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 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 cc aliquots and each was reconstituted immediately before use with 5.0 cc 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 cc 0.85 g/100 cc NaCl and 0.2 cc 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 cc3
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 mμ in a
Unicam Sp 600 spectrophotometer.

Identical procedures were followed for the stan-
dard plasma in each case. The fibrinogen concentra-
tion (mg/100 cc3) 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 cc3.

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
cogaulum 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></th>
<th>Plasma R</th>
<th>Serum R</th>
<th>D</th>
<th>Clottable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmas in 280 mg% range</td>
<td>23</td>
<td>4</td>
<td>19</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4</td>
<td>21</td>
<td>280</td>
</tr>
<tr>
<td>Plasmas in 440 mg% range</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>456</td>
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<td></td>
<td>24</td>
<td>4</td>
<td>20</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>5</td>
<td>22</td>
<td>432</td>
</tr>
</tbody>
</table>

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

1D = difference between plasma and serum reading

Stability of the heat coagulum and good cor-
relation 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>
<tr>
<th></th>
<th>OD</th>
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<tbody>
<tr>
<td>Before centrifugation</td>
<td>0.16</td>
</tr>
<tr>
<td>After centrifugation</td>
<td>0.06</td>
</tr>
<tr>
<td>Top one third</td>
<td>0.12</td>
</tr>
<tr>
<td>Middle one third</td>
<td>0.12</td>
</tr>
<tr>
<td>Measurement after single inversion after centrifugation (parallel specimen)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th></th>
<th>Fibrinogen Content of Standard Test Plasmas4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td>Standard plasma</td>
<td>5</td>
</tr>
<tr>
<td>Test plasma 1</td>
<td>8</td>
</tr>
<tr>
<td>Test plasma 2</td>
<td>4</td>
</tr>
</tbody>
</table>

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

1The 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.

2D = difference between plasma and serum readings.

3Determined by the clot weight method.
Table IV Stabilization of heat coagula by heat treatment in 10% NaCl with light absorption measured at 365 m\(\mu\)^1

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 7</th>
<th>Fibrinogen Content*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard plasma</td>
<td>0.388</td>
<td>0.388</td>
<td>320</td>
</tr>
<tr>
<td>Test plasma 1</td>
<td>0.647</td>
<td>0.67</td>
<td>560</td>
</tr>
<tr>
<td>Test plasma 2</td>
<td>0.466</td>
<td>0.57</td>
<td>468</td>
</tr>
</tbody>
</table>

1The diluted plasma and sera are the same as shown in Table II but were read directly after heating, i.e., 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.

* Determined by clot weight.

**CHOICE OF OPTICAL MEASUREMENT**

The Thorpe nephelometer uses a red filter and the light source is placed at a 90° angle to the detector. This instrument therefore is confined to measuring light scatter within an incident wavelength of between 600 and 700 m\(\mu\).

With spectrophotometric measurement the light source and detector are placed opposite each other and here the wavelength of the light source is variable. With spectrophotometry loss of incident light is induced by light absorption as well as by light scatter. It was therefore necessary to find the optimum wave length of incident light which would effect maximum sensitivity in respect of light scatter and minimum sensitivity in respect of light absorption which in our samples was mainly determined by protein content. Figure 2 records the optical density measurements of A, a heated plasma, B the plasma control (not heated), and C the corresponding heated serum in relation to a saline blank through the range of visible light. The results show that below the 400 m\(\mu\) range increase of OD by light scattering was prominent in relation to light absorption, bearing in mind that protein contents of A and B were established by a single inversion of the tube. Storage of the heated specimens at room temperature (20°C) for a period of seven days induced no visible precipitation and no significant changes in optical properties of the heat coagulum. This is illustrated by the results in Tables III and IV. Figure 1 shows that plasma minus serum readings after the high salt heating procedure gave absorption values which correlated very well with thrombin clottable fibrinogen levels in the plasmas, as estimated by the method of Farrell and Wolf (1971).

**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 m\(\mu\) incident light.
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**Fig 3** Measurement of light absorption and light scattering of heat coagula at various wavelengths of incident light.

\[ A = \text{heated plasma}, \ B = \text{unheated plasma}. \]

Read against heated serum blank.

Arrow indicates 365 nm incident light

Note predominance of light scattering at 305 and at 240 nm.

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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 lipaemic 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
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 (Thorpe, 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 technically 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 disfibrinopenemias 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 mJ 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.

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References
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