Semi-automatic method for routine evaluation of fibrinolytic components

D. COLLEN, G. TYTGAT, AND M. VERSTRAETE

*From the Laboratory of Physiopathology, Section Blood Coagulation,
Department of Medicine, University of Louvain, Belgium*

SYNOPSIS A semi-automatic method for the routine evaluation of fibrinolytic activity is described. The principle is based upon graphic recording by a multichannel voltmeter of tension drops over a potentiometer, caused by variations in the influence of light upon a light-dependent resistance, resulting from modifications in the composition of the fibrin fibres by lysis.

The method is applied to the assessment of certain fibrinolytic factors with widespread fibrinolytic endpoints, and the results are compared with simultaneously obtained visual data on the plasmin assay, the plasminogen assay, and on the euglobulin clot lysis time.

The assessment of precursors, activators, or inhibitors of the fibrinolytic system is finally based upon the proteolytic breakdown of a substrate by varying amounts of the active enzyme, plasmin. The indicator substrate may be fibrin (in a standard clot system or in fibrin plates), casein, or such synthetic esters as TAME or LMe (Johnson, 1964).

The amount of plasmin, present or formed in the test mixture, can be calculated by the time necessary to degrade to a fixed point a known amount of indicator substrate (fibrin clot method) or can be calculated by the amount of substrate degraded in a fixed period of time (casein digestion, fibrin plates, esterolysis).

The assay of fibrinolytic components by a fibrin clot lysis method with the endpoint based upon visual observation has been proved to be a reliable and reproducible method for routine laboratories (Johnson, 1964). However, this procedure is time consuming and based upon a subjective definition and often difficult reading of the lysis endpoint, and several authors have therefore sought an automatic registration of these lysis times. Most of the proposed methods are based upon changes in optical density (Kuhnke, Gill, and Brilla, 1960; Kuhnke and Gill, 1963; Bouvier and Gründlinger, 1963; Harrower and Brook, 1964; Nanninga, Zeller, and Maynes, 1964; Ferreira, 1965; Cash and Leask, 1965, 1967), in viscosity (Peeters, Steenhoudt, and Decroix, 1964), in tensile strength (Hartert, 1951), or in light reflection upon air bubbles incorporated in the clot (Newman, 1964).

Several methods do not seem to comply with all

the major recommendations for routine fibrinolytic determinations, namely: (1) the registering of the lysis time should be automatic. (2) The method must be reproducible. (3) The procedure has to allow of the determination of several samples simultaneously. (4) The method should be sensitive for short as well as for long lysis times. (5) It should be possible to perform the test on mixtures with a wide range of optical densities. (6) The validity of the proposed method as a fibrinolytic assay should be demonstrated.

This paper describes apparatus for automatically recording lysis times, and demonstrates some of the possible applications.

PRINCIPLE AND DESCRIPTION OF THE APPARATUS

PRINCIPLE Lysis of a fibrin clot can be measured by observing the rising by gravity of air bubbles which are incorporated in the clot and freed by dissolution of the fibrin framework. These air bubbles are formed by the escape of air which is less soluble in the fibrin gel at 37°C than in the separate fluid reagents kept on ice. In the proposed method, a light beam rising through the bottom of the test tube is scattered by the air bubbles and fibrin fibres and falls partly on a photo-resistor, facing the test mixture in its lateral middle third (Fig. 1).

Modifications of the light-scattering properties of the air bubbles containing clot cause a change in light reaching the photoresistor (light-dependent resistance, LDR), resulting in a variation in current in the chain which can be measured by the tension drop over a potentiometer. This tension drop is recorded by a 12-channel graphic voltmeter (Philips multichannel voltrecorder type PR 3210 A/00). The use of a variable

FIG. 1. Circuit diagram of the apparatus.

potentiometer has the advantage that the variation in tension can be adapted to the range in sensitivity of the voltmeter.

Three distinct parts can be recognized in the curves shown in Figure 2. There is usually an initial descending segment, followed by an ascending limb, possibly resulting in plateau formation, and a sigmoid descending limb. The first segment corresponds to abundant air bubbles escaping the mixture before its gel forms. During the second part, the mixture is warming up still more and newly formed bubbles are at once prevented from escaping because of gel forming. More light falls upon the light-dependent resistance by increased dispersion so that the resistance decreases while the current and the tension drop over the potentiometer increase. In the third segment the bubbles rise by dissolution of the clot and the curve returns with the fall of the light scattering.

Conventionally, the endpoint is taken at the midpoint of this descending part of the curve, and an accurate reading is facilitated by the relatively rapid decrease in the third part of the curve. This sharp decrease is explained by the migration of air bubbles in front before the light-dependent resistance once the fibrin framework of the clot is sufficiently digested.

FIG. 2. Graphic registration of fibrinolysis.

DESCRIPTION OF THE APPARATUS The apparatus consists of a thermostatic block, a current chain, and a multichannel volt recorder (Fig. 3).

The thermostatic block consists of a thermostat unit (A) pumping water at 37°C to a container with 12 light-dependent resistance holders¹ (B). A block (C) with 2 T.L. light sources 6 W/240 V is built under the container. The light-dependent resistance holders have a vertical cylindrical drill out to fix a test tube, prolonged to the bottom by a smaller drill out through which the light beam reaches the test mixture.

The light-dependent resistance is built in the side wall of the drill out, facing the test mixture in its lateral middle third. Glass test tubes (10 mm × 100 mm) with horizontal bottoms are used.

The current chain consists of, in series connexion, a transformer of 220 V alternating current into 20 V

FIG. 3. Apparatus for semi-automatic registration of fibrinolysis.

bridge rectifier, the light-dependent resistance with a resistance varying from about 3.2×10^6 ohms at rest to 10^6 ohms at maximal bubble formation, a safety resistance of 330×10^3 ohm, and a variable potentiometer of 2.7×10^3 ohm.

The channels of the voltage recorder (Philips multichannel recorder type PR 3210 A/00) are connected to the potentiometers. As the voltmeter is a dot volt recorder, every curve consists of dots plotted at a distance of 4 mm from each other. As every dot is accompanied by a number and as the apparatus prints in different colours, every curve can easily be distinguished from the others. The start of the reaction is marked by an event marker. The speed of the graph paper is variable (4, 8, 30, or 60 cm/h). The sensibility range of the recorder is 0-100 mV over a maximal resistance of 20×10^3 ohm.

APPLICATIONS AND EXPERIMENTAL RESULTS

The sigmoid endpoint in our experiments is conventionally taken at the midpoint of the decrease of the curve. Visual controls established that this endpoint corresponded to the moment when all the bubbles had reached the upper half of the 1 ml test mixture.

¹ Supplied by Kabi AB Stockholm, Sweden.

FIG. 4. Correlation between visual and automatic results.

The half rising of the air bubbles is also frequently taken as the endpoint in visual assessments.

To check the comparability of the 12 channels and the reproducibility of the method in comparison with visual assessments, five technicians performed plasminogen determinations by the method described by de Vreker (1965) during three days on four pool plasma dilutions (1/500, 1/1,000, 1/2,000, 1/4,000) with lysis times varying from 11 to 35 minutes. Each day two series of each dilution were prepared and assessed visually in triplicate (total of 36 readings). The endpoint was situated where the bubbles rose into the upper half of the height of the volume and lysis times were read to the nearest 0.5 minute.

Four series of each dilution were performed in triplicate in the automatic assessment (total of 720 readings) and divided equally between the 12 channels. Lysis times were calculated at 0.1 minute.

The standard deviation of the results obtained by different channels is 0.6% of the mean value, the reproducibility of the endpoint in the three simultaneous readings is 2.97% for the visual assays and 3.2% for the automatic assays. The reproducibility of the plasminogen assay was 8.1% with the visual method and 6.5% with the automated assay.

Figure 4 demonstrates the correlation between the visual and semi-automatic assessments of different

fibrinolytic entities over a wide range of lysis endpoints: streptokinase assay with a lysis time ranging from five to 10 min (Christensen, 1949), plasminogen assay with a lysis time ranging from 15 to 40 min (de Vreker, 1965), and euglobulin lysis time with a lysis time ranging from 15 to 450 min (Blix, 1961). The procedure for plasmin and antiplasmin determinations with the apparatus will be described elsewhere (Tytgat and Collen, 1968). The fact that visual readings for streptokinase determinations are consistently shorter than the automatic, and in plasminogen determinations consistently longer, is caused by the different endpoints taken in the visual assessments as performed routinely in the laboratory. The scattering of the visual versus automatic euglobulin lysis times is mainly due to the difficulty in reading the lysis endpoint, especially for the longer times, and the unstandardized frequency of tilting of the test tube in the visual determination.

CONCLUSIONS

Twelve samples can be processed simultaneously. The sensitivity is sufficient for it to be applicable to most fibrinolytic assays except for whole blood clot lysis. The results can be interpreted objectively and are reproducible.

The graphic method has many advantages over the visual method in the determination of euglobulin fibrinolytic activity, in particular because visual methods need a long observation time and the endpoint is difficult to determine as very often a small amount of clot remains, highly resistant to lysis floating upon the surface of the lysed liquid.

We are much indebted to R. Peeters for skilful technical assistance and to E. Meulepas for statistical advice.

REFERENCES

- Blix, S. (1961). *Scand. J. clin. Lab. Invest.*, **13**, suppl. 58.
- Bouvier, C. A., and Gründlinger, J. (1963). *Schweiz. med. Wschr.*, **93**, 1451.
- Cash, J. D., and Leask, E. (1965). *J. clin. Path.*, **18**, 821.
- , — (1967). *Ibid.*, **20**, 209.
- Christensen, L. R. (1949). *J. clin. Invest.*, **28**, 163.
- Ferreira, H. C. (1965). *Blood*, **25**, 258.
- Harrower, H. W., and Brook, D. L. (1964). *Ann. Surg.*, **160**, 870.
- Hartert, H. (1951). *Z. ges. exp. Med.*, **117**, 189.
- Johnson, A. J. (1964). In *Blood Coagulation, Hemorrhage and Thrombosis*, edited by L. M. Tocantius and L. A. Kazal. Grune and Stratton, New York and London.
- Kuhnke, E., and Gill, I. S. (1963). *Thrombas. Diathes. haemorrh. (Stuttg.)*, **10**, 247.
- , —, and Brilla, G. (1960). *Pflügers Arch. ges. Physiol.*, **272**, 191.
- Nanninga, L. B., Zeller, R., and Maynes, C. (1964). *J. Lab. clin. Med.*, **64**, 706.
- Newman, R. L. (1964). *J. clin. Path.*, **17**, 194.
- Peeters, H., Steenhoudt, G. V., and Decroix, G. (1964). *Ibid.*, **17**, 320.
- Tytgat, G., and Collen, D. (1968). To be published.
- de Vreker, R. A. (1965). *Acta haemat. (Basel)*, **34**, 305.