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COMBINATION OF HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY AND SPECIFIC DETECTION OF PROENZYME APPLICABLE TO THE ANALYSIS OF THE FIBRINOLYTIC SYSTEM OF HUMAN PLASMA

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SUMMARY

A procedure for the analysis of the fibrinolytic system in human blood was devised by combining high-performance affinity chromatography (HPAC) and specific detection of proenzyme. Components of the fibrinolytic system were separated by HPAC using Asahipak GS gel coupled with *p*-aminobenzamidine, and they were specifically detected by means of an on-line enzyme assay system. This system made it possible to quantitate not only Glu-plasminogen (Glu-Plg) but also Lys-plasminogen (Lys-Plg) in human plasma in a short time without pre-treatment. The effect of urokinase on the state of components of the fibrinolytic system in blood was studied. It was clearly shown that Lys-Plg is more susceptible to activation by urokinase than Glu-Plg (both *in vitro* and *in vivo*).

INTRODUCTION

One of the important applications of affinity chromatography is the analysis of biological molecules related to disease states. We have already reported the high-performance affinity chromatography (HPAC) of trypsin-family proteases using microparticulate polymer gels such as Toyopearl[1] and Asahipak GS gel[2,3]. In order to develop this procedure into a new clinical analysis system, it was essential to devise a sensitive, rapid and specific detection system. For enzymes, specific detection is possible by the use of an appropriate substrate. However, it was extremely difficult to analyse proenzymes in blood specifically because of the lack of enzyme activity. The problem was solved by developing a new analytical system[4].

The state of plasminogen (Plg), a proenzyme of plasmin which is a major fibrinolytic enzyme[5], is an important index of diseases caused by disorders in the

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fibrinolytic system. It has been very difficult to analyse its subspecies, Glu-plasminogen (Glu-Plg) and Lys-plasminogen (Lys-Plg), specifically, in plasma in a short time. As Lys-Plg is more susceptible than Glu-Plg to activation by plasminogen activators, information on the state of Lys-Plg is required for more reliable diagnosis. We devised a specific detection system for plasminogens and combined it with affinity chromatography[4]. In brief, plasminogens in the column effluent were activated by urokinase and the activity of the plasmin thus formed was monitored by using a fluorogenic substrate. Application of a small volume (40 μl) of plasma without any pre-treatment allowed the detection of plasminogens and plasmin in less than 1 h. In this work, the state of components of the fibrinolytic system was studied by the use of the new analytical system.

EXPERIMENTAL

Materials

Asahipak GS-520 gel (exclusion limit molecular weight $3 \cdot 10^5$; particle size 9 $\pm$ 0.5 μm) was a product of Asahi Chemical Industry (Tokyo, Japan). 7-(*tert.*-Butyloxycarbonyl-L-glutamyl-L-lysyl-L-lysineamido)-4-methylcoumarin (Boc-Glu-Lys-Lys-AMC) was a product of the Protein Research Foundation (Osaka, Japan).

Preparation of affinity adsorbent (Asahipak GS-520–AHA–ABA)

The activation of Asahipak GS gel with 1,1′-carbonyldiimidazole (CDI) and the coupling of spacer and ligand were carried out by methods similar to those reported elsewhere[3,4]. In brief, Asahipak GS gel was suspended in dry acetone and activation with CDI was carried out for 15 min at room temperature. The activated gel was treated with 6-aminohexanoic acid (AHA) overnight at 4°C and pH 10, and *p*-aminobenzamidine (ABA) was immobilized as a ligand on the carboxyl group by using water-soluble carbodiimide. The product contained 347 μmol of AHA and 96 μmol of ABA per gram of dry gel.

Procedures for chromatography and specific detection of plasmin and plasminogens

Chromatography and specific detection were carried out by using a system described elsewhere[4].

Asahipak GS-520–AHA–ABA was suspended in 0.2 *M* sodium sulphate solution and packed in a stainless-steel column (100 $\times$ 6 mm I.D.). The final flow-rate was 1.8 ml/min. The following eluents were used[3]: eluent 0, 0.05 *M* sodium phosphate (pH 6.5); eluent 1, 0.05 *M* sodium phosphate–0.1 *M* sodium chloride (pH 7.4). Other eluents contained the components described below in addition to those of eluent 1. Eluent 2, 0.02 *M* AHA; eluent 3, 0.02 *M* AHA + 3 *M* urea. Eluent 0 was used for the adsorption of Glu-Plg, Lys-Plg and plasmin. When eluent 1 was used as the initial eluent, Glu-Plg was eluted immediately after pass-through materials and Lys-Plg and plasmin were trapped by the column. Lys-Plg was eluted with eluent 2. Plasmin was eluted with eluent 3. The eluents were changed by the use of an eight-way connector, and were pumped at a flow-rate of 1 ml/min.

The column effluent was first passed through an RF-530 fluorescence spectrophotometer (Shimadzu, Kyoto, Japan) with excitation at 285 nm and emission at 340 nm to detect protein. A portion of the effluent was sampled at a point just after

the protein monitor by a peristaltic pump, which simultaneously pumped a urokinase solution and a substrate solution. The following urokinase solutions were used: urokinase solutions 1 and 1′ (for the activation of Glu-Plg), 500 and 50 U/ml, respectively, in eluent 2; urokinase solutions 2 and 2′ (for the activation of Lys-Plg), 100 and 50 U/ml in eluent 1. As the addition of AHA greatly enhanced the activation of Glu-Plg, Glu-Plg was activated by urokinase dissolved in eluent 2. The substrate solution consisted of 20 μM Boc-Glu-Lys-Lys-AMC in 0.5 M sodium phosphate buffer (pH 7.4). The effluent and the urokinase solution were mixed just after the peristaltic pump and passed through an activation coil (400 × 0.25 mm I.D.) immersed in a water-bath at 37°C, where plasminogens were converted into plasmin. After the activation, the substrate solution was mixed with the plasmin solution and the enzymatic reaction was allowed to proceed in an enzyme reaction coil (400 × 0.25 mm I.D., 37°C). 7-Amino-4-methylcoumarin (AMC) produced by the active plasmin was detected with an FD-110C fluorescence spectrophotometer (JASCO, Tokyo, Japan) with excitation at 365 nm and emission at 460 nm. The time periods required for the activation and the enzyme reaction were *ca.* 1.2 and 1.7 min, respectively.

RESULTS

Effect of urokinase on the state of the components of fibrinolysis (in vitro)

A small volume of plasma from a healthy person without any pre-treatment (40 μl) was injected into the column. Although, under the conditions employed, the Glu-Plg peak overlapped completely with large amounts of other proteins, our detection system demonstrated its presence clearly (Fig. 1A). Almost all activity was observed at the position of Glu-Plg (at 4 min) and a small but distinct peak of activity

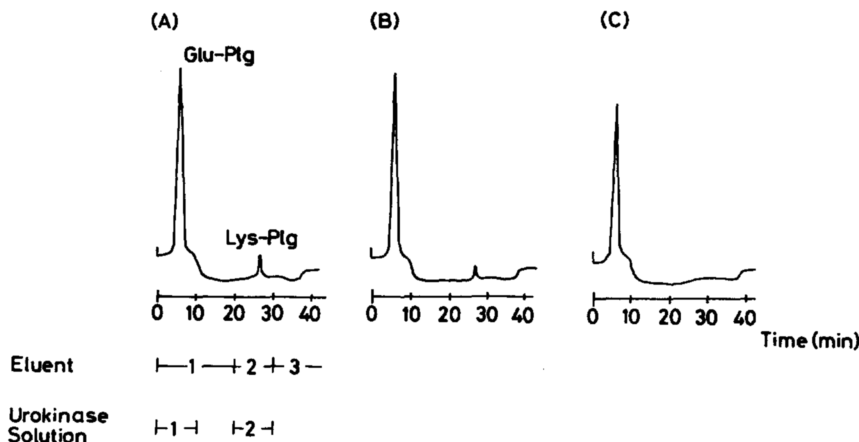

Fig. 1. Effect of urokinase (*in vitro*). Samples were injected into the column (Asahipak GS-520–AHA–ABA) equilibrated with eluent 1 (time 0). The eluents and urokinase solutions were changed as indicated in A. The compositions of the eluents and urokinase solutions are described under Experimental. (A) Normal plasma (40 μl). (B) Urokinase (9 U) was added to the same plasma as A, and the mixture was incubated for 3 min at 37°C. The activation mixture was immediately applied to the column (time 0). (C) The mixture was incubated for 9 min and was applied to the column. Detection of protein is omitted.

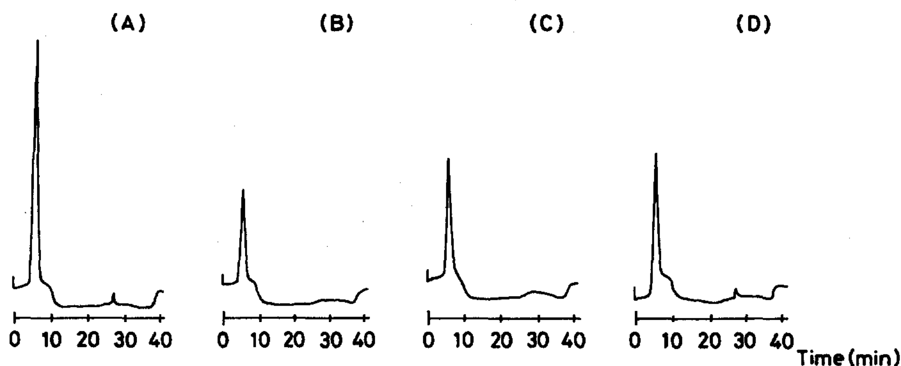

Fig. 2. Effect of urokinase (*in vivo*). Plasma samples from a patient with an acute myocardial infarction were injected into the column (Asahipak GS-520–AHA–ABA), equilibrated with eluent 1. The eluents and urokinase solutions were changed, as indicated in Fig. 1A. (A) Before urokinase treatment; (B) 3 h after urokinase treatment; (C) 9 h after urokinase treatment; (D) 72 h after urokinase treatment. Detection of protein is omitted.

corresponding to Lys-Plg (at 24 min) was observed. On the other hand, active plasmin, which should have been eluted at about 35 min if it existed in the free form, was not detected. The plateau observed at 35 min was solely due to elevation of the baseline.

Urokinase (9 U) was added to the same plasma sample and the mixture was incubated at 37°C and applied to the column. Fig. 1B is the chromatogram of the plasma sample treated with urokinase for 3 min. Although the content of Glu-Plg seemed to be unchanged, the peak of Lys-Plg decreased slightly. After incubation for 9 min the peak of Lys-Plg disappeared and a significant decrease in Glu-Plg was also observed (Fig. 1C). This was consistent with the observation that Lys-Plg can be activated much more easily than Glu-Plg by plasminogen activators[6]. In the two chromatograms in Fig. 1B and C the plasmin which should have been formed by urokinase treatment could not be detected. This shows that the active plasmin was immediately trapped by inhibitors, such as α_2-plasmin inhibitor.

Hence the proposed system proved to be useful in various studies for observing directly and immediately the state of the components of the fibrinolytic system.

Effect of urokinase (in vivo)

Plasma samples from a patient who had suffered an acute myocardial infarction and had been treated with urokinase (400 000 U injected *in vivo*) were analysed (Fig. 2).

The change in the amount of Glu-Plg was consistent with that measured by the conventional method, using streptokinase and a chromogenic substrate[7].

Analysis of plasma samples from a patient suffering from disseminated intravascular coagulation

Fig. 3 shows the change in the chromatographic pattern of plasma samples from a patient suffering from disseminated intravascular coagulation (DIC). In this series, the chromatographic conditions were set so that both Glu-Plg and Lys-Plg

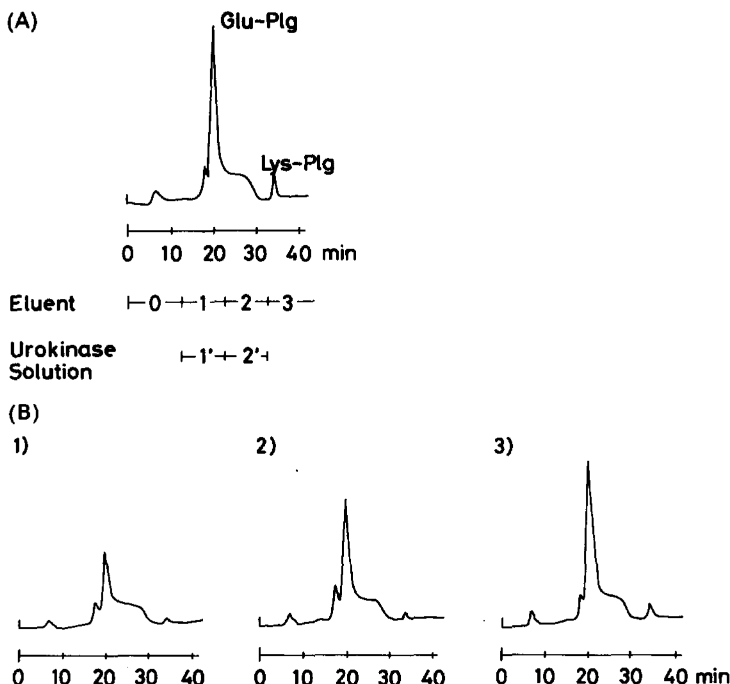

Fig. 3. Analysis of plasma from a patient with DIC on Asahipak GS-520–AHA–ABA. Plasma was injected into the column equilibrated with eluent 0. The eluents and urokinase solutions were changed as indicated in A. (A) Normal plasma (40 μl). (B) Plasma from a patient of disseminated intravascular coagulation (40 μl); (1) September 11th; (2) September 14th; (3) September 24th. Detection of protein is omitted.

were adsorbed, *i.e.*, eluent 0 [0.05 *M* sodium phosphate (pH 6.5)] was used as the initial eluent. The change in the content of Glu-Plg coincided with the recovery of the patient. On September 11th [Fig. 3B (1)], the condition of the patient was poor, and this was reflected in the very low Glu-Plg content. As the patient improved (September 24th), the Glu-Plg content became normal [Fig. 3B (3)].

DISCUSSION

As was shown in our previous study[4], the system described proved to be extremely effective for the direct analysis of plasminogens in human blood. It was directly and clearly observed that Lys-Plg in human plasma could be activated by plasminogen activators much more easily than Glu-Plg and that active plasmin was immediately trapped by plasmin inhibitors. As the conventional method for quantitation of plasminogen, which uses streptokinase and chromogenic substrates, can only give the total amount of plasminogen[7], it is extremely difficult to obtain the same information as that obtained in this work. As a very small amount of Lys-Plg was detected in human plasma, it was possible to monitor the behaviour of Lys-Plg directly. Hence new information about the fibrinolytic system can be obtained and the diagnosis of fibrinolytic diseases is much improved.

Observations on plasma from a patient with DIC were consistent with incomplete data obtained by a conventional assay method (data not shown), which was unable to provide information about Lys-Plg. Although we have applied the proposed method only to the fibrinolytic system in this work, this method will be applicable to other important regulatory systems in the blood, such as those of the coagulation system and kinin formation; we have only to devise appropriate conditions for activation and detection. Such systems would be very useful for both basic biochemical studies and medical applications.

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