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A METHOD USING FIBRIN-FIXED BLUE DEXTRAN FOR DETERMINING THE PLASMIN AND PLASMIN INHIBITOR ACTIVITIES IN HUMAN PLASMA

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Summary

The fibrinolytic activity of plasmin was determined by incubating with fibrin-fixed Blue Dextran as a substrate, the Blue Dextran released being proportional to the plasmin activity. The applicability of this method for rapid and accurate evaluation of fibrinolytic activity was demonstrated by dose-response curves with purified plasmin, plasmin generated by urokinase in human plasma and euglobulin. The method can also be used to determined plasmin inhibitors in plasma.

Introduction

Several methods have been reported for measuring the fibrinolytic activity of plasma, such as the fibrin plate method [1—3], the euglobulin lysis time method [4,5] and thromboelastography [6,7]. The euglobulin lysis time is performed on a fraction of plasma which has been rendered void of normal plasmin inhibitors and can only be semiquantitative. Recently, a new fibrin plate technique [2,3] was reported for measuring what might be termed "fibrinolytic potential". This is that plasminogen in excess of plasma plasmin inhibitors, which is available for conversion into active plasmin. Also this method is sufficiently sensitive, but somewhat time-consuming.

Barta [8] developed a Congo Red fibrin plate test that is more sensitive and rapid than the fibrin plate method, applying a solution of proteolytic enzyme to the entire surface of the dyed fibrin film. However, we found that this method could not be used for assay of the fibrinolytic activity of whole plasma.

This report describes a method using powdered fibrin containing Blue Dextran for estimation of the plasmin and plasmin inhibitor activities in human plasma and the advantages of this method over the other methods.

Materials and Methods

Materials. Human blood was obtained in the early morning (6:00 a.m.) from healthy adults before breakfast, and the plasma was separated by centrifugation at 3000 $\times g$ for 20 min at room temperature. During this procedure no anticoagulant was used. Bovine fibrinogen (Type I) and albumin were purchased from Sigma Chemical Co.; human thrombin, plasmin, and urokinase were from the Green Cross Corporation, Osaka, Japan; Blue Dextran 2000 was from Seikagaku Kogyo Co., Ltd., Tokyo, Japan; Methylene Blue and Congo Red were from Wako Pure Chemical Industries, Ltd., Osaka, Japan; Toyo filter paper, No. 6, was from Toyo Roshi Co., Ltd., Tokyo, Japan. *N,N*-dimethylamino-*p*-(*p'*-guanidinobenzoyloxy)-benzilcarbonyloxy glycolate (*p'*-GB-DBiG) was prepared in the Research Laboratories of Ono Pharmaceutical Co., Osaka, Japan. The euglobulin was obtained as follows. Human plasma was diluted 20-fold with cold H_2O. The diluted plasma was adjusted to pH 5.3 with 5% acetic acid at 0°C and stood overnight at 4°C. Then the mixture was centrifuged at 3000 $\times g$ for 10 min at 4°C and the precipitate was suspended in 0.1 M sodium phosphate buffer (pH 7.4).

Fixation of Blue Dextran, Methylene Blue and Congo Red in the fibrin clot. Bovine fibrinogen was dissolved in 0.1 M sodium phosphate buffer (pH 7.4) at concentration of 1% clottable protein. Solutions of Blue Dextran, Methylene Blue, and Congo Red were added at final concentration of 1% to separate samples of the fibrinogen solution with gentle shaking. Then thrombin (50 N.I.H. units per ml) was added to each fibrinogen solution and the mixtures were shaken until clotting was complete. The clots of fibrin containing Blue Dextran, Methylene Blue, and Congo Red thus obtained were homogenized in an Ultra Turrax (Janke & Kunkel KG.) and centrifuged at 3000 $\times g$ for 20 min at room temperature. The precipitates were washed with 0.2 M sodium phosphate buffer (pH 7.4) and incubated in the same buffer at 37°C for 30 min. Untrapped dyes and Blue Dextran were removed by centrifugation. The washing was repeated until no color was detected in the supernatant solution.

Photocolorimeter. A Jasco Uvidec 2 spectrophotometer was used.

Results

Fixation of Blue Dextran, Methylene Blue, and Congo Red in fibrin clots

The fixation of Blue Dextran in fibrin clots was examined colorimetrically. After washing the clots several times with 0.2 M sodium phosphate buffer (pH 7.4) no material with absorption at 625 nm was detected in the final supernatant, but, when 4 M urea/1 M NaOH was added to dissolve the fibrin clots, the Blue Dextran was solubilized. Plasmin also solubilized the Blue Dextran by dissolving the fibrin clots. On the other hand, Methylene Blue was completely released by washing the fibrin clots several times with 0.2 M sodium phosphate buffer (pH 7.4) and further treatment with 4 M urea/1 M NaOH caused

no additional release of dye. Congo Red adsorbed to fibrin in the clots was not washed out with 0.2 M sodium phosphate buffer (pH 7.4) but was released with 4 M urea/1 M NaOH or plasmin.

These results indicate that Blue Dextran and Congo Red are strongly fixed in the fibrin clots. Therefore, attempts were made to use clots with these dyes for determining fibrinolytic activity. Well washed Blue Dextran- and Congo Red-fixed fibrins were lyophilized and powdered for further studies. 10 mg Blue Dextran- or Congo Red-fixed fibrin powder was used conventionally for their characterization. 1.8 mg Blue Dextran was trapped in 8.2 mg of fibrin clot.

Samples of 10 mg Blue Dextran- or Congo Red-fixed fibrin powder were suspended in 2 ml 0.1 M sodium phosphate buffer (pH 7.4) containing test and control solutions and incubated at 37°C for 1 h with shaking; 4 ml 0.05 M acetic acid was then added, the resulting suspension was rapidly filtered by Toyo No. 6 filter paper and the absorbance of the filtrate was measured at 625 nm or 488 nm with distilled water as a control.

Table I shows the release of dyes from Blue Dextran- and Congo Red-fixed fibrin powder under various conditions. Plasma alone caused an absorbance of 0.017 at 625 nm but, after activation with urokinase, the absorbance increased to 0.495 with the Blue Dextran-fibrin method. This activation was completely inhibited by 10^{-3} M *p'*-GB-DBiG. As reported in our previous paper [9] with about the same concentration of *p'*-GB-DBiG, the hydrolysis of L-tosyl arginine methyl ester and fibrinogen by plasmin were completely inhibited. When Congo Red-fixed fibrin powder was used, an absorbance of 0.388 at 488 nm was observed in reaction plasma alone and this value did not change on addition of *p'*-GB-DBiG. On addition of urokinase, the absorbance only increased 2-fold and *p'*-GB-DBiG did not cause complete inhibition. Addition of albumin instead of plasma caused the development of an absorbance of 0.367.

Blue Dextran-fixed fibrin powder (10 mg) was suspended in 2 ml 0.1 M

TABLE I

RELEASE OF DYES FROM BLUE DEXTRAN- AND CONGO RED-FIXED FIBRIN POWDER UNDER VARIOUS CONDITIONS

The experimental procedures are described in the text. Values are means of 2 replicate experiments.

Blue Dextran-fibrin powder	Absorbance at 625 nm
Plasma 0.2 ml	0.017
Plasma 0.2 ml + *P'*-GB-DBiG *	0.020
Plasma 0.2 ml + urokinase **	0.495
Plasma 0.2 ml + urokinase + *P'*-GB-DBiG	0.025
Albumin 16 mg	0.020
Congo Red-fibrin powder	**Absorbance at 488 nm**
Plasma 0.2 ml	0.388
Plasma 0.2 ml + *P'*-GB-DBiG *	0.396
Plasma 0.2 ml + urokinase **	0.794
Plasma 0.2 ml + urokinase + *P'*-GB-DBiG	0.447
Albumin 16 mg	0.367

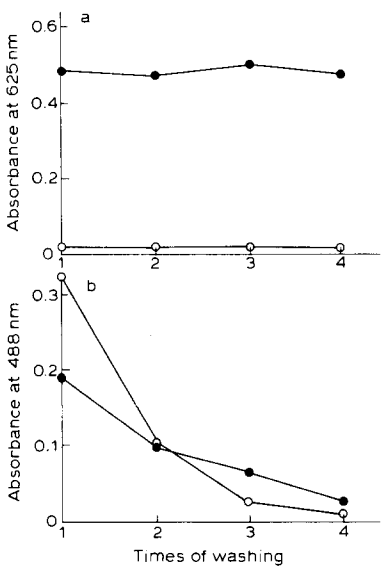
* *P'*-GB-DBiG; 10^{-3} M *N,N*-dimethylamino-*P*-(*P'*-guanidinobenzoyloxy)-benzylcarbonyloxyglycolate.
** Urokinase; 500 I.U. per ml.

soidum phosphate buffer (pH 7.4) containing 0.1% albumin and incubated at 37°C for 30 min with shaking. The mixture was centrifuged at 3000 $\times g$ for 10 min at room temperature, the precipitate was solubilized in 4 ml 4 M urea/1 M NaOH and the absorbance of the resulting supernatant and the solubilized precipitate were measured with distilled water as a control. No Blue Dextran was released on washing the fibrin powder 4 times with albumin solution (Fig. 1a). However, Congo Red was completely released from the dyed fibrin powder by repeated washing and the amount of residual Congo Red in the precipitate solubilized with 4 M urea/1 M NaOH also decreased on repeated washing (Fig. 1b).

Thus, it was demonstrated that Congo Red was transferred non-specifically from fibrin to plasma proteins and added albumin. On the other hand, Blue Dextran was transferred from the fibrin to neither plasma proteins nor added albumin. Therefore, the Blue Dextran fibrin method was suitable for assay of the fibrinolytic activity of plasmin in plasma.

Determination of the plasmin activity in human plasma by the Blue Dextran-fibrin method

The fibrinolytic activities of plasmin, plasma and euglobulin were determined by estimating the release of Blue Dextran from fibrin powder. The assay mixture contained 58 mg Blue Dextran-fixed fibrin powder. 10.4 mg Blue Dex-



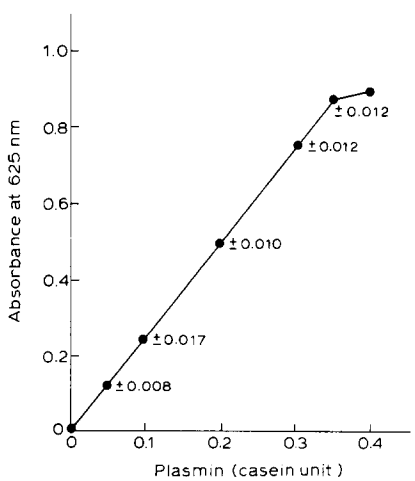


Fig. 1. Effects of washing with albumin solution on Blue Dextran- and Congo Red-fixed fibrin powders. The method for washing the Blue Dextran- or Congo Red-fixed fibrin powder with albumin solution is decribed in the text. Values are means of 2 replicate experiments. a, Blue Dextran-fixed fibrin powder; b, Congo Red-fixed fibrin powder. ○———○, Blue Dextran or Congo Red in the supernatant after washing with albumin solution; ●———●, residual Blue Dextran or Congo Red solubilized with 4 M urea/1 M NaOH in the precipitate after washing with albumin solution.

Fig. 2. Dose vs. response curve of the dye-release activity with plasmin (means of 3 experiments). Details of the assay of dye-release activity by plasmin are described in the text. The standard deviation of each point is shown.

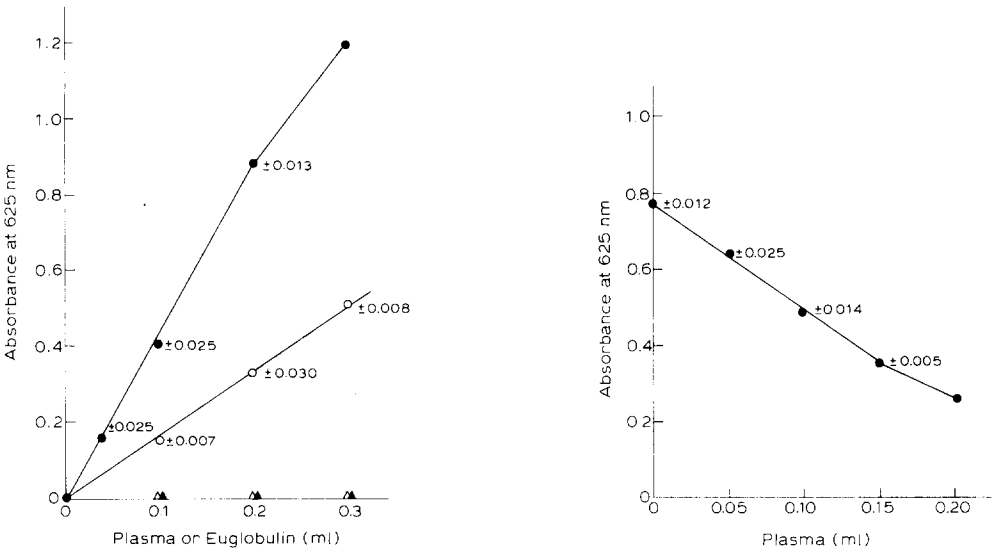


Fig. 3. Dose vs. response curve of the dye-release activity with plasma and euglobulin (means of three experiments). Details of the assay of dye-release activity by plasma and euglobulin are described in the text. The standard deviation of each point is shown. ○———○, urokinase-activated plasma; ●———●, urokinase-activated euglobulin; △———△, untreated plasma; ▲———▲, untreated euglobulin.

Fig. 4. Dose vs response curve of the plasmin inhibitor activities of plasma (means of three experiments). Details of the assay of the plasmin inhibitor activities of plasma are described in the text. The standard deviation of each point is shown.

tran was trapped in 47.6 mg of fibrin clot. Samples of 58 mg Blue Dextran-fixed fibrin powder in 2 ml proteolytic enzymes were incubated at 37°C for 1 h and then 4 ml 0.05 M acetic acid were added, the suspension was filtered by Toyo No. 6 filter paper and the absorbance of the filtrate was measured with distilled water as a control.

A linear increase was obtained up to an absorbance of 0.880 and plasmin concentration of 0.35 casein unit (Fig. 2). The standard deviations did not exceed an absorbance of 0.017. Plasma and euglobulin were activated by urokinase (500 I.U. per ml) at 37°C for 10 min and the dye-release adtivities were determined by the Blue Dextran-fibrin method. Plasma or euglobulin alone had almost no dye-release activity, but after activation with urokinase, up to 0.2 ml euglobulin was used and up to 0.3 ml plasma in the assay for fibrinolysis (Fig. 3). The standard deviations did not exceed an absorbance of 0.030.

Determination of the plasmin inhibitor activities in human plasma by the Blue Dextran fibrin method

2 ml mixtures of 0.3 casein unit of plasmin and various amounts of plasma were incubated at 37°C for 15 min. Then, 58 mg Blue Dextran-fixed fibrin powder were added and the mixture was incubated at 37°C for 1 h. A linear relationship was obtained between the absorbance (in a range of 0.350—0.770) and the amount of plasma (up to 0.15 ml) as shown in Fig. 4. The standard deviations did not exceed an absorbance of 0.025.

Discussion

The final step in coagulation, the thrombin-fibrinogen reaction, involves the transformation of fibrinogen to fibrin, which is the physical basis of all blood clots. The fact that this final step traps blood corpuscles and platelets led us to suppose that other large molecules, such as Blue Dextran (mol. wt. $2 \cdot 10^6$) might also be trapped in this final step.

In this work we found that Blue Dextran was trapped in insoluble fibrin, but, that small molecules, such as Methylene Blue (mol. wt. 373.9) were neither trapped nor adsorbed to the insoluble fibrin. On the other hand, fibrin was dyed by Congo Red and the dye was not released by washing with 0.2 M sodium phosphate buffer (pH 7.4). However, we found that Congo Red was transferred non-specifically from fibrin to plasma proteins and added albumin (Table I and Fig. 1b). Thus, the Congo Red fibrin method described in this paper is not suitable for assay of the fibrinolytic activity of plasma. Barta [8] reported a Congo Red dyed-fibrin plate assay for fibrinolysis and measured the activity of purified fibrinolytic enzyme spectrophotometrically. However, he tested only plasmin and did not check whether the method was applicable to whole plasma. In our studies Blue Dextran was transferred from the fibrin to neither plasma proteins nor added albumin (Table I and Fig. 1a).

The applicability of this method for rapid and accurate evaluation of fibrinolytic activity was demonstrated by dose vs. response curves with purified plasmin, plasmin generated by urokinase in human plasma and euglobulin. The method can also be used to determined the plasmin inhibitors in plasma. The amount of plasminogen present was 3.97 absorbance units per ml plasma (from Fig. 3). Plasmin spontaneously present in plasma could not be detected by this method (Fig. 3). Plasmin inhibitor activities were calculated at 2.8 absorbance units per ml plasma from Fig. 4.

We are now measuring the fibrinolytic activities of plasma of patients with various disorders by this method.

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