pharmaceutical powders was not a freely reversible process. Procaine base was cited as an example of powder exhibiting an unusual sorption of water vapor which can be accounted for by formation of a crystalline dihydrate.

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Standardization of Fibrinolytic Preparations

Measurement of Proteolytic and Activator Activity of Streptokinase-Activated Human Plasminogen

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Methods for the determination of proteolytic and activator activity of streptokinase-activated human plasminogen are presented. These assay procedures extend previously published methods by the incorporation of a suitable standard and improved experimental design. The experimental bias is minimized and, by test of validity, differences in preparations can be realized.

IN THE LAST few years fibrinolytic agents have been developed which can assist in the clinical dissolution of venous and arterial clots. Several of these agents have become commercially available and, consequently, standardization of these preparations has become of considerable interest.

A fibrinolytic preparation[1] has been made commercially available by our laboratory. This is a streptokinase-activated human plasminogen preparation. The activity of fibrinolysin has been determined by a fibrinolytic assay procedure (1) which measures the total activity of the preparation. It has been established, however, that fibrinolysin possesses two distinct properties which contribute to its enzymatic activity (2–4). Streptokinase reacts with a proactivator present in human plasminogen producing an activator. The resulting activator then converts the proteolytic precursor which is also present in the human plasminogen into the active enzyme.

In the fibrinolytic assay the total activity is measured; however, it is possible to determine the proteolytic and activator activity separately.

Several procedures (5, 7–9) have been described in the literature; however, they are lacking in statistical treatment and omit the use of an appropriate standard. In our laboratory a self-contained slope ratio assay for the caseinolytic and activator activity of fibrinolysin preparations has been developed. A self-contained assay includes a reference standard and provides statistical procedures for testing the validity of the assay and methods for estimating the relative potency and error of the assay.

Over a limited range of concentration, fibrinolysin preparations give a linear response and in the assay, concentrations are chosen which fall on this linear portion of the curve.

Measurement of the proteolytic activity is done by the direct caseinolytic assay in which the degree of hydrolysis of the casein substrate is determined. In the activator assay added plasminogen is first converted into the active proteolytic enzyme by the activator present in fibrinolysin. The amount of activator which is required for the enzymatic conversion of the

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[1] Thrombolysin is the registered trademark of Merck & Co., Inc., for fibrinolysin.

proteolytic precursor to the active enzyme does not produce measurable casein hydrolysis if plasminogen has been omitted from the reaction mixture. Thus, the hydrolysis of the casein substrate by the activated enzyme is, for all practical purposes, a measure of the activator activity of the fibrinolysin preparation.

MATERIALS AND METHODS

Reagents.—*Preparation of Casein.*—Fifty grams of casein (Hammarsten)[2] is suspended in 1 L. of distilled water and stirred vigorously for 1 hour. The mixture is centrifuged for 10 minutes at 1500 r.p.m. and the supernatant discarded. The casein is resuspended in 1L. of water and the process repeated. The casein is now suspended in 800 ml. of 95% ethanol (3A) and the mixture filtered by suction. The casein is washed twice on the filter with 95% ethanol followed by three washings with absolute ether. Finally, the casein powder is air dried.

Phosphate-Saline Buffer.—Potassium dihydrogen phosphate, 13.6 Gm.; sodium chloride, 9.0 Gm.; *q.s.* to 1L. with distilled water; adjust pH to 7.4 with 5 *N* sodium hydroxide.

Trichloroacetic Acid (TCA), 5% and 10%.

Sodium Carbonate, 20%.

Folin-Ciocalteu Phenol Reagent, Fisher Scientific Co., King of Prussia, Pa.

Preparation of Casein Solution for Assay.—Five grams of dried casein is suspended in 100 ml. of phosphate-saline buffer, pH 7.4, and heated for 15 minutes in a boiling water bath. After cooling, the mixture is centrifuged for 10 minutes at 1500 r.p.m., the supernatant is decanted, and its pH adjusted to 7.4 with 5 *N* sodium hydroxide. The casein solution may be kept in the refrigerator for one week prior to use.

Fibrinolysin Standard.—A number of vacuum sealed vials of fibrinolysin were stored in the refrigerator and designated "standard fibrinolysin preparation."[3] This standard is employed in the caseinolytic as well as in the activator assay.

In the caseinolytic assay a vial of fibrinolysin is quantitatively restored to 10 ml. with distilled water, and aliquots of 0.2 and 0.4 ml. are employed at the two levels of the assay.

For the activator assay the vial of fibrinolysin is restored to a final volume of 5,000 ml. As in the caseinolytic assay, 0.2 and 0.4 ml. of this solution are employed for the two concentrations of the standard.

Assay Procedure.—*Proteolytic Assay.*—The fibrinolysin standard (50,000 fibrinolytic units per vial) is dissolved in distilled water and brought to 10 ml. in a volumetric flask. The standard 0.2 and 0.4 ml. quantities, are pipetted into $^5/_8 \times$ 5-in. test tubes and the volume is adjusted to 1 ml. with phosphate-saline buffer. Tubes containing 0.2 ml. are labeled S_L and tubes 0.4 ml. are labeled S_H. In order to minimize loss of enzymatic activity on standing the tubes are immediately immersed into an ice bath.

The concentration of the test preparation is adjusted by an initial dilution in order to give responses similar to those for the standard. This requires a prior knowledge of the approximate activity of the test preparation. Aliquots of the test solution with the low concentration at one-half that of the high are added to assay tubes labelled T_L and T_H and brought to a volume of 1 ml. with phosphate-saline buffer.

Tubes can be set up in replicate. If duplicate determinations are judged sufficient, a total of three test preparations can be assayed simultaneously against the standard.

While in the ice bath the assay tubes are arranged in the order schematized in Figs. 1, 2, or 3, corresponding to quadruplicate, triplicate, and duplicate tubes, respectively. Figure 3 represents a restricted random allocation for three test preparations and a standard, so that the same duplicate tubes do not appear in the same row or column. An incubated blank containing 1 ml. of phosphate-saline buffer is included in every caseinolytic assay and may be placed at random in the test tube rack.

At zero time, 4.0 ml. of the 5% casein solution is added to the first tube starting at the lower left-hand corner, the solution is mixed, and the tube is placed into the water bath at 37° and incubated for 60 minutes. Casein is added to the remaining tubes at 60-second intervals, the tubes are mixed and placed in the water bath according to the designated scheme. Casein is added to the tubes going from left to right and ending with the tube in the upper right-hand corner. All subsequent manipulations follow the same order as above. The blank is added last. After an incubation of 60 minutes, 2 ml. of the incubated casein mixture is withdrawn from each tube and pipetted into 2 ml. of 10% trichloroacetic acid. The suspension is mixed well, allowed to stand at room temperature for at least 15 minutes, and filtered through S & S

A. Caseinolytic Assay (Blank—at Random)

	P_1	P_2	P_3	P_4
R_1	S_L	T_H	S_H	T_L
R_2	T_H	T_L	S_L	S_H
R_3	S_H	S_L	T_L	T_H
R_4	T_L	S_H	T_H	S_L

Fig. 1.—For quadruplicate determinations.

	P_1	P_2	P_3	P_4
R_1	S_L	T_H	S_H	T_L
R_2	T_H	T_L	S_L	S_H
R_3	S_H	S_L	T_L	T_H

Fig. 2.—For triplicate determinations.

	P_1	P_2	P_3	P_4
R_1	U_H	V_L	T_H	S_L
R_2	S_H	T_H	V_L	V_H
R_3	V_H	U_L	S_H	T_L
R_4	S_L	T_L	U_H	U_L

Fig. 3.—For duplicate determinations and three test preparations.

[2] Nutritional Biochemicals Corp., Cleveland 28, Ohio.
[3] Thrombolysin, 50,000 units per vial (1).

No. 576 filter paper by gravity.[4] The following mixture is prepared: 10 ml. distilled water, 3.0 ml. 20% sodium carbonate, and 1.5 ml. 5% TCA. A 0.5-ml. quantity of the filtered digestion mixture is added, followed by 1.0 ml. of the phenol reagent, and the contents of the tubes are mixed thoroughly.

Twenty minutes after the addition of the phenol reagent, the absorbance of the solution is read on a Beckman spectrophotometer at the wavelength of 650 mμ in a 5-cm. absorption cell with a light path of 50 mm. The readings are made against the casein blank.

Activator Assay.—This differs from the proteolytic assay. Human plasminogen is added to each tube and is allowed to react with the fibrinolysin standard or test preparation.

To each assay tube is added 3 mg. (0.3 ml.) of human plasminogen (fraction III-3) (9) and a predetermined volume of phosphate-saline buffer calculated to give a total volume of 1 ml. after taking into account the volume of the activator solution. The tubes are arranged according to the scheme in Fig. 4, which represents the same type of restricted random allocation as Fig. 3 except that only two test preparations are being assayed. This represents the maximum number of tubes that can be used practicably for this type assay.

B. Activator Assay (Blank—at Random)

	P_1	P_2	P_3	P_4
R_1	S_L	T_H	S_H	T_L
R_2	U_L	U_H	S_L	T_H
R_3	T_L	S_H	U_L	U_H

Fig. 4.—For duplicate determinations and two test preparations.

If determinations are desired in triplicate, the scheme in Fig. 2 may be utilized.

At 90-second intervals precooled 0.2-ml. and 0.4-ml. aliquots of the standard and appropriate aliquots of the test solution with the low concentration at one-half that of the high are added to the appropriate assay tubes, followed immediately by 0.3 ml. of the casein solution, going from left to right, starting in the lower left-hand corner and ending with the tube in the upper right corner. All subsequent manipulations follow the same order as above. Each tube is immediately incubated for 18 minutes at 37°. At that time 3.7 ml. of casein solution is added, and after an additional incubation of 2 minutes, a 2-ml. aliquot is pipetted into 2 ml. of 10% trichloroacetic acid. A second aliquot is removed after an additional 60-minute incubation.

The suspension is mixed well, allowed to stand at room temperature for at least 15 minutes, and filtered. The filtrate is treated as described under proteolytic assay.

Statistical Evaluation of a 4-Point Slope Ratio Assay.—Preliminary trials showed that absorbance was linearly related to concentration of fibrinolysin (i.e., followed the Beer-Lambert law) at least up to an absorbance of 0.8. Variation among replicate tubes was small and constant up to an absorbance of about 0.4; above this the variation became much larger and was less constant. We decided, therefore, to limit the concentration of test materials to such that the absorbance would not exceed 0.5.

A uniformity trial was run in which a standard preparation was assayed against itself. The concentrations tested were S_L = 0.2 ml., S_H = 0.4 ml., T_L = 0.15 ml., and T_H = 0.3 ml. Four tubes of each concentration were tested. The order of placement of the tubes in the water bath was, according to Fig. 1, completely randomized in order to minimize possible positional bias. A protocol illustrating the calculations is shown in Table I. This represents a simplified approximate procedure and the formulas are valid only for the standardized conditions of this assay; i.e., two concentrations of each preparation with the low concentration at one-half that of the high, and four tubes at each concentration. Similar computations are used for triplicate and duplicate tubes, except that the formulas for F and P* are as shown in Table II.

Before proceeding with the estimation of relative potency, it is necessary to make a test for validity of the assay. This is done by comparing the absolute value of $|P|$ with 7W/3. If $|P| \geq 7W/3$, this would indicate that the two response lines do not have a common intercept, and the assay would be fundamentally invalid at $P \leq 0.01$. If $|P| \geq 5W/3$, this would show fundamental invalidity at $|P| \leq 0.05$; however, for most assays, a lack of validity at this level may, for practical reasons, either be ignored or the assay repeated.

A significant lack of common intercept would indicate either that the response(s) for one of the preparations is outside the linear range of response, or else that other factors such as inhibitors or potentiators are present which may modify the response of that preparation. If the apparent lack of common intercept is due to the former, the situation may be corrected by a better selection of concentrations.

The 95% confidence limits estimated from the intrinsic error of the assay may not necessarily reflect the "true" error of the assay. Weighing, dilution, and other technical errors may be larger than the intrinsic error. A more realistic estimate would require the combination of estimates from several replicate assays.

DISCUSSION

Kunitz (10) developed a method for the determination of trypsin based on its ability to digest a casein substrate. Remmert and Cohen (5) adopted this procedure and developed an assay for streptokinase activated human plasminogen. Succeeding authors introduced minor modifications.

The present assay procedure is patterned after that of Remmert and Cohen. It was found that results of identical samples submitted for assay to several independent laboratories varied considerably and it was thought advisable to standardize the assay procedures.

Arbitrarily, a standard fibrinolysin preparation was assigned a unit value and this preparation serves as a house standard in our laboratory. This fibrinolysin is prepared by streptokinase activation of human plasminogen. Earlier authors have ex-

[4] The suspension or filtrate may stand overnight in the refrigerator.

TABLE I.—PROTOCOL FOR 4-POINT SLOPE RATIO ASSAY

Preparation	Standard		Test	
Concentration	0.2 ml.	0.4 ml. = X_S	0.15 ml.	0.3 ml. = X_T
Absorbances	0.155	0.363	0.105	0.269
	0.149	0.378	0.103	0.260
	0.140	0.356	0.096	0.277
	0.146	0.366	0.109	0.273
Sum	$S_L = 0.590$	$S_H = 1.463$	$T_L = 0.413$	$T_H = 1.079$
Range	0.015	0.022	0.013	0.017

$X = X_S/X_T = 0.4/0.3$

W = sum of ranges $= 0.015 + 0.022 + 0.013 + 0.017 = 0.067$

$N = 3S_H - 6S_L + 7T_H - 4T_L = (3)(1.463) - (6)(0.590) + (7)(1.079) - (4)(0.413) = 6.750$

$D = 7S_H - 4S_L + 3T_H - 6T_L = (7)(1.463) - (4)(0.590) + (3)(1.079) - (6)(0.413) = 8.640$

$K = N/D = 6.750/8.640 = 0.78125 \quad K^2 = (0.78125)^2 = 0.61035$

$P = 2S_L - S_H - 2T_L + T_H = 2(0.590) - 1.463 - (2)(0.413) + 1.079 = -0.030$

$$F = \frac{5.5W}{D}\sqrt{1 - 1.64K + K^2} = \frac{5.5(0.067)}{8.640}\sqrt{1 - 1.64(0.78125) + 0.61035} = 0.02431$$

$P^*_{0.05} = 5W/3 = 5(0.067)/3 = 0.112 \quad P^*_{0.01} = 7W/3 = 7(0.067)/3 = 0.156$

test for validity of assay

since $|P| = 0.030$ is less than $7W/3 = 0.156$, assay is valid

estimation of relative potency

$R = XK = (0.4/0.3)(0.78125) = 1.04$

estimation of 95% confidence limits

$R_L, R_U = X(K \pm F)$

$= (0.4/0.3)(0.78125 \pm 0.02431)$

$= (0.4/0.3)(0.75694, 0.80556)$

$= 1.01, 1.07$

TABLE II.—FORMULAS FOR F AND P* FOR REPLICATE TUBES

Replicates	F	P* 0.05	P* 0.01
2	$\frac{8.75W}{D}\sqrt{1 - 1.64K + K^2}$	13W/5	22W/5
3	$\frac{6W}{D}\sqrt{1 - 1.64K + K^2}$	9W/5	8W/3
4	$\frac{5.5W}{D}\sqrt{1 - 1.64K + K^2}$	5W/3	7W/3

pressed their results in caseinolytic units. This unit was defined as the increase in absorbance at 280 mμ after 60 minutes of incubation at 37° and conversion of the absorbance to micrograms of acid-soluble tyrosine by reference to a standard curve. This procedure possesses definite disadvantages. Changes in experimental condition such as temperature of incubation, presence of inhibitors, and variations in casein substrate do not necessarily become apparent due to the lack of comparison to a standard response.

The new procedure evaluates fibrinolytic preparations by a self-contained statistical assay. The validity of the assay is evaluated and confidence limits are determined.

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