

Evaluation of a rapid method for the determination of plasma fibrinogen

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SYNOPSIS In this study the method of Ellis and Stransky (1961) to determine plasma fibrinogen is evaluated, and modifications are described which have been introduced to improve the sensitivity and reduce the time taken for the test.

Hypofibrinogenaemia has become increasingly recognized as a major factor in the bleeding disorders of pregnancy during the past decade. Most of the methods for the measurement of fibrinogen are lengthy and require considerable technical skill and experience to obtain reliable results. Schneider (1951) introduced the thrombin titre method which has the advantage of simplicity, and a modification of this is now generally used in emergencies.

Ellis and Stransky (1961), using a thrombin reagent to polymerize fibrinogen, measured the turbidity produced spectrophotometrically and expressed the results as fibrinogen concentration. Bottema and Vervoort (1964) later extended the technique to demonstrate fibrinolysis.

Experience with these two methods has shown that accurate fibrinogen determinations are difficult at low levels due to low sensitivity and that the time taken for the test may lead to a delay in treatment.

Materials and Methods

SAMPLE

Venous blood is anticoagulated with one tenth

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its volume of 3.8% sodium citrate. The plasma is separated by centrifugation at 3,000 rpm for three minutes.

REAGENTS

1 Barbitone-saline buffer, 0.1 M, pH 7.2: 2.93 g sodium chloride and 5.71 g sodium di-ethyl barbiturate are dissolved in about 900 ml deionized water. The solution is titrated with 1.0 N hydrochloric acid to pH 7.2 using a pH meter. About 22.5 ml is required. Make the volume to 1 litre with water.

2 Calcium chloride, 3.38 M

3 Sodium chloride, 0.9%

4 Thrombin (Parke-Davis) is reconstituted in saline to a concentration of 1,000 NIH units per ml.

5 Calcium-thrombin reagent, for which 0.1 ml reconstituted thrombin is diluted with 1.9 ml 0.9% sodium chloride and 2.0 ml 3.38 M calcium chloride, is added. After mixing this reagent is stored at 4°C when not in use and has a useable life of at least 24 hours.

TECHNIQUE

The recording spectrophotometer (Unicam SP800A) is programmed to read changes in

absorbance against time at a fixed wavelength of 300 nm.

Plasma, 0.5 ml, is diluted with 5.5 ml barbitone-saline buffer in a test tube and 3.0 ml of the mixture carefully transferred to a 1 cm silica cuvette for the test. The remainder of the mixture is decanted into a similar cuvette as a blank. Both cuvettes are placed in the spectrophotometer and the instrument is adjusted to zero absorbance.

Of the calcium-thrombin reagent, 0.015 ml is added to the contents of the test cuvette and mixed rapidly and carefully to minimize the production of air bubbles. The cuvette is replaced in the spectrophotometer and the programme started. The trace is drawn for at least 10 minutes.

CALCULATION

The absorbance at 300 nm at the end of the 10 minutes is noted and substituted in the following

formula $\left(E_{300}^{10'}\right)$:-

$$\text{Plasma fibrinogen} = \left(E_{300}^{10'} \times 325\right) + 16 \times \frac{10}{9} \text{ mg/100 ml.}$$

The factor $\frac{10}{9}$ is to correct for anticoagulant dilution.

Experimental and Results

WAVELENGTH

Initial experiments with the Ellis-Stransky technique confirmed that at 470 nm, the wavelength used by these workers, sensitivity was inadequate for the measurement of low levels of fibrinogen.

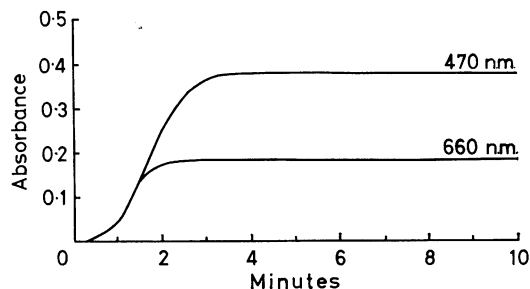


Fig 1 Comparison of sensitivity by the methods of Ellis and Stransky at 470 nm and Bottema and Vervoort at 660 nm on a sample of plasma with a fibrinogen level of 315 mg/100 ml determined chemically.

A further experiment carried out at 660 nm, as used by Bottema and Vervoort (1964), showed less sensitivity (Fig. 1).

A fibrinogen determination was carried out according to our technique and after 20 minutes a complete spectral scan was recorded over the range 215-700 nm (Fig. 2). There was no peak of absorbance but a general increase in absorbance with decrease in wavelength. Below 280 nm the trace became very 'noisy', and therefore 300 nm was selected as the working wavelength, since this provides maximum sensitivity.

TIME

Ellis and Stransky (1961) used the absorbance readings obtained after 20 minutes to calculate fibrinogen levels. A series of experiments was carried out to establish the optimum time at which to take readings. It was confirmed that a true plateau was not reached at 60 minutes but the rate of increase in absorbance at this time was very slow. The average of the readings at 10 minutes was 95% of the readings obtained at 60 minutes and at 20 minutes the average reading was 98% of the 60-minute values. We consider that the 10-minute readings provide sufficient accuracy consistent with the need for a rapid result and all subsequent readings are made at this time.

LINEARITY

A number of serial dilutions of plasma in saline were examined by the technique and the results show that the readings are linear below an absorbance value of 1.00 unit (Fig. 3).

REPRODUCIBILITY

A series of 20 plasma samples at varying fibrinogen levels was analysed singly on two separate

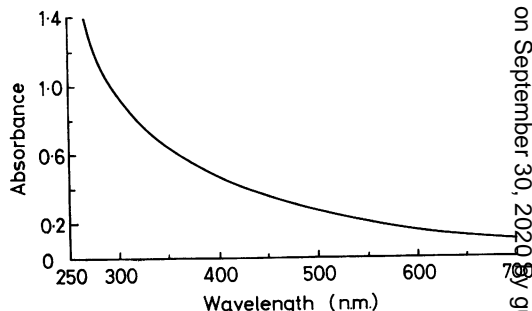


Fig 2 Absorption spectrum of the reaction mixture after 20 minutes.

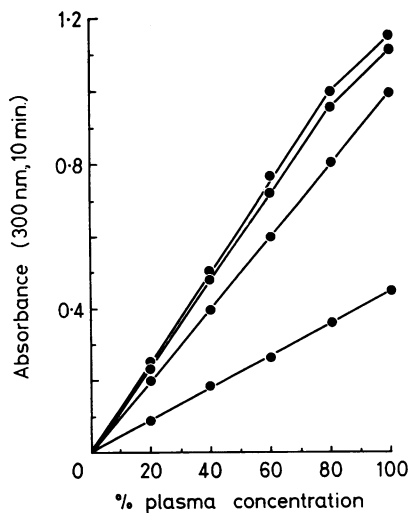


Fig 3 Linearity of the modified technique. Each curve is derived from a different specimen of plasma.

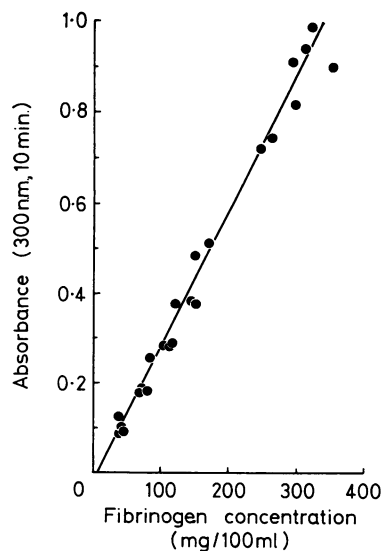


Fig 4 Correlation between the results of fibrinogen assay on 23 plasma samples by the proposed modification (ordinate) and the method of Ratnoff and Menzie (abscissa). Each point represents the means of duplicate analyses by each method.

occasions. The results are shown in Table I. The standard deviation (SD) of the duplicates is ± 0.018 absorbance units. The 95% confidence limits of ± 2 SD is ± 0.036 absorbance units (equivalent to ± 12 mg fibrinogen). The chart of the Unicam SP800A can be read to 0.005 absorbance units.

CALIBRATION

The reference method was a modification of the

Sample No.	Absorbance (300 nm at 10 minutes)		
	First Analysis	Second Analysis	Difference (d)
1	0.925	0.875	0.050
2	0.515	0.515	0.000
3	0.260	0.250	0.010
4	0.280	0.275	0.005
5	0.130	0.130	0.000
6	0.900	0.935	0.035
7	0.095	0.090	0.005
8	0.390	0.390	0.000
9	0.275	0.280	0.005
10	0.370	0.375	0.005
11	0.845	0.800	0.045
12	0.180	0.185	0.005
13	0.090	0.090	0.000
14	0.275	0.290	0.015
15	0.980	0.995	0.015
16	0.185	0.185	0.000
17	0.380	0.370	0.010
18	0.085	0.085	0.000
19	0.370	0.385	0.015
20	0.185	0.185	0.000
	Sum of differences		0.220
	Mean of differences		0.011
	Standard deviation of difference		± 0.018

Table I Reproducibility of the technique at different fibrinogen levels

procedure of Ratnoff and Menzie (1951). In this modification protein was precipitated, collected and washed as in the original technique, and then dissolved in 4 ml 0.2 N sodium hydroxide by heating in a boiling water bath for 15 minutes. After cooling, 0.2 ml 4% copper sulphate was added and the tubes were shaken vigorously for about a minute. The colour was developed by incubating the tubes at 37°C for 20 minutes and the optical density at 560 nm was observed after centrifuging at 2,500 rpm for two minutes. A standard was prepared with each batch of analyses by diluting 0.1 ml standard protein solution with 3.9 ml 0.2 N sodium hydroxide, adding 4% copper sulphate, and developing the colour as in the tests. As a blank, 4 ml 0.2 N sodium hydroxide was treated with 4% copper sulphate and the colour developed.

Twenty-three plasma samples were analysed in duplicate by this procedure and the same samples were analysed, again in duplicate, by our method.

The results are shown in Table II and illustrated in Figure 4.

Statistical analysis of these results shows a high degree of correlation between the two methods, ($r = 0.9899$). The regression data for the line of best fit as derived from the least squares formula is:—

$$b = 324.68 \text{ and } c = 16.06$$

where b = slope of x on y and c = intercept.

Sample No.	Absorbance (300 nm at 10 min) by Modified Ellis and Stransky Method	Fibrinogen (mg/100ml) by Ratnoff and Menzie Method
1	0.745	257
2	0.255	86
3	0.375	123
4	0.918	290
5	0.390	147
6	0.130	42
7	0.900	352
8	0.515	172
9	0.715	244
10	0.098	47
11	0.278	109
12	0.183	85
13	0.378	149
14	0.940	310
15	0.085	41
16	0.373	154
17	0.185	73
18	0.090	48
19	0.988	322
20	0.283	119
21	0.278	113
22	0.823	296
23	0.185	73

Table II Results of fibrinogen assay by the proposed modification and by the method of Ratnoff and Menzie¹

¹Each value is the mean of duplicates.

Therefore, from the formula for a straight line, $y = bx + c$, plasma fibrinogen =

$$\left(324.68 \times E_{300}^{10'} \right) + 16.06.$$

In practice the formula is simplified to

$$\left(325 \times E_{300}^{10'} \right) + 16.$$

Discussion

From the results of these experiments we conclude that the original method as described by Ellis and Stransky (1961) can be modified so that both sensitivity and the time required to complete an analysis are improved. The fact that readings made after only 10 minutes give a value which is 95% of the value reached at 60 minutes does not appear to be a major disadvantage since it has been demonstrated that there is a very close correlation obtained with the reference procedure. Consequently no attempt has been made to correct for this difference. The experiments also show that there is linearity below an absorbance value of 1.00 unit, which is equivalent to a plasma fibrinogen level of 380 mg/100 ml. Although the lowest level of fibrinogen which can be determined is 18 mg/100 ml, differences of 2 mg above this level can be detected. The value for 2 SD of ± 12 mg is well within the requirements of the technique

and in clinical practice sufficiently accurate direct treatment.

With regard to the use of the method of Ratnoff and Menzie (1951) as a calibration procedure, this was selected because it measures 'thrombin clottable protein' by the Biuret reaction as distinct from direct spectrophotometry and because the method has been authenticated and is used extensively in clinical investigations. The main advantage of all the methods which use thrombin to separate fibrinogen from other plasma proteins is that they provide a measure of 'functional' fibrinogen unlike the methods depending upon chemical precipitation which may be affected to a varying degree by the split products of fibrinolysis (Sharp, Howie, Biggs, and Methuen, 1956).

The use of a recording spectrophotometer for our technique is not essential, but a reliable ultraviolet spectrophotometer is necessary to produce the sensitivity although the calibration data would need to be verified for each instrument before routine use. One advantage of using a recording instrument is that it can detect fibrinolysis, since the curve shows a demonstrable fall after reaching a maximum absorbance value in some cases where plasmin is present in high concentrations. A similar fall can be obtained *in vitro* by adding streptokinase to the reaction mixture as described previously by Bottema and Vervoort (1964) and this can be neutralized by the addition of one of the fibrinolytic inhibitors.

We have used this modification of the method of Ellis and Stransky in obstetrical practice in the routine assessment of patients with antepartum haemorrhage, intrauterine death, and with bleeding after delivery. The main practical advantage is that an accurate and reliable result can be rapidly obtained by a relatively simple procedure and therefore an initial assessment of the condition and adequate monitoring of the response to therapy is readily available.

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