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Virus Inactivation of Fresh Frozen Plasma by a Solvent Detergent Procedure: Biological Results

Abstract

In order to increase the safety of blood products, we have developed a procedure for the virus inactivation of fresh frozen plasma. Several batches have been prepared and with the first 10 batches, each of them composed of 60 litres of plasma, we have determined a set of biological parameters. Virus inactivation was realised using TnBP (1%) and Octoxynol 9 (1%). After their elimination with castor oil using chromatography on insolubilized C18 resin, glycine was added and the pH of the plasma was adjusted to 7.4. Plastic bags were aseptically filled with a mean volume of 200 ml of plasma. The mean levels of coagulation factors were all over 0.7 U/ml and their recovery from initial plasma was nearly the same as total protein except for factor VIII:C. The net loss in factor VIII:C was 16%, when including the dilution of plasma. In vivo and in vitro tests demonstrated that in the final product there were no activated factors. As in fresh frozen plasma, the protein concentration was over 50 g/l and the potassium level lower than 5 mmol/l. According to these results, virus-inactivated plasma has the same qualities of fresh frozen plasma and could now replace it.

The heating of albumin for 10 h at 60°C [1] has proved its efficiency in eliminating the risk of transmission of viruses. The safety of other blood-derivative proteins, such as coagulation factors, could be improved, first by the incorporation of a heating procedure in the lyophilized state to kill HIV viruses [2], and second, by the introduction of a solvent detergent method to inactivate all enveloped viruses [3]. Other methods can be used including heating of coagulation factors in the liquid phase [4] or β -propiolactone treatment [5]. Today almost all plasma derivatives are virus-inactivated.

Unfortunately, with labile products including fresh frozen plasma, the risk of virus transmission is not com-

pletely eliminated, despite screening tests. Nevertheless, Piet et al. [6] have shown that plasma could be treated by solvent detergent mixtures, especially with TnBP and Triton X-45.

This procedure provides an inactivation of various human pathogenic viruses added to plasma of more than 10^5 TCID₅₀. This was confirmed by Horowitz et al. [7] by testing an inactivation procedure using 1% TnBP and 1% triton X-100 (Octoxynol 9) on viruses such as HIV, HBV and HCV.

Based on these findings, a virus-inactivating procedure for pooled plasma has now been established by Octapharma Laboratories. We decided to use the Octapharma pro-

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cedure in our blood bank and currently 27 batches of virus-inactivated plasma have been prepared. In 10 batches we have performed coagulation and biochemical parameters which are presented in this report.

Material and Methods

Fresh Frozen Plasma Collection

Fresh frozen plasma was isolated from whole blood donations. The plasma was frozen at -40°C within 6 h after collection and stored at -30°C . The mean volume of plasma in each bag was 250 ml. Fresh frozen plasma was also obtained from plasmapheresis. In this case, the plasma was immediately frozen after collection. The mean volume of plasma in each bag was 600 ml.

Each batch of plasma can be treated with respect to blood group specificity, but our first 10 lots were prepared without taking blood group into consideration.

Virus-Inactivation Procedure

Ten batches of 60 litres of pooled plasma were studied in this work. Each batch was composed of 50% of plasma from routine whole blood donations and 50% from plasmapheresis.

Plasma was thawed in a thermostatic tank and filtered with 1- μm filters into an other tank. The pooled plasma was held at a temperature of 30°C . Then 1% (w/w) TnBP (Fluka Laboratory) and 1% Octoxynol 9 (w/w) (Serva Laboratory) were added and gently mixed for 4 h at 30°C . Viral inactivation and all the operating procedures after inactivation were performed in a strictly separate and controlled room.

The virus-inactivating agents were removed by first adding 5% (vol/vol) castor oil (Cooper Pharmaceutical Laboratory) and then by chromatography on insolubilised C18 resin obtained from the Waters division of Millipore. Plasma was collected in a tank without making any concentration. Glycine (3%, w/w, Merck Laboratory) was then added, solubilized by mixing and the pH of the plasma was adjusted to 7.4.

After this last step, plasma was filtered through a 0.22- μm filter and the plastic bags (Maco Pharma Laboratory) were aseptically filled with a nominal volume of 200 ml of plasma. Then the plasma was frozen to -40°C with liquid nitrogen and stored at -30°C .

Coagulation Parameters

Factors VIII, IX:C, XI and XII were tested using a one-stage method [8] on a semi-automated coagulometer (KC 10, Baxter). The content of factor VIII was calculated and compared with a national standard. The levels of factor IX:C, XI and XII were determined and compared with those of a pool of citrated plasma from 20 normal donors, with coagulation values considered to be 1 U/ml.

The prothrombin time method [9] was used to measure coagulation factors V, II, X and VII:C, with a pooled plasma as standard. AT III was evaluated using a chromogenic substrate (Stago Laboratory [10]). Clottable fibrinogen was quantified by a method previously described [11]. Von Willebrand factor activity (vWF:RiCof) was measured with an aggregometer using washed human platelets from blood donors [12] and vWF:Ag by an ELISA method [13].

Assays of Factor-VIII Stability

Stability of Factor VIII at 37°C . Three bags of fresh frozen plasma and 3 bags of virus-inactivated plasma were placed in a water bath at 37°C . After thawing, samples of plasma were taken from each bag at 1, 2, 3, 4 and 5 h and factor VIII was measured immediately.

Stability of Factor VIII at Room Temperature. Three bags of fresh frozen plasma and 3 bags of virus-inactivated plasma were thawed at 37°C and then placed at room temperature. Factor VIII assays were done on each bag at 1, 4, 12, 24 and 48 h.

Stability of Factor VIII at -30°C . We have begun a stability test on 5 batches after 3 months of storage at -30°C .

Thrombogenicity Tests and Evaluation of Biological Parameters to Determine Factors X, VII and IX Activators

An in vivo thrombogenicity test was carried out as described elsewhere [14]. A dose of 5 ml body weight was injected to male Wistar rats. Activated factor Xa [15] was measured by an amidolytic assay (Stago). Factor-VII antigen (VII:Ag) and factor-IX antigen (IX:Ag) were evaluated by an immunoenzymatic assay [16] to obtain VII:Ag/VII:C and IX:Ag/IX:C ratios,

Biochemical Parameters

Total protein was determined by the biuret method [17]. This test was validated by comparing it with those tests for total protein set out by the European Pharmacopea. Free haemoglobin was quantitated with an assay using tetramethyl benzidine [18], while potassium and sodium were determined by flame photometry.

TnBP was measured by gas chromatography [19] and Octoxynol 9 by high pressure liquid chromatography. The main steps of the assay were as follows.

First, the separation of plasmatic proteins from Octoxynol 9 was carried out on a C18 resin. After loading the column with the sample or the standards, the gel was washed with water and the fraction containing Octoxynol 9 was eluted with 75% isopropanol, and then concentrated to 0.2 ml with an analytical evaporator. Water was added to bring the volume to 1 ml. Second, samples were chromatographed on a 3.9 nm $\times$ 30 cm Bondapak C18 column (Waters). A linear gradient with isopropanol was used. It was performed with 100% water (initial solution) and 100% isopropanol (final solution). The flow rate was 1 ml/min and 0.25 ml of sample was applied on the top of the column. Octoxynol 9 was detected at 230 nm by UV absorption. For each sample the retention time was noted, the area of the peak measured and the quantity of Octoxynol 9, evaluated comparatively to standards.

Electrophoresis on initial and final products was done on cellulose acetate. The endotoxin level was estimated using a chromogenic test after a 1/20 dilution of plasma [20].

The pyrogen test was done by infusion of 3 ml/kg of plasma which had previously been heated for 30 min at 56°C to avoid rabbit reactions due to human complement. The specifications of this test are described in the British Pharmacopea for dried human plasma.

Results

The majority of coagulation factors were not affected by the viral-inactivation procedure. Except for factors VI-II and XI, all values were over 0.80 U/ml (table 1).

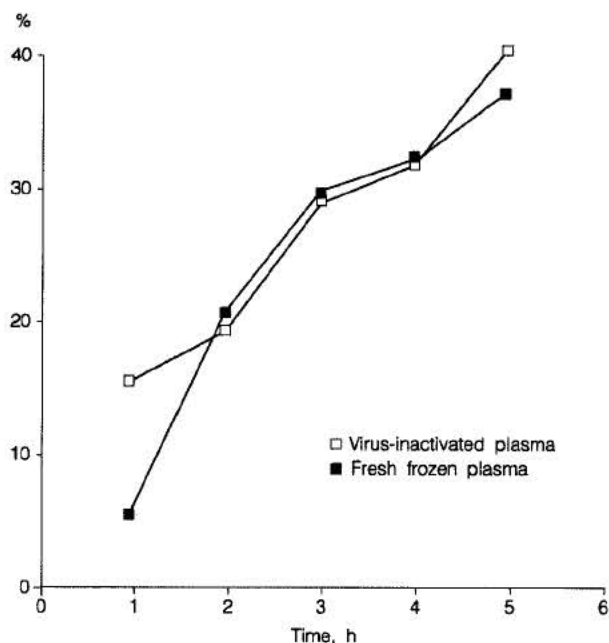


Fig. 1. Loss of factor VIII after a 5-hour storage period at 37°C.

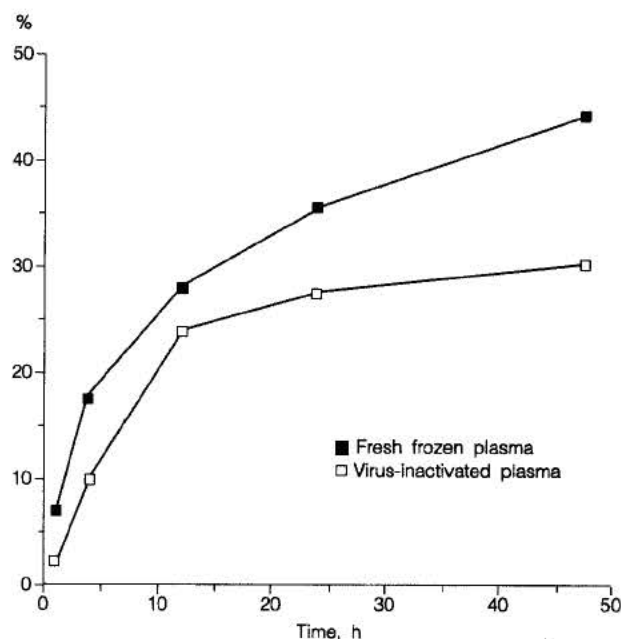


Fig. 2. Loss of factor VIII storage for 48 h at room temperature.

Table 1. Mean levels and recovery of coagulation factors in virus-inactivated plasma (n = 10)

Coagulation factors	Levels, U/ml	Recovery, %
II	0.90±0.05	90.78±4.06
V	0.83±0.06	84.82±6.9
VII	0.99±0.08	98.20±3.41
VIII	0.76±0.07	75.66±6.06
vWf:Ag	0.92±0.14	97.87±4.42
vWf:RiCof	0.93±0.11	90.95±6.06
IX	0.82±0.08	88.23±7.59
X	0.87±0.06	91.55±6.11
XI	0.75±0.07	91.71±7.33
XII	1.04±0.08	95.52±4.61
AT III	0.94±0.09	91.34±5.51
Total proteins		92.02±2.60

Table 2. Stability study of factor VIII after a 3-month storage period at -30°C

Number	Levels of factor VIII, U/ml, in virus-inactivated plasma	
	day 1	day 90 at -30°C
1	0.67	0.64
2	0.70	0.65
3	0.65	0.70
4	0.70	0.73
5	0.86	0.75
Mean	0.71	0.69
±SD	0.08	0.05

In our study of the factors measured, factor VIII:C was the most labile with an average decrease of about 24% during the whole process. As there was a small dilution of plasma in the chromatographic step, the real decrease in factor VIII, after correction for dilution, was about 16%.

After thawing, the decrease in factor VIII:C at 37°C in virus-inactivated plasma was the same as in fresh frozen plasma (fig. 1).

The same results were found when plasma bags were kept for 48 h at room temperature (fig. 2) and no clot was seen in all the bags checked at room temperature after thawing.

Until now, stability trials at -30°C have shown no loss of coagulation factor VIII (table 2).

Coagulation factor V was slightly affected in our process where 84.82% was recovered (table 1). The decrease

Table 3. Results of in vitro tests to study the activation of coagulation factors

Batch No.	Factor VIIC/factor VII Ag, U/ml		Factor IXC/factor IX Ag, U/ml		Factor Xa, U/ml	
	before inactivation	after inactivation	before inactivation	after inactivation	before inactivation	after inactivation
1	1	1.03	1.03	1.17	0.045	0.046
2	0.96	1.06	1.09	0.98	0.043	0.041
3	0.95	1.05	1.11	0.96	0.040	0.042
4	1.03	1.03	1.11	0.96	0.040	0.042
5	1.02	1.12	1.13	1.28	0.040	0.043
6	1.02	1.08	1.09	1.01	0.038	0.043
7	1.03	1.12	1.02	1.06	0.041	0.045
8	0.80	0.82	0.92	0.88	0.040	0.044
9	0.80	0.84	1.80	0.85	0.032	0.037
10	0.85	0.98	1.40	1.60	0.034	0.033
Mean	0.94	1.01	1.19	1.07	0.039	0.042
SD	0.09	0.10	0.25	0.22	0.004	0.004

Table 4. Mean biochemical parameters and electrophoretic profile on virus-inactivated plasma (n = 10)

Biochemical parameters	Values
pH	7.45±0.06
Total proteins, g/l	54.9 ±1.78
Sodium, mmol/l	157.66±9.04
Potassium, mmol/l	3.28±0.11
Osmolarity, mosmol/l	323.9 ±18.79
Plasmatic haemoglobin, mg/l	63.1 ±12.26
Endotoxins, EU/ml	0.88±0.43
TnBP, µg/l	<5
Octoxynol 9, µg/ml	<5

Electrophoretic profile	Before viral inactivation	After viral inactivation
Albumin, %	59.96±3.33	59.78±4.87
α ₁ -Globulins, %	2.41±0.45	2.49±0.45
α ₂ -Globulins, %	8.08±1.39	8.07±2.05
β-Globulins, %	10.38±1.55	11.15±1.47
γ-Globulins, %	19.19±2.24	18.51±1.51

in the other factors was much the same as those of total protein. The in vivo activation of coagulation factors was negative and no thrombogenic effect was observed.

In vitro assays for activation of coagulation factors were negative. There was no statistical difference between VII:Ag/VIII:Ag and IX:Ag/X:Ag ratios and fac-

tor Xa when performed on initial plasma and on the viral-inactivated plasma (table 3).

The protein concentration was consistently over 50 g/l and the distribution of proteins in virus-inactivated plasma was the same as in the initial plasma (table 4).

The mean value of the pH is 7.45 and the mean potassium level was less than 5 mmol/l. Very small amounts of TnBP and Octoxynol 9 were found. These were lower than 5µg/ml. Both the limulus test and pyrogenicity testing in rabbits gave no evidence for the presence of pyrogen in any batch.

Discussion

These studies show that the virus-inactivated plasma described in this report has the same characteristics as our fresh frozen plasma, with respect to the coagulation factors (mean level ≥0.7 U/ml), potassium (<5 mmol/l), protein concentration (>50 g/l). These observations confirm our first results [21] and demonstrate that the quality of biological parameters in the final product depends not only on the preparation method but also on the initial plasma quality.

In all our batches, we included some plasma from plasmapheresis, because the factor-VIII level is higher than in plasma from whole blood donations [22]. If it is necessary for each batch of virus-inactivated plasma to achieve a factor-VIII level of over 0.7 U/ml, then plasma from plasmapheresis should be used exclusively.

Large volumes of virus-inactivated plasma can induce haemolytic disorders due to haemagglutinins and as a consequence we have decided that future batches will be prepared to take blood group specificity into account.

We have found the same results as Toby and Simon [23] on factor-VIII molecule stability. After 3 months of storage, no loss of activity has been observed and we expect that the expiry date will be similar to fresh frozen plasma. For one batch, a study of a 6-month storage has been achieved and the levels of factor VIII was the same as that found on the first day after manufacture.

One important question is whether the inactivation procedure induces activation of coagulation factors. According to our *in vitro* biological results, the products are characterized by the absence of activation of coagulation factors. As SD treatment is mainly directed towards lipids and lipophilic proteins [24], virus-inactivated plasma may deplete phospholipids with procoagulant activity. This conclusion was supported by the results obtained with the *in vivo* test, which did not reveal any thrombogenic activity in the rat stasis model.

Other methods for improving the safety of plasma have been reported. Some have used UV irradiation [25], gamma radiation [26] and others have proposed filtration

methods [27]. Cambrecht et al. [28] have described a method using phenothiazine dyes in combination with visible light. This latter approach necessitates the manipulation of individual donations of plasma. We believe the processing of plasma pools may have some advantages: standardization of biological parameters is facilitated. Thus all plasma bags from the same batch have the same quality. Pyrogen tests are done on each batch and the load of endotoxins can be determined. This latter facility may be a very important consideration when virus-inactivated plasma is used in large volume plasma exchange. Finally, this solvent detergent approach to virus inactivation in plasma is unlikely to give rise to the development of neo-immunogens [7].

The safety of blood products is a priority for blood transfusion services and, according to the studies reported above, we believe that it should be possible to replace fresh frozen plasma by virus-inactivated plasma.

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