

## Stability of prothrombin and factor VII in freeze-dried plasma

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**SYNOPSIS** The stability of prothrombin and factor VII was studied using accelerated degradation tests in three preparations of freeze-dried pooled normal plasmas. In a previous report (Brozović, Gurd, Robertson, and Bangham, 1971) factor X was shown to be relatively unstable in these preparations of freeze-dried plasma: it was calculated that up to 8% of the original factor X activity would be lost after 10 years at  $-20^{\circ}\text{C}$ , up to 54% at  $4^{\circ}\text{C}$ , and up to 90% at room temperature.

The losses of factor VII activity were estimated to be negligible at  $-20^{\circ}\text{C}$ , between 2 and 18% at  $4^{\circ}\text{C}$ , and between 20 and 70% of the original activity at  $20^{\circ}\text{C}$ , after 10 years of storage. Prothrombin was found to be less stable than factor VII: the expected loss in 10 years at  $-20^{\circ}\text{C}$  may be up to 4%, at  $4^{\circ}\text{C}$  up to 30%, and at  $20^{\circ}\text{C}$  up to 83% of the initial activity. These findings indicate that in freeze-dried plasma prothrombin as well as factor X may be insufficiently stable for plasma to serve as long-term reference material for the standardization of the one-stage prothrombin time. Moreover, the loss of prothrombin and factor X in freeze-dried plasma stored at  $4^{\circ}\text{C}$  may be so high that when it is required to preserve these factors it may be necessary to store freeze-dried plasma at lower temperatures.

During the past several years attempts have been made to devise a generally acceptable method of standardization of one-stage prothrombin times. There is now general agreement that the thromboplastin sensitivity ratio of Biggs and Denson (1967) obtained with characterized thromboplastin preparations can be employed to compare prothrombin time results obtained in different laboratories using different thromboplastin preparations. Another approach has been proposed by Miale and LaFond (1967). Freeze-dried reference plasmas of determined clotting factor content are used to test the performance of different thromboplastins. It has been suggested that the two approaches might be combined, and characterized thromboplastin used in conjunction with freeze-dried reference plasma preparations to test different thromboplastins in a simplified system, as outlined by Biggs and Bangham (1971). The long-term stability of both reference thromboplastin and reference plasmas is of the utmost importance if such a system were to be relied upon over a period of years.

Preparations of two thromboplastins, one human and one bovine, were characterized in a collaborative

study (Bangham, Biggs, Brozović, and Denson, 1970) and shown to be very stable in accelerated degradation tests over a period of two years. The stability of three additional thromboplastins (two rabbit and one human preparations) is currently being studied. Loeliger and colleagues (Loeliger Menwisse-Braun, Buitendijk, Veltkamp, and Hemker, 1970) have shown that pooled normal plasma and pooled plasma from patients stabilized on long-term anticoagulant treatment, stored in small aliquots at  $-70^{\circ}\text{C}$ , gives reproducible results over a period of some years. Miale and LaFond (1969) tested their freeze-dried reference plasmas after 10 months' storage (temperature not specified), and showed that little change in prothrombin times occurred.

We have recently studied the stability of factor X in some preparations of freeze-dried plasma and found that factor X activity decreased on storage. When degradation constants were calculated and the loss of potency estimated, it became apparent that up to 8% of the initial factor X activity could be lost in 10 years if the ampoules were stored at  $-20^{\circ}\text{C}$ . As much as 50% could be lost at  $4^{\circ}\text{C}$  and about 90% at  $20^{\circ}\text{C}$  after the same period of time (Brozović *et al.*, 1971).

No data on the stability of prothrombin and factor VII in freeze-dried plasma were found in the literature. Preliminary results (Bangham *et al*, 1970) indicated that prothrombin and factor VII activity also decreased on storage. We have therefore studied the stability of these factors in plasma preparations freeze dried under the optimal conditions used to prepare international standards.

## Materials and Methods

### FREEZE-DRIED HUMAN PLASMA PREPARATIONS

Three preparations of freeze-dried human plasma, labelled 65/153, 66/355, and 68/413, were used. Each was prepared from a pool of approximately 4 litres of plasma, collected into ACD from 20 donors at the Oxford Blood Transfusion Centre in 1965, 1966, and 1968 respectively. The individual blood donations were promptly centrifuged at 4°C to get rid of cells and then pooled.

The same day the pooled plasma was distributed at room temperature in equal amounts into hard glass ampoules. Vessels used for the plasma were previously rinsed out with sodium citrate; the ampoules were steamed in acid, extensively rinsed with distilled water and citrate, and sterilized by dry heat in a clean oven. Approximately 10-12 hours after the blood had been obtained from the donors the plasma in the ampoules was frozen at -35°C, placed in liquid nitrogen and then freeze-dried as one batch at -35°C. Thereafter the ampoules were fitted with plastic plugs designed to limit gaseous diffusion and subjected to secondary desiccation for 13 days. After the ampoules had been filled with pure dry nitrogen they were sealed by fusion of the glass.

The moisture content was estimated as loss of weight by the material after heating to 56°C over P<sub>2</sub>O<sub>5</sub> in a vacuum for five hours. The oxygen content of the ampoules was estimated by mass spectrometry. The results obtained for the three plasmas are shown in Table I.

Plasma	Moisture Content (%)	Oxygen Content (%)
68/413	Negligible	0.09
66/355	Less than 1	0.15
65/153	2.59	4.39

Table I *Moisture and oxygen content of freeze-dried plasmas*

After the ampoules had been tested for pinholes and cracks they were stored at -20°C in the dark.

### PROTHROMBIN DETERMINATIONS

The prothrombin content of the three plasmas was measured by a modification of the method of Ware and Seegers (1949). The reaction mixture consisted of 3 parts Michaelis buffer, 2 parts 25 mM CaCl<sub>2</sub>, and 3 parts phenolized freeze-dried human brain thromboplastin reconstituted in distilled water. The bovine factor V used was obtained from Diagnostic Reagents Ltd. Fibrindex thrombin (Ortho Pharmaceutical Ltd) was used to determine the standard curve for each assay.

### FACTOR VII DETERMINATIONS

Factor VII was estimated by a one-stage method according to Denson (1967). An artificial factor VII-deficient plasma (kindly provided by Dr Swart, Academisch Ziekenhuis, Leiden) and phenolized freeze-dried human brain thromboplastin were used throughout.

### ACCELERATED DEGRADATION TESTS

These tests were performed as described by Jerne and Perry (1956). The samples of plasma were stored at -20°C, 4°C, 20°C, and 37°C; plasma 68/413 was investigated after 16 and 22 months, plasma 69/355 after 39 months, and plasma 65/153 after 49 and 56 months of storage at these temperatures.

## Results

### PROTHROMBIN CONTENT

The prothrombin content of the three freeze-dried plasmas, 68/413, 66/355, and 65/153, stored at -20°C, was estimated to be equivalent to 255, 210, and 150 NIH thrombin units per ml respectively. The prothrombin content of the plasmas decreased on storage as shown in Table II.

Plasma	Time of Storage (mth)	Prothrombin Content at:				Number of Assays
		-20°C	4°C	20°C	37°C	
68/413	22	255 <sup>1</sup>	225	157	50	2
66/355	39	210	—	—	64	4
65/153	56	150	138	85	—	2

Table II *Prothrombin in freeze-dried plasmas*

<sup>1</sup>Mean of two assays expressed as NIH units per ampoule

When degradation constants were calculated and the loss of potency estimated for plasmas 68/413 and 65/153, it became apparent that in 10 years at -20°C up to 4% of the original activity may be lost. If stored for the same period at 4°C and 20°C the loss could be up to 30 and 80% respectively (Table III).

Plasma	Estimated Potency Loss (as % of the Original Activity) in 10 Years at:		
	-20°C	4°C	20°C
68/413	0.89	23.84	83.08
65/153	3.90	29.35	69.98

Table III Estimated potency loss in prothrombin after 10 years of storage

#### FACTOR VII CONTENT

The factor VII content of the three plasmas was studied at the same time and expressed as a percentage of activity in plasma 68/413 stored at -20°C. It was estimated to be 93% and 72% for plasma 66/355 and 65/153 respectively, as shown in Table IV.

Plasma	Time of Storage (mth)	Factor VII Content at:				Number of Assays
		-20°C	4°C	20°C	37°C	
68/413	22	100 <sup>1</sup>	95 <sup>1</sup>	76	17	2
66/355	39	93	101	63	5	3
65/153	56	72	60	41	—	2

Table IV Factor VII in freeze-dried plasmas

<sup>1</sup>Mean of two assays expressed as % factor VII activity of plasma 68/413 stored at -20°C.

Factor VII activity decreased on storage although to a lesser extent than prothrombin or factor X activity. The estimated loss after 10 years at -20°C would be less than 2%, and at 4°C less than 18% of the original activity as shown in Table V. At room temperature, the plasmas were estimated to lose between 20 and 70% of factor VII activity in 10 years (Table V).

Plasma	Estimated Potency Loss (as % of the Original Activity) in 10 Years at:		
	-20°C	4°C	20°C
68/413	0.02	2.82	32.46
66/355	0.75	17.53	70.79
65/153	1.85	17.05	51.25

Table V Estimated potency loss in factor VII after 10 years of storage

#### Discussion and Conclusions

The three preparations of plasma studied had been ampouled under the conditions developed to provide international standards for various biological substances. These studies show that even in plasma ampouled under these conditions neither prothrombin nor factor X (Brozović *et al*, 1971) were as

stable at -20°C as would normally be required for the preparations serving as long-term reference materials. Factor VII was found to be more stable than the other two factors.

The stability of all three factors in these plasma preparations suggests that plasma could be used as a working standard for a few months or years providing it was stored below 4°C.

Plasma 65/153, stored for over four years, had a higher moisture and oxygen content than plasmas 66/355 and 68/413. The content of prothrombin and factor VII in this plasma was low when compared with that of the other two plasmas. However, the stability of the two factors was of the same order as in plasmas 66/355 and 68/413.

No buffer was added to the three preparations of plasma before freeze-drying. The addition of a buffer, such as N-2 hydroxyethyl-piperazine N-2-ethanesulphonic acid (HEPES), may improve the stability of these factors. HEPES is known to stabilize the pH of the solution after reconstituting the freeze-dried material (Zucker, Cathey, and West, 1970), but little is known about its effect on the long-term stability of individual factors. Some preliminary results suggest that it improves the stability of factor VIII in freeze-dried plasma on short-term storage at 37°C (Denson, 1970).

The results of this study indicate that prothrombin and factor X may be insufficiently stable in freeze-dried plasma to allow the use of plasma as long-term reference material for the standardization of the one-stage prothrombin time, and that for use as a working standard it should be stored at -20°C, and not at 4°C. Further investigations into the effect of various buffers added to plasma before freeze-drying are needed.

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