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# The Effect of Dilution, pH and Ionic Strength of Plasma on t-PA Precipitation in Euglobulin Fraction

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*In order to evaluate the influence of dilution, pH and ionic strength on the precipitation of t-PA and PAI-1 during euglobulin precipitation, we measured t-PA Ag, PAI-1 Ag and fibrinolytic activity in the euglobulin fraction made of pooled plasma from liver cirrhosis patients, under various conditions by changing pH, ionic strength and degree of dilution. The precipitation of t-PA Ag in the euglobulin fraction was enhanced by decreasing the ionic strength and greatest at pH 6.0. The fibrinolytic activity in the euglobulin fraction showed consistent changes with t-PA Ag under varying pH and ionic strength. The precipitation of t-PA Ag was not influenced by the dilution factor but the larger the dilution factor, the greater the PAI-1 and the smaller the fibrinolytic activity in the euglobulin fraction. PAI Ag in euglobulin fraction showed consistent changes with t-PA Ag in the euglobulin fraction regardless of the changes in ionic strength and pH. The amount of precipitation of t-PA and PAI-1 was increased by the presence of dextran sulfate, under varying pH, ionic strength and dilution conditions. Our results show theat the currently used conditions for standard euglobulin precipitation are the most favorable for t-PA precipitation into the euglobulin fraction. The fibrinolytic activity exerted in the euglobulin fraction seems to depend on the amout of t-PA-PAI-1 complex rather than minimized protease inhibitor in the euglobulin fraction.*

**Key Words:** *t-PA, PAI-1, Euglobulin fraction, Fibrinolytic activity*

## INTRODUCTION

The rationale for the study of fibrinolytic activity in the euglobulin fraction has been based on the fact that euglobulin contains a considerably smaller amount of fibrinolytic system inhibitors than does plasma, thus allowing the detection of fibrinolytic activity normally suppressed by the inhibitors in plasma. The standard procedure used currently was developed in the late fifties[1,2] and is based on studies of fraction conditions during euglobulin fractionation. The procedure resulting in the most active fibrinolytic activity in the euglobulin fraction as measured by the fibrin plate assay has been chosen as the standard procedure[1,2]. In the circulation there are many kinds of protease inhibitors which interfere with plasminogen activators; $\alpha_1$-antitrypsin[3], $\alpha_2$-macroglobulin[4], inter-$\alpha$-trypsin inhibitor[4], C1 inactivator[3] and antithrombin III[5]. Dilution, adjustment of pH, decreasing ionic strength at low temperatures have been accredited with minimizing these inhibitors in euglobulin fraction. Recently, many studies have established the presence of a variable concentration of rapid-acting inhibitor (PAI) of both t-PA and urokinase in the plasma[6~10]. It has been reported that PAI-1 is one of the key enzymes modulating fibrinolytic activity[11~14]. Kepping this in mind, it is necessary to reassess the effect of ionic strength, changes in pH and the dilution on the t-PA and PAI-1 precipitation into the euglobulin fraction.

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## MATERIALS AND METHODS

### 1. Reagents

**Barbital Buffers**: Buffer containing 0.05 M sodium diethylbarbiturate, 0.093 M NaCl, 1.66 mM $CaCl_2$ and 0.69 mM $MgCl_2$ was used to prepare the fibrin plates. The buffer was adjusted to pH 7.8 with HCl solution.

**Fibrinogen Solution**: Plasminogen-rich fibrinogen was obtained from SIGMA. Bovine thrombin (SIGMA), 5000 units, was dissolved in 250 ml of saline (0.15 M NaCl).

**Pooled Plasma of Liver Cirrhosis Patients**: For this study, the euglobulin fraction was made using pooled plasma from liver cirrhosis patients instead of normal pooled plasma, in the hopes that the higher t-PA or PAI-1 concentration in liver cirrhosis patients would make it easier to discern the differences in t-PA or PAI-1, precipitated in euglobulin fraction, by the modification of pH, dilution and pH. t-PA (15 ng/ml vs 10 ng/ml) and PAI-1 (45 ng/ml vs 25 ng/ml) levels were higher in liver cirrhosis patients than in normal pooled plasma. FDP was negative in the pooled plasma from the liver cirrhosis patients.

**Fibrinolytic Activity**: The fibrinolytic activity of the euglobulin fractions was measured by the fibrin plate method.

**Preparation of Fibrin Plate**: A fibrinogen solution with a final fibrinogen concentraton of 0.1% (w/v) and ionic strength of 0.15 was prepared. 6 ml of this solution was pipetted into petri dishes and after mixing with 0.2 ml thrombin solution (20 NIH units/ml), allowed to stand for at least 30 min on a carefully leveled surface at room temperature.

### 2. Preparation of Euglobulin Fraction

To make te standard dextran sulfate euglobulin fraction, plasma (0.5 ml) was diluted with 4.0 ml of cold distilled water and 0.5 ml of dextran sulfate solution was added. The solution was then mixed, placed on ice and with a constant stirring motion, the pH was adjusted to 5.9 (5.85-5.9) with an acetic acid solution. It was left to stand on ice for 30-60 minutes, then centrifuged at 2,000 g for 10 minutes. The precipitate was dissolved in a 0.5 ml saline barbital buffer. After incubation of the plates at 37 °C for 18 hr on carefully leveled shelves in an incubator, two perpendicular diameters of each lysed zone were determined. The mean of the diameters was taken as the diamter of the zone.

### 3. Modification of Standard Euglobulin

**Modifications of the dilution factor, pH and ionic strength were obtained as follows**: Dilution of plasma at ratio of 1:5, 1:10, 1:15 were obtained using cold distilled water and pH values ranging from 5.

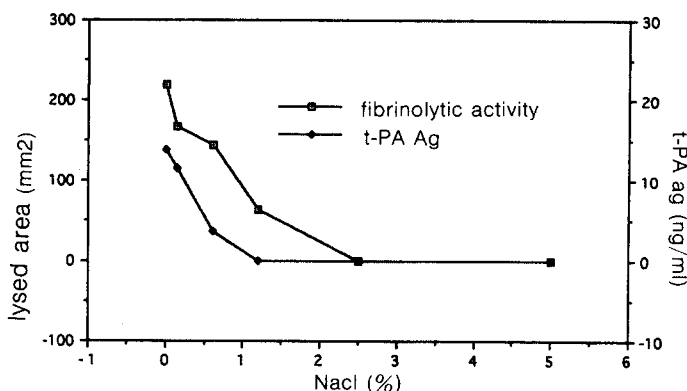


Fig. 1. The effect of ionic strength on the relationship between fibrinolytic activity and t-PA Ag in the euglobulin fraction. The dextran sulfate euglobulin fraction prepared at a constant pH (5.8-6.0) and 1:10 dilution. Variations in ionic strength is recorded on the abscissa as final concentration of added NaCl in 1:10 diluted plasma. t-PA Ag and fibrinolytic activity show a consistent change at low ionic concentration state.

0 to 9.0 were obtained using acetic acid and NaOH. Variation in ionic strength was obtained by addition of appropriate amounts of sodium chloride solution. All of the conditions were duplicated with or without dextran sulfate. The euglobulin fractions and supernatant from the euglobulin precipitation, obtained under the above conditions, were then subjected to t-PA antigen and PAI-1 antigen.

**t-PA antigen**: Commercially available enzyme-linked immunosorbent assay kit (ASSERACHROM t-PA of Diagnostica Stago) was used to determine t-PA antigan.

**PAI-1 antigen**: Commercially available enzyme-linked immunosorbent assay kit (IMUBIND PAI-1 ELISA kit, product 822/5, American Diagnostica inc.) was used to determine PAI-1 antigen.

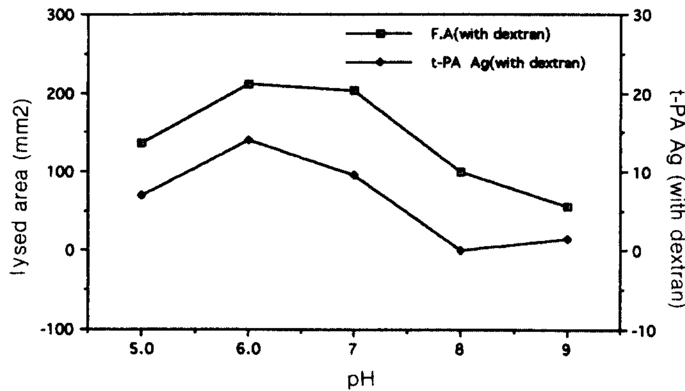


**Fig. 2.** The effect of pH on the relationship between fibrinolytic activity and t-PA Ag in euglobulin fraction. The dextran sulfate euglobulin fraction prepared at a constant ionic strength (0.15) and 1:10 dilution. Variation in pH is recorded on the abscissa. t-PA Ag and fibrinolytic activity sow a consistent change.

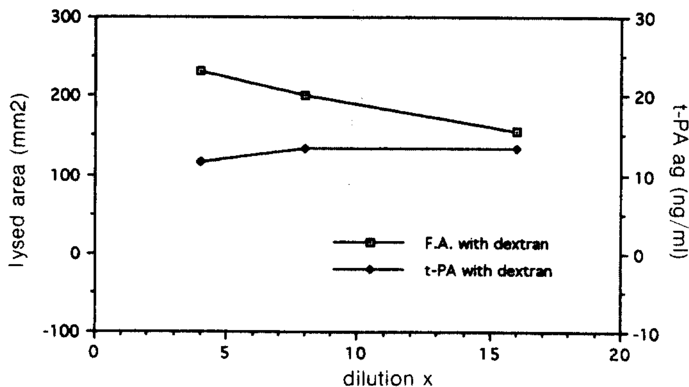


**Fig. 3.** The effect of dilution on the relationship between fibrinolytic activity and t-PA Ag in euglobulin fraction. The dextran sulfate euglobulin fraction prepared at a constant ionic strength (0. 15) and at pH 5.8-6.0. Variations in dilution is recorded on the abscissa. t-PA Ag show no significant change but the fibrinolytic activity decreases as the dilution factor increases.

## RESULTS

The precipitation of t-PA in the euglobulin fraction was greatest at a pH of 6.0 and enhanced by decreasing the ionic strength (Fig. 1, 2). The fibrinolytic activity in the euglobulin fraction showed consistent changes with t-PA Ag.

Although the precipitation of t-PA Ag in the euglobulin fraction was not influenced by the

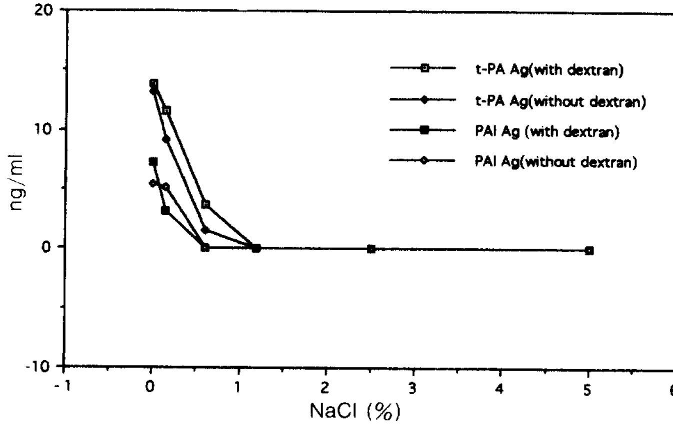


**Fig. 4.** The effect of ionic strength on the relationship between PAI-1 Ag and t-PA Ag in the euglobulin fraction with or without dextran sulfate. Euglobulin fraction prepared at a constant pH (5.8-6.0) and 10 times dilution with or without dextran sulfate. Variations in ionic strength is recorded on the abscissa as final concentration of added NaCl in 1:10 diluted plasma. t-PA Ag, PAI-1 Ag, with or without dextran sulfate show a consistent change at low ionic concentration state.

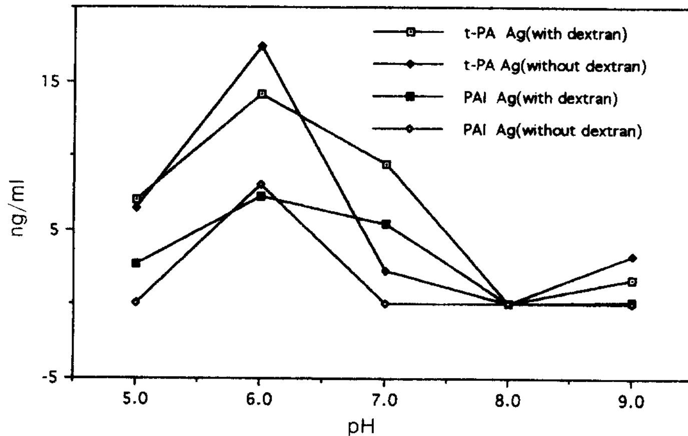


**Fig. 5.** The effect of pH on the relationship between PAI-1 Ag and t-PA Ag in euglobulin fraction with or without dextran sulfate. Euglobulin fraction prepared at a constant ionic strength (0. 15) and 1:10 dilution with or without dextran sulfate. Variation in pH is recorded on the abscissa. t-PA Ag, PAI-1 Ag, with or without dextran sulfate, show a consistent change.

dilution factor, there was an inverse relationship between the dilution factor and fibrinolytic activity in the euglobulin fraction; the greater the dilution factor, the smaller the fibrinolytic activity in euglobulin fraction (Fig. 3).

The precipitation of PAI Ag in the euglobulin fraction was greatest at pH 6.0 and enhanced by decreasing the ionic strength (Fig. 4) and showed consistent change with t-PA Ag in the euglobulin fraction (Fig. 5).

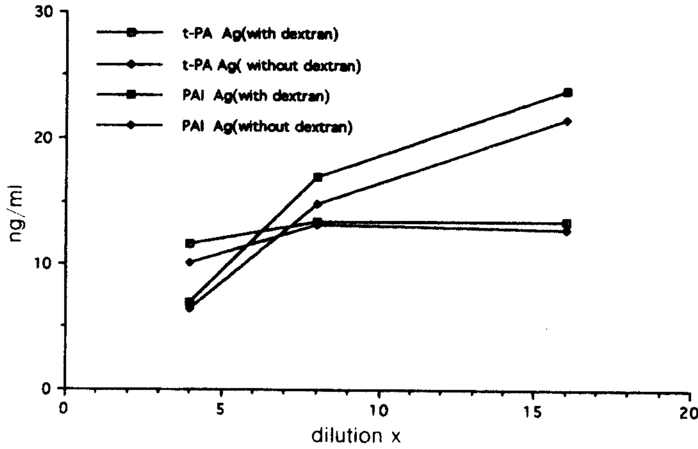


Fig. 6. The effect of dilution on the relationship between PAI-1 Ag and t-PA Ag in euglobulin fraction with or without dextran sulfate. Euglobulin fraction prepared at a constant ionic strength (0. 15) and pH 5.8-6.0, with or without dextran sulfate. Variation in dilution is recorded on the abscissa. t-PA Ag shows no significant change but the PAI-1 Ag increases as the dilution factor increases.

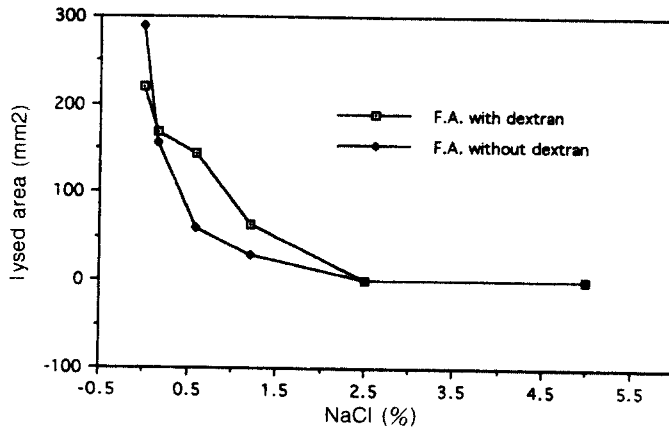


Fig. 7. The effect of ionic strength on the euglobulin fibrinolytic activity, with or witout dextran sulfate. The euglobulin fraction prepared at a constant pH (5.8-6.0). Variation in ionic strength is recorded on the abscissa as final concentration of added NaCl in 1:10 diluted plasma. The fibrinolytic activity is higher in dextran sulfate euglobulin fraction regardless of the change in ionic strength.

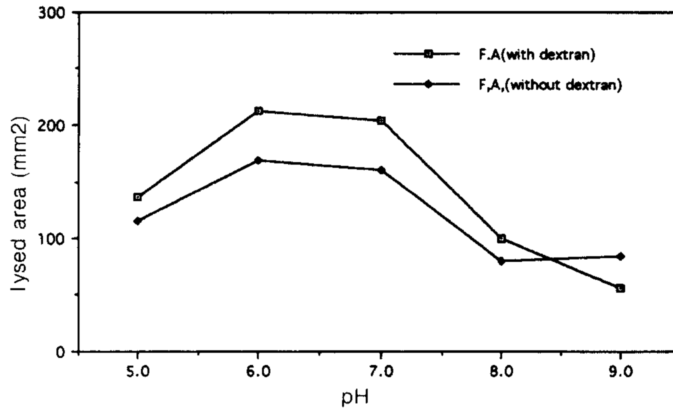


**Fig. 8.** The effect of pH on the euglobulin fibrinolytic activity, with or without dextran sulfate. The euglobulin fraction prepared at a constant ionic strength (0.15) and 1:10 dilution. The fibrinolytic activity is higher in dextran sulfate euglobulin fraction at a pH between 5.0-9.0.

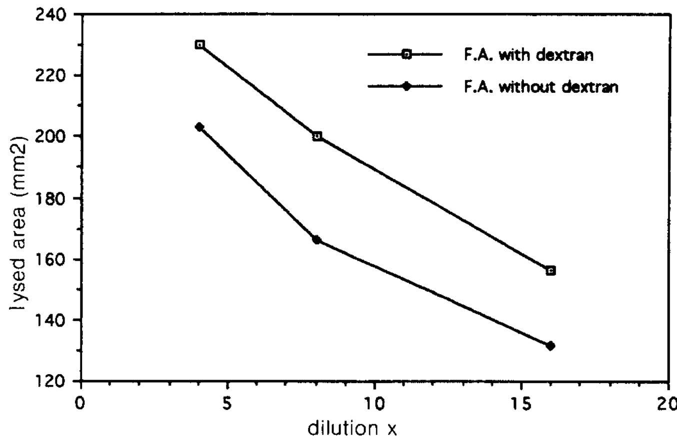


**Fig. 9.** The effect of dilution on the euglobulin fibrinolytic activity, with or without dextran sulfate. The euglobulin fraction prepared at a constant ionic strengt (0.15) and pH 5.8-6.0. The fibrinolytic activity is higher in dextran sulfate euglobulin fraction regardless of the change of dilution ratio.

The precipitation of PAI-1 Ag in the euglobulin fraction was enhanced by increasing the dilution factor (Fig. 6). Dextran sulfate increased the amount of precipitation by t-PA and PAI-1 under various pH, ionic strength and dilution (Fig. 7, 8, 9). More than 95% of t-PA (14/15 ng/ml) in plasma precipitated down in the euglobulin fraction, but PAI-1 was 45 ng/ml in plasma and about 8 ng/ml in euglobulin. This finding suggests that the standard method is more favorable to the precipitation of t-PA than PAI-1.

## DISCUSSION

Euglobulin fraction of plasma is widely used in studying plasma fibrinolytic activity. But, what exactly is measured in euglobulin fractions during fibrinolytic assay has not yet been established. It is

assumed that the measured activity represents primarily the concentration of circulating plasminogen activators. The procedure for euglobulin formation consists of diluting the plasma, adjusting the pH and decreasing the ionic strength, with or without dextran sulfate, in a cold environment in the hopes of minimizing inhibitors. The rationale for the most commonly used procedure of-1:10 dilution, ionic strength of 0.15 and pH of 5.8-5.9[15,16], seems to be drawn from experience as it is the condition which yields the strongest fibrinolytic activity measured on a fibrin plate or by the clot lysis time. In circulation, there are many kinds of plasminogen activator inhibitors of $\alpha_1$-antitrypsin[3], $\alpha_2$-macroglobulin[4], inter-$\alpha$-trypsin inhibitor[4], C1 inactivator[3] and antithrombin III[5]. Literature[1~3] has shown that these inhibitors remain in the supernatant and are minimized in euglobulin fraction during euglobulin precipitation. But in the last decade, many studies have established the presence of variable concentrations of rapid-acting inhibitor (PAI) of both t-PA and urokinase[11~14] in plasma.

Although there is evidence that the intrinsic fibrinolytic pathway is an important fibrinolytic component in euglobulin[15~17], it also has been reported that t-PA is the main factor initiating euglobulin lysis[18,19]. Since the amount of t-PA correlated well with the amount of total PAI-1 and t-PA-PAI-1 complex in euglobulin fraction[20], and the plasma concentration of total PAI-1 was almost twice that of t-PA[20], most of the t-PA must have existed as t-PA-PAI-1 complexes and excess amounts of PAI-1 may have existed as free PAI-1.

In our study, the precipitation of t-PA Ag in the euglobulin fraction was enhanced by decreasing ionic strength and greatest at pH 6.0 while the fibrinolytic activity in the euglobulin fraction showed consistent change with t-PA Ag through out the changes in ionic strength and pH.

The amount of PAI-1 was proportional to the amount of t-PA at various ionic strengths and pH. This finding suggests that the t-PA-PAI-1 complex does not dissociate with changes in ionic strength and pH.

It is not clear whether the conditions for standard euglobulin favors only the t-PA-PAI-1 complex or whether it also favors free form t-PA. In our preliminary study, addition of purified t-PA in plasma resulted in increased fibrinolytic activity and t-PA in euglobulin fraction showed a dose dependant pattern (data is not presented), especially with dextran sulfate. This finding suggests that both free form t-PA and t-PA-PAI-1 complex tend to be precipitated in standard dextran sulfate euglobulin fraction.

Concerning the dilution factor, t-PA in euglobulin fraction was relatively constant regardless of the dilution factor, but the larger the dilution factor, the more PAI-1 and the smaller the fibrinolytic activity in the euglobulin fraction. The reason as to why PAI-1 increased disproportionately to t-PA in euglobulin fraction as th dilution factor increased is not clear, but it shows that the larger the dilution factor the smaller the fibrinolytic activity in the euglobulin fraction. Considering that in plasma, the concentration of PAI-1 is higher than t-PA and some portion of PAI-1 exists as free form, excessive dilution of plasma, greater than 15 times, may favor the precipitation of free form PAI-1. In agreement with previous reports[20,21], PAI-1 looks like the key factor in determining the fibrinolytic activity in euglobulin fraction. Both t-PA Ag, PAI-1 Ag and the fibrinolytic activity in the euglobulin fraction are higher when dextran sulfate is presented under varying conditions of ionic strength, pH and dilution. This implies that the increased fibrinolytic activity by dextran sulfate is due not only to the activation intrinsic factor[17] but also to the increased t-PA Ag in euglobulin fraction. Our data shows that standard method of euglobulin precipitation favors the precipitation of t-PA and/or PAI-1. Considering that the t-PA concentration was 15 ng/ml in plasma and 14 ng/ml in euglobulin, more than 95% of t-PA precipitated down in euglobulin. But PAI-1 was 45 ng/ml in plasma and about 8 ng/ml in euglobulin. So, the standard method is more favorable to the precipitation of t-PA than PAI-1. The ratio of molecular weight of t-PA and PAI-1 is about 3:2 (M.W. 72,000:50,000). Otherwise, the ratio of t-PA and PAI-1 in euglobulin fraction was about 3:2 (t-PA; 14 ng/ml: PAI-1; 8 ng/ml) in standard euglobulin method which suggests that t-PA and PAI-1 are stoichiometric complexes. These results imply that the precipitation of PAI-1 plays a passive role following active precipitation of t-PA in euglobulin fraction. t-PA-PAI-1 complex did not seem to be dissociated by the changes in ionic strength, pH and dilution and the fibrinolytic activity in euglobulin fraction was proportional to the amount of t-PA and/or PAI-1. It is interesting to note how t-PA exerted its fibrinolytic activity in euglobulin fraction with PAI-1. Our study does not explain why, but there are two possibilities: First,

the t-PA-PAI-1 complexes are dissociated in the euglobulin fraction or second, t-PA-PAI-1 complex exerts fibrinolytic activity. But it is difficult to dissociate the covalent binding of t-PA-PAI-1 in euglobulin fraction. So, in agreement with a recent report[22], we believe that the t-PA-PAI-1 complex exerts fibrinolytic activity on fibrin film.

In conclusion, the currently used conditions for standard euglobulin precipitation are the most favorable for t-PA precipitation into euglobulin fraction. The fibrinolytic activity exerted in the euglobulin fraction seems to depend on the amount of t-PA-PAI-1 complex rather than minimized protease inhibitor in the euglobulin fraction. Further study is necessary to investigate how the t-PA exert it's fibrinolytic activity with PAI-1 in euglobulin fraction.

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