1623

KINETIC ANALYSES OF THE ACTIVATION OF GLU-PLASMINOGEN BY TISSUE PLASMINOGEN ACTIVATOR IN THE PRESENCE OF FIBRIN, FIBRINOGEN, FRAGMENTS D AND E. A. Takada(1), Y. Sugawara(1), Y. Watahiki(2) and Y. Takada(1). Departments of Physiology (1) and Surgery (2), Hamamatsu University, Sch. Med., Hamamatsu-shi, Shizuoka, Japan

Kinetic analyses were made for the activation of Glu-plg (Glu-plasminogen) by tissue plasminogen activator (t-PA: two chain t-PA obtained from Bowes melanoma cell line) in the presence of fibrin, fibrinogen, fragment D or E. Glu-plg was activated by t-PA in the presence of various concentrations of fibrinogen, fibrin, fragment D or E, and the activation rate was measured by the hydrolysis of S-2251 with plasmin generated. Straight Line-weaver-Burk plots were obtained by changing the concentration of Glu-plg. In the absence of fibrin, t-PA activated Glu-plg with Km of 1.11 μM, k_{cat} of 0.005 sec^{-1}, k_{cat}/Km of 0.0045 $\mu M^{-1} \cdot sec^{-1}$. The presence of catalytic amounts (0.0002 μM) of fibrin resulted in 11.4 fold decrease in Km, thus 15.6 fold increase in k_{cat}/Km. The presence of larger amounts of fibrin resulted in increase in k_{cat} and decrease in Km, which resulted in 142 fold increase in k_{cat}/Km. There seems to be two phases in the enhancement of the activation of Glu-plg by t-PA, one induced in the presence of catalytic amounts of fibrin, and the other induced in the presence of higher concentration of fibrin (up to 0.05/0.2 of the molar ratio of fibrin to plasminogen). When effects of fragment D or E were examined for the enhanced activation of Glu-plg by t-PA, the presence of increasing amounts of E resulted in increase in k_{cat}, but the presence of D resulted in increase in k_{cat} and decrease in Km. Since plasminogen was shown to bind to both D and E domains of fibrin, and t-PA was shown to bind to D domain, we hypothesize that the role of D in the activation of Glu-plg by t-PA was to provide a site to t-PA and plasminogen to form a ternary complex, which facillitates the activation of plasminogen by t-PA, and that the role of E was to provide a site to bind to plasminogen, where t-PA activates plasminogen more effectively. The results suggest that roles of D and E domains of fibrin may be different in the enhanced activation of plasminogen by t-PA.

1624

SENSITIVE AND QUANTITATIVE ASSAYS OF FUNCTIONALLY ACTIVE PLASMINOGEN ACTIVATORS IN PLASMA. M. Mahmoud and P.J. Gaffney. National Institute for Biological Standards and Control, London, U.K.

A previously described (Mahmoud, M. and Gaffney, P.J., Thrombos.Haemostas. 53: 356-9, 1985) bioimmunoassay for tissue plasminogen activator (t-PA) has been modified in terms of catcher antibody concentration and conditions of incubation of various of the steps in this assay to achieve a sensitivity range of 5 - 500 x 10^{-3} iu/ml of t-PA. A similar assay has been developed for urokinase (UK) achieving a sensitivity range of 5-500 x 10^{-4} iu/ml. The sensitive t-PA assay has been demonstrated to be a valuable replacement of the traditional euglobulin clot lysis time (ECLT) assay and has the added advantage of avoiding loss of t-PA during formation of a euglobulin fraction and of calibration in terms of an International Standard for t-PA. Using both these assays it has been demonstrated that the enhanced fibrinolytic activity observed following exercise is due to increased levels of t-PA while the level of UK in plasma remains unaltered. This was further confirmed by quenching experiments using specific antibodies to t-PA and UK. In contradiction to an earlier report from this laboratory, free t-PA (10 - 60 x 10^{-3} iu/ml) has been demonstrated in resting plasma, while the level of free UK activity was 50 - 100 x 10^{-3}.

These sensitive t-PA and UK assays suggest that physiological and drug induced (heparin fraction CY 222 from Laboratoire Choay, Paris, and SP54 from Benechemie, Munich) enhancement of fibrinolytic potential in the human circulation is mainly mediated by t-PA. Venous occlusion of matched groups of subjects with and without deep venous thrombosis (DVT) showed a wide variation in response in t-PA levels in each group and no change in u-PA levels. There was no difference between the DVT and non DVT groups and this suggests that the notion of the 'non-responder' being more prone to thrombosis needs re-examination. Further experimentation with these sensitive assays may help to elaborate whether the levels of UK in plasma play any role in the haemostatic balance in man.

1625

EFFECT OF pH DURING PREPARATION OF EUGLOBULIN PRECIPITATES ON t-PA OR UK ASSAY IN PLASMA. O. Kunitomi (1), E. Kakishita (1), K. Nagai (1), and O. Matsuo (2). Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya 663, Japan (1), and Department of Physiology, Kinki University School of Medicine, Sayama, Osaka 589, Japan (2).

A more sensitive method for the commonly used euglobulin method is proposed for examining plasminogen activators (PAs) in blood, since little attention has been paid to the conditions of production of euglobulin precipitates, especially the pH during their preparation. In the present study, the effect of pH on measurement of the activity of tissue-type plasminogen activator (t-PA) or urokinase (UK) in plasma was investigated and compared by enzymography, bio-immunoassay (BIA) and the fibrin plate method. The antigenicity of PAs was observed as follows. Polyclonal anti-t-PA or UK rabbit IgG previously purified with aprotinin-Sepharose to remove protease activity in the IgG fraction (final concentration, 0.375 mg/ml of 0.01% Triton X-100) was reacted with euglobulin precipitate at 4°C for 1 hr. After incubation, enzymography was preformed, and the residual fibrinolytic activity was measured qualitatively. Euglobulin precipitates were prepared by dilution (1:20) of fresh venous occlusion plasma obtained from healthy volunteers (n = 5) with ice cold, distilled water, and adjusted to pH 4.5 - 7.5 with 0.1% (v/v) acetic acid. The euglobulin precipitates were collected by centrifuge at 4°C, and dissolved in Veronal buffer (0.1 M NaCl, 0.05 M barbital Na, pH 7.75) to the original volume. The PA activities in these solutions were examined by the above three methods. The fibrinolytic activities between pH 5.0 and 6.8 were found to be almost the same (no statistical difference) by the fibrin plate method. However, a stronger t-PA activity as determined from the molecular weight as well as antigenicity, was detected at pH 6.7 by enzymography and BIA. On the other hand, a stronger UK activity as determined from the molecular weight and antigenicity was recognized at pH 5.6 by enzymography. The present results suggest, therefore, that it is necessary to consider the effect of pH during the preparation of euglobulin precipitates for the measurement of t-PA or UK assay in plasma. The suitable pH value for t-PA and UK assays in plasma is 6.7 and 5.6, respectively.

1626

SENSITIVE AND SPECIFIC ENZYME-LINKED IMMUNOSORBENT ASSAY FOR UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN HUMAN PLASMA. J. Grøndahl-HANSEN, N. Agerlin, L.S. Nielsen and K. Danø. The Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, DK-2100 Copenhagen Ø. Denmark.

An enzyme-linked immunosorbent assay (ELISA) was developed for the measurement of human urokinase-type plasminogen activator (u-PA) in plasma and serum. Microtiter plates were coated with a monoclonal antibody and incubated with standard or sample. Bound u-PA was quantitated with polyclonal antibodies conjugated with biotin, followed by avidin-peroxidase. The assay was 10-fold as sensitive as other previously reported ELISAs, the detection limit being approximately 1 pg of u-PA in a volume of 100 µl with a linear dose-response up to 15 pg of u-PA. The assay detected active u-PA and its inactive proenzyme form equally well and the recovery of both forms was higher than 90% in plasma. A variety of structurally related proteins, including t-PA, were tested, but no reaction with proteins other than u-PA and its amino-terminal degradation product were observed. The intra-assay and inter-assay coefficients of variation for determination of u-PA in plasma were 7.6% and 8.4%, respectively. The assay was equally applicable to serum. The values obtained with plasma and serum were similar, and the results were not affected by small variations in the preparation of the samples. The ELISA was used to measure the concentration of u-PA in plasma from 34 healthy donors. The mean values for u-PA in plasma from healthy donors was 1.1 ng/ml $\pm$ 0.3 ng/ml (SD) (range 0.6 - 1.5 ng/ml). No significant differences were found between men and women and no correlation between u-PA concentration and age could be demonstrated. The mean u-PA concentration in plasma from healthy donors obtained in this study is substantially lower than that reported by others. This might be due to different methods of determination of the protein content of the standard preparations or to differences in the specificity of the assays.