise resolution of precipitated factor VIII is inevitable. These two essential requirements are in conflict with one another and the extent of this is related to the scale of manufacture. Hence, though good recovery may be possible at an analytical scale [6], loss is already significant in the routine preparation of single-donation cryoprecipitate [7] and increases further at the industrial scale [8], where plasma pools of up to 1,000 litres may be processed as a single batch [9].

The essential process design problem is to maximise the rate of thaw within the temperature constraint provided by the solubility of factor VIII. To achieve this, the surface area available for heat transfer must be max-

[1] A preliminary report of this work was presented at the VIIth International Congress on Thrombosis and Haemostasis, London 1979 [27].

imised and the heat input and distribution must be carefully controlled [10]. However, in practice there is often little attempt at size reduction of the frozen plasma block and thawing is inevitably carried out in a batch or semi-batch mode during which the temperature of the thawed plasma is particularly difficult to control.

Process control is more easily achieved in continuous steady-state operation and the unvarying conditions also allow more efficient heat transfer and mixing operations to be designed.

We have utilised a hammer mill for the size reduction of frozen plasma for a number of years and, to solve the problems of mixing and temperature control during thawing, a change from batch to continuous processing has been studied. In this report we present the results of a pilot-scale design study and the subsequent full-scale application of the process.

Materials and Methods

Plasma

Frozen plasma was received from Scottish Regional Blood Transfusion Centres where whole blood was collected into CPD anti-coagulant and plasma separated into single-donation, 2-litre or 5-litre plastic packs and frozen on the same day of collection (6-hour plasma) or after overnight storage (24-hour plasma). Immediately prior to thawing for fractionation, the frozen blocks were reduced in size to a coarse 'snow' using a stainless steel hammer mill (Scott Reitz Extructor, Balfour Ltd., Leven, Fife, UK). The resultant plasma 'snow' was then thawed in either a batch or in a continuous mode to produce cryoprecipitate.

Batch Thawing

Batch thawing was carried out using a 200-litre jacketed stainless steel vessel heated with water at 15 °C. A single wide-blade helical ribbon of small pitch was used for mixing as this design had previously been found to be preferable [11]. Thawing was considered to be complete when only a thin layer of ice remained at the surface and at this point the thawed plasma was discharged into another vessel held at +4 °C and the cryoprecipitate recovered using a refrigerated tubular bowl centrifuge (Sharples 6-P).

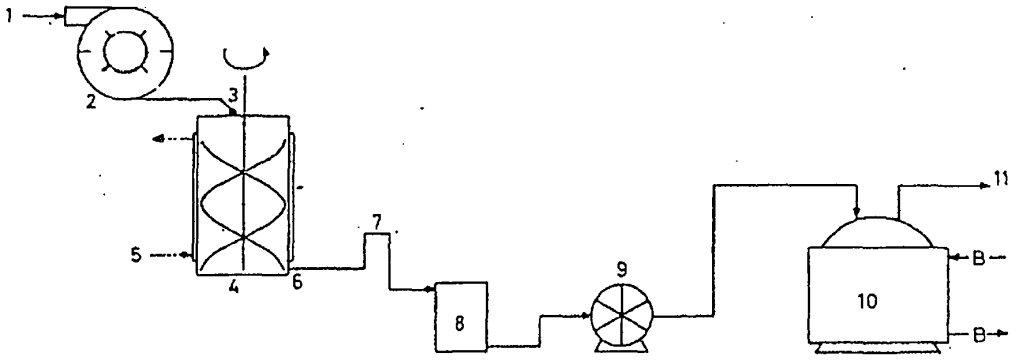
Continuous Thawing

For pilot-scale experiments a 20-litre jacketed stainless steel vessel was fitted with thermocouples so that the temperature of the plasma film at the wall could be monitored. The flow of water through the jacket was set at 60 litres/min after initial experiments indicated that at lower flow rates heat transfer in the jacket was limiting the rate of thaw. The water temperature was varied in the range 12–35 °C and mixing studies were carried out using a variety of single and double helical ribbon impellers of different dimensions and rotating at speeds in the range 40–80 rpm.

In the process (fig. 1) plasma 'snow' from the hammer mill entered the thawing vessel where mixing was designed to circulate the frozen plasma within the vessel while the thawed liquor and suspended cryoprecipitate drained off by gravity. A raised weir was required at the outlet to provide a bottom zone of recirculating liquor and ice so that thawed plasma, running down the heated wall, could be prevented from overheating in the exit region. The thawed suspension was then pumped from a 2-litre holding vessel directly into a refrigerated multichamber centrifuge (Westfalia BKA6) for cryoprecipitate recovery.

The change in centrifuge was introduced to achieve a higher refrigeration capacity. This was particularly important for pilot-scale thawing as the centrifuge feed rate of 60–70 litres/h would have resulted in inadequate temperature control in the tubular bowl machine.

Scale-up of the thawing vessel was based on the area of heated wall, with the aim of achieving a throughput of approximately 200 litres plasma/h. Following pilot-scale experience the full-scale unit comprised a 55-litre thawing vessel, with a height/diameter ratio of 2.67, agitated with a close-fitting, narrow-blade, double helical ribbon impeller rotating at 55 rpm [12; fig. 2]. The throughput of water in the jacket was 130 litres/min to retain a constant Reynolds number, but the temperature was varied in the range 15–28 °C to determine the optimal conditions for this precise design of agitator. For operation at full scale the thawed liquor flowed



1 Frozen plasma feed
2 Hammer mill
3 Frozen plasma 'snow'
4 Jacketted thawing vessel with agitator
5 Warm water feed to jacket
6 Thawed plasma outlet
7 Weir
8 Thawed plasma feed vessel
9 Peristaltic pump
10 Refrigerated multichamber centrifuge
11 Cryosupernatant for FIX recovery

Fig. 1. Continuous-flow process for cryoprecipitate preparation.

continuously via a weir and 9-litre holding vessel to a refrigerated multichamber centrifuge (fig. 1).

Processing Cryoprecipitate

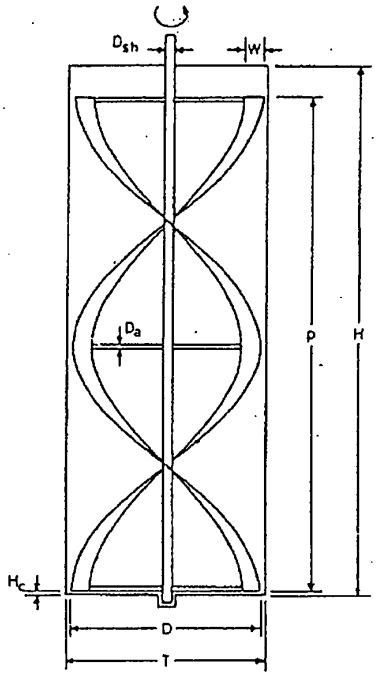
The processing of cryoprecipitate to an intermediate-purity concentrate was based on the methods of *Newman* et al. [8] and *James* et al. [13]. The recovered cryoprecipitate was suspended in 0.02 *M* Tris buffer (30 ml/l of plasma) and extracted at pH 7.0 and 20 °C using either high-shear (Silverson, model L2R; Chesham, UK) or low-shear (Vibromixer, model E2; Chemapec, Abingdon, UK) agitation. Aluminium hydroxide (Alhydrogel, 1.3% oxide, Superfos, Copenhagen) was mixed into the suspension (3.5 ml gel per litre of plasma) to adsorb coagulation factors II, VII, IX and X and other proteins which inhibit membrane filtration. Aluminium hydroxide gel and insoluble protein were removed together by batch centrifugation at 4,500 *g* for 15 min followed by filtration through a glass-fibre disc to ensure complete removal of any traces of gel.

The citrate content of the solution was increased to 0.02 *M* using 0.5 *M* trisodium citrate and 0.02 *M* citric acid was added to readjust the final concentrate to pH 7.0. The solution was filtered for clarification (to 0.45 µm) and for removal of bacteria (0.22 µm) and then dispensed aseptically into vials, frozen to −40 °C and freeze-dried.

Assays

Factor VIII:C in plasma and cryoprecipitate was assayed using a one-stage method with congenitally deficient plasma as substrate [14]. These assays were standardised against British Plasma Standards (77/520, 78/506) calibrated [15] according to both one-stage and two-stage [16] methods. The factor VIII:C content of the intermediate-purity concentrate was determined from results of local one-stage assays and one-stage and two-stage assays carried out at an external laboratory, all of these being calibrated against a concentrate standard [76/540; 15].

Factor-VIII-related antigen (VIIIR:Ag) was measured by both the standard method of *Laurell* [17] and as modified by *Prowse* et al. [4]. In the latter procedure, 0.1% mercaptoethanol was added to the gel to reduce the factor VIII molecule to a constant size so that interference from molecular size changes due to processing



	D	T	H	p	W	D_{sh}	D_a	H_c	θ
Pilot, cm	23.5	24.5	42	36.8	2.3	2.1	0.9	1.5	25°
Full scale, cm	29.0	30.0	80	75.0	2.8	1.6	0.6	0.6	45°

Fig. 2. Thawing vessel and agitator dimensions.

could be avoided, as this affects the rate of migration through the gel. This method was designated VIIIR:Ag reduced (VIIIR:Ag_R) and the standard method VIIIR:Ag non-reduced (VIIIR:Ag_N). For both of these assays a 24-donor pool of frozen plasma was used as a standard.

Total protein was measured by the biuret method [18] and the reconstitution time of the freeze-dried concentrate was determined at room temperature (22 °C) by adding distilled water at half the dispensed volume of concentrate and swirling gently until all of the solids had dissolved.

Statistics

Results were expressed as the mean ± standard deviation. For data involving a measurement of factor VIII activity, such as factor VIII recovery or specific activity, the calculation was carried out in the geometric rather than arithmetic form [19]. The significance of differences was established using Student's t test.

Results

Mixing and Mechanical Operation

The most important aspect of the mechanical design was the need to achieve steady-state operation without either blockage of the vessel outlet, by ice or cryoprecipitate, or excessive loss of ice into the pump feed vessel. The characteristic mixing pattern of the double helical ribbon agitator [20, 21] was found to be particularly suitable. The rotating ice mass produced a 'wiped-film' effect at the wall giving good heat transfer while, with upwards pumping, a secondary flow of ice up the wall and down the central axis retained the plasma 'snow' within the vessel; however, the exact design of the agitator and vessel outlet were important in achieving this [12]. The agitator blades had to be sufficiently close to the vessel wall to pick up ice particles and the pitch and blade angle were set to give maximum pumping capacity [21]. The blade width was limited by the need to allow a free downward flow of plasma ice but, when too narrow, pumping capacity was inadequate. With both of these factors taken into account, a width of approximately 0.1 × vessel diameter was found to be optimal. A speed of rotation of 55 rpm was also found to be optimal in both the pilot and full-scale designs as at higher speeds the circulation velocity tended to inhibit the flow of liquor leaving the tangential outlet.

Temperature Control

In continuous thawing, control of temperature is primarily achieved by removing plasma from the point of heat input as soon as it thaws. Even with this mode of operation there is a thin film of thawed plasma at the heated surface which is being continuously renewed and further control is therefore important. Monitoring at different points at the vessel wall has shown that this temperature is largely determined by that of the water flowing through the jacket. Typical temperature profiles illustrate this (fig. 3), with the highest temperature (T_H) being found near the jacket inlet port.

A comparison between batch and continuous thawing was made using a jacket temperature of 16 °C. During the batch thaw, the highest film temperature (T_H)

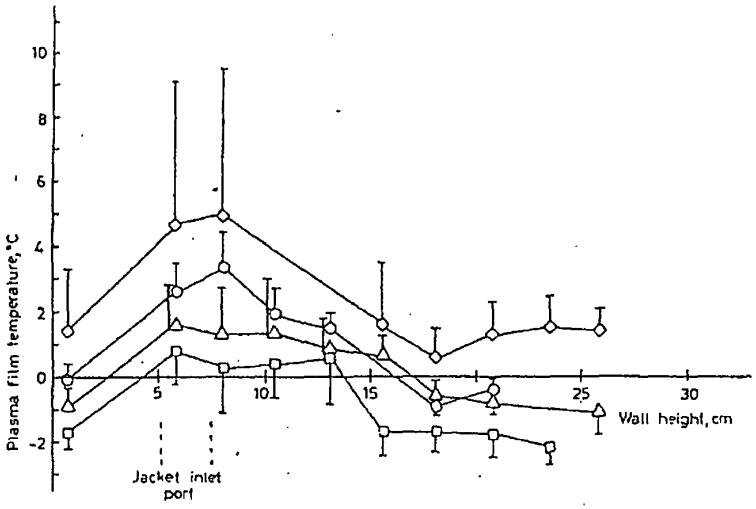


Fig. 3. Plasma temperature at the wall of the pilot vessel during continuous thawing. Results are the mean and standard deviation from measurements taken at 10-min intervals throughout 4 h of thawing. Mean jacket water temperature: ◇=32.1 °C; ○=19.1 °C; △=14.7 °C; □=12.5 °C.

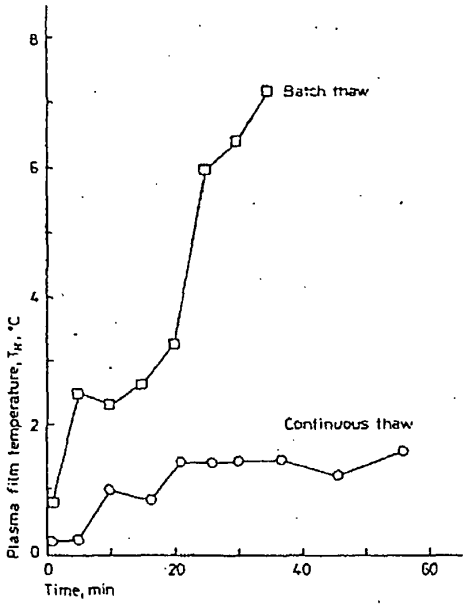


Fig. 4. Plasma film temperature for batch and continuous thawing. □=Batch thaw; ○=continuous thaw.

rose rapidly even though the bulk of the solution in the vessel remained close to 0 °C. In contrast, continuous thawing resulted in T_H values remaining close to 1.5 °C after the initial start-up period (fig. 4).

The factor VIII content of cryoprecipitate was measured from runs lasting 3–4 h over a range of jacket temperatures (table I). All three factor VIII activities were higher in the low-temperature group ($p < 0.025$ for VIII:C and VIIIR:Ag_N) suggesting that T_H should be held close to 2 °C and that this could be achieved in the pilot vessel with a mean jacket temperature close to 15 °C. On scale-up, the more precisely designed agitator gave better mixing, allowing an optimal jacket temperature of 20 °C to be used.

Product Recovery

Having determined the optimal thawing conditions at pilot-scale, the factor VIII recovery in cryoprecipitate was compared with parallel lots thawed by the batch method, all utilising 6-hour plasma (table II). As well as giving a significant yield improvement ($p < 0.001$), the continuous method also appeared to produce a more readily soluble cryoprecipitate. The fibrous nature of routine batch-thawed cryoprecipitate was such that severe agitation (Silverson) was required for 20–30 min to ensure full extraction of factor VIII. With a more

Table I. Pilot-scale thawing. Effect of temperature on the recovery of factor VIII activities in cryoprecipitate

	n	Wall temperature	Plasma pool volume	Factor VIII content, u/l plasma		
		T_H °C	litres	VIII:C	VIIIR:Ag_N	VIIIR:Ag_R
High-temperature thaw	9	3.7 ± 1.9	227 ± 50	361 ± 54	1,042 ± 403	661 ± 160
Low-temperature thaw	10	2.9 ± 1.1	189 ± 25	429 ± 53	1,449 ± 242	800 ± 266

T_H is the mean wall film temperature at the warmest position. The jacket temperatures were in the range 22 – 32 °C for the high-temperature group and 15 – 17 °C for the low-temperature experiments.

Table II. Factor VIII: C recovery in cryoprecipitate prepared by different methods

Method of preparation	No. of lots	Plasma		Cryoprecipitate	
		pool volume litres	thaw rate l/h	cryoprecipitate weight g/l plasma	FVIII:C recovery IU/l plasma
Batch thaw					
Full scale: high-shear extraction	25	149 ±23	(140)	8.8 ± 1.6	317 ± 66
Continuous thaw					
Pilot scale: high-shear extraction	44	230 ± 44	66.8 ± 8.9	10.0 ± 1.5	424 ± 51
Pilot scale: low-shear extraction	8	286 ± 6	78.8 ± 6.2	9.4 ± 0.7	485 ± 88
Full scale: low-shear extraction	9	387 ±84	194.5 ± 26.4	8.9 ± 1.3	498 ± 42

The batch thaw rate, in parentheses, is an estimate. Factor VIII recovery was assessed in the clarified cryoprecipitate extract, before addition of aluminium hydroxide.

soluble cryoprecipitate being produced, a low-shear agitator (Vibromixer) was reassessed and complete extraction was achieved in 10 min with a further increase in factor VIII yield ($p<0.005$) at this stage. Better discrimination between factor VIII and less soluble protein was also evident, improving both the specific activity ($p<0.001$) and solubility ($p<0.005$) of the final intermediate-purity concentrate (table III). Following scale-up of the continuous thaw process, the desired throughput has been achieved with a mean residence time of 17 min and improvements in yield and specific activity have been maintained giving a 55% increase in factor VIII recovery in comparison to the batch method ($p<0.001$).

18-Hour Plasma

Continuous thawing was also tested using complete pools of 18-hour plasma processed under the same conditions as the 6-hour fresh-frozen plasma (table IV). There was an improvement in factor VIII yield at the cryoprecipitate ($p<0.01$) and final product ($p<0.001$) stages and the introduction of low-shear extraction was

Table III. Factor VIII:C recovery as intermediate-purity concentrate

Method of preparation	No. of lots	FVIII:C recovery IU/l plasma	Specific activity IU/mg protein	Reconstitution time, min
Batch thaw				
Full scale: high-shear extraction	25	206 ± 34	0.25 ± 0.06	9.3 ± 5.8
Continuous thaw				
Pilot scale: high-shear extraction	44	277 ± 33	0.26 ± 0.05	11.1 ± 4.7
Pilot scale: low-shear extraction	8	290 ± 39	0.33 ± 0.06	5.9 ± 3.0
Full scale: low-shear extraction	9	320 ± 48	0.38 ± 0.07	6.3 ± 2.2

Table IV. Factor VIII recovery from 18-hour plasma

Method of preparation	n	Plasma pool volume	Cryoprecipitate	Intermediate-purity concentrate	
		litres	FVIII:C recovery IU/l plasma	FVIII:C recovery IU/l plasma	specific activity IU/mg
Batch thaw					
Full scale: high-shear extraction	11	135 ± 19	290 ±90	180 ±50	0.22 ±0.06
Continuous thaw					
Pilot scale: high-shear extraction	8	249 ± 33	346 ± 68	259 ± 31	0.25 ± 0.04
Pilot scale: low-shear extraction	1	288	448	324	0.37
Full scale: low-shear extraction	5	435 ±7	415 ± 52	289 ± 32	0.37 ± 0.03

again associated with an increased specific activity ($p < 0.001$).

Discussion

Numerous studies have been carried out to try and improve the recovery of factor VIII in cryoprecipitate, especially at the small pool scale. A particularly successful approach has been the thaw-siphon technique developed by *Mason* [22]. The high factor VIII:C recoveries reported [22–24] are almost certainly a consequence of an improvement in temperature control, achieved by removing the liquid phase from the point of heat input as soon as the plasma thaws; but the scale of operation is probably also important.

Although the thaw-siphon technique may hold considerable promise for the preparation of single-donation cryoprecipitate, its potential for scale-up may be rather limited. The frozen plasma block within the plastic pack has a relatively small surface

area, limiting the rate of thaw to about 0.3 litres/h for a single donation. At the same time, even though the thawed liquor is continuously removed, the frozen block is essentially thawed in a batch mode and some reduction in temperature control might be expected on scale-up. Extension to 1-litre and 2-litre packs of plasma has been proposed [23], but the surface area for heat transfer will be further reduced and the aim of increased throughput may be offset by a reduced rate of thaw. With temperature control probably becoming more difficult, then factor VIII yield is unlikely to be maintained even on moderate scale-up.

Large-scale processing is required not only to handle a greater plasma throughput but also because it is only at this scale of manufacture that proper pharmaceutical quality control can be applied.

Attempts to resolve the problem of poor cryoprecipitate factor VIII yield at this scale have concentrated on the addition of reagents, such as ethanol [8] or polyethylene glycol [3], which lower factor VIII solubility, thereby reducing the loss from dissolution into the cryosupernatant. Unfortunately, the solubility changes are not specific for factor VIII. Consequently, ethanol-assisted cryoprecipitation has been found to result in a lower specific activity with no overall improvement in yield [13, 25] and we have made similar observations for both ethanol- and PEG-assisted cryoprecipitations.

During the development of the continuous thaw process, other factors were noted which appeared to influence both factor VIII yield and the extent of contamination with residual protein. In one set of experiments frozen plasma was removed from −40°C cold storage and immediately crushed in the hammer mill and thawed. In all of these runs, the recovery of factor VIII:C in the extracted cryoprecipitate was poor (less than 300 IU/l) and a terminal cold precipitation step [25] was required before the solution could be properly filtered. In contrast, much better factor VIII:C recoveries and filtration characteristics were observed when −40°C plasma was allowed to warm at +4°C for a few hours before crushing and thawing.

The reasons for this behaviour are not yet fully understood but the nature of the thawing process itself may well be important. The particle size of the crushed plasma varied according to the temperature of the frozen block, with colder plasma producing larger particles. When using −40°C plasma, the thawing process was therefore challenged with a colder feedstock and a reduced surface area for heat transfer thereby giving a slower and less regular rate of thaw. The optimal temperature of plasma for crushing was found to be in the range −15 to −10°C, corresponding to a median particle size after crushing of approximately 0.2 cm diameter, compared to about 1.0 cm at −40°C; however, further studies are in progress to resolve this feature more precisely.

Cryoprecipitate produced by ourselves over many years using the batch thaw process was usually rather difficult to resuspend, and a Silverson agitator was needed so that the fibrous mass could be broken down and fully dispersed, but the continuous-thaw cryoprecipitate consistently appeared to be easily resuspended. Although the change to continuous processing also included the introduction of a different centrifuge for cryoprecipitate collection, it is unlikely that the consistency of the solids could have been so markedly changed by this, especially as cryoprecipitate sediments readily and temperature control in the Sharples centrifuge

was adequate at the feed rate from the batch process. It is more likely that the change was due in some way to the more uniform thawing conditions of the continuous process and perhaps because there was also much less opportunity for freshly cryoprecipitated particles to aggregate together to form large fibrous lumps.

The Vibromixer agitator previously recommended for cryoprecipitate extraction [8] was therefore reevaluated and it was apparent from the increase in yield and specific activity that the Silverson was inactivating FVIII:C, probably by shear or shear-associated effects, while at the same time promoting the dissolution of less soluble protein into the factor VIII extract.

Immunological measurements of factor VIII can be used to monitor process losses and comparisons with factor VIII:C can help to distinguish between the various loss mechanisms, such as inactivation or dissolution into the cryosupernatant. The modified VIIIR:Ag assay, VIIIR:Ag_R, is considered to give a more accurate measure of the true antigen levels during processing [4]. For the continuous thaw process, the mean cryoprecipitate VIIIR:Ag_R value of 800 U/l plasma, for the lower-temperature pilot-scale experiments (table I), compares favourably with a mean figure of 570 U/l previously reported for the batch process [4]. As about 25% of plasma factor VIII remains in the cryosupernatant [23, 26], this recovery of antigen activity is probably close to the maximum that can be achieved suggesting that, with continuous thawing, solubility losses are close to zero and that further improvement in cryoprecipitate yield will be possible only if inactivation of factor VIII:C can be reduced during the routine collection of plasma for fractionation.

Factor VIII was not inactivated during the size reduction of the frozen plasma blocks [4] and representative samples of the crushed plasma have provided an estimate of the factor VIII content of the plasma fractionated. A mean VIII:C value of 0.71 ± 0.18 IU/ml (n=22) was obtained for the pools of 6-hour plasma used in the full-scale continuous-thaw process, giving a yield of 70% at the cryoprecipitate stage and 45% as freeze-dried intermediate-purity concentrate.

However, the most important feature of the process design is that the steady-state characteristics of the continuous process are independent of the plasma pool size, and factor VIII recovery is therefore no longer dependent on the scale of processing.

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