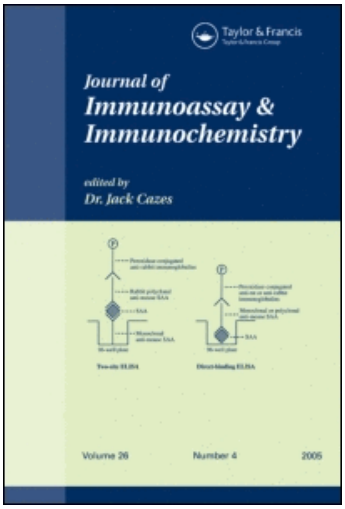

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Enzyme-Linked Immunosorbent Assay for Human Urokinase-Type Plasminogen Activator and its Proenzyme Using a Combination of Monoclonal and Polyclonal Antibodies

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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR HUMAN UROKINASE-TYPE PLASMINOGEN ACTIVATOR AND ITS PROENZYME USING A COMBINATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES

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ABSTRACT

A sensitive and specific enzyme-linked immunosorbent assay (ELISA) for human urokinase-type plasminogen activator (u-PA) and its inactive proenzyme (pro-u-PA) was developed. A monoclonal antibody was used as solid-phase antibody, while rabbit antibodies against human u-PA followed by peroxidase-conjugated third antibody were used for detection of bound u-PA. No reaction was observed with tissue-type plasminogen activator or with a variety of other human proteins. The assay was used for quantitation of u-PA in human urine and in culture fluid from human tumor cells. The recovery of added pro-u-PA was > 95%. A good agreement with the results obtained by enzymatic assays was found. The detection limit was less than 0.1 ng per ml, both for u-PA and pro-u-PA. The advantages of the use of ELISA compared with enzymatic assays and radioimmunoassays for quantitation of u-PA and pro-u-PA in biological samples are discussed.

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INTRODUCTION

Two main types of plasminogen activator have been found in mammals, differing in molecular weight, immunological reactivity, amino acid sequence and presumably in function. Urokinase-type plasminogen activator (u-PA) has an M_r of approximately 50,000 and is among other functions believed to be involved in tissue degradation in a variety of normal and pathological conditions, including cancer. Tissue-type plasminogen activator (t-PA) has an M_r of approximately 70,000 and is supposed to be a key enzyme in thrombolysis (for reviews, see 1-5).

Assays for quantitation of plasminogen activators in biological fluids should exhibit a high degree of sensitivity in order to measure the often very low concentrations present. Furthermore, such assays should be able to discriminate between the two types of activator. Most plasminogen activator assays measure enzyme activity. However, the interpretation of enzyme assays is complicated by the facts that many biological fluids contain inhibitors of plasminogen activators and/or plasmin (4, 6-8) that may interfere with the assay, and that both types of plasminogen activator exist as proenzymes with little or no enzymatic activity (9-12). Immunological assays may therefore be useful alternatives or supplements.

We previously reported the derivation of monoclonal antibodies against human u-PA (13). We now report the combined use of these monoclonal antibodies and rabbit polyclonal antibodies for a sensitive enzyme-linked immunosorbent assay (ELISA) for the activator and its proenzyme.

MATERIALS AND METHODS

Materials

Peroxidase-conjugated swine anti-rabbit immunoglobulins were obtained from Dakopatts, Glostrup, Denmark; o-phenylenediamine, Tween-20, and hydrogen peroxide from Merck, Darmstadt, West Germany; urokinase from LEO, Ballerup, Denmark; bovine serum albumin (BSA) from Behring, Marburg, West Germany; Immuno Plate I from NUNC, Roskilde, Denmark. Bowes melanoma and HT-1080 fibrosarcoma cell lines were gifts from D.B. Rifkin and A. Vaheri, respectively. All other reagents were as described previously (11, 13, 14, 15) or of the best grade commercially available.

Cell Cultures

Hybridomas were grown as described (15) in Dulbecco-modified Eagle's medium supplemented with 10% fetal bovine serum. Serum-free cell culture fluid from human tumor cell lines was prepared as previously reported (11, 15).

Plasminogen Activator Preparations

Human u-PA for immunization of rabbits (active enzyme containing a mixture of $M_r \sim$ 52,000 and $M_r \sim$ 36,000 forms) was purified from a commercial urokinase preparation, and pro-u-PA from the conditioned culture fluid from a human glioblastoma cell line as described (16). The purified preparations were pure as evaluated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS--PAGE) followed by staining with Coomassie Blue (detection limit

for contaminating proteins ~ 5%). Pro-u-PA was activated by plasmin as described (11). The activated form consisted solely of the two-chain 52,000-M_r form, as evaluated by SDS-PAGE under reducing conditions (11). t-PA was purified as described from conditioned culture fluid from the human Bowes melanoma cell line by immunosorbent chromatography on a Sepharose column with monoclonal antibody against t-PA (16). Protein content of the plasminogen activator preparations was determined by the method of Lowry et al. (17) as modified by Bonsall and Hunt (18) with BSA as a standard.

Antibodies

Five hybridomas producing different monoclonal antibodies directed against human u-PA were obtained from a previously reported fusion (13). Monoclonal antibodies were purified from hybridoma culture fluid by chromatography on Protein A-Sepharose (19), modified as described (15). The antibody originally described (clone 1) does not bind the M_r ~ 36,000 degradation product of u-PA (13), while the remaining four antibodies (clones 2-5) do (unpublished results).

Rabbit antibodies against human u-PA were prepared by immunization with purified diisopropylfluorophosphate-inhibited u-PA. Seven µg of u-PA was used per injection, according to a previously reported scheme (14). Anti-sera were compared by ELISA using purified u-PA attached to the solid phase. Serial dilutions of rabbit serum were used and bound rabbit antibody was detected by peroxidase-conjugated swine anti-rabbit immunoglobulins (see below). The one showing the highest titer was chosen for ELISA experiments.

The IgG fraction was isolated by chromatography on protein A-Sepharose (19). Purified IgG reacted strongly with the murine monoclonal antibody used for immunosorbent purification of u-PA (see Results section). It was therefore absorbed against monoclonal anti-u-PA as follows: 6.6 mg of IgG in 2 ml of 0.1 M TrisHCl, pH 8.1, was applied to a 0.75 ml column of Sepharose coupled with 1.5 mg of monoclonal anti-u-PA, clone 2. The run--through was collected and the IgG-concentration determined from the absorbancy at 280 nm using an extinction coefficient $E^{1\%}_{280\ nm}$ = 14.0 (20).

ELISA

Based on a number of optimization experiments in which the concentrations of reagents, washing procedures and incubation periods were varied (see Results section), the following optimal standard conditions were chosen and used for all experiments unless otherwise stated:

Immuno Plates were coated overnight at 37°C with 2 µg/ml of monoclonal anti-u-PA IgG, clone 2, in 0.1 M Na_2CO_3, pH 9.8, 200 µl per well, and used immediately or stored at 4°C for up to two weeks. The plates were then washed and incubated with 0.25% BSA in PBS (0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl), 200 µl per well for at least 15 min at room temperature in order to block remaining protein binding sites. After washing, 100 µl of standard dilutions of a pro-u-PA standard preparation or samples to be tested was added to each well. The plates were incubated for 1 hour at

37°C with gentle shaking, washed, and again while shaken incubated for 1 hour with rabbit anti-u-PA IgG (2 µg/ml, 100 µl per well), washed, incubated for 1 hour while gently shaken with peroxidase--conjugated swine anti-rabbit immunoglobulins (diluted 1:800, 100 µl per well), and washed again. PBS with 0.1% Tween-20 (PBS-Tween) was used for all dilutions and washings. All the preceding washings between incubations were performed four times using a NUNC Immunowash 8 apparatus. After the last washing, the plate was emptied completely by shaking, and finally washed once with H_2O. The peroxidase reaction was performed for 10 min at room temperature with 0.1% o-phenylenediamine, 0.01% H_2O_2 in 0.1 M citrate-phosphate buffer, pH 5.0, 100 µl per well. The reaction was stopped by adding 100 µl 1M H_2SO_4 per well, and absorbancy was measured at 492 nm using a Dynatech Microelisa Minireader.

Electrophoresis and Immunoblotting

SDS-PAGE was performed in a stacking system of slab gels as described (21). Zymography for plasminogen activators and immunoblotting were performed as described (22-24).

Enzyme Assay for Plasminogen Activator

Plasminogen activators were assayed by the $[^{125}I]$-fibrin plate method as described (22). Each well contained 1 µg human Glu--plasminogen and 8 ng of plasmin in a total volume of 0.55 ml. With this amount of plasmin added, all pro-u-PA present is converted to the active form during the assay, allowing estimation of the sum

of the pro-u-PA and active u-PA content of the samples (11). Assays were linear up to approximately 0.2 ng/ml.

RESULTS

Absorption of Rabbit Antibodies

With non-absorbed IgG from rabbits immunized with affinity--purified u-PA, very high absorbance values ($A_{492} > 2$) were obtained in the ELISA (performed as described in the Materials and Methods section), independent of the concentration of u-PA or pro-u-PA used. It was found that the non-absorbed rabbit IgG bound strongly to the solid-phase monoclonal antibody, while no binding was observed with IgG from non-immunized rabbits or when the solid phase antibody was replaced by BSA. SDS-PAGE followed by immunoblotting analysis of the purified u-PA preparation with non-absorbed IgG from the immunized rabbits revealed a strong band with an $M_r \sim 52,000$ (indistinguishable from that of u-PA) and a faint band with an $M_r \sim 150,000$, comigrating with mouse IgG (results not shown). These findings suggested that the u-PA, which had been purified by immunosorbent chromatography using a mouse monoclonal antibody coupled to Sepharose, contained small amounts of monoclonal antibody leaking from the column during the elution of bound u-PA, and that the rabbit antibodies raised against this preparation contained antibodies against mouse IgG. The antibody preparation was therefore passed through a Sepharose column coupled with the monoclonal anti-u-PA antibody. Rabbit IgG absorbed by this procedure did not react with

the solid-phase monoclonal antibody, and this absorbed preparation of rabbit anti-u-PA was used as second antibody for all this subsequent experiments.

Following these preliminary experiments, each step of the ELISA was investigated separately in order to optimize the procedure, i.e. to achieve a low background, high sensitivity and linearity between the absorbancy and the amount of u-PA added.

Monoclonal Solid-Phase Antibody

Five different monoclonal antibodies were compared as solid--phase antibody (Figure 1). Clone 4 gave a high background, while clones 1, 2, 3 and 5 all proved to be useful. The monoclonal antibodies designated clones 2 and 5 resulted in highest assay sensitivity, and clone 2 was chosen because of slightly lower background values. Using increasing concentrations of this antibody for coating the solid phase it was found that both sensitivity and background values increased (results not shown). A coating concentration of 2 µg/ml resulted in good sensitivity and low background ($A_{492} < 0.2$) and was used in the following experiments.

Concentration of Second and Third Antibody

Various concentrations of the second antibody (rabbit anti-u-PA) were tested. Using increasing concentrations of this antibody up to 2 µg/ml led to an increase both in sensitivity and background values (Figure 2). Higher concentrations of the second antibody did not increase the sensitivity, while the background increa-

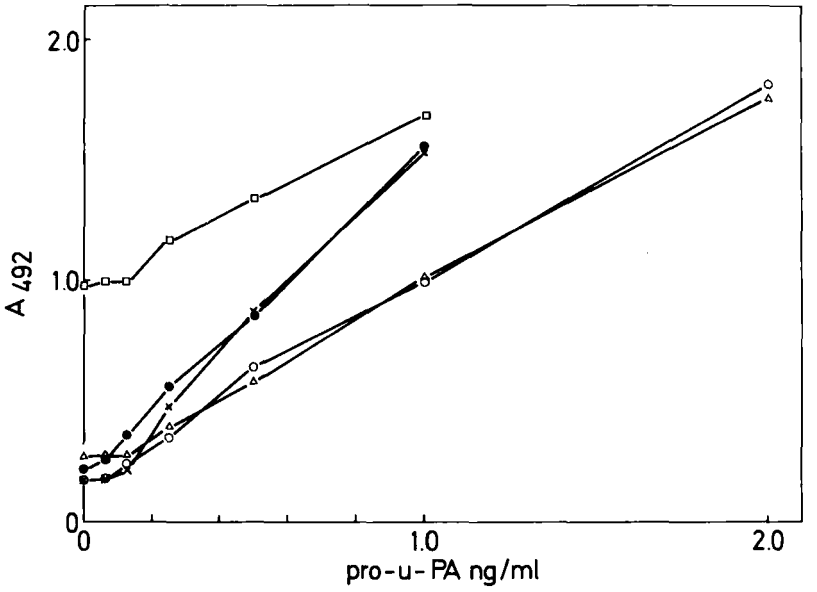

FIGURE 1. Dose-response curves for ELISA of pro-u-PA with different monoclonal anti-u-PA antibodies as solid-phase antibody. Assays were performed under standard conditions except that the following monoclonal IgG antibodies were used: (o) anti-u-PA-clone 1; (x) anti-u-PA-clone 2; (Δ) anti-u-PA-clone 3; (□) anti-u-PA-clone 4; and (●) anti-u-PA-clone 5. Each point represents the average of two determinations.

sed strongly. A concentration of rabbit anti-u-PA of 1 μg/ml was therefore chosen.

Different concentrations of the third antibody: peroxidase--conjugated swine anti-rabbit antibody were tested. Increasing concentrations of this antibody resulted in increasing sensitivity as well as background values (results not shown). A 1:800 dilution of this antibody was found to be optimal and was selected for the standard assay.

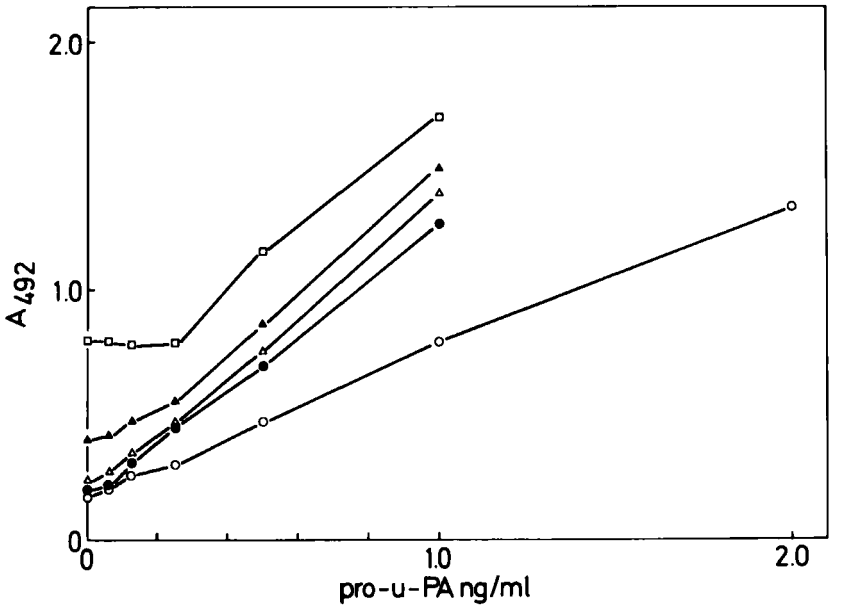

FIGURE 2. Dose-response curves for ELISA of pro-u-PA with different concentrations of second antibody. Standard conditions were used except that polyclonal rabbit anti-u-PA was used in the following concentrations: (o) 0.08 µg/ml; (●) 0.4 µg/ml; (△) 2 µg/ml; (▲) 10 µg/ml; and (□) 50 µg/ml. Each point represents the average of two determinations.

Time of Incubation with Antigen and Antibodies

The incubation period for serial dilutions of pro-u-PA was varied between 15 min and 240 min (Figure 3). Increasing the incubation period to more than 1 hour caused the sensitivity of the assay to increase only slightly. For convenience, a one-hour incubation period was chosen for the standard assay. The effect of varying the incubation period with second and third antibody was investigated. Only a slight increase in sensitivity with prolonged incubation periods was seen (results not shown), and for convenience incubation periods of 1 hour were chosen for both these antibodies.

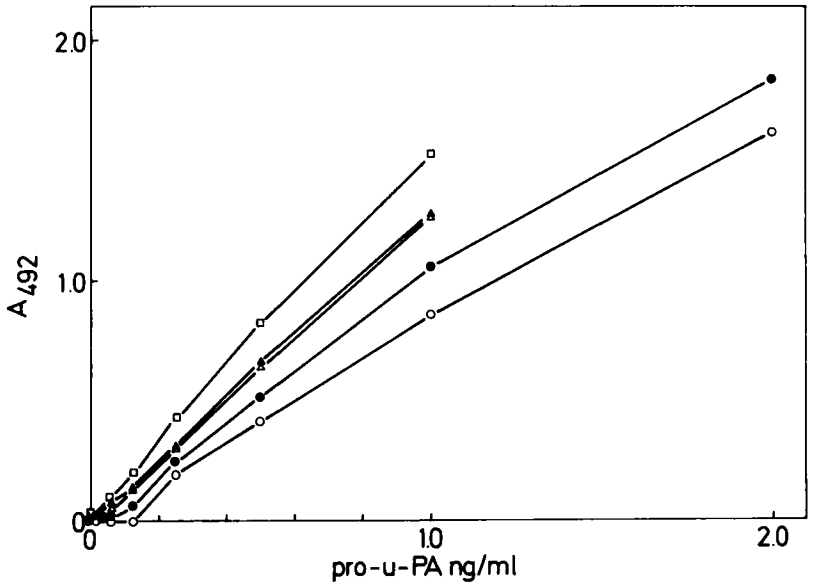

FIGURE 3. Dose-response curves for ELISA of pro-u-PA with different incubation periods. Standard conditions were used, except that the incubation period with pro-u-PA was: (o) 15 min.; (●) 30 min.; (△) 60 min.; (▲) 120 min.; and (□) 240 min. Each point represents the mean of 4 determinations. Background values in wells without pro-u-PA added (A_{492} < 0.2 in all cases) did not change significantly with the incubation period. These values have been subtracted.

Characteristics of the Standard ELISA

Based on the results described above, the standard procedure described in Materials and Methods was adapted. A typical standard curve for pro-u-PA was as shown in Figure 4. Controls included: omission of the solid phase antibody bottom layer and substitution of rabbit anti-u-PA IgG with preimmune IgG. None of these gave absorbancy readings above background (results not shown). It was found that A_{492}-values were quite constant from assay to assay; the interassay standard deviation of A_{492}-values for a pro-u-PA concen-

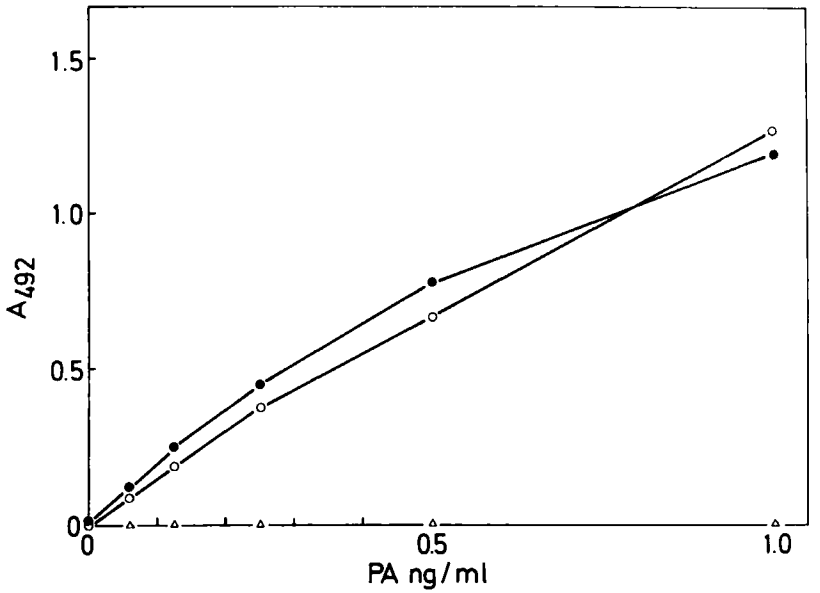

FIGURE 4. Dose-response curves for ELISA of purified (●) pro-u-PA, (o) u-PA and (△) t-PA. Standard conditions were used. Additional control experiments with t-PA (up to 1 µg/ml), human plasminogen (up to 100 µg/ml), and human serum albumin (up to 1000 µg/ml) did not show any increase in A_{492} (results not shown). Each point represents the average of two determinations. Background values in wells without plasminogen activator added ($A_{492} < 0.2$ in all cases) have been subtracted.

tration of 1 ng/ml in 12 assays was < 14% of the mean. A_{492} was linear with concentrations of pro-u-PA and u-PA up to approximately 1 ng/ml. The detection limit of the assay was less than 0.1 ng/ml of pro-u-PA. t-PA, plasminogen and serum albumin did not increase A_{492} above background (Figure 4).

Purified standards of pro-u-PA and active u-PA prepared in parallel were tested in the ELISA. The results were almost identical for the same dilutions, indicating that the two molecular forms react identically in the assay (Figure 4).

TABLE 1

Comparison of ^{125}I -fibrin Plate Assay and ELISA for u-PA

A urine sample and a sample of HT-1080 cell culture fluid were assayed enzymatically by the radiolabelled fibrin plate assay for plasminogen activator and by ELISA. The same purified pro-u-PA preparation was used as a standard in all assays. Each number represents mean ± standard deviation of 5 determinations performed in separate assays. Using Student's t-test, neither urine nor HT-1080 cell culture fluid showed any significant difference between the values obtained by the two methods ($P > 0.20$ in both cases).

Sample	u-PA concentration ng/ml ± SD	
	^{125}I -fibrin plate assay	ELISA
Urine	296 ± 77	330 ± 46
HT-1080 cell culture fluid	230 ± 29	216 ± 21

The assay was used for quantitation of u-PA in human urine and cell culture fluid from cultured human fibrosarcoma cells of the line HT-1080. When samples of urine and cell culture fluid were assayed 5 times each the interassay standard deviations for the calculated concentrations of both samples were < 12% of the mean (Table 1). As evaluated by zymography (22), both urine and culture fluid from HT-1080 cells contained u-PA, but no t-PA or other plasminogen activator activity (results not shown). This made possible a comparison of the enzymatic [^{125}I]-fibrin plate assay (22) and ELISA. The results of this comparison gave similar values

and also similar coefficients of variation with the two types of assay (Table 1). A linear relationship was found between concentrations of urine and cell culture fluid and absorbancy. Addition of pro-u-PA and u-PA to urine and conditioned culture fluid from the HT-1080 cells resulted in a complete recovery in the ELISA (consistently > 95%, results not shown). Culture fluids from Bowes melanoma cells (130 µg protein/ml) which, as judged by zymography, contained t-PA but no u-PA, did not increase the absorbancy values above background.

DISCUSSION

The present results demonstrate that the sandwich ELISA described meets the basic requirements for quantitating the total amount of pro-u-PA and u-PA in the biological samples tested: 1) the sensitivity was sufficient (detection limit less than 0.1 ng/ml) to measure the concentrations of the activator in the samples; 2) it measured pro-u-PA and u-PA equally well; 3) internal standards of pro-u-PA and u-PA could be recovered; 4) it distinguishes between u-PA and t-PA. The specificity of the assay was further supported by the findings that no reaction was seen with any other of the human proteins tested, including the variety of proteins present in conditioned culture fluid from Bowes melanoma cells (see ref. 15).

It is noteworthy that a prerequisite for the use of the polyclonal rabbit anti-u-PA IgG in the ELISA was found to be an absorp-

tion of these antibodies with the murine monoclonal anti-u-PA IgG, because the u-PA preparation used for immunization had contained small amounts of the monoclonal antibody which was used for its purification. This might well be a general problem in the use of monoclonal antibody-based immunosorbents for purification of antigens for immunization. The severe elution conditions used with antibody columns (low pH or high concentrations of a chaotropic agent) cause leakage of minute amounts of the coupled antibody (unpublished results), probably because a small amount of agarose material is dissolved (see also ref. 25). This amount of monoclonal antibody in the eluate will normally go undetected by the conventional staining methods for SDS-polyacrylamide gels, but the content may be high enough to elicit an antibody response when subsequently injected into rabbits.

A variety of enzymatic assays for plasminogen activators have been described. Some of these assays use plasminogen as a substrate and measure the enzyme activity of the plasmin formed, or utilize the fact that plasmin migrates differently from plasminogen in SDS-PAGE to measure the amount of plasmin formed, e.g. by the use of radiolabelled plasminogen. Other enzymatic assays for plasminogen activators use low molecular weight substrates (for reviews, see refs. 1 and 5). Common to these enzymatic assays is that they do not measure the inactive form of u-PA unless it is converted to its active form, e.g. by plasmin. This conversion can be inhibited by small amounts of plasmin inhibitors (9, 11, 12) that may be present in biological samples. The present ELISA

circumvents this complication because it measures pro-u-PA and u-PA equally well. Immunological detection methods also circumvent the interference of plasmin inhibitors that may complicate detection of active u-PA by the enzymatic methods that measure the enzymatic activity of plasmin formed. Enzymatic assays of u-PA will also be complicated by the presence of u-PA inhibitors. This may or may not be the case for immunological assays, depending on whether the antibodies used are directed against epitopes that are covered or modified in the inhibitor/u-PA complex. Internal standards of pro-u-PA and u-PA should therefore always be used in the immunological assay to evaluate this possibility, and modifications may be necessary to obtain sufficient recoveries. By testing different monoclonal anti-u-PA antibodies known to bind to different epitopes as well as different preparations of polyclonal antibodies, it is likely that conditions may be found in which complexes between u-PA and inhibitors or other molecules are detected equally well as free u-PA.

The sensitivity of the present ELISA for u-PA is similar to that of the most commonly used enzymatic assays for u-PA, i.e. the fibrin plate assay (26) and the [^{125}I]-fibrin plate assay (27, see ref. 5 for further discussion).

Several radioimmunoassays (RIAs) for human u-PA have been described using polyclonal rabbit antibodies against the enzyme (28-31). Most of these assays have a detection limit between 3-10 ng/ml (28-30), while one had a detection limit of approximately 0.01 ng/ml (31). Besides being faster and more easy to perform,

the present ELISA offers the advantage compared to these RIAs that the possible interference of cross-reacting antigens can be readily controlled by the parallel use of monoclonal antibodies directed against different epitopes of the u-PA molecule. An ELISA for human u-PA using two different monoclonal antibodies has been reported (32). This ELISA has a detection limit of 2-5 ng/ml and requires an incubation period of 16 hours. The reactivity of pro-u-PA and u-PA has not been compared in any of the previous reports on immunoassays.

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