A batch method for estimating thrombin clottable fibrinogen

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SYNOPSIS A method for estimating thrombin clottable fibrinogen in a large number of plasma samples is described. This method is based on measuring the optical density difference of a diluted plasma and its corresponding serum after incubation at 59°C in the presence of 10% NaCl. The method gave excellent correlation with previously described thrombin clottable estimates, but was easier to perform and more accurate when estimating friable thrombin clots.

The method is compared with measurements of heat-coagulable plasma protein described by Thorp (1967) and by Millar, Simpson, and Stalker (1971), and direct optical density measurement of plasma after thrombin treatment (Burmester, Aulton, and Horsfield, 1970).

The feasibility of automating the proposed technique together with immunoreactive fibrinogen estimates is discussed.

Immunological estimation of plasma proteins including fibrinogen has now been developed to allow batch estimation of large numbers, especially by use of light scattering techniques (Farrell and Wolf, 1972). However, thrombin clottable fibrinogen measurement, which has proved to be a diagnostically valuable complement to immunological fibrinogen estimation (Wolf, Farrell, and Walton, 1972), is not suitable for batch estimation in bulk.

Here we describe a method of estimating thrombin clottable fibrinogen suitable for large batches of plasma samples. The limitations of this new technique are discussed in relation to the inherent errors of thrombin clottable fibrinogen estimation in general.

Principle of Estimation

Light scattering as described by Thorp (1967) or light absorption measurements of a diluted plasma and its corresponding serum at the same dilutions were compared after heating for 20 minutes at 59°C. The difference between the plasma and serum readings was directly proportional to the concentration of thrombin clottable fibrinogen.

Materials and Methods

The following solutions are required: 0·85 g/100 ml NaCl; 10·0 g/100 ml NaCl; 20·0 g/100 ml NaCl.

HUMAN THROMBIN

Human thrombin was prepared by the method of Biggs and Macfarlane (1957). The thrombin was stored freeze-dried in 0·2 cc3 aliquots and each was reconstituted immediately before use with 5·0 cc3 distilled water. One volume of this reconstituted thrombin clotted an equal volume of normal citrated plasma in 11 to 13 seconds.

PREPARATION OF PLASMA

Venous blood, 4·5 ml, collected into a disposable syringe, was added to 0·5 ml 3·8 g% trisodium citrate. The blood was centrifuged at 3000 rpm for five minutes at +4°C and the plasma transferred to a glass universal container.

REPLICATE ESTIMATION OF THROMBIN CLOTTABLE FIBRINOGEN

These were estimated by the method of Farrell and Wolf (1971b).

PROCEDURE OF TEST

1 Into two tubes labelled 'serum' and 'plasma' were pipetted 1·8 cc3 0·85 g/100 cc3 NaCl and 0·2 cc3 fresh citrated plasma.

2 The contents of the 'serum' tube were clotted with 0·2 ml thrombin solution and an equivalent volume of 0·85% saline was added to the contents of the plasma tube.

3 The contents of both tubes were mixed and incubated at 37°C for 20 minutes and the fibrin clot...
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which formed in the 'serum' tube was removed by
winding on to a wooden applicator stick. All the
liquid included in the fibrin was drained back into
the tube by pressing the clot on the side of the tube.

4 Next an equal volume, ie, 2.0 cc, of 20 g/100 cc NaCl
was added to the 'plasma' and 'serum' tubes to
bring the concentration of NaCl to approximately
10% in each tube.

5 Both tubes were then incubated at 59°C for
15 minutes.

6 Before measurement by light scattering, test and
standard 'sera' and 'plasmas' were further diluted by
the addition of 9 volumes of 10% NaCl. The tubes
were then read immediately in the Thorpe micro
nephelometer set at maximum sensitivity to obtain
the difference between the matched serum and
plasma specimens.

7 Before measurement by light absorption, the
tubes were cooled to room temperature. The
difference between 'serum' and 'plasma' tubes was
measured without additional dilution at 365 m\u00b4 in a
Unicam Sp 600 spectrophotometer.

Identical procedures were followed for the stan-
dard plasma in each case. The fibrinogen concen-
tration (mg/100 cc²) of the test sample was given by:
OD (or light scattering) difference between test
'plasma' and 'serum'/OD (or light scattering)
difference between standard 'plasma' and 'serum' ×
fibrinogen content of standard plasma in mg per
100 cc².

Experimental

STABILIZATION OF OPTICAL PROPERTIES OF
PLASMA HEAT COAGULUM BY RAISING THE SALT
CONCENTRATION DURING HEAT COAGULATION
Initially, plasma and serum dilutions were made in
0-85 g/\% NaCl. Under these conditions the heat
coaugulum was partly particulate to the naked eye,
heterogeneous in size and unstable. Many of the
heat precipitates settled out of solution during the
heat treatment and subsequent cooling of these
specimens to room temperature induced further
precipitation. As a result, optical measurement
correlated poorly with predetermined fibrinogen
concentration in plasma as illustrated by the results
in table I.

<table>
<thead>
<tr>
<th>Plasma R</th>
<th>Serum R</th>
<th>D</th>
<th>Clottable</th>
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<td>23</td>
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<td>19</td>
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</tr>
<tr>
<td>25</td>
<td>4</td>
<td>21</td>
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Plasmas in 280 mg/\% range

<table>
<thead>
<tr>
<th>Plasma R</th>
<th>Serum R</th>
<th>D</th>
<th>Clottable</th>
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<td>456</td>
</tr>
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<td>24</td>
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<tr>
<td>27</td>
<td>5</td>
<td>22</td>
<td>432</td>
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</table>

Plasmas in 440 mg/\% range

Table I Nephelometric readings on heat coagula
produced in 0-85 g/\% NaCl

1 D = difference between plasma and serum reading

Stability of the heat coagulum and good corre-
lation of optical measurement with fibrinogen
concentration in plasma was achieved by adding
2 ml of 20% NaCl to both plasma and corresponding
serum after removal of fibrin in the serum tube.
This step brought the NaCl concentration in both
tubes to just over 10% and induced particularly
desirable properties in the heat coagulum in the
plasma tube. A colloidal type of turbidity was
induced in the plasma with no visible particulate
precipitate. In fact, as the results in table II illustrate,
centrifugation at an RCF of 6000 for 20 minutes at
+4°C only partially cleared the fibrin coagulum
from the top layer of the centrifuged tube, and a
uniform distribution of the coagulum was re-

<table>
<thead>
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<tr>
<td>0-14</td>
</tr>
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<td>0-16</td>
</tr>
</tbody>
</table>

Table II Distribution of heat coagulum (formed in high
salt concentration) after centrifugation and redistribu-
tion by a single inversion

1 Centrifugation 20 minutes at +4°C, Rotor-radius 9 cm, rpm 5000
(RCF 6000).

<table>
<thead>
<tr>
<th>OD</th>
</tr>
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<tbody>
<tr>
<td>280</td>
</tr>
<tr>
<td>560</td>
</tr>
<tr>
<td>468</td>
</tr>
</tbody>
</table>

Table III Stabilization of heat coagula by heat treatment in 10% NaCl nephelometric readings

1 The diluted heat-treated plasma and sera were stored for six days at 20°C. The table shows no significant change in reading on day 7. The
raised NaCl concentration during heating, therefore, stabilized the coagula which remained in colloidal suspension.

2 D = difference between plasma and serum readings.

3 Determined by the clot weight method.
Table IV  Stabilization of heat coagula by heat treatment in 10% NaCl with light absorption measured at 365 mμ1

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 7</th>
<th>Fibrinogen Contenta</th>
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<tbody>
<tr>
<td>Standard plasma</td>
<td>0-388</td>
<td>0-388</td>
<td>320</td>
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<tr>
<td>Test plasma 1</td>
<td>0-647</td>
<td>0-67</td>
<td>560</td>
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<tr>
<td>Test plasma 2</td>
<td>0-466</td>
<td>0-57</td>
<td>468</td>
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1The diluted plasma and sera are the same as shown in table II but were read directly after heating, ie, not given a second dilution in 10% NaCl. The readings are of the OD difference between each plasma and corresponding serum on days 1 and 7.

aDetermined by clot weight.

Fig 1  Correlation of optical density of heat coagulated fibrin in plasma with thrombin clottable fibrinogen levels estimated by the method of Farrell and Wolf (1971).

The continuous line is the linear regression line (r = 0-997 p = > 0-001).

Fig 2  Measurement of light absorption and light scattering of heat coagula at various wavelengths of incident light. A = heated plasma, B = unheated plasma, C = heated serum.

Read against saline blank in a Unicam Sp 8000. Arrow indicates 365 mμ incident light.
equal. Thus the relative increase of OD in A over B was ascribed to the presence of light scatter of the particulate heat fibrin in A. In fig 3 the optical densities of A and B were compared in the 200-450 m\mu range with the heated serum C set as the 0 blank. Figure 3 shows that at 305 m\mu and at 250 m\mu light scatter was markedly predominant over protein absorption.

Our choice of measurement at 365 m\mu was determined by availability of a suitable instrument, 365 m\mu being the lower limit of availability to us. Our investigation, however, suggests that with suitable apparatus incident light at 305 and 250 m\mu might achieve higher sensitivity at low fibrinogen levels.

Measurement of the heat precipitates in the Thorpe machine and by spectrophotometry at 365 m\mu gave equally good correlation with clottable fibrinogen levels as is indicated by the results shown in tables II and III. More extensive experience led us to prefer the use of the spectrophotometer. The Sp 600 measurement showed less daily variation in the reading of the standard plasma. It also proved more sensitive in measuring fibrinogen in lipaeemic plasma, giving relatively lower serum blank readings than those obtained in the Thorpe machine.

STORAGE AND HANDLING OF STANDARDS AND SPECIMENS BEFORE ESTIMATION

Preparation of standards

The best standard was found to be intact acid-citrate-dextrose blood bank plasma, or plasma collected from blood from a normal donor in 3·8% trisodium citrate (1 volume citrate/1 volume blood). The most stable plasmas had a fibrinogen level of below 400 mg/100 ml. The plasmas were separated, making all efforts to preserve sterility, and were frozen in 1 ml quantities without preservatives by placing in a −30°C cabinet in which they were also stored. These standards gave stable readings over the maximum period of storage investigated, a period of four months.

Plasmas with relatively high fibrinogen content usually provided unstable standards and showed deposition of cryofibrin after relatively short periods of storage at −30°C. Some of this material could not be resolubilized on thawing. Denatured cryofibrinogen also formed in all solutions of partially purified fibrinogen preparations when these were stored at −30°C and we cannot recommend these preparations for use as standards.

Blood collected in sequestrene also provided unsuitable standards. Plasma prepared from mixtures of 9 volumes of blood with 1 volume of either 0·75, 1·0, or 1·5 g% solution of dipotassium sequestrene and stored at +4°C or −30°C showed signs of fibrinogen denaturation. Within hours of plasma preparation all the specimens gave progressive loss of thrombin coagulability without, however, showing concomitant cryofibrin formation.

Storage

Whilst control normal plasmas could be stored at −30°C, plasmas with high fibrinogen content, and particularly those from patients affected by thrombosis, gave irreversible fibrin precipitates at −30°C. These usually induced substantial underestimates of thrombin clottable fibrinogen. The fibrinogen in these plasmas also precipitated out after short periods of storage at +4°C within one half to three hours and these changes were also observed with room temperature storage (+20°C) albeit at a slower rate.

To obviate underestimates associated with irreversible fibrin formation in these plasmas, citrated whole blood specimens were not stored for longer than
Advantage of the heat coagulability estimation in dealing with specimens showing delay in thrombin generation and functionally defective fibrinogen

Many postoperative plasma specimens, especially from very ill patients, show delay in clotting and functional fibrinogen defects. These abnormalities produce a fragile coagulum which is difficult to harvest. Here methods of estimating thrombin clottable fibrinogen which depend on harvesting and washing the coagulum are at a serious disadvantage. When the clot is very fragile, fibrin loss and underestimates are inevitable. Moreover, even in normal plasma, clot recovery procedures are tedious and time consuming.

The heat coagulation process obviates any special care in clot harvesting. The plasma tube required no clot recovery, and in the serum tube complete recovery was not necessary after thrombin action for 20 minutes at 37°C. Any residual coagulum not initially harvested floated to the top of the cuvettes in the 10% NaCl after the 59°C incubation. Further, when there was delay in thrombin coagulation a floating compact coagulum formed at 59°C and completed the coagulation. In both instances the coagulum was not in the way of the light path and gave a true serum reading.

Limitations in use of method

Jaundiced plasma

In jaundiced plasma the measurement of difference in optical density at 365 mµ between dilute heated plasma and dilute heated serum gave substantial underestimates of thrombin clottable values for reasons which were not elucidated.

To obviate this our choice lay between an alternative optical density measurement at 600 mµ which was less sensitive in detecting small changes in fibrinogen concentration than reading at 365 mµ (see fig 2), or estimating the clottable values by our original method (Farrell and Wolf, 1971). The latter alternative was preferred.

Estimation of thrombin clottable values in heparinized plasma

In the presence of heparin, thrombin clottable fibrinogen estimations proved to be unreliable. An attempt was made to obtain a value by replacing thrombin with reptilase obtained from Pentaphorm, Basle, which clots fibrinogen in the presence of heparin. However, since reptilase in non-heparinized plasmas produced fibrin yields 14% below that obtained with thrombin this approach was abandoned. At present in heparinized patients we have abandoned thrombin clottable estimates, replacing this by a procedure which compares the OD difference between 59°C heated and unheated diluted plasma, dilution also being effected in 10% NaCl. This estimation resembles that described by Miller et al (1971) but differs in being an optical density measurement rather than visual estimation of precipitate volume.

Discussion

The method described here incorporates features of three previously described fibrinogen estimations: (1) the spectrophotometric method of Ellis and Stranski (1961) and its recent modification by Burmester et al (1970); (2) estimation of plasma fibrinogen using a nephelometric estimate of heat coagulum in plasma (Thorp, 1967); and (3) estimation of plasma fibrinogen by a visual estimate of the volume of plasma heat coagulum (Millar et al, 1971).

The Ellis and Stranski technique is based on the principle of following the optical changes after thrombin fibrinogen interaction in dilute plasma by changes of OD at 300 mµ. Using this technique, thrombin fibrinogen interaction in normal plasma produces a relatively rapid increase in measurement of optical density which slows down after 10 minutes. The modification of Burmester et al recommends calibration of fibrinogen concentration based on reading the OD after exactly 10 minutes of thrombin fibrinogen interaction. With this method it is technologically difficult to estimate a large number of test samples in batches, ensuring a 10-minute thrombin fibrinogen interaction time for each test. When plasma fibrinogen is partially denatured by trace thrombin and plasmin action, delay in fibrin polymerization induces substantial underestimates of thrombin clottable fibrinogen.

Both Thorp’s method and the method of Millar et al measure plasma protein which is heat coagulable at 56°C. The Thorp method gives a poor correlation with thrombin clottable values, mainly, we think, because the buffered saline used in his method as plasma diluent induces the optically unstable heat coagulates which we have already observed for 0.85% NaCl diluent.

and can be expected to give values close to thrombin clottable fibrinogen in normal plasma.

In the disfibrinogenaemias of pregnancy or following prostate gland surgery, where anticoagulant fibrin degradation products prevent thrombin-induced fibrin polymerization, enough fibrinogen and macromolecular fibrinogen derivatives may be present to give high values by measurements relying only on heat precipitable protein in plasma. By measuring the difference in OD of heated thrombin-treated plasma (serum) with a heated parallel sample not treated with thrombin, this anomaly of results disappears and the high reading of OD of both heated plasma and serum tubes reveals and measures the concentration of polymeric functionless fibrinogen.

When only part of the circulating fibrinogen is susceptible to thrombin-induced polymerization, measurement of heat-coagulable protein in plasma alone may produce variable overestimates of potentially functionally intact fibrinogen. The degree of correlation will depend on (1) the concentration of non-functional heat precipitable fibrinogen derivatives, and (2) the extent to which these are occluded in the clot formed by functionally intact protein producing the overestimates of thrombin coagulable protein to which we have previously referred (Wolf and Farrell, 1972).

We have previously shown (Wolf et al, 1972; Wolf and Farrell, 1971) that discrepancy between clottable and immunoreactive fibrinogen estimates is an index of thrombosis and thrombolysis, and a more reliable indicator of the latter than isolated measurement of fibrin degradation products in serum. Both immunoreactive and thrombin-clottable determination have now been adapted to provide for estimates on multiple samples. We have found this discrepancy helpful when screening postoperative patients for either abnormal or excessive postoperative thrombosis. The immunonephelometric method of measuring immunoreactive fibrinogen by light scattering (Farrell and Wolf, 1972) has now been modified by us for light absorption measurement at 365 mμ simply by doubling the concentration of both antigen and antibody (Farrell and Wolf, 1972, unpublished). Thus both thrombin-clottable and immunoreactive estimations are measured by the same optical system of light absorption using apparatus which is widely available.

Both methods have the potential to be fully automated, especially since the test samples show an optical stability extending over many hours.

Our thanks are due to Professor K. W. Walton for suggestions and criticism.

References