

Studies on Plasminogen Activators

II. A Method for the Separation of Purified Urokinase from Small Samples of Urine

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The estimation of urokinase activity has been studied employing relatively small (10 ml) samples of fresh human and rabbit urine. Gel filtration on Sephadex G-200 at pH 8 with 1 M NaCl was found to be applicable for the determination of urokinase in the eluates by fibrinolytic activation of bovine plasminogen, without the disturbance of proteolytic inhibitors. The isolated enzyme was shown to have esterase activity on lysine methyl ester. Identity with a highly purified urokinase was obtained by comparative gel filtration and sedimentation rate using sucrose density gradient ultracentrifugation in a stabilizing medium with buffer and 1 M NaCl. An elution profile after gel filtration was standardized for urokinase activity with small samples of fresh urine including the absorbancy at 280 and 260 $m\mu$ of materials removed by the procedure. An addition of 15 units of purified urokinase to 10 ml of fresh female urine was recovered in the active fractions. All preparative operations were performed in a cold room or at 0° to +5°C. It is also suggested that the gel filtration procedure may be useful for a preparative isolation of crude urokinase with concentrated samples of fresh urine. The specific activity of 210 units per mg of protein was obtained after recycling of an arbitrarily collected ultrafiltrated 250 ml sample of female urine.

Recent successful purifications and the crystallization of urokinase¹ isolated from human urine by Lesuk *et al.*² emphasize the importance of the physiological aspects concerning this enzyme.³ Urokinase excretion has generally been estimated from 24-h urine pools using urine directly or diluted samples for activity measurements by activation of plasminogen.⁴⁻⁶ The presence in urine of proteolytic inhibitors has been studied by Astrup and Sterndorff⁷ who found that a partially purified urokinase preparation had slight inhibitory effect on chymotrypsin and plasmin, in amounts that would completely inhibit trypsin. While proteolytic inhibitors in urine may be accidental excretory products,⁷ alterations from normal may occur,⁸ at the same time as differences in urokinase excretion have been found.⁴ While urokinase

excretion rates are considered independent of age and urine volume,⁴⁻⁶ variations occurred in certain states of disease⁴ and between the sexes.⁶ Caution has been paid to the possible interference of antagonistic activities in urine^{9,10} which may inhibit urokinase activity at neutral pH. In the present work a method is presented by which urokinase output may be studied as a homogeneous material purified by gel filtration starting with small amounts of fresh urine. Experimental conditions are described by which the antiplasmin and antitrypsin effects do not interfere with the determination of urokinase activity.

MATERIAL AND METHODS

Fresh human urine from healthy females was used in experiments non-relating to the daily output volume. In most cases the more concentrated morning urine was collected. Samples were stored in ice until used in gel filtration experiments the same day (deep frozen urine was not used). The pH 5.5–6.0 of the urine was kept unchanged, any precipitate present was removed by filtration through cotton before the sample was used for gel filtration. *Concentrated urine* was prepared by ultrafiltration at 5°C for 24 h by the procedures described before. The ultrafiltrated urine had an ionic strength of averagely $\mu = 0.31$ with 6–7 times concentrated urine, measured with 1:200 diluted samples and interpolated from a standard curve with 0.003 M NaCl.¹¹

A *crude urokinase* was prepared according to Astrup and Sterndorff.⁷ The preparation had an activity of 1.69 Ploug units¹² per mg and corresponded to 1130 ml of urine per g.

Purified urokinase (2220 units per ampoule, Leo Pharmaceutical Products, Denmark) was utilized as a standard. A linear dose response curve was obtained with 0.01–1.33 Ploug units per 0.03 ml dissolved in 0.05 M Tris-HCl buffer pH 7.8 and $\mu = 0.15$ with NaCl, by the bovine fibrin plate analysis (unheated).^{11,12} The activity range 65–600 mm² was used in measurements. All urokinase activity in this work is expressed in Ploug units relative to this purified urokinase as obtained by the plate method.

Rabbits (female) urine was collected during narcosis with urethane, 1 g per kg body weight (2.76 kg). It was kept at 0°C until submitted to gel filtration the same day. Diuresis was effected by intraperitoneal injection of 20 ml of physiological NaCl solution (0.9 %).

Urokinase solutions in buffer 8.5–11 ml, or *fresh urine*, 10 ml samples, were submitted to gel filtration with Sephadex G-200 (Pharmacia, Uppsala, Sweden), 40–120 μ ; dimensions of columns were 3.8 \times 38–40 cm. Runs were made with 0.05 M Tris-HCl pH 8 containing 1 M NaCl. The elution rate was 7–8 ml per half hour at 5°C. Absorbancy of eluates was read at 280 m μ and 260 m μ . Recycling was performed with concentrated fractions of urokinase. The elution of urokinase activity was measured on unheated bovine fibrin plates. The incubation time used was 16 h with concentrated eluates 0.03 ml per spot, and 20 h and 0.06 ml with eluates from fresh urine. Measurements of urokinase activity and absorbancy were performed separately from each fraction. Symbols for these single determinations are omitted from figures presenting the *urokinase elution profile* (Fig. 2–4, 6).

The *trypsin* and *plasmin inhibiting capacity* in eluates from concentrated gel filtrated urine was measured according to the methods described in a separate publication.¹¹ Trypsin inhibition was also determined on heated fibrin plates,¹¹ using 0.2 μ g of trypsin (Worthington, 2 \times crystallized) per spot (0.03 ml), dissolved in 0.05 M Tris-HCl buffer pH 8 with 1 M NaCl.

Urokinase esterase activity was measured according to the method for serum trypsin activity described by Siegelman *et al.*¹⁴ as applied for urokinase by Sherry *et al.*¹⁵ Lysine methyl ester dihydrochloride (LYME) (Mann Research Laboratories) 0.015 M in 0.1 M phosphate buffer pH 6.5, 3 ml was mixed with 0.5 ml enzyme solution and incubated for 30, 60, and 90 min at 37°C. The spontaneous hydrolysis of the ester was measured in parallel incubations and used for correction of enzyme activity together with the proper control of enzyme solution.

Aliquots of 1 ml of the incubation mixtures were withdrawn to stop the reaction and added to 0.5 ml 15 % trichloroacetic acid. A 1 ml sample was mixed with 0.1 ml 2 % potassium permanganate (Merck, *p.a.*) for the colour reaction. After 1 min 0.1 ml 10 % sodium sulfite (Merck, *p.a.*) was added together with 4 ml chromotropic acid working reagent. The mixture was refluxed for 15 min at about 100°C. The final volume was 5.0 ml. Optical density was measured at 580 $m\mu$ against a water blank. Methanol (Merck, *p.a.*) refluxed with calcium oxide and redistilled at 65°C was used for a standard curve, diluted to different concentrations in the phosphate buffer pH 6.5 containing 0.9 % NaCl. Activity is expressed as the equivalent μM of LYME hydrolyzed.

Protein determinations were made according to the Folin-Cu method.¹⁶

Density gradient ultracentrifugation was performed according to the method described by Kunkel.¹⁷ Details concerning the technique have been described before.¹¹

RESULTS AND DISCUSSION

The gel filtration behaviour of fresh human urine compared with crude and purified urokinase is presented in Fig. 1. From 8.5 times concentrated urine 1069 units were recovered as calculated by fibrin plate assay from single eluates. This corresponds to 13.7 units per ml of the original urine (not tested for activity). Correction was not made for eventual losses occurring during the ultrafiltration procedure. The recovery after gel filtration was 95 % with purified urokinase (525 units). The crude urokinase corresponded approximately to 700 ml of urine. After one run through the column, concentration was made by ultrafiltration to 50 ml which was recycled. As shown in Fig. 1 the activity peak from all three preparations was eluted after approximately 250 ml and the total elution volume was about 130 ml.

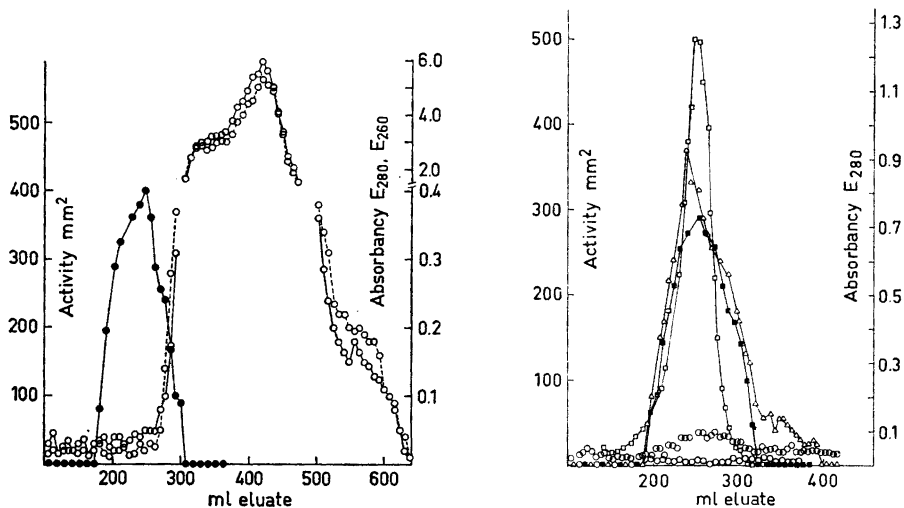


Fig. 1. Gel filtration on Sephadex G-200 with fresh human urine and different preparations of urokinase. Absorbancy at 280 $m\mu$ (O—O) and 260 $m\mu$ (O---O) was measured directly from the eluates. a) 9 ml concentrated urine corresponding to 78 ml fresh urine (●). b) Purified urokinase (■), 555 Ploug units from a preparation with 2220 units per ampoule, recycling of 600 mg crude (Astrup) urokinase (▲), and human albumin (Kabi, protein standard) 110 mg of protein (□). Bed volume 430 ml in all runs.

Human serum albumin (mol. weight 69 000) was used for approximate comparison of the respective molecular sizes. As shown in Fig. 1 the albumin peak (280 m μ) coincided with the activity peaks obtained with urokinase, and the total elution volumes were the same. This would indicate similar molecular size with human serum albumin (>50 000). In recent works various molecular weights have been reported for urokinase.^{2,18,19} As found by Lesuk *et al.*,^{2,18} crystalline urokinase has a molecular weight of 54 000 with Sephadex G-100 and 53 000 ultracentrifugally estimated. In agreement with these authors we obtained single symmetrical peaks with urokinase preparations of different degrees of purity. Lesuk *et al.*¹⁸ testing different preparations obtained the similar molecular weight (54 000) in spite of protein heterogeneity. Differences between crystalline urine and serum albumin have been reported²⁰ with the sedimentation constants 2.6 and 4.2, respectively. The urinary albumins were found to be more negatively charged at pH 8.6 than human serum albumin. By similar charges urokinase may have associated with other substances excreted in the urine and appear after gel filtration at pH 8 as a larger sized molecule, particularly when fresh urine was filtered directly through the gel.

When urokinase obtained by gel filtration of a concentrated urine sample was recycled on the Sephadex gel after ultrafiltration (Fig. 2), 841 units were recovered. A total of 4 mg protein was found in the active eluates yielding the approximate specific activity of 210 units per mg. This corresponded to an estimated 50 % of the total protein put through the gel. The loss can be explained by assuming that ultrafiltrable peptide material and not only protein contaminated the preparation, although the peak of UV absorptive material was excluded after the first gel filtration procedure. As indicated by the absorption curves obtained in Fig. 1 a, the impurities consisted mostly of smaller molecular weight (> 50 000) materials.

The esterase activity of purified urokinase was measured using samples of a gel filtrated concentrated urine with 120 units per ml. An activity corresponding to 0.17 μ M of methanol released per ml of the reaction mixture per h was obtained (see methods). The higher sensitivity towards urokinase made the plate method more suitable at present for tracing small amounts of urokinase activity.

Bomgaard *et al.*⁶ report activities for the 24 h output of 5770 ± 1420 units (female) of urokinase. Since variations of urokinase output occur in samples of urine taken at different times during the day, the urokinase excretion is considered relatively constant throughout the 24 h period.⁶ On the other hand Von Kaulla and Riggenbach²¹ found that the first urine specimen in the morning has lower urokinase content. Since in some of our experiments (Fig. 1 a, 3) morning urine was used, a strict comparison cannot be made on the basis of present results. Our activity related to volume was a maximum of 13.7 units of urokinase per ml in gel filtrated fresh urine (Fig. 1 a).

Concentrations by ultrafiltration of fresh urine were made at pH 5.5–6 prior to the gel filtration experiments. When eluated fractions were ultrafiltrated at pH 8 heavy losses of activity (40–60 %) occurred with fresh urine and purified urokinase. A change of pH to 6 during the ultrafiltration procedure increased the yield to 83 %. This is in agreement with the findings by Plough and Kjeldgaard¹³ who reported the highest stability at pH 5.

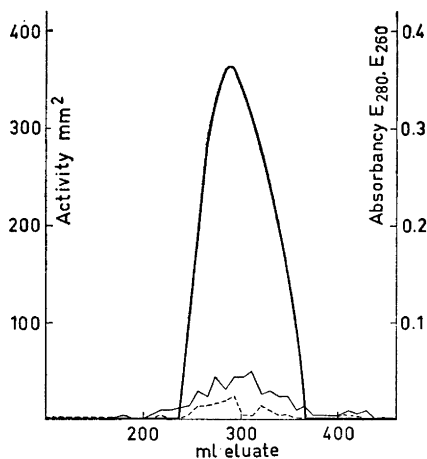


Fig. 2. Concentrated urine recycled on Sephadex G-200. Sample corresponds to 250 ml of original fresh urine. The specific activity was 210 units per mg of protein. Elution profile of urokinase activity (—). Absorbance at 280 μ (---) and 260 μ (· · ·).

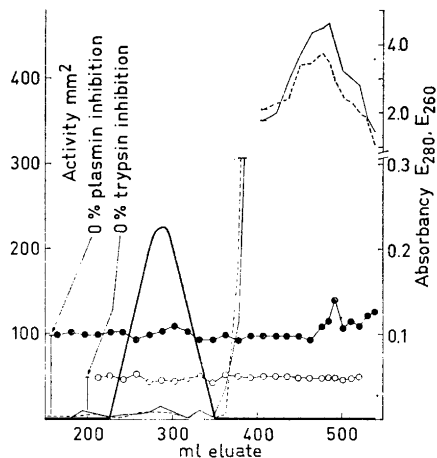


Fig. 3. Estimation of antitrypsin (O) and antiplasmin (●) in fractions obtained by gel filtration with 77 ml of concentrated fresh urine recovering 220 units of urokinase (2.9 units per ml urine). Inhibition of trypsin was measured on BAPA, 0.002 M, using 250 μ l of eluates; antipiasmin was determined on heated fibrin plates using 0.112 units of purified plasmin¹¹ per 0.03 ml in 0.05 M Tris-HCl pH 8 with 1 M NaCl, running a control with plasmin alone. The elution profile of urokinase and absorbances are similar to those obtained in Fig. 2.

Crude urokinase, however, could be ultrafiltrated at pH 8 without losing more than about 13 % of activity. According to Astrup and Sterndorff⁷ crude urokinase prepared by ethanol precipitation is most stable at alkaline and neutral pH, sustained in our experiments. Due to these circumstances urokinase solutions as well as the fresh urine used in experiments were kept in ice and all procedures were performed in a cold room. A lower pH (<6) was not found advisable to use due to the uropepsin activity in fresh urine.^{7,10}

Fig. 4 presents the gel filtration with 10 ml fresh urine alone and with added purified urokinase. The high recovery obtained indicated that urokinase is not destroyed nor inhibited in the gel filtrated fractions.

The eventual presence in the gel filtrated fractions of a proteolytic inhibitor was tested as shown in Fig. 4, from the single eluates of a gel filtrated sample of concentrated fresh urine. When measured from the single eluates no inhibitory effect could be detected. When the fractions were concentrated by ultrafiltration corresponding to the sieve ranges I, 100—226 ml (20-fold); II, 347—540 ml (19-fold); and III, the active fractions (52-fold), no trypsin inhibitory effect was detected with 250 μ l. When the same fractions were tested (0.03 ml)

for antiplasmin effect with 0.112 units of plasmin on heated plates an activation occurred with a maximum with fraction III (139 % activity), presumably due to an earlier observed plasminogen content in the plasmin preparation used.¹¹ While trypsin inhibition occurred (to about 65 %) with male urine in a similar experiment, plasmin inhibition was not obtained in either case in the active region. The estimation of urokinase output as shown in Fig. 3 will therefore not be influenced by proteolytic inhibitors in normal urine, unless alterations caused by other circumstances would cause an output of plasmin inhibitor in urine.

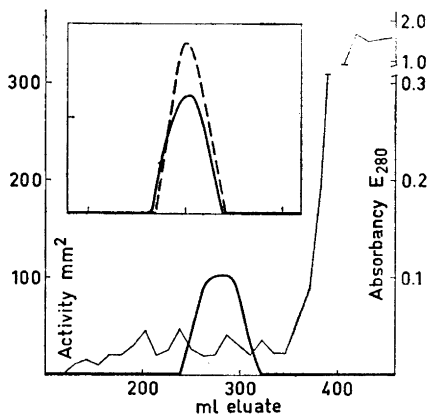


Fig. 4. Elution profiles of urokinase obtained with 10 ml fresh urine (—) corresponding to 15 and 17 (inset) units of urokinase. The recovery of 15 units of purified urokinase was 113 % (34 units) after the addition to 10 ml of fresh urine (---). Absorbance at 280 $m\mu$ (—).

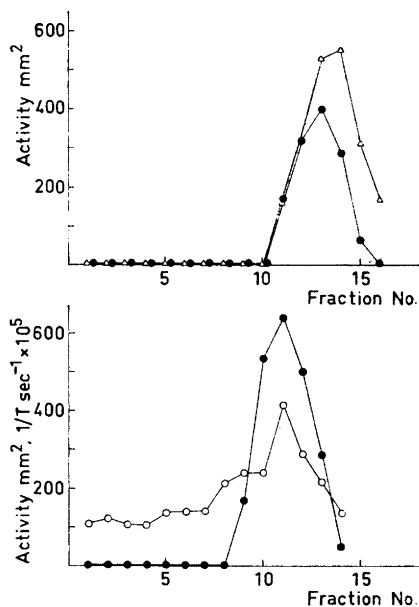


Fig. 5. Identification of urokinase from fresh human urine by comparative ultracentrifugation against a linear gradient of 5 to 20 % sucrose in 0.05 M Tris-HCl buffer pH 8 with 1 M NaCl (volume 4.5 ml). Runs were made for 21.5 h (lower) and 18.5 h (upper) at 38 000 rpm (Beckman Spinco Model L ultracentrifuge SW 39). Concentrated gel filtrated fresh urine 0.35 ml (●) was spun simultaneously with a highly purified urokinase (25 000 units per ampoule) 5000 units/0.2 ml (○) (lower), and 0.35 ml of recycled crude urokinase (cf. Fig. 1 b) (△). Urokinase activity was determined on heated fibrin plates with 0.06 ml samples from fractions collected icecold by the drop method (20 drops per fraction). The activity of purified urokinase (○) was measured by the clot lysis method.

Further identification of the plasminogen activator activity obtained with fresh urine samples by gel filtration was performed by testing samples on heated fibrin. No proteolytic activity was obtained with freshly filtrated urine from the urokinase fractions.

Ultracentrifugation experiments (Fig. 5) gave closely similar distribution between the three different preparations of urokinase (*cf.* Fig. 1 a, b). This indicates that the plasminogen activator obtained from freshly filtered urine is identical also by molecular weight with the purified urokinase. In our experiments a similar distribution was obtained by ultracentrifugation with urokinase from freshly filtered urine and human serum albumin (mol. weight 69 000). This discrepancy as compared with the lower molecular weights obtained by Lesuk *et al.*¹⁸ cannot be explained on the basis of the present experiments. Results reported by Merler *et al.*²⁰ describe urinary albumin which differs from serum albumin having a lower sedimentation constant, S_{w20} 2.6. This was explained by the possibility of denaturation of the urinary albumin following the renal filtration or a splitting of the peptide bonds *in vivo* with formation of smaller fragments held together with disulphide bonds. If denaturation takes part in the molecule of urokinase through the many manipulations used for preparations of the highly active and crystallized preparations, the comparatively untreated and more truly physiological urokinase obtained by direct gel filtration and subsequent ultracentrifugation may still be undenatured. Some differences in the methods used at ultracentrifugation^{2,12} may explain the results. In any case our estimation of molecular weight for presumably undenatured physiological urokinase must be considered as preliminary.

The filtration method was finally tested with a 10 ml sample of freshly taken rabbit urine. As shown in Fig. 6, the urokinase activity showed a comparable elution profile as obtained with the human urine samples.

The experiments described illustrate that a column made up with Sephadex G-200 can be applied in the analysis of urokinase output, needing only small urine samples for the estimation of fibrinolytic activity by activation of plasminogen. Due to the variations in enzyme contents urine samples collected over longer periods, particularly when animal experiments are concerned, are

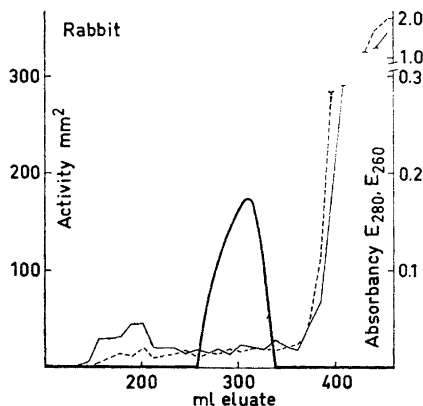


Fig. 6. Elution profile of urokinase obtained with 10 ml of fresh rabbit urine. Elution profile of urokinase activity (—). Absorbance at 280 $m\mu$ (---) and 260 $m\mu$ (· · ·).

not reliable sources of study since change of pH or deepfreezing may not be recommended. The rapid sieving of small samples of urine collected cold under controlled conditions may be useful to detect enzyme activity otherwise partly or totally escaping through destruction during collections or storage over longer periods.

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