Technical method

An improved procedure for determining thromboplastin generation

T. EXNER AND K. A. RICKARD *From the Haematology Department, Royal Prince Alfred Hospital, Sydney, Australia*

Thromboplastin or thrombin generation tests involve preincubating dilute plasma-serum-phospholipid-calcium chloride mixtures and subsampling aliquots at regular intervals into either recalcified plasma or fibrinogen solutions for determining clotting times (Biggs and Douglas, 1953; Pitney and Dacie, 1953). Since the clotting endpoints can occur during the subsampling procedure, such testing may even require a second person. Although automatic equipment could be used, several machines would be needed to handle successive subsamples if their clotting times overlapped the sampling times.

The sedimenting bead technique is suitable since the test system requires no attention after the initial mixing (Exner and Koppel, 1972a, b). The application of this method to the screening thromboplastin generation test (Hicks and Pitney, 1957) modified by the addition of kaolin (Macpherson and Hardisty, 1961) is described.

Materials and Methods

Potassium ferricyanide (100 ml, 0.2 M) was completely absorbed in Sephadex G-200¹ (5 g) and the gel added with stirring to 500 ml of 0.2 M ferrous ammonium sulphate solution. After one hour the dark blue beads were allowed to settle and repeatedly washed by decantation until the supernatant became colourless. The Prussian Blue-stained Sephadex G-200 was stored at 4°C in a volume of 250 ml.

Fresh citrated platelet-free plasma collected from normal volunteers was thawed once and redistributed as 0.1 ml aliquots into plastic tubes before being stored at -20°C before use.

Platelet substitute phospholipid was prepared from rabbit brain thromboplastin by the method of Bell and Alton (1954).

¹Pharmacia, Uppsala, Sweden.

²Dow Chemical Co, Midland, Michigan, USA.

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Methocel 60HG² was dissolved in water to give a 1% stock solution.

Kaolin was included as activator, but only at a relatively low final concentration to limit the background turbidity.

Typical Hicks-Pitney incubation mixtures contained the following:

Veronal-buffered saline	..	..	..	0.7 ml
Platelet-poor plasma	..	..	..	0.1 ml
Bell and Alton reagent	..	..	..	1.0 ml
Kaolin (5 mg/ml)	..	..	..	0.2 ml
Calcium chloride (0.025M)	..	..	..	1.0 ml

The following assay-substrate mixture was stirred (kept suspended) when dispensed by syringe as 0.5 ml aliquots into selected 7 × 75 mm glass tubes.

Normal platelet-poor plasma	..	..	2.0 ml
Methocel 60HG (1% solution)	..	..	1.5 ml
Stained Sephadex G-200 suspension (2%)	..	..	2.0 ml
Veronal-buffered saline	..	..	4.5 ml

At 30-second intervals 0.1 ml samples were removed from the incubation mixture at 37°C and added together with 0.4 ml 0.025M calcium chloride (also at 37°C). The beads were evenly suspended through each sample immediately on mixing all required components. Each tube was then allowed to stand vertically until after coagulation occurred.

A Coleman Junior II³ spectrophotometer was used to measure optical density at 650 nm of each tube.

Results

Dilutions of a normal Hicks-Pitney mixture which had been incubated for four minutes at 37°C were used to calibrate the assay system, as shown in figure 1.

As an example of the use of the method we investigated the effect of preincubating the dilute normal plasma with kaolin and phospholipid *before* the addition of calcium chloride. Results are shown in figure 2. It is apparent that this preincubation decreased the time required for peak thromboplastin generation after the addition of calcium chloride.

Comment

Due to the more frequent sampling of the incubated

³Perkin-Elmer Corporation, Maywood, Illinois, USA.

Fig 1 Final optical density at 650 nm plotted against volume of Hicks-Pitney thromboplastin used in the assay system.

Fig 2 Hicks-Pitney thromboplastin generation tests for normal plasma preincubated with phospholipid and kaolin for times shown.

0 = 0 minutes, x = 2 minutes, + = 5 minutes

mixture, the method gives a more accurate estimate than the manual method of the time at which thromboplastin is being generated most rapidly.

It may be less accurate for the estimation of final thromboplastin level, but it may still be used for this purpose if samples are diluted. This would give a final absorbance in a more sensitive range of the calibration curve.

As well as being useful for investigating factor deficiencies (Biggs and Douglas, 1953) and action of inhibitors (Margolius, Jackson, and Ratnoff, 1961), thromboplastin generation tests appear to be a sensitive indicator of the tendency to thrombosis (Duckert, Flückiger, Isenschmid, Matter, Vogel-Meng, and Koller, 1954). This is especially true when more dilute plasmas are used (Thompson, Owen, Spittell, and Pascuzzi, 1962). However, the methods are usually tedious, involved, and not accurately reproducible. The adaptation of the sedimenting bead method for the assay of thromboplastin generated in these tests would remove one of their difficult features.

The shortening of the time required for maximal thromboplastin generation by preincubating dilute plasma with phospholipid and kaolin apparently results from the slow activation of factors XII and XI in the absence of calcium.

The method would be improved if beads with a wider range of sedimentation rates were used. The viscosity-increasing agent (Methocel) was only included to modify the settling rate of Sephadex G-200 beads.

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