New Method for Determining Thrombin-Clottable Fibrinogen

Alberto Frigola, Sandro Angeloni, and Anna Rita Cerqueti

We describe a new method for determination of thrombin-clottable fibrinogen, which eliminates the systematic error caused by occlusion of other serum proteins in the fibrin clot and reduces the sensitivity to high concentrations of fibrin degradation products. Essentially, the method consists of densitometric quantitation of the fibrin band after a standard electrophoresis run of plasma, thrombin fixation of the fibrinogen, and removal of the non-clotted proteins by washing in saline. The procedure shows good precision and gives results that are accurate, significantly correlate with results for the classical thrombin clotting method \((r = 0.92, P < .001)\), and are not affected by fibrin degradation product concentrations up to 900 mg/liter. These characteristics make the method especially valuable in establishing fibrinogen concentration in patients who are undergoing thrombolytic therapy.

Additional Keyphrases: analytical error · fibrin degradation products · electrophoresis and densitometry

Plasma fibrinogen concentration is usually determined by converting fibrinogen to fibrin and quantitating the fibrin content of the isolated clot.

Despite the great variety of procedures developed, this technique suffers from several sources of error. It is well known that proteins other than fibrinogen may be occluded in the fibrin clot, producing a considerable degree of error—especially at low fibrinogen concentrations, when the recovery of fibrin is very difficult and often incomplete \((I)\). Furthermore, in the presence of high concentrations of fibrin and fibrinogen degradation products (FDP), as during thrombolytic treatment for deep-vein thrombosis, the thrombin clotting method gives an underestimate of the true clottable fibrinogen \((2, 3)\).

These sources of error, disregarded in normal or near-normal conditions, may lead to extremely inaccurate determinations in situations similar to those described above. To overcome these difficulties, we have developed a new procedure for the quantitation of thrombin-clottable fibrinogen. Essentially, the method consists of three steps: first, a standard electrophoresis run of plasma is performed on cellulose acetate; second, by thrombin action, fibrinogen is converted to fibrin, which is entrapped on the electrophoretic support; and finally, after the removal of the other plasma proteins by washing with saline solution, the cellulose acetate strip is stained and fibrin quantitated.

This procedure combines the resolution given by the electrophoretic separation with the specificity of the thrombin activity; the result is a complete removal of proteins occluded in the clot and a remarkable reduction of the susceptibility to high FDP concentrations.

Materials and Methods

Blood Collection

Blood samples were collected by venipuncture with a plastic syringe, after minimal venous stasis, from 67 adult hospital outpatients (35 men and 32 women). Blood was anticoagulated by adding nine volumes to one volume of trisodium citrate solution (38 g/liter) containing aprotinin (Trasylo1, Bayer) 500 kilounits/liter (these are kallikrein inhibitor units). Plasma obtained by centrifuging at 4000 rpm for 20 min was used immediately or stored at \(-20^\circ\text{C}\).

Apparatus and Reagents

Electrophoresis. The cellulose acetate strips we used were Cellogel strips, 5.7 × 14 cm (Chemetron Chimica, Milan). The electrophoretic apparatus consisted of a cell with a 8.5-cm membrane support bridge and a suitable power supply giving a constant voltage output of 0–500 V. Densitometric readings were done with a Digiscan-M-Scanner Model 39397 connected to the Digiscan Recorder Model 39405 (Gelman Instrument Co.).

Buffer. Tris(hydroxymethyl)methylamine 8.91 g, barbital 3.68 g, and sodium barbital 14.55 g were dissolved in 500 ml of warm distilled water and diluted, after cooling, to 1 liter, giving a pH of 8.8 and an ionic strength of 0.085.

Thrombin solution. Thrombin solution was prepared by reconstituting Fibrindex (Ortho Diagnostics, 50 NIH

Laboratory of Clinical Pathology, B. Eustachio Hospital, 62027 San Severino Marche, Italy.
Received July 8, 1977; accepted Aug. 15, 1977.
units/vial, lot. no. 10N215) with 13 ml of Tris-saline solution (0.154 mol/liter NaCl, 0.2 mol/liter tris(hydroxymethyl)methylamine buffer, pH 7.4) containing 2.5 mmol of CaCl₂ in final concentration per liter.

Washing solution. Isotonic saline was used to remove non-fixed proteins.

Staining, destaining, and clearing solutions. Ponceau S dye, 0.5 g, was dissolved in 100 ml of aqueous trichloroacetic acid, 50 g/liter. As destaining solution we used acetic acid/water (5/95 by vol). Clearing was done by drying the strip in an oven at 90–100 °C for 5 min after baths in absolute methanol for 1 min and in acetic acid/absolute methanol (13/87 by vol) for 3 min.

Electrophoretic Procedure

Cellulose acetate strips were soaked in cold buffer for at least 15 min and excess buffer removed by blotting between sheets of absorbent paper. Plasma was applied with a wire applicator delivering 1.5 μl; four samples were applied to every strip. After the power supply was adjusted to give an initial current of 4.5 mA for every 5.7-cm width strip (180 V), electrophoresis was continued for 25 min. At the end of the run, the strip was incubated in the thrombin solution for 20 min at room temperature. To remove the non-clotted proteins, we then washed the strip by continuous agitation for 20 min in saline. Residual proteins were stained with Ponceau S solution and destained with three successive baths in acetic acid/water (5/95 by vol). Clearing was done as described above. All reagents used were discarded after every electrophoretic run. Figure 1 shows a typical electrophoregram.

For fibrinogen quantitation, cleared strips were scanned at 510 nm with the integrating densitometer; from the integration count the concentration was obtained from the calibration curve.

Preparation of the calibration curve. Fibrinogen from bovine plasma (General Diagnostics, lot no. 2969103) was reconstituted to obtain respectively 6, 3, and 1.5 g/liter. After an electrophoretic run with thrombin fixation, the calibration curve was constructed by plotting fibrinogen concentration vs. integration count obtained from each fibrin band (Figure 2).

Quality Control

Accuracy and precision of the electrophoretic method were evaluated by using a commercial reference plasma, Ortho Plasma Coagulation Control (Ortho Diagnostics; lot no. 2P81; fibrinogen value, 2620 ± 150 mg/liter), fibrinogen from bovine plasma (General Diagnostics, lot no. 2969103), and a pool of fresh plasma from 17 healthy subjects.

Reference Procedure

As reference method we used the thrombin clotting method as described by Cannon et al. (4).

FDP Preparation

An “in vitro” plasmin digest of fibrinogen was prepared by adding 1000 U of streptokinase (Streptase, Behringwerke) per milliliter to a normal plasma pool. Fibrinogen lysis was stopped when the thrombin time of the incubation mixture exceeded 120 s, by adding 0.2 mol of ε-aminocaproic acid per liter (final concentration) and freezing. The presence of early and late FDP’s was assessed by immunoelectrophoresis in agarose gel (10 g/liter) as described by Marder and Shulman (5). FDP were quantitated by the tanned-erythrocyte hemagglutination inhibition immunoassay, with use of the FR-Ag Test (lot no. 621R06A1, ICL Scientific).

Results

Comparison between the Electrophoretic Procedure and the Reference Method

The mean values for plasma fibrinogen concentration
Table 1. Mean Values for Plasma Fibrinogen Concentration (n = 67)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Electrophoretic</th>
<th>Thrombin Clotting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/liter</td>
<td>method</td>
</tr>
<tr>
<td>Mean</td>
<td>2800</td>
<td>3070</td>
</tr>
<tr>
<td>SD</td>
<td>620</td>
<td>610</td>
</tr>
<tr>
<td>SE of the mean</td>
<td>76</td>
<td>75</td>
</tr>
</tbody>
</table>

found in the population under study by the electrophoretic procedure and the thrombin clotting method are reported in Table 1. There is a statistically significant difference between the two mean values as shown by *t*-test (*t* = 2.92, *P* < 0.01); and also the estimated normal ranges (mean ± 2 SD) are different, being respectively 1560 to 4040 mg/liter for the electrophoretic procedure and 1850 to 4290 mg/liter for the reference method. However, results by the two methods correlate well (*r* = 0.92, *P* < 0.001). The scatter diagram and the resulting regression line obtained by plotting the fibrinogen concentration evaluated by the electrophoretic procedure and the thrombin clotting method are shown in Figure 3.

Interference by FDP

The interference by FDP with fibrinogen assay was assessed by adding, in the fixed volumetric ratio of 0.5 to 1, a variable amount of FDP from the fibrinogen digest to a normal plasma pool containing 0.2 mol of ε-aminocaproic acid (final concentration). The resulting fibrinogen value was determined by the electrophoretic procedure and by the thrombin clotting method, with reference to a control plasma to which saline was added.

Results of a typical experiment are shown in Figure 4. With the thrombin clotting method we observed fibrinogen values artifically decreased at high concentrations of FDP; the electrophoretic procedure, on the other hand, was unaffected by FDP. With regard to the data in Figure 4, the results obtained have been interpreted as being due to the potent anticoagulant effect of high FDP concentrations. Derepression of plasmin by the 1/30 dilution of ε-aminocaproic acid can not be definitively excluded, but such reactivation should affect fibrinogen determination, even at low FDP concentration.

Precision and Accuracy Evaluation, Electrophoretic Method Precision and Accuracy

Within-day precision was evaluated by performing 21 replicate determinations on a fresh plasma pool. The mean value obtained was 2790 mg/liter with a standard deviation of 116 mg/liter and a coefficient of variation of 4.1%.

Day-to-day precision and accuracy were assessed by performing 32 replicate determinations over a three-month period on a commercial lyophilized plasma (Ortho Plasma Coagulation Control). The reconstituted plasma was not cleared before any determination, because turbidity minimally affects the electrophoretic procedure. The mean fibrinogen value obtained, 2638 mg/liter, was very close to that reported by the manufacturer, 2620 mg/liter; the standard deviation and the coefficient of variation were respectively 101 mg/liter and 3.8%.

Finally, to evaluate the accuracy at low fibrinogen concentration, we diluted bovine fibrinogen from General Diagnostics with saline to obtain 500 mg/liter, and assayed it. The mean value obtained for 26 replicate determinations was 510 mg/liter, with a standard deviation of 72 mg/liter and a coefficient of variation of 14%.

Discussion

The above results show clearly that the electrophoretic method is suitable for fibrinogen quantitation, even if it is too time-consuming for use in emergency cases.
situations. However, it should be stressed that the accuracy and precision of the electrophoretic method strictly depend on two basic conditions: use of freshly made reagents, which must be discarded after every assay, and application of an exactly constant volume of sample on the cellulose acetate strip. If either of these conditions is disregarded the method reliability may be seriously affected.

Beyond these technical limitations the electrophoretic procedure shows two major advantages. The electrophoretic run, by removing from the fibrinogen band the proteins that may be occluded in the fibrin clot, eliminates an important source of systematic error. This fact could explain the difference between the mean value of fibrinogen concentration and the normal-range limits obtained on using the classical thrombin clotting method and those obtained by the electrophoretic procedure. The electrophoretic procedure, moreover, does not lead to artifactually decreased fibrinogen values in the presence of high FDP levels. The insensitivity to FDP is especially valuable in establishing the fibrinogen value in conditions under which thrombin clottable fibrinogen is depleted and FDP titer is high, as in patients who are undergoing thrombolytic therapy for deep-vein thrombosis.

References