Assay of Prekallikrein in Human Plasma: Comparison of Amidolytic, Esterolytic, Coagulation, and Immunochemical Assays

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Using the substrate H-o-Pro-Phe-Arg-p-nitroanilide-HCl, an amidolytic assay was designed to measure prekallikrein in plasma. At a substrate concentration of 1 mM ($K_m = 0.2$ mM), the amidolysis of purified prekallikrein at 1 coagulant unit/ml was observed to be 2.47 $\mu$moles/min/ml. Conditions for plasma prekallikrein activation were optimized to approach complete activation when compared to the amidolytic activity of the purified plasma kallikrein. Plasma treated with chloroform to destroy inhibitors of kallikrein was activated with dilute kaolin (final concentration 1 mg/ml) for 1 min at 25°C. Activated plasma prekallikrein had 78% (1.92 $\mu$moles/min/ml) of activity of purified kallikrein at plasma concentration. Comparison of this amidolytic assay with immunochemical, esterolytic, and coagulant assays of three subject populations (normals, women on birth control pills, and patients with hepatocellular disease) showed good correlation both in normals and in the patient groups between the amidolytic and esterolytic assays ($r = 0.89$). Each enzymatic assay correlated with the immunochemical assay ($r = 0.72$, $r = 0.68$, respectively). However, comparison of each of these assays with the coagulant assay showed no significant correlation due to the large inherent error of the latter assay. This standardized plasma prekallikrein amidolytic assay should facilitate studies of plasma prekallikrein concentration in physiologic and pathologic conditions and help identify activation of the contact phase of coagulation in disease states.

Previous functional assays of prekallikrein have measured the ability of the activated enzyme, kallikrein, to liberate bradykinin from crude preparations of kininogen, to hydrolyze synthetic ester substrates, to correct the abnormal activated partial thromboplastin time (APTT) of prekallikrein-deficient (Fletcher factor) plasma, or to hydrolyze chromogenic tripeptide substrates. The liberation of bradykinin depends on measurement of this peptide with a sensitive but semiquantitative bioassay or the time-consuming radioimmunoassay. The use of synthetic esters has been helpful, but these substrates have a $K_m$ that is about tenfold higher than the tripeptide substrates, and the assay requires many steps, each one multiplying the pipetting error. The correction of the mildly abnormal partial thromboplastin time of prekallikrein deficiency yields a relatively flat standard.

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curve limiting the accuracy of the coagulation assay. The time of incubation is critical, since correction of the defect occurs with prolonged incubation.16 Recent investigations have utilized amidolytic14,15 assays for measuring the kallikrein derived from prekallikrein but no comparative studies of the functional and immunochemical assays have been performed.

This study compares three functional assays for prekallikrein using coagulation, esterolytic, and amidolytic substrates with a radial immunodiffusion reference assay in three subject populations—normals, women receiving oral contraceptives, and patients with severe liver disease. In addition, this study directly compares our plasma prekallikrein amidolytic assay with several different plasma prekallikrein amidolytic assays from other laboratories against a purified kallikrein standard. Our findings allow us to recommend a rapid and simple plasma prekallikrein amidolytic assay that measures 78% of the absolute amount of prekallikrein in plasma.

MATERIALS AND METHODS

Materials

H-D-Pro-Phe-Arg-p-nitroanilide-HCl (PPAN) (S-2302, Ortho Diagnostics, Raritan, NJ) was used as a synthetic substrate of kallikrein. H-D-Val-Leu-Lys-p-nitroanilide-HCl (VLLN) (S-2251, Ortho Diganostics) was used as a synthetic substrate of plasmin. Tris and p-tosyl-L-arginine methyl ester (TAME) (Sigma Chemical); kaolin (Fisher Scientific Co., Pittsburgh, Pa.); HSA agarose (Lex, Denmark); sodium barbital buffer, pH 8.6 (B-2) (Harleco, American Hospital Supply Corp., Gibbstown, N.J.); inosithin (Associated Concentrates, New York, N.Y.); dextran sulfate (Sigma Chemical); and Thrombofax (Ortho Diagnostics), were purchased from the designated supplier. All other materials used were reagent grade.

Plasma and Protein Preparations

All plasma samples to be assayed for prekallikrein determination regardless of methodology employed (enzymatic, immunologic or clotting) were prepared as follows. Prior to venipuncture informed written and verbal consent were obtained from each donor. Twenty milliliters of blood were drawn from a clean venipuncture site through a 19-gauge siliconized butterfly needle into a plastic syringe containing 3.8% trisodium citrate (1:9 v/v). After adequate mixing, blood was immediately centrifuged at 2500 g for 15 min at 4°C to produce platelet-poor plasma and then frozen in 1-ml aliquots in plastic tubes at −70°C for later analysis. In the experiments where fresh plasma was used, it was collected in a similar manner and used within 30 min after collection.

Plasma was exposed to chloroform (CHCl₃) to inactivate inhibitors of plasma kallikrein as previously described.17 Each sample was processed just prior to its assay. An equal volume of CHCl₃ cooled to 4°C was added to freshly thawed plasma, mixed for 1 min using a vortex mixer, and then centrifuged at ≥2000 g for 5 min at 4°C.17 The centrifuged CHCl₃-treated plasma, which separated into two phases, was not transferred to another plastic tube, and only the top half was used for the assay by carefully removing the amount necessary for each test.

Prekallikrein-deficient plasma was donated by Dr. C Abildgaard (University of California, Davis, Calif.). Pooled normal human plasma (lot 02), which was used for the comparison of the different amidolytic assays and as a pooled normal reference plasma, was purchased from George King Biomedicals Inc., Overland Park, Kans. Kallikrein was prepared by the method of Bagdasarian et al.2 It contained no detectable HMW kinogen, CI-inhibitor, alpha-2-macroglobulin, alpha-1-antitrypsin, plasminogen, or plasmin, but contained IgG, which on radial immunodiffusion was found to be 8% of the total protein present in the kallikrein preparation.5 This kallikrein preparation was used for the production of antisera. Antiserum to kallikrein and prekallikrein cross-reacts with both the zymogen and the enzyme.2,14 Prekallikrein for functional studies was prepared by the method of Scott et al.19 and was activated to kallikrein by incubating it with Hageman factor fragments that were prepared as previously reported.19 The specific activity of the kallikrein was 29 coagulant units/mg.

Assays for Prekallikrein and Kallikrein

Radial Immunodiffusion

Radial immunodiffusion was performed according to the method of Mancini et al.20 using 1% agarose in B-2, 1/2 - 0.0375. Kallikrein antiserum was used at final concentrations of 3%. Diffusion proceeded for 24 hr. No further increase in ring diameter was observed at 48 or 72 hr. In our laboratory, the concentration of plasma prekallikrein antigen has been determined to be 39 μg/ml.19 This value correlates well with those reported by two other laboratories.21,22

Kallikrein Coagulant Activity

This was measured by preparing a standard curve from normal pooled plasma diluted in sodium barbital buffer in saline (VBIS), pH 7.4. The dilutions ranged from 1/40 to 1/320. In this assay, a modified one-stage APTT,2,17 50 μl of diluted pooled normal plasma or test sample was added to a mixture of 50 μl of prekallikrein-deficient plasma, 50 μl of 0.1% inosithin in VBIS, and 50 μl of 10 mg/ml kaolin in normal saline. This mixture was then incubated for 1 min at 37°C before recalcification with 50 μl of 0.025 M CaCl₂. One coagulant unit was defined as that amount that is present in 1 ml of normal pooled plasma.

Kallikrein Esterolytic Activity

Kallikrein esterolytic activity was assayed for its ability to hydrolyze the synthetic substrate TAMe according to the method of Siegelman et al.23 as modified by Colman et al.24 using kaolin to convert prekallikrein to kallikrein. Kallikrein inhibitors were removed by choloroform prior to activation. Briefly, prekallikrein in plasma is activated by kaolin. The amount of activated kallikrein was then permitted to react with a known amount of the substrate at 37°C. The amount of esterase activity generated was then determined colorometrically by quantitating the release of methanol, which was oxidized to formaldehyde with KMnO₄ and finally coupled to chromotropic acid to yield a purple color. The absorption of this purple solution was determined at 580 nm spectrophotometrically. Spontaneous arginine esterase activity was determined as above, substituting phosphate buffer for kaolin. Without an activating agent, plasma prepared under these conditions hydrolyzed less than 0.05 μmole TAMe/min/ml. This value was subtracted from the value with kaolin to yield the plasma prekallikrein concentration.
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Kallikrein Amidolytic Activity

This was determined using the chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide-HCl (PPAN). Plasma (50 μl) in a plastic test tube at 25°C was mixed with 50 μl of 0.1 M sodium phosphate, pH 7.65, containing 0.15 M NaCl and kaolin (final concentration 1 mg/ml) and incubated at 25°C for 1 min. Ten microliters of this mixture was then added to a cuvette that contained 60 μl of PPAN stock solution (4 mM) and 170 μl of sodium phosphate-NaCl buffer, pH 7.65, at 37°C. The final concentration of PPAN in the assay was 1 mM. After mixing, the initial rate of absorbance at 405 nm was recorded in a Gilford 240 Spectrophotometer with a Gilford 6015 chart recorder (Gilford Instr., Oberlin, Ohio). The initial rate without kaolin activation was less than 5% of the rate with kaolin added and was subtracted to calculate the prekallikrein concentration. The micromoles of PPAN hydrolyzed/min/ml plasma was calculated using an assumed molar extinction coefficient of 10,600 for p-nitroaniline at 405 nm.

Initial studies were performed with purified kallikrein to determine the optimal buffer and substrate concentration for the assay. All dilutions of purified kallikrein were performed with buffer containing 3 mg/ml bovine serum albumin (BSA). Initial studies with kaolin-activated plasma were performed to determine the necessity of treatment of plasma with CHCl₃ to remove inhibitors and the optimal temperature of activation. Subsequent studies were performed with CHCl₃-treated plasma to determine the optimal kaolin concentration and the sensitivity of the assay over a wide range of plasma dilutions.

Studies to Compare Plasma Spectrophotometric Assays

The present kaolin-activated plasma kallikrein assay was compared with other published plasma kallikrein assays and against purified kallikrein. The assay of Kluft using dextran sulfate as the activator was performed as described and in a modified version that increased the final concentration of the substrate (PPAN) to 1 mM and decreased the plasma dilution to 1:48. The assay of Friberger et al. using ellagic acid as the activator was performed as written. All comparison studies were performed with one lot of commercial pooled normal human plasma. As performed with the kaolin assay, the initial rate of amidolysis without the activator was determined and was subtracted to calculate the prekallikrein concentration. Moreover, spectrophotometric studies were performed on dextran-sulfate-activated plasma according to Kluft to see if plasmin was generated. Plasma plasmin was assayed using the substrate VLLN according to the procedure of Teger-Nilson et al.

Statistical Methods

Mean, standard deviation, and standard error of the mean were determined for all assays performed on every patient group. Correlation coefficients were determined between assays in each patient group. Students' t test (unpaired) was performed to determine significance between particular assays in all groups.

RESULTS

Standardization of the Amidolytic Assay With Purified Kallikrein

Initial studies were performed with purified kallikrein to define the optimal conditions of the chromogenic assay. Using purified kallikrein, the Kₘ for kallikrein on PPAN was determined by a Lineweaver-Burke plot to be 0.2 mM. In accordance with the findings of Friberger, the Kₘ for this protein on this substrate was not altered in buffer systems using 0.1 M sodium phosphate containing 0.15 M NaCl, pH 7.65, or 0.01 M Tris-Cl containing 0.15 M NaCl, pH 8.0. Moreover, the amidolytic activity of kallikrein on the substrate PPAN was proportional to the enzyme concentration from 0.1 to 1.0 coagulant units in the original sample.

Standardization of the Amidolytic Assay With Plasma

Studies were performed with undiluted plasma, native or chloroform treated, at 0°C and 25°C. As shown in Fig. 1, activation of native plasma with kaolin (final concentration 1 mg/ml) at 25°C showed an initial rapid formation of amidolytic activity with a 40% decrease at 5 min, similar to the previously
described activation using the synthetic ester TAME.\textsuperscript{12}

The maximum activity observed was only 65\% of that in CHCl\textsubscript{3} treated plasma as previously observed.\textsuperscript{8}

Activation with kaolin at 0°C, the same temperature used in the dextran sulfate assay,\textsuperscript{14} resulted in suboptimal amidolytic activity, reaching only 30\% of the maximum value in CHCl\textsubscript{3}-treated plasma at 5 min. In contrast, activation with kaolin of CHCl\textsubscript{3}-treated plasma gave the highest values at 1 min and only a 20\% decrease in activity at 5 min.

Proper handling of CHCl\textsubscript{3}-treated plasma required assaying the material directly from the tube that was centrifuged. Transferring the supernatant of CHCl\textsubscript{3}-treated plasma to another plastic tube resulted in a 50\% decrease in the measured amidolysis.

The optimum concentration of kaolin was next determined (Fig. 2A). Activity was maximal at a final concentration of kaolin from 1 to 5 mg/ml in CHCl\textsubscript{3}-treated plasma. For all subsequent studies, the final kaolin concentration in the activation mixture was 1 mg/ml. The amidolytic activity after kaolin activation of CHCl\textsubscript{3}-treated plasma was linear for plasma volumes in the assay from 0.5 \(\mu\)l to 7.5 \(\mu\)l (Fig. 2B).

Comparison of Kallikrein Amidolytic Assays

Using commercially available pooled normal human plasma, a direct comparison was performed among purified kallikrein, the proposed kaolin-activated plasma kallikrein assay with normal plasma, and prekallikrein-deficient plasma reconstituted with purified prekallikrein (39 \(\mu\)g/ml), and previously published plasma kallikrein assays (Table I). With purified kallikrein (1 unit coagulant activity/ml), the amount of amidase activity was 2.47 \(\mu\)mole/min/ml. The presence of BSA in the buffer used to dilute the stock kallikrein stabilized the amidase activity of the diluted kallikrein for at least 1 hr. The BSA in the buffer at this concentration (3 mg/ml) prevented surface adsorption of the kallikrein.\textsuperscript{19} With native plasma and kaolin (final concentration 1 mg/ml), only 39\% of the available plasma prekallikrein was measurable on the amidase assay. The partial measurement of the available prekallikrein must have been due to the presence of plasma inhibitors, since pretreatment of plasma with chloroform resulted in activation of 78\% of the available plasma prekallikrein, as calculated from the amidolytic activity of purified kallikrein when used in a concentration of 1 unit coagulant activity/ml. Moreover, reconstitution of prekallikrein-deficient plasma with purified prekallikrein (39 \(\mu\)g/ml), followed by CHCl\textsubscript{3} treatment and kaolin activation, lead to amidolytic measurement of 71\% of the available prekallikrein.

The dextran sulfate assay of Kluft\textsuperscript{14} when performed as written exhibited 76\% of the available plasma prekallikrein activation. Modifying this assay by decreasing the plasma dilution to 1:48 from 1:162 and increasing the substrate concentration to 1 mM from 0.16 mM, the same plasma prekallikrein activity as determined with the kaolin-activated assay was expressed. Moreover, in agreement with Kluft,\textsuperscript{14} the measured amidase activity with dextran sulfate as the
and decreasing the final plasma dilution 1:48.

Kallikrein used in the assay was equivalent to 1 coagulant unit/mL.

The concentration of purified prekallikrein used in the assay was performed as outlined in Fig. 1. The assay was performed as described in reference 14.

The mean prekallikrein antigen concentration in 19 normal individuals ranged from 29 to 44 μg/ml. There was no significant difference between the 10 male subjects (94.2 ± 4.0% of normal pooled plasma) and the 9 female subjects (97.8 ± 4.3%). The 21 women tested using birth control pills had a value of 107.8 ± 4.8%. The value was not significantly different from the value observed in the 9 normal female subjects. Seven patients with severe liver disease showed markedly decreased immunochemical levels of prekallikrein 40.1% ± 2.7% (p < 0.001).

Functional Prekallikrein Assay

The mean of 19 normal individuals was 2.35 ± 0.09 μmole TAME hydrolyzed/min/ml (83.7% ± 3.2% of normal pooled plasma) (Table 2). The actual amount of esterolytic activity measured in the 19 normal individuals ranged from 1.43 to 3.16 μmoles TAME hydrolyzed/min/ml. There was no difference between male (2.41 ± 0.14) and female (2.28 ± 0.11) subjects. The 21 women receiving oral contraceptives had elevated esterase activity 2.50 ± 0.12 μmoles TAME hydrolyzed/min/ml (89.2% ± 4.2% of normal pooled plasma), but this did not differ significantly from the normal women. Nevertheless, 8 of these 21 women exceeded 2 standard deviations of the mean of the normal group.

Functional Prekallikrein Amidolytic Assay

The mean for 19 normal individuals was 1.60 ± 0.1 (mean ± SEM) μmoles PPAN hydrolyzed/min/ml (83.3% ± 5% of normal pooled plasma) (Table 2). The actual amount of amidolytic activity measured in the 19 normals ranged from 0.98 to 2.21 μmoles PPAN hydrolyzed/min/ml. There was no difference between male (1.60 ± 0.16) and female (1.60 ± 0.16) subjects. The 21 women receiving birth control pills had a mean of 1.94 ± 0.7 μmoles PPAN hydrolyzed/min/ml (101% ± 3.9% of normal pooled plasma), which was significantly greater than the normal controls (p < 0.02) and the normal women (p < 0.05). The liver disease patients had markedly lower levels of prekallikrein amidolytic activity 0.58 ± 0.1 μmoles PPAN

Immunologic Concentration of Prekallikrein

The mean prekallikrein antigen concentration in normal individuals was 95.9% ± 2.9% (mean ± SEM) of normal pooled plasma (Table 2). The actual amount of prekallikrein antigen measured in these 19 normal individuals ranged from 29 to 44 μg/ml. There was no significant difference between the 10 male subjects (94.2 ± 4.0% of normal pooled plasma) and the 9 female subjects (97.8 ± 4.3%). The 21 women tested using birth control pills had a value of 107.8 ± 4.8%. The value was not significantly different from the value observed in the 9 normal female subjects. Seven patients with severe liver disease showed markedly decreased immunochemical levels of prekallikrein 40.1% ± 2.7% (p < 0.001).

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I, Correlation coefficient; TAMe, kallikrein esterolytic assay; PPAN, kallikrein amidolytic assay; NS, not significant.

![Graph](image_url)

**Fig. 3.** Correlation of amidolytic and immunologic prekallikrein determinations (data not shown). However, when the two enzymatic assays and the immunologic assays in any patient group were compared to the coagulation assay, there was no significant correlation of the coagulant assay with any of the other assays (Table 2). The absence of correlation, especially between the coagulant and immunologic assays, may be due to the large standard error of the coagulant assay (Table 2).

The amidolytic and immunoochemical assays of all three clinical groups are compared in Fig. 3. An excellent correlation ($r = 0.86, p < 0.001$) exists.

**DISCUSSION**

A functional assay described over 10 yr ago by this laboratory measures the hydrolysis of tosyl arginine methyl ester (TAMe) of plasma exposed to kaolin for 1 min. When the spontaneous activity (0 min) is subtracted, the difference was taken to represent functional prekallikrein. Recently, we have identified two variables (HMW kininogen, C1 inhibitor) that affect this assay. In both HMW-kininogen deficient and normal native plasma, the addition of 0.2 U/ml HMW kininogen increases the mean arginine esterase activity (kallikrein) 30%. Moreover, when the esterase activity is measured in HMW-kininogen deficient plasma after CHCl₃ treatment, the mean activity also rises 30%. However, in CHCl₃-treated normal plasma, the esterase activity does not rise after supplementation with HMW kininogen. The effect of HMW kininogen in normal concentrations to increase apparent kallikrein activity is due to its ability to protect kallikrein against plasma protease inhibitors. Thus, it appears that CHCl₃ treatment of normal plasma to inactivate inhib-
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The assays have also been compared in two patient groups. As reported previously,26 women on birth control pills have a somewhat higher plasma prekallikrein concentration than women not receiving this drug. This finding was confirmed in both the amidolytic and immunochemical assays when the treated women were compared with normal controls. However, when women receiving the birth control pills were compared with normal women, the means did not differ significantly (immunochemical) or were of borderline significance (amidolytic). The increase in prekallikrein concentration when it occurs is probably due to estrogen stimulation of hepatic synthesis, since an increase in antigen protein as well as functional activity was observed. Certain women seem to hyperrespond, since a number have levels well beyond two standard deviations higher than normal (Fig. 3). Conversely, the levels in liver disease are low, similar to previous studies from this2,12 and other laboratories. Since the percent of normal by the antigenic, (40%), amidolytic (31%), and coagulant assays (34%) all show proportional decreases, impaired liver synthesis is probably responsible, although increased turnover cannot be excluded at this time. Patients with other signs of decreased hepatic cell synthesis, such as low albumin and long prothrombin time, seem to have lower plasma prekallikrein concentrations than those with normal prothrombin times.26

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