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 $-70^\circ$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 $+4^\circ$C. Reconstituted plasmas showed a marked shortening of clotting times when stored at 22 and $37^\circ$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 $37^\circ$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 $+4^\circ$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, Velinkamp, 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.

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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-N'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 (2H2O).....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 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 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 plasma 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.
Stability of freeze-dried plasma prepared from patients on oral anticoagulants

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Plasma Pool</th>
<th>Laboratory</th>
<th>1</th>
<th>2</th>
<th>Normal</th>
<th>Abnormal 1</th>
<th>Abnormal 2</th>
<th>Abnormal 3</th>
<th>Abnormal 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen (mean)</td>
<td>18-5</td>
<td>19-5</td>
<td>33-5</td>
<td>34-7</td>
<td>44-5</td>
<td>470</td>
<td>51-5</td>
<td>53-5</td>
<td>58-5</td>
</tr>
<tr>
<td>Freeze dried</td>
<td>18-5</td>
<td>19-5</td>
<td>34-0</td>
<td>35-9</td>
<td>45-0</td>
<td>450</td>
<td>52-5</td>
<td>55-9</td>
<td>58-5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Laboratory</th>
<th>1</th>
<th>2</th>
<th>Normal</th>
<th>Abnormal 1</th>
<th>Abnormal 2</th>
<th>Abnormal 3</th>
<th>Abnormal 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen</td>
<td>1-81</td>
<td>1-79</td>
<td>2-40</td>
<td>2-33</td>
<td>2-78</td>
<td>2-78</td>
<td>3-16</td>
<td>3-14</td>
</tr>
<tr>
<td>Freeze dried</td>
<td>1-83</td>
<td>1-87</td>
<td>2-43</td>
<td>2-48</td>
<td>2-83</td>
<td>2-92</td>
<td>3-16</td>
<td>3-23</td>
</tr>
</tbody>
</table>

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

CLOTTING TIMES AND CLOTTING TIME RATIOS IN RECONSTITUTED FREEZE-DRYED 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°C 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 thromboplatin 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 thromboplatin 70/115. In contrast, the clotting times invariably shortened with thromboplatin 67/40 and in particular with thromboplatin 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.
**BEHAVIOUR OF FACTOR VII IN RECONSTITUTED FREEZE-DRIED PLASMA**

As the cold-promoted shortening of the thrombotest 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.
**Stability of freeze-dried plasma prepared from patients on oral anticoagulants**

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

**Table IV Prothrombin**

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

**Table V Factor VII**

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

**Table VI Factor IX**

<table>
<thead>
<tr>
<th>Plasma Pool</th>
<th>Normal 72/411</th>
<th>Abnormal 72/425</th>
<th>Abnormal 72/427</th>
<th>Abnormal 72/429</th>
<th>Abnormal 72/431</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory</td>
<td>1 2</td>
<td>1 2</td>
<td>1 2</td>
<td>1 2</td>
<td>1 2</td>
</tr>
<tr>
<td>Frozen</td>
<td>101 100</td>
<td>76 64</td>
<td>53 52</td>
<td>41 45</td>
<td>32 40</td>
</tr>
<tr>
<td>Freeze dried (-20°C)</td>
<td>109 108</td>
<td>60 76</td>
<td>41 48</td>
<td>29 38</td>
<td>24 24</td>
</tr>
<tr>
<td>Freeze dried (+37°C)</td>
<td>90 82</td>
<td>39 30</td>
<td>30 40</td>
<td>26 40</td>
<td>22 21</td>
</tr>
</tbody>
</table>

**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°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°C for two months were invariably slightly longer than those with plasma stored at -20°C, as shown in table VIII.
Table VII  Factor X

<table>
<thead>
<tr>
<th>Plasma Stored at</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
</tr>
<tr>
<td>67/40</td>
<td>18.5</td>
</tr>
<tr>
<td>69/223</td>
<td>13.5</td>
</tr>
<tr>
<td>68/434</td>
<td>36.5</td>
</tr>
<tr>
<td>70/115</td>
<td>27.5</td>
</tr>
<tr>
<td>70/178</td>
<td>14.0</td>
</tr>
</tbody>
</table>

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 activation 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 prae-}

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.

References


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