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Reçu le 9 novembre 1956

UROKINASE
AN ACTIVATOR OF PLASMINOGEN FROM HUMAN URINE

I. ISOLATION AND PROPERTIES

JØRGEN PLOUG AND NIELS OLE KJELDGAARD

Leo Pharmaceutical Products, Hormone Department, Copenhagen/Skovlunde (Denmark)

INTRODUCTION

The recognition of the physiological significance of the fibrinolytic system during thrombotic conditions has promoted a growing interest in the problems concerning the formation in the blood of the fibrinolytic enzyme, plasmin, from its precursor, plasminogen. Several activators of this process have been isolated from bacteria, body fluids and tissues. The presence of an activator in human urine acting upon both human and animal plasminogen was described by WILLIAMS[1], ASTRUP AND STERNDORFF[2] and GUEST *et al.*[3]. The activator was named "urokinase" by GUEST *et al.*

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Attempts to isolate urokinase by the usual protein precipitation methods[2] or by foaming[4] have been rather unsuccessful and only adsorption on $BaSO_4$[5] seems to give good purification.

The therapeutic importance of obtaining a plasminogen activator from a human source, made us attempt to isolate urokinase in pure form[6]. By adsorption on silica gel, followed by chromatography on Amberlite IRC-50, urokinase has been obtained as a very stable, colorless protein. The preparations are still inhomogeneous as judged by electrophoresis.

MATERIALS AND METHODS

Phosphate buffer 0.1 *M*, pH 7.2.

Bovine fibrinogen was isolated from oxalated plasma by ammonium sulphate fractionation according to ASTRUP AND MÜLLERTZ[7] and dissolved in phosphate buffer to give a fibrinogen concentration of about 0.8%. These preparations contained plasminogen in varying amounts.

Thrombin: Prepared by this factory from bovine plasma, about 30 NIH-units/mg, dissolved in buffer to 100 NIH-units/ml.

Silica gel: To commercial waterglass (8.1% Na_2O, 25.4% SiO_2) 35° Beaume, was added a little more than the equivalent amount of 25% sulfuric acid. The mixture was washed with 5 vol. water, allowed to settle, and the supernatant decanted. The washing was repeated four times. Excess of water was sucked off on a Büchner funnel, the silicic acid pre-dried, granulated, and completely dried at 140°C.

Amberlite IRC 50 (*XE-97*) was obtained from Rohm and Haas, Philadelphia, Penn., and screened with a 200 mesh sieve to remove the finest particles, then treated as described by HIRS, MOORE AND STEIN[8] and equilibrated at pH 6.2 in 0.1 *M* phosphate buffer (5.4 g $NaHPO_4$. 2 H_2O, 8.5 g NaH_2PO_4 and 5.8 g NaCl/liter).

Urokinase assay and unit

Two methods have been used to determine urokinase activity, both based on measurements of the fibrinolytic activity brought about by the interaction of plasminogen and urokinase.

Test tube method. In this method the glass ball principle of FLETCHER'S plasmin assay[9] has been used. In a 9 × 100 mm test tube, 0.5 ml phosphate buffer, 0.1 ml thrombin, 0.01–0.1 ml urokinase solution and 1.0 ml fibrinogen were mixed. The solutions were ice cold at the time of addition. The tube was then placed in a 37°C waterbath and a stopwatch was started. After 1 min a firm coagulum was formed and a glass ball (7 mm in diameter and about 500 mg in weight) was placed on the top. When fibrin dissolution was near completion, the ball fell through the clot. The time required for the ball to reach the bottom of the test tube was taken as the time of lysis. Since the plasminogen content of the fibrinogen preparations changed from one lot to another, urokinase units could not be defined on the basis of lysis time alone. A crude preparation of urokinase was therefore chosen as standard and its activity was defined in arbitrary units. Tests with serial dilutions of a standard solution were run daily. When lysis time was plotted against number of urokinase units on log-log paper a straight line could be drawn within a lysis time range of 3 to 30 min, roughly corresponding to 100–1 units. When the plasminogen content of the fibrinogen was too low, slight deviations from linearity were seen. The reproducibility of the method was approximately *ca.* 10%. Urine could be determined only semi-quantitatively by this method.

Fibrin plate method. Low activity solutions were determined by a modification of the plate method of ASTRUP AND MÜLLERTZ[7]. Stainless steel frames 10 × 40 cm and 2 cm high were fixed with grease on plane glass plates placed on a levelled bench, and the plates covered with mixtures of 50 ml fibrinogen, 50 ml phosphate buffer and 5 ml thrombin. Uniform thick fibrin layers (about 2 mm) were obtained. Aliquots (10 μl) of serial dilutions of sample and standard solution of urokinase were placed alternately on the fibrin layer in two rows in order to minimize the influence of small thickness variations in the fibrin plate (Fig. 1). The plates were incubated at 37°C overnight. The product of two diameters, at right angle to each other, of the lysed circular areas were plotted against dilution, and the activity of the unknown samples read on the curves. The lytic activity of normal pooled male urine determined by this method was found to correspond to 5–8 units of urokinase per ml. The sensitivity of this method is very great giving clear lysis zone with as little as 1/100 unit of urokinase. However, owing to the long incubation time and the very complex reaction sequence with the activator placed on top of the solid media, the method has both practical and theoretical shortcomings.

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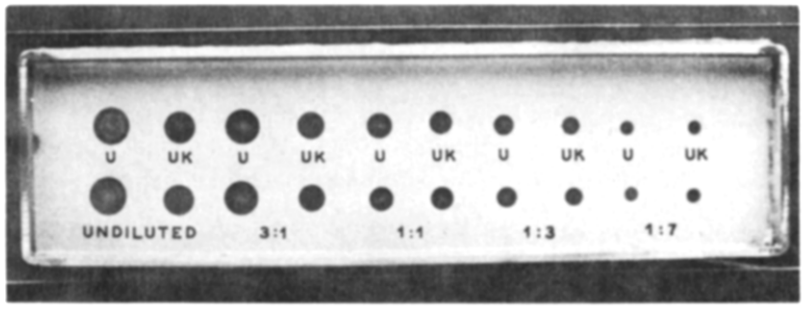

Fig. 1. Fibrin plate. Urine and urokinase standard (6 units/ml) applied alternately in 10 μl aliquots on a fibrin plate, in the dilutions indicated.

RESULTS

Isolation of urokinase

Adsorption on silica gel. To 300 l of normal male urine was added about 1.3 l of 28% sodium hydroxide to bring the pH to 7.5. A heavy precipitate was formed and removed by filtration. The filtered urine was passed through a 3" × 25" column of silica gel at a flow rate of about 500 ml/min. The column was previously treated with 5 l 5% hydrochloric acid, 5 l water and 5 l 10% sodium chloride, in that order. Most of the fibrinolytic activity was adsorbed on the silica gel, with only about 10% of the activity passing through. The column was washed with about 30 l of water and the activity eluted with 4% ammonia at a flow rate of about 150 ml/min. The first slightly yellow fractions contain no activity and were discarded. Subsequently about 2 l of brownish eluate with *ca.* 70% of the original activity was collected.

Sodium chloride precipitation

Sodium chloride (20% by weight) was added to the eluate and the pH adjusted with 5 *N* hydrochloric acid to about 1.5, whereby a heavy precipitate containing all the activity was obtained. The collected precipitate was dissolved in water by addition of alkali to about pH 8. Any undissolved material (mostly silicic acid) was removed by centrifugation and the clear red-brown supernatant dialyzed overnight against distilled water. The dialyzed solution was lyophilized, giving 2–3 g crude urokinase with an activity of 400–700 units/mg.

Chromatography on IRC 50

Enough resin to form a 41 × 2.0 cm column was suspended in phosphate buffer pH 6.2, filled into the chromatographic tube and allowed to settle by gravity. Excess liquid was drained off and about 4 g crude urokinase dissolved in 40 ml phosphate buffer pH 6.2 was applied to the column. When the solute had drained into the column, 100 ml phosphate buffer pH 6.2 was applied as washing agent, followed by 0.5 *M* sodium chloride as eluting agent. Most of the proteins, including the colored material, passed through the column. The activity was adsorbed on the resin and reappeared in the front of the eluting agent with very little accompanying color. The active fractions were dialyzed and lyophilized, giving about 150 mg urokinase with an activity of about 10,000 units/mg. Chromatography was performed at 2° C.

Fig. 2 shows a chromatogram with measurements of u.v.-absorption at 280 mμ

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and urokinase activity of the effluent fractions. By repeating the chromatography on IRC 50, preparations with an activity of about 18,000 units/mg were obtained. Since rather limited amounts of these highly purified preparations were at our disposal, further purification has been without success.

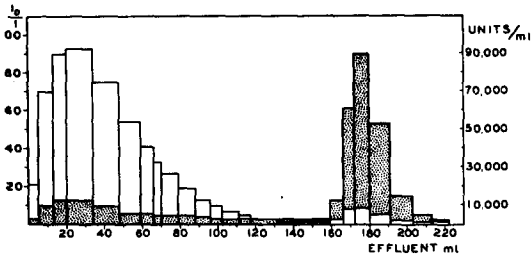

Fig. 2. Chromatography of urokinase on IRC 50 (XE-97). 4.02 g crude urokinase (740 units/mg) was applied to the chromatographic column as described in the text. Fractions from 166–213 ml effluent were dialyzed and lyophilized, giving 140 mg urokinase with an activity of 12,500 units/mg. The stippled columns refer to urokinase activity, the blank columns to density at 280 mμ calculated from absorption measured in 1.0 cm cells in appropriate dilutions.

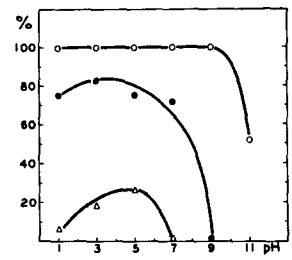

Fig. 3. Effect of temperature and pH on urokinase stability. 0.1 ml samples of a solution of urokinase in water (9,000 units/ml) were diluted with 0.3 ml of buffer solutions at pHs varying from 1 to 11. The mixtures were heated in a waterbath to 50°, 70° and 100°C and the activity determined at zero time and after 30 min. Abscissa: pH of sample during heating. Ordinate: activity at 30 min in % of original activity. O—O 50°C. ●—● 70°C. △—△ 100°C.

Stability

Lyophilized urokinase, recovered from all stages of purification, is perfectly stable for months, and probably years, when kept at 2° C. Solutions of urokinase are very stable with respect to heating and pH. Fig. 3 shows the activity remaining after 30 min heating of urokinase dissolved in buffers at pH varying from 1 to 11. At 50° C urokinase is stable below pH 10. Increase in temperature to 70° C shows a marked stability change in the region about pH 9. At this temperature heating for 20 min is sufficient for complete destruction of urokinase at pH 9. Heating in boiling waterbath showed clearly the acid stability of urokinase with maximum stability about pH 5. These results contradict those of ASTRUP AND STERNDORFF[2] who found that the fibrinolytic activator in urine was easily destroyed in acid even at 30° C. This dis-

TABLE I

Urokinase (5300 units/mg) was dissolved in water to give an activity of 12,000 units/ml and 0.1 ml samples were mixed with 0.3 ml of the following solutions: 1. urine at pH 7.2; 2. 0.1 *M* phosphate buffer pH 7.2; 3. urine acidified to pH 3.0 by addition of *N* HCl; 4. citric acid-disodium phosphate buffer at pH 3.0; 5. urine heated for 20 min at pH 7.2 in boiling water-bath, then adjusted to pH 3.0 with *N* HCl. The mixtures were incubated at 37°C. 10 μl aliquots were withdrawn for activity determinations at zero time and after 30 min.

Time *min*	*Activity (units/ml)*				
	pH 7.2		*pH 3.0*		
	urine	*buffer*	*urine*	*buffer*	*boiled urine*
0	2800	2800	2800	2800	2800
30	2800	2800	<100	2800	2600

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crepancy may be explained by the presence of uropepsin in urine which destroys urokinase in acid. Incubation experiments with mixtures of urine and urokinase were performed. As shown in Table I it was found that urokinase incubated at 37° C for 30 min with urine at pH 3 was completely destroyed. No loss in activity was observed after incubation of the mixture at pH 7.2 or with urine adjusted to pH 3 after destruction of uropepsin by boiling at pH 7.2 This clearly shows the destructive action of acid urine, and therefore it seems unnecessary to assume the existence of two types of urine activators which differ with respect to acid stability.

Elementary composition

Elementary analysis of urokinase (with an activity of 18,000 units/mg) gave the following composition:

N: 14.11%, C: 46.55%, H: 7.35%, S: 1.50%, P: 0.30%, and ash: 0.61%.

*Electrophoresis**

Electrophoresis at pH 4.63 showed that urokinase (with an activity of 18,000 units/mg) consists of three components A, B and C in the relative concentrations 22, 37, and 41% (Fig. 4).

Fig. 4. Electrophoresis diagram of 0.8% urokinase (18,000 units/mg) in buffer pH 4.63, (NaAc + HAc + NaCl $\Gamma/2$ = 0.1 + 0.1) after 300 min at 4.02 V/cm followed by 613 min at 1.94 V/cm. Mobility in 10^{-5} cm^2 volt^{-1} sec^{-1}: A 3.18, B 2.35, C *ca.* 1.0.

Analysis of samples taken from different compartments of the electrophoresis cell show that the urokinase activity was located in component B.

SUMMARY

1. Urokinase was isolated from urine by adsorption on silica gel and elution with ammonia.
2. Crude urokinase preparations were further purified by chromatography on IRC 50 at pH 6.2.
3. Urokinase is stable at 50°C in the pH range 1–10. No decrease in activity was observed in 30 minutes. At higher temperatures urokinase is most stable in the acid range. Urokinase is rapidly destroyed by acid urine.

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Received November 20th, 1956

* Electrophoretic analysis was kindly, performed by Dr. NIELS HARBOE, Electrophoresis Laboratory, Institute of General Pathology University of Copenhagen.