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ANALYSIS OF HUMAN GLUTAMYL- AND LYSYLPLASMINOGEN BY HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

NAOFUMI ITO* and KOHJI NOGUCHI

Gel Separation Development Department, Asahi Chemical Industry Co., 1-3-2, Yako, Kawasaki-ku, Kawasaki 210 (Japan)

MUTSUYOSHI KAZAMA

School of Medicine, Teikyo University, Itabashi, Tokyo 173 (Japan)

and

KEN-ICHI KASAI

Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01 (Japan)

SUMMARY

An analytical system for the fibrinolytic system in the blood is described, based on high-performance affinity chromatography and a newly devised specific proenzyme detection procedure. Plasminogen subspecies in human plasma without any pretreatment (less than 100 μl) were specifically separated on an high-performance affinity column. For detection they were continuously activated by urokinase, and their elution profile was determined by assay of the plasmin activity using a specific fluorogenic substrate. Preliminary data on plasma from a patient are presented.

INTRODUCTION

The fibrinolytic system is composed of a network of various precursors, activators and inhibitors. The state of plasminogen (Plg), a proenzyme of plasmin, a major fibrinolytic enzyme[1], is an important index in the diagnosis of fibrinolytic diseases. However, it has been extremely difficult to determine specifically and rapidly its subspecies, glutamylplasminogen (Glu-Plg) and lysylplasminogen (Lys-Plg), in plasma because they lack enzymatic activity. Since Lys-Plg can be activated much more easily than Glu-Plg by plasminogen activators[2], its plasma concentration seems to have greater significance in the function of the fibrinolytic system.

We have developed a new analytical method for both Lys-Plg and Glu-Plg by the use of high-performance affinity chromatography (HPAC)[3–5] in combination with a specific detection system for the proenzyme[6,7]. A small volume of human plasma (less than 100 μl) was applied to a column of affinity adsorbent (*p*-aminobenzamidine, coupled to Asahipak GS gel) without any pretreatment. Plasminogen subspecies were separately eluted and detected by using a specific and sensitive fluorogenic substrate.

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Since the presence of Lys-Plg in human plasma has clearly been demonstrated[7], the application of this system to clinical analysis has become feasible. The detection method in the prototype system was inadequate. In the present work, considerable improvement was effected. The peristaltic pump, which had been used for sampling of the effluent and delivery of urokinase and substrate, was replaced with an high-performance liquid chromatography (HPLC) pump. As more stable and reproducible chromatograms were obtained, the peak areas were quantified by an integrator. The relationships between the concentrations of Glu-Plg and Lys-Plg in plasma and disease are now more amenable to analysis.

EXPERIMENTAL

Materials

Asahipak GS-520 gel (exclusion limit molecular weight, $3 \cdot 10^5$; particle size, 9 ± 0.5 μm) is a product of Asahi Chemical Industry Co. (Tokyo, Japan), urokinase for therapeutic use (60 000 units per vial) was obtained from the Green Cross (Osaka, Japan) and 7-(*tert.*-butyloxycarbonyl-L-glutamyl-L-lysyl-L-lysineamido)-4-methylcoumarin (Boc-Glu-Lys-Lys-AMC) from the Peptide Institute (Osaka, Japan).

Affinity adsorbent (Asahipak GS520-AHA-ABA)

Asahipak GS520-AHA-ABA used as an affinity adsorbent was prepared as reported previously[6]. It contained 347 μmol of 6-aminohexanoic acid (AHA) as a spacer and 96 μmol of *p*-aminobenzamidine (ABA) as a ligand, per gram of dry gel.

Features of the improved system and chromatography

Chromatography and specific detection were carried out by using the system shown schematically in Fig. 1. Asahipak GS520-AHA-ABA was suspended in 0.2 *M* sodium sulphate solution and packed into a stainless-steel column (100 mm × 6 mm I.D.). The following eluents were used: eluent 1, 0.05 *M* sodium phosphate–0.1 *M* sodium chloride–0.4 *M* glycine (pH 7.4); eluent 2, 0.05 *M* sodium phosphate–0.1 *M* sodium chloride–0.02 *M* AHA. The glycine in eluent 1 had the effect of stabilizing the baseline. When eluent 1 was used as an initial eluent, Glu-Plg was eluted immediately after the void volume, while Lys-Plg and active plasmin were retained by

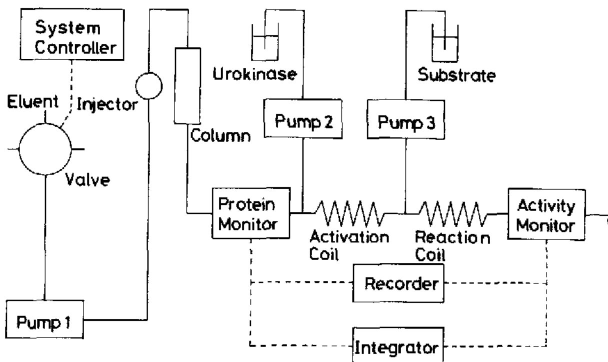

Fig. 1. Schematic diagram of the chromatographic system.

the column. Lys-Plg was eluted with eluent 2. Active plasmin was eluted with an eluent containing 3 *M* urea in addition to the components of eluent 2[4]. It is usually trapped by inhibitors in blood, and is therefore difficult to detect by assay of the plasmin activity. The eluents were changed by the use of a low-pressure valve (FCV-3AL; Shimadzu, Kyoto, Japan), controlled by a system controller (SCL-6A, Shimadzu). They were pumped at a flow-rate of 1 ml/min by pump 1 (LC-6A, Shimadzu). The column effluent was first passed through a fluorescence spectrophotometer (Shimadzu RF-530, flow-cell volume 12 μl, excitation at 285 nm, emission at 340 nm; indicated as a protein monitor in the figure) to detect protein.

The improvements in the specific detection system for plasminogens were made after this step. The entire effluent was introduced into the specific detection system instead of sampling only one-tenth volume of the effluent, as had been done previously. Just after the protein monitor, an urokinase solution (500 U/ml in eluent 2) was added to the effluent at a flow-rate of 0.4 ml/min by pump 2 (LC-6A, Shimadzu). The solutions were mixed and passed through an activation coil (10 m $\times$ 0.5 mm I.D.) immersed in a water-bath at 37°C, where the plasminogens were converted into plasmin. Since addition of AHA greatly enhanced the activation of Glu-Plg[2], AHA was present in the urokinase solution. When AHA was absent, Glu-Plg could scarcely be detected while the same amount of Lys-Plg could be clearly detected. After activation, the substrate solution was pumped into the activated plasmin solution at a flow-rate of 0.5 ml/min by pump 3 and the enzymatic reaction was allowed to proceed in a reaction coil (33 m $\times$ 0.5 mm I.D.) at 37°C. The substrate solution comprised 100 μM Boc-Glu-Lys-Lys-AMC in 0.05 *M* sodium phosphate buffer (pH 7.4). 7-Amino-4-methylcoumarin (AMC) produced by the active plasmin was detected with another fluorescence spectrophotometer (Shimadzu activity monitor RF-530, excitation at 365 nm, emission at 460 nm). The times required for the activation and the enzyme reaction were *ca.* 1.4 and 3.4 min, respectively. The outputs from the two monitors were recorded on a two-channel recorder and processed by an integrator (SIC-7000A; System Instruments, Kyoto, Japan).

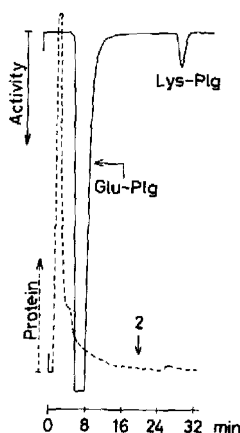

Fig. 2. Chromatogram of plasma from a healthy person. Plasma (90 μl) was injected into the column (Asahipak GS520-AHA-ABA) equilibrated with eluent 1 (time 0). The eluent was changed as indicated by the arrow numbered 2. The output from the activity monitor is shown by the solid line; the dotted line indicates the output from the protein monitor. For details, see Experimental section.

RESULTS AND DISCUSSION

The chromatogram of the plasminogens, produced by the specific detection system, was greatly improved as shown in Fig. 2 (see solid line). Plasma from a healthy person (90 μl) was injected into a column without any pretreatment. A flat baseline and sharp peaks were obtained. More than 300 injections of plasma (usually 90 μl per injection) could be made on a single column. As we have previously shown[7], the main peak (8.5 min) corresponds to Glu-Plg and the minor peak (30.8 min), eluted with eluent 2 (containing AHA), corresponds to Lys-Plg. The total time between injections was about 1 h including the time for equilibration of the column. Peak areas processed by the use of the integrator are shown in Table I. Since the coefficients of variation for peak height had been 38% for Glu-Plg and 45% for Lys-Plg (n = 7) in the previous system (detailed data not shown), the reproducibility in the new system was greatly improved.

These values do not correspond directly to the absolute amount of plasminogens, because Lys-Plg can be activated 5–15 times faster than Glu-Plg[2], and consequently gives a much higher value per amount of protein. The amount of Lys-Plg was roughly estimated as 100 ng per ml plasma[7] based on a comparison with an authentic sample of Lys-Plg in electrophoresis. Since the accuracy of the procedure was improved, it should be possible to study the relationships between the content of plasminogen subspecies in plasma and disease.

For example, plasma samples from a patient suffering from acute myelocytic leukaemia were analyzed. The levels of both Glu-Plg and Lys-Plg were low compared with those of an healthy person. On September 9th, the complication of disseminated intravascular coagulation syndrome was observed. It might be important that the decrease in Lys-Plg on that day was larger than that in Glu-Plg. These analytical data (not shown) were reproducible. Thus, considerable improvement was attained by the present system: more accurate information about plasminogens in plasma is now obtainable from a single and rapid experiment.

TABLE I

PEAK AREAS OF Glu-Plg AND Lys-Plg IN PLASMA FROM AN HEALTHY PERSON

Plasma (90 μl) was injected into the column. The chromatogram obtained is shown in Fig. 2. Outputs from the activity monitor are given. Analysis was carried out twice on days 1 and 4.

Day of analysis	*Glu-Plg*	*Lys-Plg*
1	3766528	45055
	3704074	45307
2	4242796	60368
3	4305271	50789
4	4147810	50214
	4113932	52932
5	4008515	62398
Average (n = 7)	4041275	52438
S.D. (n = 7)	212870	6272

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