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Streptokinase Assay on Large Agar Diffusion Plates*

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A description is given of a quantitative assay method for streptokinase activity on large plates by which seven samples can be compared on one plate with a standard at two potency levels. The influence on the zone definition of the buffer system and concentration and of the incubation temperature have been studied. To obtain an agar-fibrinogen-thrombin-mixture that is poured easily to produce a homogeneous layer, the influence of the concentration of these substances has been investigated. A device by which the zone diameters can easily be determined with high precision is described. A statistical method for distribution of test solutions, previously used for microbiological assays, has been applied.

In the last decade, increasing industrial interest in streptokinase has enhanced the need for an assay by which it is possible to compare several samples with each other, in order to check the different purification steps.

The first quantitative method for the determination of streptokinase was a tube test procedure devised by Christensen [1] and later modified by Wasserman, Ciminera, Hayflick and Verwey [2] so that the results could be treated statistically. However, it has been the present author's experience that only a highly trained personnel can obtain reproducible results with the tube test method.

Astrup and Müllertz [3] have designed a plate method which is more easily performed, and by which it is possible to achieve better reproducibility. When a large number of samples are to be assayed as routine procedure, the Astrup technique is less tedious than the tube test. However, it requires a longer time.

In this paper, a description will be given of a modification of the Astrup technique which makes it possible to compare seven samples with a standard preparation on one large plate at two different potency levels. The statistical design described by Lees and Tootill [4] for microbiological assays has been applied.

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MATERIALS AND METHODS

Fibrinogen. A one per cent fibrinogen solution was prepared from commercial human fibrinogen (a freeze-dried product, coagulability 93 %, kindly supplied by AB Kabi, Stockholm). The clear solution was distributed into test tubes, and stored at −20°C. Storage up to 6 months did not affect this solution markedly. This human fibrinogen preparation was chosen because it contains enough contaminating plasminogen to be in excess for the test.

Thrombin. A stock solution was prepared by diluting 10 mg thrombin (Parke, Davis and Co., "Topical") in 25 ml physiological saline. The stock solution was never stored longer than 3 days at + 4°C. Fresh working solutions were prepared each day by diluting the stock solution 1:500 in saline (approx. 0.02 NIH units/ml).

Tris-buffer solutions. Two stock solutions were prepared. A, containing 18.2 g tris-(hydroximethyl)aminomethane in 1000 ml 0.15 M NaCl and B, containing 18.2 g tris-(hydroximethyl)aminomethane in 1000 ml 0.15 M HCl. To prepare a buffer of pH 7.5, 40 ml A and 460 ml B were mixed. This buffer has an ionic strength of 0.30. For the routine assays a buffer with an ionic strength of 0.20 was used.

Agar gel. A gel containing 1 % of Bacto agar (Difco) in buffer was prepared. When not used immediately the agar gel was stored at + 4°C.

Merthiolate. A 10 % solution of merthiolate (Thiomersal, Lilly & Co.)was prepared according to Kilduffe and DeBakey.[5]

Streptokinase standard. The streptokinase standard was dissolved in the same buffer as used for the agar gel. The preparation used was a freeze-dried product supplied by AB Kabi, its potency being standardized against a streptokinase preparation from The National Institutes of Health called "streptokinase reference standard, tentative preparation".

Preparation of samples. The samples were diluted in buffer to roughly the same potencies as those of the standard solutions.

Glass plates. The glass plates used were constructed according to Lees and Tootill [4] with the inner dimensions 30 × 30 cm. The adhesive used was Araldite (equal amounts of Araldite type 106 and Hardener 953 U, Ciba). This glue gives a strong adhesion glass to glass which has been found to be very resistent to hot water. The glass frame pieces were glued under pressure over night at room temperature.

Agar hole puncher. The agar hole punching apparatus described by Callieri [6] has been slightly modified. The puncher, made of stainless steel, has a punch-hole diameter of 9 mm. The conveyer is made of stainless steel blade 9 × 60 mm that was twisted while stressed, and split at the lower end. (Fig. 1).

Application of test solution. Four drops of test solution were added to each hole from a pipette consisting of a glass tube tipped with a 20 mm long Pt-Ir tube (inner diam. 0.70 mm, outer diam. 1.25 mm). The standard error of the applied volumes was less than ±1.2 %. Soliconation of the pipette will diminish the adsorption of streptokinase to the glass wall.

Zone comparator. A special device was used for measuring zone diameters with a high degree of precision. Fig. 2 shows the main principle of the apparatus (constructed by Ing. J. Engblom at the LKB Research laboratories, Bromma, Sweden). An improved contrast on the screen was obtained when the condenser system was replaced by a round sooted plate in order to produce a kind of dark field illumination.

Statistical design. According to Lees and Tootill,[4] the large plates have been used for 8 × 8 quasi latin square designs in ordinary assays. For the determination of standard curves, 8 × 8 latin square designs were used. The relative potencies of samples were calculated as described by Lees and Tootill.[4] However, the values were calculated with the aid of semilogarithmic paper plots instead of being obtained from a nomogram.

Proposed assay method. For each plate, 210 ml 1 % agar-buffer-solution is melted and then cooled to 39°−41°C. 7 ml of fibrinogen and 0.2 ml of merthiolate solution is added and thoroughly mixed with the agar. 7 ml thrombin solution is added and the solution is carefully mixed. Precautions must be taken to avoid air bubbles in the substrate as they will produce inhomogeneities in the agar layer. Immediately after mixing, the solution is poured onto the carefully levelled and disinfected glass plate. Warming the plate to 37°C facilitates the homogeneous casting of the agar layer.

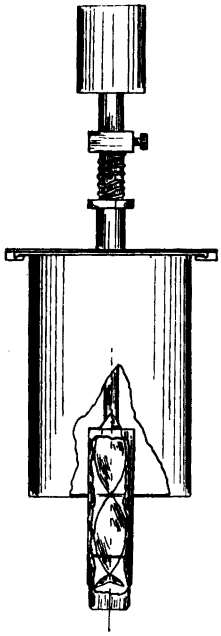

Fig. 1. Agar hole puncher showing in detail the conveyer.

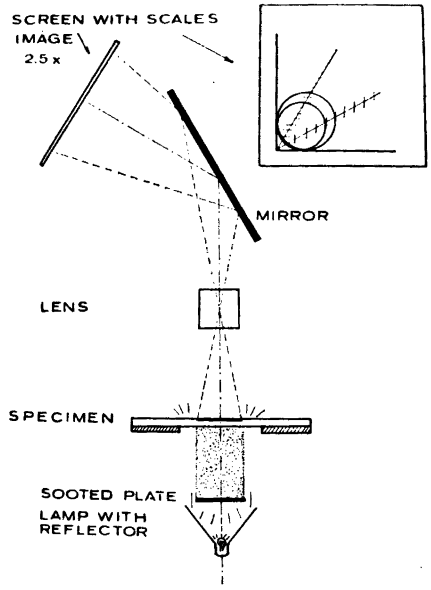

Fig. 2. Main principles of zone comparator used for measuring the zone diameters in streptokinase assays.

After 5 to 10 min the agar is solid and the plate can be removed from the horizontal surface. After another 30 min at room temperature the plate is placed in the refrigerator for at least 1.5 h in an inverted position to permit any condence which may form to settle on the cover and not on the agar surface. At a low temperature, the agar is harder and thus easier to punch. (It has been possible to store the poured plates for several days in the refrigerator without any detectable change in the assay). 64 holes are punched in the agar gel, 8 rows with 8 holes in each. The test solutions are added to the holes in the plate with the aid of the pipette. After incubation for 16 — 18 h (over night) at 30°C, clear circular zones are formed around the holes with solutions containing streptokinase. Two perpendicular diameters are measured with the aid of the zone comparator and the mean value is calculated. The potencies of the samples are determined. For routine work the sample is tested on two plates.

RESULTS

When the streptokinase diffuses from the test solution into agar gel the plasminogen is activated to plasmin. This enzyme will lyse the fibrin in the gel. This reaction is visualized by a clarification of the turbid gel surrounding the holes. The area thus clarified is dependent upon the concentration of streptokinase in the solution to be tested.

To obtain reproducible assays, it is of prime importance to have clear and distinct zones, so that the zone diameter can be estimated with a high degree of accuracy. This means that the contrast between the clear zones and the

turbid areas surrounding them should be as marked as possible. Following factors have been found to affect the turbidity of the agar gel containing fibrin: a) fibrin concentration, b) ionic strength of the buffer used in the agar gel, c) pH of the buffer in the agar gel, d) incubation temperature.

The turbidity of the agar-fibrin gel increases with increasing concentration of added fibrinogen. The sensitivity of the assay decreases, however, with increasing concentration of fibrinogen. Astrup[1] used 0.1 % fibrinogen, the concentration needed to produce a stable gel. When the gel is stabilized with agar it is possible to decrease the fibrinogen concentration to 0.03 % and still have a sufficiently high turbidity in the gel to produce good contrast between turbid background and lysed clear zones. The amount of thrombin added was chosen so as to clot the fibrinogen after the agar was poured onto the plate.

The tris-buffer has been found to give the best contrast, thus the best reproducibility, with an ionic strength of 0.20—0.25. When the ionic strength was changed by altering the concentration of the buffer, it could be shown that an ionic strength of less than 0.10 or higher than 0.30 resulted in poor clotting of the fibrinogen thus decreasing the turbidity in the gel.

In the pH range 7.2—7.8, the zones are distinct and sharply defined and allow an accurate measurement of the diameters. At pH 6.9 the zones are relatively diffuse but it is still possible to measure the diameters. At lower pH or at a pH above 8.0 the clotting of the fibrinogen is incomplete, resulting in no turbidity in the agar gel.

The influence of different pH on the regression line at constant temperature 30°C, has been studied. The results are shown in Fig. 3. By analysis of variance it can be shown that the pH has an effect on the regression coefficient.

The influence of different incubation temperatures on the regression line is shown in Fig. 4. Even though the regression line is steeper at 30°C than at 37°C, the lower temperature was chosen as a standard since small variations in the pH and ionic strength were found not to affect the zone definition at this temperature as much as at 37°C.

The agar concentration used was the lowest possible which allowed holes to be punched. Higher concentrations gave smaller zones and thus lessened sensitivity of the assay.

DISCUSSION

During the isolation procedure for streptokinase, the purpose of the assay is often to compare different purification methods. It is then more essential to compare the samples under the same conditions than to obtain the results within a short time. Åstrup's plate technique is well suited for these determinations, although it has certain disadvantages. Since only small plates can be prepared, not more than six zone determinations can be made on one plate and the differences between plates have to be considered when estimating the potencies. Because of the irregular shape of the zones it is often difficult to determine their accurate diameters.

Stabilizing the fibrin gel with agar facilitates the preparation of large plates so that several samples can be compared on the same plate. Unevenness in the agar depth or of temperature in the incubation chamber is compensated

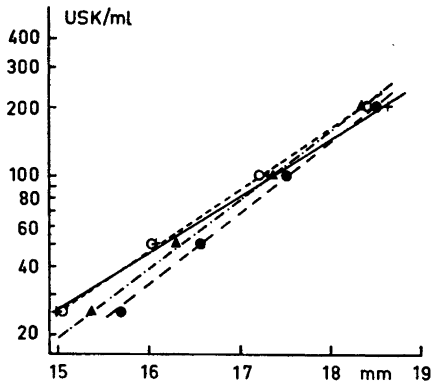

Fig. 3. The influence of different pH on regression line at constant temperature, 30°C.

pH	Symbols	Regression coefficient
6.9	+ —— +	4.05 ± 0.47
7.2	O - - - - - O	3.69 ± 0.21
7.5	▲ — · — ▲	3.29 ± 0.27
7.8	● — — ●	3.13 ± 0.31

Each experimental point represents at least 120 zone determinations and the use of 15 different plates. (USK = unit streptokinase).

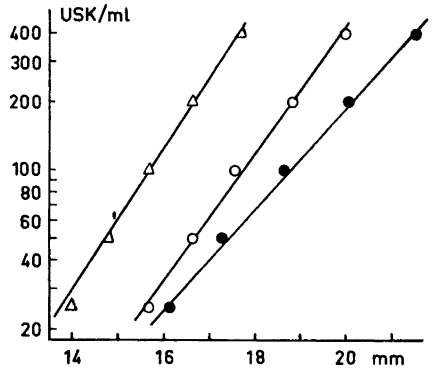

Fig. 4. The influence of different temperature of regression line at constant pH, 7.5.

Temp. °C	Symbols	Regression coefficient
23	△	3.08 ± 0.19
30	O	3.60 ± 0.23
37	●	4.50 ± 0.28

Each experimental point represents at least 110 zone determinations and the use of 14 different plates (USK = unit streptokinase).

by the distribution of the test solutions according to a quasi latin square design.

It is also possible to punch holes in the agar gel. When the test solutions are added to these holes, the diffusion, and hence the zones obtained, will be completely circular.

The standard error in this method has been estimated as described by Lees and Tootill.[4] The following result was obtained: Standard error of each potency determination per plate (one determination requiring 8 holes) ± 4.5 %.

The agar plate method has also been used for the determination of plasminogen activator activity in extracts from tissues. The extraction method described by Astrup[7] employs a 2 M KSCN solution. With this solution alone (without activator) Astrup did not obtain any lysis zones when no agar was added to the fibrin gel. Contrary to this finding, the agar plate method gives clear zones with KSCN. For instance, a 2 M KSCN solution results in zones corresponding to about 100 NIH units streptokinase/ml. Use of a 1 M KSCN solution results in zones corresponding to about 20 units streptokinase/ml. Even a 0.25 M KSCN solution produces small lysis zones. Before KSCN extracts from tissue can be assayed by the agar plate method they must be dialysed against tris-buffer.

The described method gives reproducible results with solutions containing as little as 25 units streptokinase/ml. However, it is our experience that the most convenient range lies between 250 and 500 units streptokinase/ml where the zones have a diameter of about 20 mm.

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