Stability of freeze-dried plasma prepared from patients on oral anticoagulants

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SYNOPSIS The suitability of freeze-dried plasmas from patients on oral anticoagulants to serve as reference material in the calibration of thromboplastins used in the control of oral anticoagulant treatment was assessed in two centres. One pooled normal plasma and four pooled plasmas from patients at different levels of anticoagulation were collected into HEPES-citrate containing Trasylol. The plasmas, in 1 ml volumes, were freeze dried in siliconized ampoules under the optimal conditions used for biological standards. Small aliquots of the same plasmas were stored at -70°C .

Five different thromboplastins were used to carry out the tests on these plasmas. There was little difference in the clotting times obtained with five reference thromboplastins (two human, two rabbit, and one bovine reagent) on frozen and freeze-dried plasma, if the latter was tested immediately after complete reconstitution or within six hours of reconstitution if kept at $+4^{\circ}\text{C}$. Reconstituted plasmas showed a marked shortening of clotting times when stored at 22 and 37°C if thromboplastins sensitive to activation of factor VII were used. In contrast, when thromboplastins sensitive to factor V were used, prothrombin times in plasmas stored at 22 and 37°C became prolonged due to the loss of factor V.

Freeze-dried plasmas from patients on oral anticoagulants can be used to calibrate thromboplastins, provided they are used immediately after complete reconstitution or kept at $+4^{\circ}\text{C}$ for use within four to six hours of reconstitution.

The standardization of the one-stage prothrombin time has been extensively studied and frequently reviewed (Biggs, 1965, 1969; Biggs and Denson, 1966; Biggs and Denson, 1967a, b; van Horn, 1968; Denson, 1969; Miale and LaFond, 1969; Loeliger and Hemker, 1970; Loeliger, Meuwisse-Braun, Buiterdijk, Veltkamp, and Hemker, 1970; Poller, 1970a and b; Zucker, Cathey, and West, 1970; Poller, Thomson, and Alderson, 1971; Loeliger, 1972; Miale and Kent, 1973). A number of different schemes for defining the therapeutic range and for standardizing the prothrombin time test are used in different countries. All these schemes are based on the use of one of the two types of reference materials, thromboplastins (Denson, 1966, 1967; Bangham, Biggs, Brozović, and Denson, 1970, 1973; Poller, 1970b, 1971; Biggs and Bangham, 1971) or plasma (Miale and LaFond, 1969; Miale and Kent, 1972; (Bangham, Biggs, Brozović, and Denson, 1973).

The suitability of thromboplastins to serve as long-term standards has been studied and documented (Denson, 1966, 1967; Poller, 1970b, 1971; Bangham *et al.*, 1970, 1973; Biggs and Bangham, 1971). A similar but much larger international collaborative study to investigate the use of reference plasmas is being organized. Two types of reference plasmas will be studied: first freeze-dried plasmas prepared from blood of patients on oral anticoagulants and secondly freeze-dried artificially prepared abnormal plasmas designed to mimic the situation in patients on oral anticoagulants. As a preliminary to this international trial we have studied the suitability of freeze-dried plasma collected from patients to serve as reference material and the results are reported here.

Materials

PLASMAS

Five plasmas were studied, one normal and four plasmas collected from patients on oral anticoagulants.

Plasma pool 72/411 consisted of plasma from 20 normal individuals, 10 men and 10 women; 18 ml of blood was collected into 2 ml HEPES-citrate solution in plastic containers.

Plasma pools 72/425, 72/427, 72/429, 72/431 consisted each of plasma from 30 patients stabilized on long-term anticoagulants. From each patient 4.5 ml of plasma was collected into 0.5 ml HEPES (N-2-hydroxyethylpiperazine-N¹-2-ethanesulphonic acid) citrate solution in plastic containers.

The HEPES citrate solution was made up in the following manner:

HEPES	44.62 g
Trisodium citrate (2H ₂ O)	38.00 g
Distilled water to	1000 ml
Trasylol (1000 u/ml)	0.5 ml

Individual samples were centrifuged at 3000 rpm for 10 min at room temperature, the plasmas separated and pooled. The pools were centrifuged in plastic containers at 20 000 g at 4°C for 30 minutes. In order to prevent bacterial growth 1 ml of 10% NaN₃ was added per litre of plasma.

The plasmas were distributed in 1 ml volumes into siliconized glass ampoules: 20 ampoules from each pool were sealed and stored frozen at -70°C. The remainder were freeze-dried under optimal conditions for biological standards. Twenty ampoules from each freeze-dried pool were stored at 37°C whereas the bulk of the ampoules was stored at -20°C.

THROMBOPLASTINS

1 *Thromboplastin 67/40 human, research standard A*

This is a specially prepared freeze-dried human brain preparation containing bovine fibrinogen and factor V. It is reconstituted with 2.0 ml of 3.2 mM CaCl₂ and used in 0.4 ml volumes with 0.05 ml of plasma.

2 *Thromboplastin 69/223, human plain, MRC reagent*

This thromboplastin is a batch of freeze-dried Manchester reagent. It is reconstituted with 2 ml distilled water and used in 0.1 ml volumes with 0.1 ml of plasma and 0.1 ml of 25 mM CaCl₂.

3 *Thromboplastin 68/434 bovine, MRC reagent*

This is a specially prepared batch of Thrombotest, bovine brain reagent with bovine factor V and fibrinogen. It is reconstituted with 2.2 ml of 3.2 mM CaCl₂ and used in 0.4 ml volumes with 0.05 ml plasma.

4 *Thromboplastin 70/115, rabbit MRC reagent*

This is a specially prepared batch of Normotest, rabbit brain reagent with bovine factor V and

fibrinogen. It is reconstituted with 2.2 ml distilled water and used in 0.25 ml volumes with 0.01 ml plasma.

5 *Thromboplastin 70/178, rabbit plain, MRC reagent*

This is a freeze-dried rabbit brain suspension. It is reconstituted with 1 ml distilled water and used in 0.1 ml volumes with 0.1 plasma and 0.1 ml 25 mM CaCl₂.

Methods

The tests were performed at the Academisch Ziekenhuis in Leiden and at the National Institute for Biological Standards and Control in London. Both laboratories performed prothrombin times using five different thromboplastins on frozen and on freeze-dried plasmas. The clotting times on freeze-dried plasma were performed immediately after reconstitution, four to six hours after reconstitution in samples stored at +4°C, +20°C, and +37°C during this time, and 24 hours after reconstitution on samples stored at these temperatures. In addition, prothrombin times were carried out on freeze-dried plasma samples stored at 37°C for two months (table I).

The levels of factors VII, IX, and X and prothrombin were estimated by both laboratories in frozen samples and in freeze-dried samples stored at -20°C and +37°C for two months, using the same freeze-dried reference plasma.

The behaviour of factor VII in reconstituted freeze-dried plasma was followed for 24 hours in one laboratory. Factor VII levels were measured immediately after reconstitution and after five, 10, 30, and 60 minutes, as well as after six hours and 24 hours.

The tests with five thromboplastins were done manually, whereas the factor assays were carried out using Depex coagulometers.

Results

COMPARISON OF PROTHROMBIN TIMES AND PROTHROMBIN TIME RATIOS ON FROZEN AND FREEZE-DRIED PLASMA AFTER RECONSTITUTION

There was little or no difference in clotting times obtained with frozen plasma when compared to those obtained with freeze-dried plasma; this was reflected in clotting time ratios which were virtually the same for frozen and freeze-dried abnormal plasmas. This is shown in tables I and II using as illustration the results obtained with thromboplastin 67/40, research standard A, and in table III showing the results of the clotting times of normal plasma with all five thromboplastins.

Plasma	Plasma Pool									
	Normal		Abnormal 1		Abnormal 2		Abnormal 3		Abnormal 4	
	Laboratory									
	1	2	1	2	1	2	1	2	1	2
Clotting time										
Frozen (mean)	18.5	19.5	33.5	34.7	44.5	47.0	51.5	53.5	58.5	61.8
Freeze dried	18.5	19.5	34.0	35.9	45.0	45.0	52.5	55.9	58.5	60.7

Table I Clotting times with thromboplastin 67/40 human, research standard A

Plasma	Plasma Pool							
	Abnormal 1		Abnormal 2		Abnormal 3		Abnormal 4	
	Laboratory							
	1	2	1	2	1	2	1	2
Clotting time (fresh)	1·81	1·79	2·40	2·33	2·78	2·78	3·16	3·14
Clotting time (freeze dried)	1·83	1·87	2·43	2·48	2·83	2·92	3·16	3·23

Table II Clotting time ratios with thromboplastin 67/40 human research standard A

<i>Thromboplastin</i>	<i>Laboratory</i>			
	<i>1</i>		<i>2</i>	
	<i>Plasma</i>			
	<i>Frozen</i>	<i>Freeze Dried</i>	<i>Frozen</i>	<i>Freeze Dried</i>
67/40	18.5	18.5	19.1	19.3
69/223	13.5	13.5	14.7	13.3
68/434	36.5	37.0	39.9	41.0
70/115	27.5	27.0	23.7	23.0
70/178	14.0	14.5	15.1	15.3

Table III Prothrombin times of normal plasma with different thromboplastins

CLOTTING TIMES AND CLOTTING TIME RATIOS IN RECONSTITUTED FREEZE-DRIED PLASMA STORED AT DIFFERENT TEMPERATURES

The reconstituted plasmas were stored at +4, 22, and 37°C and the prothrombin times performed with different thromboplastins after four to six hours and 24 hours of storage.

Plasmas stored at +4°C

There was virtually no change in clotting times or clotting time ratios of plasmas stored at 4° during the first four to six hours. After 24 hours of storage the clotting times obtained with thromboplastins 67/40, 68/434, and 70/115 were occasionally shorter than immediately after reconstitution, although the clotting ratios remained similar to the initial value. The clotting times and clotting time ratios obtained

with thromboplastins 69/223 and 70/178 remained unchanged (figs 1 and 2).

Plasma stored at +20°C

Clotting times obtained with thromboplastins 69/223, 70/178, and 70/115 showed very slight prolongation, whereas with thromboplastins 67/40, and in particular 68/434, a marked shortening of clotting times was evident. After 24 hours at room temperature these effects became more pronounced. The prothrombin time ratios obtained with thromboplastins 67/40, 68/434, and 70/115 remained very similar to those of freshly reconstituted plasma. With thromboplastin 70/178, and in particular 69/223, the prothrombin time ratios were variable, but usually lower than in the freshly reconstituted plasma. This is illustrated in figures 3 and 4.

Plasma stored at +37°C

There was a marked prolongation of clotting times with thromboplastins 69/223 and 70/178 after four to six hours' incubation, becoming more marked after 24 hours. The clotting times were only slightly prolonged in tests with thromboplastin 70/115. In contrast, the clotting times invariably shortened with thromboplastin 67/40 and in particular with thromboplastin 68/434 (see figs 5 and 6). Nevertheless, the prothrombin time ratios obtained with thromboplastins 67/40, 68/434, and 70/115 remained unaffected by the storage of plasma at 37°C for up to 24 hours. The ratios obtained with thromboplastins 69/223 and 70/178 varied and were often lower than before incubation.

Fig 1

Fig 2

Fig 1 Behaviour of reconstituted plasma 72/411 (normal) at +4°C.

Fig 2 Behaviour of reconstituted plasma 72/428 (abnormal 3) at +4°C.

Fig 3

Fig 4

Fig 3 Behaviour of reconstituted plasma 72/411 (normal) at +20°C.

Fig 4 Behaviour of reconstituted plasma 72/428 (abnormal 3) at +20°C.

Fig 5

Fig 6

Fig 5 Behaviour of reconstituted plasma 72/411 (normal) at 37°C.

Fig 6 Behaviour of reconstituted plasma 72/428 (abnormal 3) at 37°C.

BEHAVIOUR OF FACTOR VII IN RECONSTITUTED FREEZE-DRIED PLASMA

As the cold-promoted shortening of the thrombo-test time is due to the activation of factor VII (Gjønnaess, 1972), the behaviour of factor VII was

investigated in reconstituted freeze-dried plasma stored in plastic containers. The results are shown in figure 7. At room temperature, and particularly at 37°C, there was a rapid activation of factor VII. There was no increase in factor VII activity at 4°C.

Fig 7 Effect of temperature on factor VII activity in reconstituted plasma 72/411 (normal) and 72/429 (abnormal 3).

LEVELS OF PROTHROMBIN AND FACTORS VII, IX, AND X IN FROZEN AND FREEZE-DRIED PLASMA

The levels of the four vitamin-K-dependent factors were measured in frozen plasma and in freeze-dried plasma stored at -20 and $+37^{\circ}\text{C}$ for two months, as shown in tables IV, V, VI, and VII. All four factors showed slight decrease in activity in samples stored at 37°C ; this fall was most pronounced for factor IX. The two laboratories, each using different methods and substrate plasmas, were in good agreement except for factor VII, where laboratory 1 measured a slight fall in factor VII activity on storage, whereas laboratory 2 detected a slight rise.

The prothrombin times of plasmas stored at $+37^{\circ}\text{C}$ for two months were invariably slightly longer than those with plasma stored at -20°C , as shown in table VIII.

Plasma	Plasma Pool									
	Normal 72/411		Abnormal 72/425		Abnormal 72/427		Abnormal 72/429		Abnormal 72/431	
	Laboratory									
	1	2	1	2	1	2	1	2	1	2
Frozen	114	100	49	47	31	39	24	35	20	28
Freeze dried (-20°C)	109	120	44	47	28	29	24	26	19	24
Freeze dried (+37°C)	100	100	41	47	27	40	21	35	19	26

Table IV Prothrombin

	Plasma									
	Normal 72/411		Abnormal 72/425		Abnormal 72/427		Abnormal 72/429		Abnormal 72/431	
	Laboratory									
	1	2	1	2	1	2	1	2	1	2
Frozen	105	100	43	25	27	20	21	19	20	15
Freeze dried (- 20°C)	100	95	35	31	24	27	22	22	19	18
Freeze dried (37°C)	84	102	33	39	24	32	20	29	18	22

Table V Factor VII

	Plasma									
	Normal (72/411)		Abnormal (72/425)		Abnormal (72/427)		Abnormal (72/429)		Abnormal (72/431)	
	Laboratory									
	1	2	1	2	1	2	1	2	1	2
Frozen	101	100	76	64	53	52	41	45	32	40
Freeze dried (− 20°C)	109	108	60	76	41	48	29	38	24	24
Freeze dried (37°C)	90	82	39	30	30	40	26	40	22	21

Table VI Factor IX

	Plasma									
	Normal (72/411)		Abnormal (72/425)		Abnormal (72/427)		Abnormal (72/429)		Abnormal (72/431)	
	Laboratory									
	1	2	1	2	1	2	1	2	1	2
Frozen	113	106	24	30	14.5	18	12	12	10	10
Freeze dried (−20°C)	114	95	24	28	13	20	12	16	10	14
Freeze dried (37°C)	96	100	20	24	13	13	12	11	11	11

Table VII Factor X

<i>Thromboplastin</i>	<i>Laboratory</i>			
	<i>1</i>		<i>2</i>	
	<i>Plasma Stored at</i>			
	<i>− 20 °C</i>	<i>37 °C</i>	<i>− 20 °C</i>	<i>37 °C</i>
67/40	18.5	19.0	19.1	19.6
69/223	13.5	14.6	14.7	14.5
68/434	36.5	40.0	41.0	41.5
70/115	27.5	28.5	23.0	24.6
70/178	14.0	15.7	15.1	16.1

Table VIII Prothrombin times of normal plasma with different thromboplastins

Discussion

It is evident from the results reported here that freeze-dried plasma collected from patients on oral anticoagulants can be used to calibrate thromboplastins, as the calibration of thromboplastins makes use of prothrombin time ratios which remain unaffected by storage of freeze-dried or reconstituted plasma.

Standardization procedures that depend on absolute clotting times are open to many disadvantages. First, as shown in tables I, II, III, and VIII, two laboratories using identical thromboplastins, identical plasmas, and identical technique did not record identical clotting times.

Secondly, the prothrombin times of reconstituted plasma stored at room temperature and 37°C showed marked prolongation of clotting time (with thromboplastins 69/223 and 70/178) due to the deterioration of factor V, or alternatively marked shortening of clotting times (with thromboplastins 68/434) due to the activation of factor VII.

Thirdly, the preliminary accelerated degradation studies showed that the prothrombin times of freeze-dried plasma were invariably prolonged. This instability of freeze-dried plasma makes it unsuitable as a basis for standardization relying on clotting times.

The activation of factor VII, in Trasylol-treated, freeze-dried plasma follows the pattern already described (Brozović and Gurd, 1972) for plasma with added soya bean trypsin inhibitor: the activa-

tion is most marked at 37°C, whereas little activation occurs at 4°C. This behaviour of factor VII makes the immediate use of freeze-dried plasma a prerogative for comparable and successful tests.

The limited degradation studies performed in this study are in good agreement with previously reported results on the stability of vitamin-K-dependent factors (Brozović, Gurd, Robertson, and Bangham, 1971a, b). It is of interest that factor IX appears to be the least stable of the four factors investigated.

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