A SIMPLE APPARATUS FOR THE DETERMINATION OF BLOOD CLOTTING-TIMES AND ITS APPLICATION TO THE ASSAY OF HEPARIN

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INTRODUCTION

DETERMINATION of the clotting time of suitable fibrinogen-containing systems forms the basis of most assays of blood coagulants and anti-coagulants. The purpose of this paper is to describe a simple apparatus whereby the precision of such determinations may be much improved. In particular, we have applied it to the method of assay which Adams[1] developed for heparin solutions. We have found this method very satisfactory and have used it also for thrombin assay. He observes the clotting process in standard glass tubes and takes for end-point the time when the clot formed remains adherent to the walls of the tube on inversion. We have not found this end-point entirely satisfactory; it is subject to considerable variation, and we do not find it convenient to examine a series of tubes in this manner according to a strict time schedule. Further, since premature inversion permanently alters the clotting system and leads to the abandonment not only of the individual result, but also of the whole series (for this is the most satisfactory way of dealing with the contingency), operators tend to be rather tardy in inverting, so that a bias creeps into the assay. For these reasons it seemed necessary to find a suitable end-point indicator, a problem which has received much attention from workers in the past.

There are two main approaches to the problem; one is to detect fibrin-fibril formation; this, in long-clotting-time systems may occur a considerable time before formation of a reasonably firm clot; the other is to detect the increase in viscosity of an incipient-clotting system.

In the very substantial literature of the subject are to be found many and ingenious devices for detecting one or other end-point. For example, of the first type, Wright[2] observes dispersion of the system in water, Trought and Riddoch[3] observe fibril formation when a capillary tube containing the system is momentarily brought into contact with a rough surface, and there is a widely-used method, probably also due to Wright, in which fibrin is detected in a micro-system by dredging with a fine glass rod. A macroscopic modification of this is described by Quick.[4] To the second type belong the Brodie-Russell-Bogg, and Dale and Laidlaw[5] coagulometers, which consist respectively of a spinning drop observed under the microscope, and a glass tube containing a metal sphere whose fall under gravitation through the liquid is observed. Measurements based on viscosity-change are capable of considerable precision. Hartert,[6] using a rotating cylinder viscometer, has shown that it is possible to determine clotting times with an "average error" of <3 per cent. Randall[7] has

described a similar type of apparatus which has the further advantage of being automatic. He quotes replicate times of 5·1, 4·8, 4·9 and 4·8 minutes for a particular sulphated-blood/ox-brain extract/heparin system. The coefficient of variation is therefore 2·9 per cent. which confirms the accuracy claimed by Hartert.

We could find no apparatus already described which seemed adaptable to the assay, chiefly because none would lend itself to multiple determinations, and so the following simple apparatus was devised. It depends on the viscosity change and yields reproducible results.

Apparatus

Description. The apparatus consists of a wooden block, drilled to hold 6 test tubes (3 × ½ inch), which slides vertically up and down 4 guides set in a base-plate. A metal plate is screwed to the top of the guides to maintain them in correct position, and this bears 6 holes, each lying on the axis of one of the test tubes (Fig. 1). The block is moved up and down by means of a lever. Indicators are hung from the top plate, each of which consists of a glass bead set on the end of a suitable length of wire, whose last cm. of length is bent through approximately 100°. Thus when an indicator is lowered through a hole in the upper plate into a test tube, its weight is carried by the terminal portions of the wire which is resting on the plate. Glass indicators prepared by allowing molten glass to elongate under gravitation were originally used, but were too fragile to permit ready cleaning. Clearly the detail of the apparatus is of little importance, all that is necessary being a block to hold the required number of tubes and impart to them a vertical motion of about 1 cm. together with some form of superstructure from which to hang the indicators.

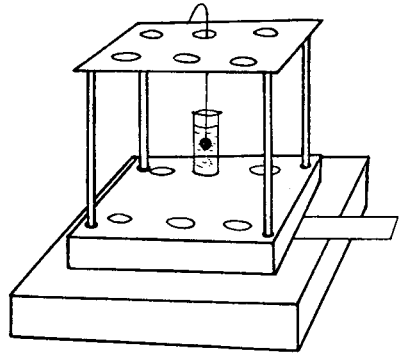

Fig. 1

Method of operation. The reagents are introduced into the tubes and are mixed by inverting, the mouth of the tube being closed with a waxed cork. The cork is removed and the tube is then placed in position in the block and an indicator lowered into it. At 10-second intervals the lever is gently depressed. As long as the system remains fluid, the indicator remains still, but on attaining a critical viscosity, the vertical motion of the tube is transmitted to the indicator which makes a distinct movement. This end-point is quite distinct and has been used to record clotting-times to the nearest 5 seconds, but 10 seconds is preferable since it allows time for recording results and is a convenient time unit for computational purposes. The critical viscosity depends on the weight and dimensions of the indicator used. For example, an indicator weighing 0·38 g.

prepared from a 4 mm. diameter glass bead is suitable for detecting the formation of a soft clot.

Accuracy. The accuracy of the apparatus may be gauged from the following experiment which was designed to detect any significant difference in the end-points shown by 6 similar indicators. The system used was heparin 4 units in 2 ml. of water, ox-brain extract 0·5 ml., sulphated blood (containing 3·6 per cent. of Na_2SO_4 10 H_2O) 2 ml., the ingredients being added in that order at 3-minute intervals, and mixed immediately after the addition of blood. The experiments were carried out in 3 randomised blocks. The indicators were coated with paraffin wax. Times are recorded in 10 second units (see Table I).

TABLE I

Blocks	Indicator number						Totals
	I	II	III	IV	V	VI	
1	34	34	35	34	36	34	207
2	38	34	36	37	37	34	216
3	40	39	39	41	38	40	237
Totals	112	107	110	112	111	108	660

Mean clotting time 36·7 seconds/10.

TABLE II

ANALYSIS OF VARIANCE

Item	Sum of squares	Degrees of freedom	Mean square	Variance ratio	Probability
Series..	79·0	2	39·5	25·2	<0·1 per cent.
Indicators	7·3	5	1·47		
Residual	15·7	10	1·57		
Total ..	102·0	17			

It is evident that the above data give no ground for supposing that there is any significant difference in the performance of the different indicators. The highly significant series mean square is attributable to the warming-up of the reagents which had been stored in a refrigerator. It is important to bear this effect in mind when conducting an assay in which the observations in each series are not randomised.

The total variance within samples is $23/15 = 1{\cdot}53$.

$$\therefore \text{coefficient of variation} = 100\sqrt{\frac{1{\cdot}53}{36{\cdot}7}} = 3{\cdot}4 \text{ per cent.}$$

which compares very favourably with that obtained with the more elaborate apparatus previously mentioned. A fully mechanised model is at present under construction and it is hoped that with this the precision will be further increased

A further experiment was carried out to determine the effect on clotting-time of an increase in the liquid/glass interface, and of varying the order of mixing the reagents. The system used was the same as that in the above experiment, but reagents were added at 2-minute intervals (see Table III).

TABLE III

	Order of adding reagents				
	Heparin—Thromboplastin—Blood		Heparin—Blood—Thromboplastin		
Series No.	With bead	Without	With bead	Without	Totals
1	42	41	39	39	161
2	36	40	36	34	146
3	35	37	33	34	139
4	37	36	35	38	146
5	38	40	33	37	148
6	38	39	36	36	149
	226	233	212	218	889

In 10-second units.

The experiment consisted in observing the clotting time of the 4 possible combinations of order of adding reagents and presence or absence of a 4 mm. diameter soda-glass bead. The experiments of each series were conducted in random order.

TABLE IV

ANALYSIS OF VARIANCE

Item	Sum of squares	Degrees of freedom	Mean square	t	Probability
Series	64·7417	5	12·94833		
Order of addition ..	35·0417	1	35·04167	4·19	<0·1 per cent.
Bead	7·0417	1	7·04167	1·87	5 to 10 per cent.
Inter-action	0·0417	1	0·04167		
Residual	30·0917	15	2·00611		
Total ..	136·9583	23			

We may therefore assert that addition of reagents in the order: heparin, blood, thromboplastin, produces a more rapidly clotting system, so that the more desirable order of mixing is heparin, thromboplastin, blood. The analysis also suggests that the presence of the additional glass surface accelerates clotting, but the level of significance is not high and the experiment is not conclusive in this respect. It does however point to the desirability of having solutions of standard and unknown of as near as possible the same potency in the assay.

The interaction term is subnormal and remarkably small but no significance is to be attached to this as the probability of such a value lies between 0·1 and 0·2.

The coefficient of variation in this data

$$= 100 \times \sqrt{\frac{2{\cdot}0061}{37{\cdot}042}} = 3{\cdot}8 \text{ per cent.}$$

In order to effect a comparison with the data published by Adams, the data was transformed into logs of clotting time in minutes and the residual variance found.

Adams data	Variance 0·0015
Above data	,, 0·000273

so that the precision of his method of assay is much improved by use of this indicator.

SUMMARY

1. An apparatus is described for the simultaneous determination of several clotting times.

2. The accuracy is compared with that obtainable by other methods.

3. Its application to the assay of heparin described by Adams is suggested.

4. The effect of glass-liquid interface and order of mixing in the assay of heparin is investigated.

The author thanks Dr. R. Maxwell Savage for many stimulating discussions and suggestions.

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DISCUSSION

The paper was presented by MR. D. M. BRYCE.

MR. K. L. SMITH (Nottingham) stated that he used the Adams method of determining end-points, but automatic methods were under consideration.

MR. J. M. MYERS (Bradford) pointed out that if the glass surface had been treated with silicones to start with, it might have prevented clotting.

MR. D. M. BRYCE, in reply, said that the real purpose of designing the apparatus was to overcome the personal factor of operators, and in its present form it gave satisfactory results. The effect of surface coating was very important. An endeavour had been made to make comparisons at the same potency level, in order to remove any error, because the variation caused by different types of surfaces was such as sometimes to prevent clotting altogether. He hoped to try the silicone covered materials.