Use of a Precision Coagulation Timer in the Biological Assay of Thrombin

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A new *in vitro* assay for the determination of thrombin potency has been studied using a precision coagulation timer. This instrument showed a more objective and quantitative clot-time determination of the human and bovine plasmas. The 2 × 2 and 3 × 3 assay designs were employed utilizing the dose-response relationship established between thrombin and the plasma. The differences between the present and proposed methods and between plasma of the two species are discussed.

THE PRESENT method used for the assay of thrombin is essentially the same as that published by the Federal Security Agency, National Institutes of Health, Bethesda, Md., 1946. In this method, two dilutions of unknown bovine thrombin are chosen so that one has a slightly higher and the other a slightly lower clotting time than a dilution of N.I.H. standard thrombin. The potencies (units/ml.) of the unknown thrombin dilutions, which are based on their assumed potencies, are plotted with the clotting times and these points are connected by a straight line. A point on this line which corresponds to clotting time of the standard thrombin is determined. A ratio is set up by comparing the potency corresponding to this point with the assigned potency of the standard. The potency of the unknown thrombin is obtained by multiplying the assumed potency by the ratio, as follows:

$$\frac{\text{assigned potency (of standard) units/ml.}}{\text{obtained potency (of standard) units/ml.}} \times \text{assumed potency (of unknown)} = \text{potency of unknown}$$

The clotting times in this method are visually determined by observing the clot in a small test tube after the plasma has been added to the thrombin and the clotting time is recorded by using a stop watch. During the observation time, the test tube is tilted once every second until coagulation occurs.

Due to its simplicity, this assay can be done rapidly. However, the dependence on subjective judgment for the end point determination and a complete lack of any statistical validity tests appear as serious disadvantages of this method. Thus, it was the purpose of this study to attempt the automation of the end point determination and to design a new assay in which the procedure will have to pass at least one validity test.

In this study we used a precision coagulation timer.[1] This instrument is being used by many clinical laboratories for the determination of prothrombin times.

For the determination of the clotting times, a plastic cup[2] containing 0.2 ml. of plasma is placed in the reaction well and the timer is automatically started when 0.1 ml. of thrombin is delivered from a pipet. After 1.5 sec. delay, the probe arm inserts the two electrodes into the plasma–thrombin mixture. The electrodes consist of a stationary electrode and a moving electrode which moves in and out of the plasma–thrombin mixture. When the moving electrode has risen above the reaction mixture, it reaches a contact point which would complete the circuit except for the gap between the two electrodes. Thus, when thrombin has converted the fibrinogen to fibrin, the latter is partially pulled out of the reaction mixture by the moving electrode and completes the electrical circuit between the electrodes. This electrical short stops the timer and the clotting time is recorded. The clotting times are obtained at 37° since the instrument is equipped with heating blocks which maintain this constant temperature.

The moving electrode completes a cycle (in and out of the reaction mixture) every 0.5 sec. Since the circuit can only be completed when the moving electrode has moved out of the reaction mixture, the clotting time end point can occur in an interval which is one-half of a cycle. Thus, in 1 sec. there are two intervals of approximately 0.25 sec. duration in which the end point can be detected. This means that the exact clotting time is not always given to the tenth of a second as the timer indicates. However, since this is a constant error, and it is the relative clotting times which are important in the biological assay, the clotting times to the tenth of a second as shown on the timer are used for the assay calculations.

Although there is some chance for error in the end point determination, this instrument has the advantage of a more objectively determined clot

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[1] Fibrometer, Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Md.
[2] Supplied with instrument.

time than the method now being used. It is anticipated that this increased objectivity will reduce the variation in obtained potencies between operators.

EXPERIMENTAL

Assay.—For the 2 × 2 assay, thrombin dilutions of 5.64 and 8.46 units/ml. (a log 1.5 interval) were prepared for both standard and production material. The dilutions were made up in 10-ml. volumetric flasks with normal saline. The individual dilutions were numbered so that a random order could be used for the collecton of data. For the 3 × 3 assay design, 5.64, 7.05, and 8.81 units/ml. dilutions of thrombin were used (a log 1.25 interval). The dilutions for unknown thrombin were based on their assumed potencies. The 50% plasma solutions were prepared by adding 5 ml. of distilled water and 5 ml. of normal saline to a vial containing 5 ml. of lyophilized human plasma.[3]

Two tenths of a milliliter of plasma solution was delivered to the plastic cup by using the autopipet. One rack of cups containing plasma was placed in the heating block. At the same time, the appropriately numbered cups containing the thrombin dilutions were also placed in the heating block. At the end of the 3-min. warm-up period, one of the cups containing plasma was placed in the well directly beneath the electrodes. One-tenth milliliter (0.1) of the thrombin solution was delivered to the plasma *via* the autopipet. The autopipet activates the timer when the plunger is depressed and the switch is in the "on" position. When coagulation occurs, the timer automatically stops, and the time of the end point is recorded.

The data for this assay were gathered as quickly as possible due to the effect of the 37° temperature on the thrombin and plasma. According to the manufacturer, plasma begins to decompose after 15 min. at this temperature. In this study it was found that effects on the plasma are insignificant if the plasma was used within 6 to 7 min. when at 37°.

The data were arranged under the appropriate dilutions (six determinations per dilution) and the clotting times in seconds were converted to logs. The logs are treated as numbers rather than as exponents in the computations of the statistics of the assay. In this laboratory the computations were carried out by an IBM 1620 computer. The computations for this 2 × 2 assay were described by Bliss (1).

Dose-Response Curve.—In order to design a new assay, the dose-response relationship was determined. Since there are many unknown variables in plasma coagulation, several dose-response curves were determined using both human and bovine plasmas and also for N.I.H. standard and production thrombins.

Although the curves shifted slightly from day to day, the shapes of the curves were the same even for the plasmas of the two different species. The dose-response relationship is a curved line. Examination of these lines led to the selection of the middle part of the curve for the assay work. The dose-response relationships in this area were somewhat linear and the sensitivity of the response in this area was more suitable for assay work. The linearity of this part of the dose-response curve was confirmed when orthogonal analysis of curvature was not significant in this area. However, the variance due to curvature was significant for the entire dose-response curve, and curvature still remained a problem in many of the assays. However, this problem was partly solved by plotting log dose *versus* log response as will be discussed later.

From the dose-response curve study it was determined that the dilutions of thrombin which resulted in clotting times between 10–22 sec. were useful dilutions for assay purposes. This useful area of the curve was determined for the different types of plasma used, *e.g.*, bovine, human plasma[3] half-strength, human plasma full-strength.

Statistical Design of Assay.—At the outset of this study it was felt that a statistical design was needed which would allow the operator to establish limits for the assay. At the beginning of this study the statistical design used for the three-dose balanced assay of corticotropin injection U.S.P. XVII (referred to as the 3 × 3 assay) was chosen since it contained four different validity tests. The present assay method contains no validity tests. Although the validity tests in the 3 × 3 assay design are far from perfect, they do serve as a source of confidence for the operator. The passing of these validity tests depends upon the "F" values obtained by dividing the mean squares resulting from deviation from parallelism, curvature, differences in curvature, and the combination of these three by the error variance. The weakness in these validity tests is that a high error variance can increase the probability that the validity test will be passed. Thus, it is up to the operator and the laboratory to limit the error variance which will be accepted. This may also be accomplished by placing limits on the "L" value. All of the computations for the statistical designs used in the study were programmed for the IBM 1620 computor.

RESULTS AND DISCUSSION

It became apparent in the early part of this study that temperature had an effect on the clotting times when the thrombin solutions remained at 37°. Thus, it was important that each dilution of thrombin be kept standing at 37° for the same length of time prior to clotting time determination. In order to insure this, tubes containing the dilutions of thrombin should be placed in the heating block at the same time. After the 3-min. warm-up period, the clotting times for the different thrombin solutions are determined using a random order design. In this design for the collection of data, the effect of standing at 37° will be distributed over the different dilutions.

For the 3 × 3 assay, the responses (clot times) are statistically compared to the log dose for the standard and unknown thrombin dilutions. In this assay, we were using a statistical design which was intended for a linear dose-response relationship for data which possessed a curved dose-response relationship. Therefore, several of the assays failed the curvature validity test. A log × log transformation of the data was attempted in order to obtain a straight line.

The portion of the dose-response curve in which we were working became more linear when log dose was plotted against log response (Fig. 1.) Another

[3] Normal Plasma Control (5-ml. size), Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Md.

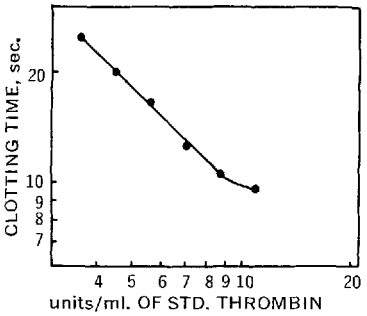

Fig. 1.—Log × log dose-response curve for N.I.H. standard thrombin and human plasma. Each point represents mean of six clotting times.

advantage of using the log × log transformation is that it tends to decrease the differences in the response variation which were often noted between the long and short clotting times.

A study to determine whether the log × log transformation reduced the sensitivity of the validity tests was conducted by intentionally making increased "errors" in one of the test dilutions in consecutive assays. The effect of these increased "errors" on the mean squares resulting from deviation from parallelism, curvature, and difference in curvature was observed and a comparison was made between semilog and log × log designs.

The results of this comparison indicated that the log × log transformation did not make the assay less sensitive than the semilog assay. The assays failed and passed in the same instances. The mean squares resulting from deviation from parallelism were increased in the assays where intentional errors were made in dilutions.

A 2 × 2 (log × log) assay (two standard and two unknown dilutions) was also used in this study and is now being used in place of the 3 × 3 design. This 2 × 2 design is thought to be more practical for routine work since it requires less time and reduces the amount of time the thrombin is kept standing at 37°.

The 2 × 2 assay has only one validity test to pass and this is deviation from parallelism. This is one of the more important validity tests since the log × log transformation reduced the problem of curvature. It was also observed that the potencies of 2 × 2 assays differed very little (0.5 to 1%) from the potencies obtained with the 3 × 3 design.

Results of many assays have shown that bovine plasma at 37° gave a high error variance. Analysis of the row effect, which represented the effect of time at 37°, revealed that the variance resulting from time was significant. It is probable that after a short time, the bovine plasma was breaking down at 37°. This temperature effect was not present when human plasma was used in the same time interval and at the same temperature. It was also revealed that in assays using human plasma, the thrombin was more efficient when the 0.2 ml. of plasma solution consisted of 50% plasma rather than 100% plasma. More thrombin was required by the 100% plasma to obtain the same clotting time as the 50% plasma. Apparently the optimal substrate concentration is closer to the 50% plasma solution than to the 100% solution.

The statistical data indicated that there was no

TABLE I.—COMPARISON OF POTENCIES OBTAINED WITH PRESENT AND FIBROMETER METHODS

Lots	Potencies, units/Vial — Present Method[a] Bovine Plasma	Present Method[a] Human Plasma	Proposed Method Bovine Plasma	Proposed Method Human Plasma
A	1130	1053	1436	1280
B	1293	1210	1612	1335
C	1115	1088	1378	1190
D	5113	4825	5665	5350
E	5538	5225	6302	6005

[a] 3 × 3 semilog assay design used.

TABLE II.—COMPARISON OF RESULTS FROM THE DIFFERENT METHODS FOR THE CALCULATION OF POTENCY

Lots	Potencies, units/Vial — Proposed Method[a]	Point Assay Proposed Method	Point Assay Present Method[b]
A	1271	1227	1130
B	1333	1263	1293
C	1191	1271	1115
D	5440	5230	5113
E	6000	5665	5538

[a] 2 × 2 (log × log) assay design used with human plasma.
[b] Bovine plasma used.

advantage in using 100% plasma solution rather than 50% solutions. The error variance remained about the same in both instances.

Samples from five production lots of thrombin, three 1000-units/vial and two 5000-units/vial, were assayed with bovine and human plasma solutions. These results were compared with the results obtained using the present N.I.H. method. The result of this study appears in Table I. Examination of these data reveals that the thrombin was more potent when assayed with bovine plasma than with human plasma. It was observed that the precision coagulation timer method employing human plasma at 37° results in a higher potency for thrombin than does the present method employing bovine plasma at 28°. This increase in assayed potency was about 50–150 units for the 1000 units/vial material and about 250–400 units/vial for the 5000 units/vial material. Part of this increase is probably due to the increased temperature from 28° to 37°. However, the results in Table II indicate that part of this difference in potency between the present and proposed precision coagulation timer methods is due to the method employed to calculate the potency. When the single point assay which is used to calculate the potency in the present method was applied to the new method, the potency values are closer together.

SUMMARY AND CONCLUSIONS

It is felt that this new assay offers advantages over the assay presently being used. Human plasma which is used at 37° is closer to the conditions which exist when thrombin is used clinically.

The automated end point eliminates the reliance on subjective observation on the part of the analyst. The statistical design which contains a validity test can be a source of increased confidence in the results.

REFERENCE

(1) Bliss, C. I., *Drug. Std.*, **24**, 41(1956).