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A CHROMOGENIC ENZYMATIC ASSAY CAPABLE OF DETECTING PROUROKINASE-LIKE MATERIAL IN PLASMA

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ABSTRACT We report a functional assay capable of quantifying prourokinase (ProUK)- like material in plasma where urokinase (UK) is also present. The assay involves inactivation of urokinase with a specific, active site directed irreversible inhibitor, dansyl-glutamyl glycyl arginine chloromethylketone (dansyl- GGACK). Excess inhibitor is subsequently quenched with dithiothreitol (DTT). The ProUK-like material in plasma is then converted to active urokinase with thermolysin, a proteolytic enzyme of bacterial origin. Alpha 2-macroglobulin in plasma inhibits thermolysin; however alpha 2-macroglobulin is inactivated with methylamine. The assay can detect as little as 20 ng of ProUK and is linear from 20 to 120 ng. The assay was applied to quantify the amount of ProUK-like material in plasma obtained from dog at various times after i.v. administration of 100,000 or 75,000 U/kg, of pro-urokinase.

INTRODUCTION

Prourokinase (ProUK) is the inactive, zymogen form of urokinase. ProUK consist of a single polypeptide chain with an apparent molecular weight of 54,000 daltons; it is the precursor of high molecular weight, two chain urokinase (1) a plasminogen activator. In the blood, proUK may have at least four fates. First, circulating ProUK/UK is rapidly cleared by the liver (1). Second, some can be bound to receptors on the surface of endothelial cells (2). Third, ProUK may be inactivated proteolytically by thrombin (3) although aminopeptidases may

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restore activity. Fourth, the proenzyme can be converted to active two-chain urokinase by plasmin (1,4) or kallikrein. Some patients treated with ProUK have shown little loss of fibrinogen while others have undergone considerable fibrinogen depletion (5). It is speculated that this is caused by individual variation in the fraction of the ProUK which has been converted into active UK before these species are rapidly cleared from the circulation. This has not been determined previously because immunoassays used to monitor pharmacokinetics do not generally discriminate between UK and ProUK or inactive forms of either of these (6) all of which are very similar in structure. Determination of the amount of ProUK in the blood maybe confounded by the continuous conversion of ProUK to UK by plasmin in the plasma, particularly if a significant amount of UK is present in a plasma sample. Because of the potential application of ProUK as a thrombolytic agent, and the need to monitor its fate in the circulation it seemed worthwhile to develop an assay procedure that would quantify the activity of both the active enzyme urokinase, as well as the potential activity of zymogen ProUK in biological samples. In this communication, we report an assay that is able to detect functional proUK-like material in plasma. The procedure is rapid and separately quantitates urokinase and proUK activity in the same sample.

MATERIALS AND METHODS

Two chain low molecular weight (32,000 daltons) urokinase (Abbokinase) was a product of Abbott Laboratories. ProUK, was purified from the culture medium of the human kidney cell line (HEK),by published procedures; high molecular weight urokinase (54,000)and plasminogen were from Abbott Laboratories. Dansyl-glutamyl glycyl arginine chloromethyl ketone (dansyl-GGACK) was prepared at Abbott Laboratories according to the method of Kettner, Shaw and Nesheim (7,8). Pyro-glu-gly-arg p-nitroanilide (acetate salt) (S2444), bovine serum albumin (Fraction V), methylamine hydrochloride, sodium chloride, calcium chloride, thermolysin, sodium citrate, trizma base (Reagent grade), and dithiothreitol were obtained from Sigma Chemical Company. Aprotinin (Trasylol) was from FBA Pharmaceuticals. Acetic acid was reagent grade from J.T. Baker Chemical Company. 96 well microtiter plates (Nunc Immuno Plate I) were from Scientific Supply Company. Plate sealer was from Costar.

Pharmacokinetic Studies:

Each of four (4) anesthetized beagle dogs received a single intravenous bolus of (100,000 U/kg) ProUK. At various times after the injection, one ml of blood taken at a distant site was collected into a tube containing 0.1 ml of a 3.8% (wt/vol) solution of sodium citrate. The sample was centrifuged for 10 minutes at 2,500 RPM at room temperature to separate the plasma from the cellular elements of the blood. Aprotinin was added to the plasma to achieve a final concentration of 30 KIU/ml and plasma samples were stored at -80° until analyzed.

Standard ProUK Assay Procedure

The standard assay for ProUK was conducted in a 96 well microtiter plate as follows: 25 μl of unknown sample or ProUK standard in dog plasma was added to a 45 μl aliquot of reaction mix

containing 555 KIU/ml Aprotinin, 34 mM Tris buffer (pH 8.0), 6.8 mM NaCl, 1.4 mg/ml BSA, and 0.9 mM freshly added dansyl-GGACK. The microtiter tray was covered with a plastic sealer and incubated for 30 minutes at 37°C. At the end of the incubation, 20 µl of 5 mM DTT was added to each well. Then, 10 µl of 1 M $CaCl_2$ 60 µl of 5 M methylamine, 30 µl of 10 µg/ml thermolysin, and 10 µl of 10 mM S2444, were added sequentially to each well. The microtiter tray was incubated for 150 min at 37°C. The incubation was terminated by adding a 10 µl aliquot of 10% (v/v) acetic acid to each well. Formation of the reaction product p-nitroaniline in each well was measured by reading absorbance at 405 nM with an automated plate reader.

The addition of aprotinin in the reaction mix was to inhibit conversion of pro-urokinase to urokinase by any free plasmin that might be present. The initial incubation in the presence of dansyl-GGACK was to irreversibly inhibit any urokinase activity (4,6). DTT was added to quench any excess dansyl-GGACK that would inhibit subsequently formed urokinase. Thermolysin was added to convert ProUK-like material to UK; methylamine was added to block inhibition of thermolysin by alpha-2 macroglobulin (7). The urokinase newly formed from ProUK catalyzed the release of para-nitroaniline whose characteristic yellow color is quantified.

Total urokinase assay:

The assay to determine total urokinase (i.e urokinase and pro-urokinase) activity was conducted in a volume of 200 µl that contained: 22.5 mM Tris buffer (pH 8.0), 0.9 mg/ml BSA, 45 mM NaCl, 125 KIU/ml aprotinin, 1.5 µg/ml thermolysin, 1.5 M methylamine, 0.5 mM S2444 and 25 µl of either standard in plasma or unknown. The assay system was incubated for 60-90 minutes at 37°C and absorbance was measured at 405 nM. No dansyl-GGACK was used.

RESULTS

The standard assay discriminated between urokinase and pro-urokinase. Table 1 shows that urokinase was not detected in the standard assay, owing to prior inactivation by dansyl-GGACK while pro-urokinase (i.e. potential UK activity) was.

TABLE 1
Standard Assay Discriminates Between Urokinase and Pro-Urokinase

Enzyme	Absorbance 405 nM
none	0.140 ± .01
20 U Abbokinase	0.152 ± .01
20 U Abbokinase + 0.125 µg ProUK	0.504 ± .01
20 U Abbokinase + 0.25 µg ProUK	0.870 ± .2
20 U Abbokinase + 2.5 µg ProUK	2.210 ± .01

The standard assay for ProUK was conducted with Abbokinase (low molecular weight UK and ProUK (indicated). The data represent mean ± SE of triplicate observations. In total urokinase assay, 20 U/ml UK will give 2.36 OD. The same result could be observe when high molecular weight urokinase is combine with ProUK.

The assay of potential ProUK activity in plasma was more complicated than the assay for this enzyme in buffer. Biological samples from animals dosed with ProUK may contain urokinase (derived from the administered proUK) that will hydrolyze the substrate S2444. Table 2 shows that as with buffer, the inclusion of dansyl-GGACK in the assay system abolished the urokinase activity present in plasma. However, plasma contained some enzyme activity, presumably other than UK, that survived the pretreatment with dansyl-GGACK and was able to hydrolyze the chromogenic substrate. This caused the plasma blank (plasma but no added ProUK) to be greater than the buffer and no added ProUK blank, (compare curves in Figure 1). Figure 1 also shows that the linear range for the standard curve for enzyme dissolved in plasma was smaller than the linear range for the standard curve in buffer. Because of these differences, the standard assay used a standard curve containing a volume of plasma equivalent to the unknown samples.

The assay was used to determine the half-life of ProUK administered to dogs. ProUK 100,000 U/kg was administered (i.v.) to 4 beagle dogs and blood samples were taken for up to 2 hours after the drug administration. Figure 2 presents data obtained on one typical experiment. An initial increase and subsequent decay in the proUK-like material activity was detectable in the 40 minutes following injection of the enzyme. In the four experiments, the mean half life of the functional proUK activity was 8.5 ± 1.5 minutes (mean ± SE), comparable with values obtained by immunoassay using polyclonal anti-UK antibody.

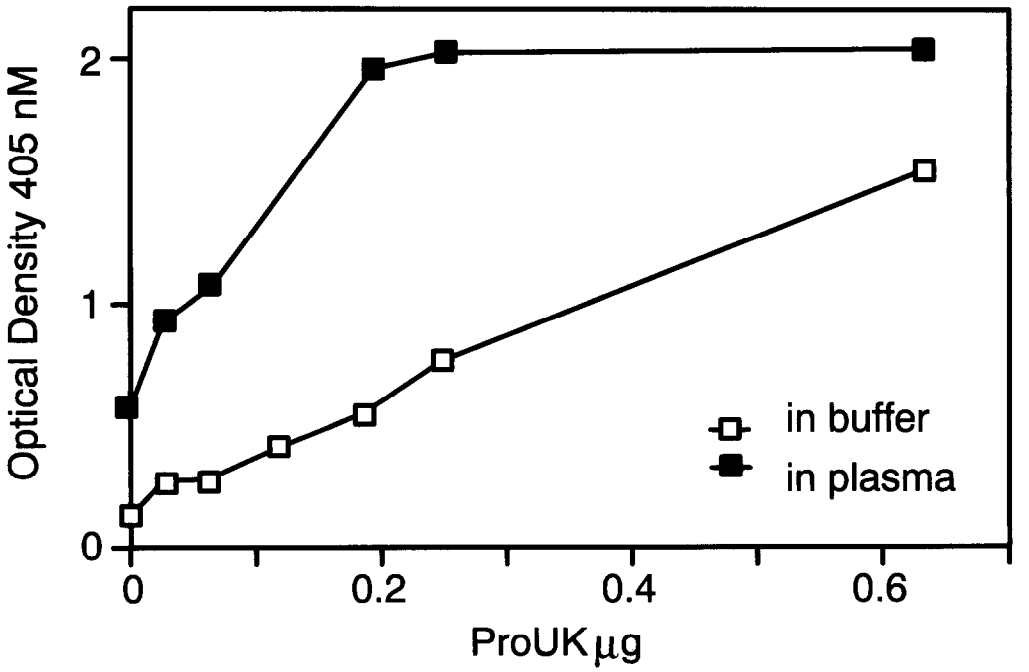

Figure 1 (facing). Comparison of standard curves in buffer and plasma. The standard assay was conducted using the indicated amounts of ProUK dissolved in either buffer (open squares) or dog plasma (filled squares). Note that the blank (no enzymes) is higher for plasma than for buffer. N.B. for assays of unknowns in dog plasma, the standard curve was constructed in dog plasma.

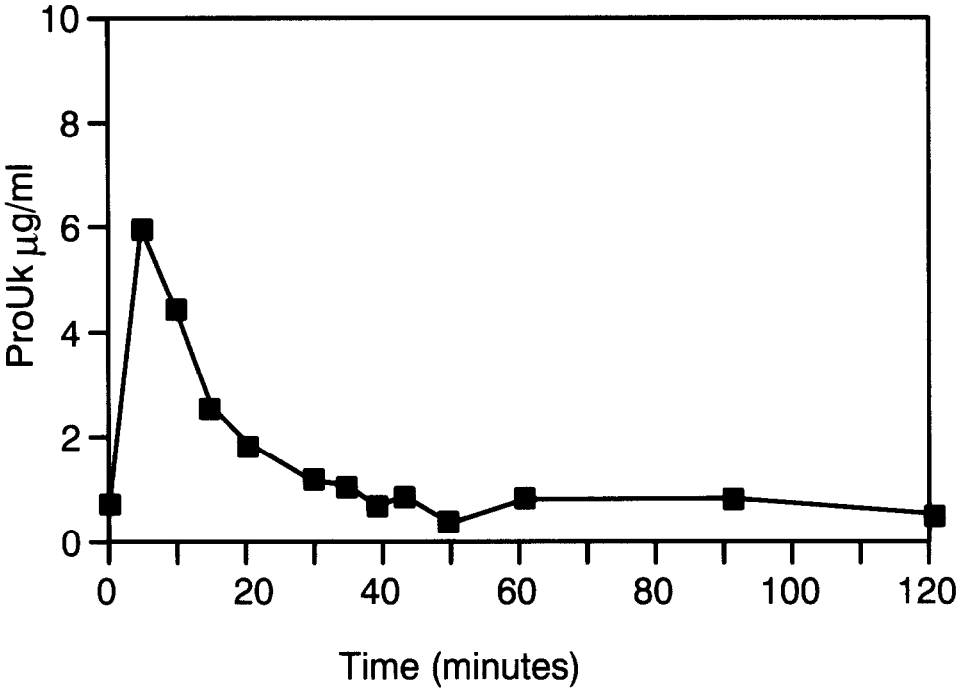

Figure 2. ProUK (100,000 U/kg) was administered i.v. to an anesthetized dog; at the indicated times a blood sample was drawn and processed as described in Methods. At each time point, the amount of functional ProUK using the standard assay procedure was determined. At the 5 minute time point, the 25 µl aliquot of plasma contained 149 ng of ProUK. In this experiment, the half life of the functional ProUK in the circulation was calculated to be 6 minutes.

TABLE 2
Dansyl-GGACK Inhibits Urokinase in Plasma

Addition	Absorbance 405 nM
none	0.26 ± .04
urokinase (50 U/ml)	1.10 ± .05
urokinase + dansyl-GGack	0.32 ± .02

Note that the addition of dansyl-GGACK totally eliminates urokinase activity.

DISCUSSION

The results present a method to quantify ProUK (potential UK activity) in plasma even if urokinase is simultaneously present. This requires stabilization of the sample by rapid inactivation of UK. Thermolysin rather than plasmin is used to activate the proUK because of the presence of large amounts of alpha-2-antiplasmin in plasma. The addition of methylamine (to prevent the inhibition of thermolysin by alpha 2-macroglobulin) is also a critical step to the assay. The method may also detect secondary forms of potential UK activity. For example, thermolysin has been shown to activate both ProUK and thrombin-inactivated ProUK (9). The latter is a two-chain species where the catalytic chain begins 2 amino acid residues proximal to its normal N-terminus (10). Restoration of this activity may be achieved by plasma aminopeptidases. This assay can be used to quantify ProUK in plasma samples from dogs given a bolus dose of the drug.

We show that it is possible to functionally assay proUK in biological samples. Because the method is based on the conversion of ProUK to UK, it is necessary to inhibit any UK activity which would destabilize the samples leading to underestimates of proUK and overestimates of UK. This is accomplished by the addition of dansyl-GGACK to the samples (4,11); followed by thiol inactivation of the excess UK inactivator. In the present experiments, ProUK was activated with thermolysin rather than with plasmin as has been described previously. Thermolysin has been used to convert prorenin to renin (12). In preliminary experiments, we found thermolysin to give a more sensitive assay than plasmin. Erdos et al. have shown that the activity of thermolysin is inhibited by α-2 macroglobulin in plasma (12); however, these authors also reported that methylamine can inactivate the alpha-2 macroglobulin. In the present system, we found that methylamine would potentiate the activation of ProUK by thermolysin in the presence of plasma (data not shown). Thermolysin can activate thrombin-inactivated prourokinase and the assay cannot discriminate between ProUK and thrombin inactivated prourokinase, however the latter may still represent potential UK activity in plasma. In the plasma samples withdrawn before ProUK infusion, there was some activity that caused hydrolysis of S2444 in the assay; this source of background remains unidentified. There was a marked rise in ProUK functional activity following the ProUK bolus injection. The clearance of this potential UK activity was similar to that measured by immunoassay for UK antigen. However, unlike the immnunoassay the present assay allows the assessment in any time point of whether a significant fraction of the zymogen has been converted into active enzyme.

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