

Improvements of the method for the preparation of an artificial prothrombin reagent

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B R I E F C O M M U N I C A T I O N

IMPROVEMENTS OF THE METHOD FOR THE PREPARATION OF AN ARTIFICIAL PROTHROMBIN
REAGENT

C.Vermeer, B.A.M.Soute and H.C.Hemker

Department of Biochemistry, State University Limburg,
Maastricht, The Netherlands

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I N T R O D U C T I O N

About two years ago we developed a method to prepare a prothrombin-free reagent for the one-stage determination of prothrombin. The reagent was devoid of any prothrombin and had an infinite buffer time (1). The preparation procedure of the reagent was:

- a. oxalate plasma was adsorbed three times with 100 mg/ml of BaSO_4
- b. serum was prepared from oxalate plasma by adding Echis Carinatus venom and CaCl_2 . The serum was adsorbed to DEAE Sephadex and after washing the Sephadex the factors VII and X were eluted by a step elution, dialyzed and added to the BaSO_4 adsorbed plasma.

During the last two years we have prepared more than 20 batches of the bovine reagent and in this communication we report several improvements of the preparation procedure which were made during these two years.

M A T E R I A L S A N D M E T H O D S

Buffer A: 0.15 M NaCl, 0.005 M Tris-HCl, pH 7.9. Buffer B: 1.0 M NaCl, 0.005 M Tris-HCl, pH 7.0. Buffer C: 0.0286 M sodium acetate, 0.0286 M sodium barbiturate, 0.1164 M NaCl, pH 7.4. Oxalate and citrate plasma were prepared by collecting blood in 0.1 M disodium oxalate (10% v/v) or in 0.1 M trisodium citrate (10% v/v) followed by centrifugation for 30 min at 4,000 x g.

Coagulation tests were performed by incubating 0.1 ml reagent, 0.1 ml thromboplastin (2) and 0.1 ml sample at 37°C for 30 sec. The reaction was started by adding 0.1 ml CaCl₂ solution 33 mM. Normal pool plasma was obtained from 30 cows and plasma dilutions were prepared in buffer C.

RESULTS

a. Improved recovery of factors VII and X

Using oxalate plasma for the preparation of the factor VII-X concentrate, we noticed that the recovery of factor X varied from one experiment to the other, and that it was always lower than we expected. As is shown in Table I, the amount of factor II (prothrombin) was independent of the anticoagulant solution, but the level of factors VII and X appeared to be higher in citrate plasma than in oxalate plasma, probably because these factors adsorb partly on the calcium oxalate, formed during the collection of blood and after recalcification of the plasma (3). Therefore citrate plasma should be used for the production of the factor VII-X concentrate.

TABLE I

The Amount of Clotting Factors in Plasma

	Concentration of clotting factor (U/ml) in	
	citrate plasma	oxalate plasma
Factor II	1.00	1.00
Factor VII	1.00	0.68
Factor X	1.00	0.87

b. The amount of Echis Carinatus venom to be added

At several occasions it appeared that a minute amount of prothrombin had not reacted with the Echis Carinatus venom. Even the addition of 5 mg of venom per litre of plasma (a five-fold amount) sometimes left residual amounts of prothrombin. The concentration of unreacted prothrombin was below the detection range but still impaired the properties of the reagent (a buffer time of 5-15 min was observed). These last traces of prothrombin can be removed from the dialyzed factor VII-X concentrate by repeating the Echis Carinatus treatment, DEAE-Sephadex adsorption and elution procedure.

c. Removal of antithrombin III

The BaSO₄ adsorbed plasma still contains thrombin inhibitors, the major part of which consists of antithrombin III. In some cases the level of inhibitor was so high that it caused deviations in the reference curve (t-D plot). As was published earlier (4), this curve should be rectilinear when the clotting time is plotted against the dilution of clotting factor. The presence of thrombin inhibitors, however, caused higher clotting times than expected,

especially at prothrombin concentrations lower than 0.003 U/ml. In the range between 0.1 and 0.003 U/ml hardly any deviation could be detected. The anti-thrombin activity could be efficiently removed by adding $\text{Al}(\text{OH})_3$ (5% w/v) to the BaSO_4 adsorbed plasma. $\text{Al}(\text{OH})_3$ is known to bind antithrombin III (5). After stirring for 30 min and centrifuging (15 min, 10,000 x g), the plasma was used to prepare a prothrombin reagent, which was compared to the normal reagent. This is shown in fig. 1. When plasma, adsorbed with both, BaSO_4 and $\text{Al}(\text{OH})_3$ was used for preparing the prothrombin reagent, the t-D plot is rectilinear, whereas the reagent from plasma, adsorbed with BaSO_4 only, gives a curved t-D plot, especially at very low concentrations of prothrombin.

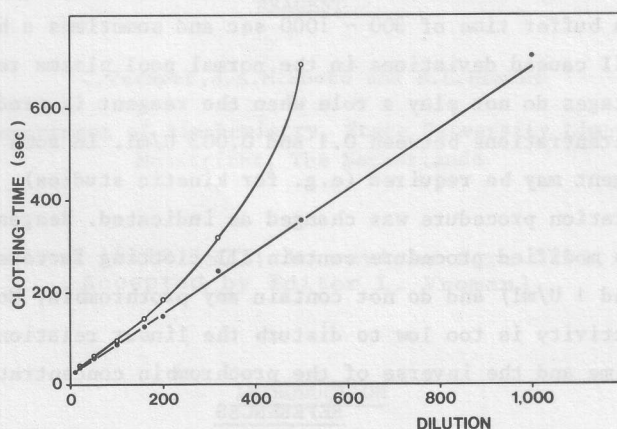


FIG. 1.

Reference curves of varying prothrombin reagents. BaSO_4 -adsorbed plasma (see text) was adsorbed with $\text{Al}(\text{OH})_3$. The plasma before and after $\text{Al}(\text{OH})_3$ treatment was used to prepare a prothrombin reagent. Reference curves of normal pool plasma were made with $\text{Al}(\text{OH})_3$ -treated (● — ●) and the non-treated (o — o) reagent.

d. Method of preparation of the prothrombin reagent

The experiments shown above lead to the following preparation procedure:

- a. oxalate plasma is adsorbed three times with BaSO_4 (100 mg/ml) and once with $\text{Al}(\text{OH})_3$ (5% w/v).
- b. citrate plasma is recalcified and Echis Carinatus venom (5 mg/l) is added. After incubation for 2 hours at 37°C the clot is disrupted and removed by centrifugation (30 min at 40,000 x g). The serum is adsorbed onto DEAE Sephadex (15 ml of slurry per litre of serum) by stirring for 1 hour and the Sephadex is brought into a column, washed with buffer A (200 ml per litre of serum) and eluted stepwise with buffer B. The eluate is dialyzed against buffer A and tested for its prothrombin content by adding BaSO_4 adsorbed plasma to a sample of the factor VII-X concentrate and measuring

its buffer time. When a buffer time is found (that means: when some prothrombin is left) a second Echi Carinatus treatment may be performed. The dialyzed factor VII-X concentrate is added to the $BaSO_4$ -adsorbed plasma in such a way that factor X ≥ 1 U/ml. (Usually about 100 ml concentrate per litre of plasma).

DISCUSSION

In 1976, we published an easy method for the preparation of an artificial prothrombin-free prothrombin reagent (1). The bovine reagent, however, was not always of a constant quality, probably due to variations of the level of some plasma proteins in different cows. Sometimes a small amount of prothrombin was left, causing a buffer time of 300 - 1000 sec and sometimes a high level of antithrombin III caused deviations in the normal pool plasma reference curve. These disadvantages do not play a role when the reagent is used for measuring prothrombin concentrations between 0.1 and 0.003 U/ml. In some cases, however, an optimal reagent may be required (e.g. for kinetic studies). Therefore, the original preparation procedure was changed as indicated. Reagents prepared according to this modified procedure contain all clotting factors in excess (between 0.4 and 1 U/ml) and do not contain any prothrombin. Moreover, the antithrombin activity is too low to disturb the linear relationship between the clotting time and the inverse of the prothrombin concentration.

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