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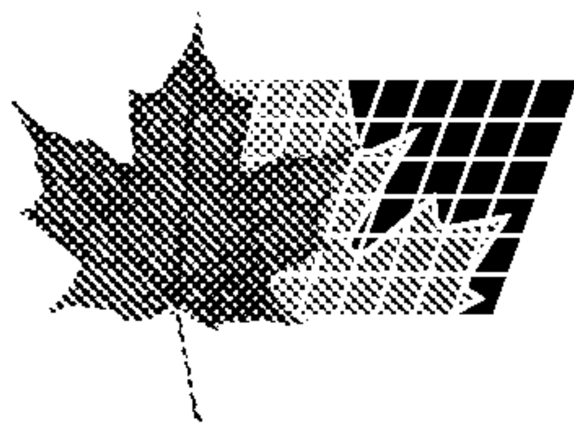
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(54) **EPREUVE GLOBALE POUR DOSER LES PRINCIPALES COMPOSANTES D'UN SYSTEME DE FIBRINOLYSE**

(54) **A GLOBAL TEST FOR MEASURING THE PRINCIPAL COMPONENTS OF THE FIBRINOLYSIS SYSTEM**

(57) A method for the global measurement of the components of the fibrinolysis system in plasma or other biological fluids is described, wherein a plasminogen activator is added to plasma or this other biological fluid, and the resulting plasmin activity is determined.

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Abstract

A method for the global measurement of the components of the fibrinolysis system in plasma or other biological fluids is described, wherein a plasminogen activator is added to plasma or this other biological fluid, and the resulting plasmin activity is determined.

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A global test for measuring the principal components of the fibrinolysis system

5 The invention relates to a method for the global measurement of the components of the fibrinolysis system in plasma or other biological fluids, entailing addition of a plasminogen activator or activation of a plasminogen activator present in the sample, and the resulting
10 plasmin activity being determined.

The human body possesses an enzymatic system capable of redissolving any blood clots that have formed: the fibrinolytic system. While it has for many years been simple to investigate the function of the coagulation
15 system by global tests such as the partial thromboplastin time or activated partial thromboplastin time, such a measurement method for functional measurement, in a comprehensive manner, of the decisive components of the system was not available for fibrinolysis. The essential
20 components of the system are plasminogen, alpha-2 antiplasmin (A2-AP) and plasminogen activator inhibitor (PAI), in this case especially of the endothelial type I. All 3 components can be determined by individual tests; a global test reflecting the interplay of the 3 com-
25 ponents was not available in the prior art.

It has surprisingly been found that plasmin activity can be generated, as a function of plasminogen, alpha-2 antiplasmin and plasminogen activator inhibitor, within a few minutes by incubation of plasma samples with a
30 plasminogen activator together with an omega-amino carboxylic acid and/or a methionine-specific oxidant, and thus the fibrinolytic system of a patient can be investigated by determining the resulting plasmin activity, e.g., via a chromogenic or fluorogenic plasmin substrate.

If t-PA is used as the activator, and a chelating agent such as EDTA in a concentration of 0.5 - 10 mM is used in the test mixture the test also detects the stimulation of t-PA by fibrin, which is a further important factor in
5 fibrinolysis.

If a contact phase activator such as ellagic acid (1-100 μg/mixture) or a sulfatide is used instead of the plasminogen activator the test measures the intrinsic pathway of fibrinolysis via plasmic prourokinase, factor
10 XIIa and kallikrein, but incubation times which are longer by a factor of 5 are required.

The invention therefore relates to a method for the global measurement of the components of the fibrinolysis system in plasma or other biological fluids, which
15 comprises a plasminogen activator being added to a sample to be investigated or plasminogen activator present in the sample being activated, and the resulting plasmin activity being determined.

The quantity of plasminogen activator added is such that
20 the concentration of t-PA is 2-200 IU/ml or that of urokinase is 1 - 100 IU/ml.

To carry out the method according to the invention 200 μl of a plasminogen activator reagent which contains 2.5 - 40, preferably 5 - 20, IU/ml urokinase or 12.5 - 100
25 IU/ml tissue plasminogen activator and, where appropriate in order to improve the activity, 100 - 2000 μg/ml of a substance stimulating t-PA activity, are added to 50 - 200 μl (preferably 100 μl) of plasma, preferably citrated plasma.

30 This stimulating substance may be fibrinogen degradation products prepared from fibrin by proteolysis with plasmin and withdrawal of Ca^{2+} and are employed as a solution in 20 - 200 mM Tris, 0-2% polygeline™, 0.01% ®Triton x 100, pH 7.5 - 9.5, preferably 8.5. If the plasminogen

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activator is urokinase the solution containing it may contain a substance stimulating it, preferably 3 mM tranexamic acid or else 30 mM epsilon-aminocaproic acid or 100 mM lysin or another omega-amino carboxylic acid. The omega-amino carboxylic acids may also be added separately to the reaction mixture.

A methionine-specific oxidizing agent such as chloramine T, chloramine B or HOCl can furthermore be added to the reaction mixture instead of the omega-amino carboxylic acid, preferably for measurements with t-PA, or in combination with the omega-amino carboxylic acid, preferably for measurements with u-PA, in order to destroy antiplasmin and other serin protease inhibitors, the concentrations in the test mixture being 0.2 - 20 mM. Methionine-specific oxidants are understood as being substances which oxidize methionine to methionine sulfoxide, preferably at pH 7 - pH 9, particularly at pH 8 - pH 8.8.

After incubation at 37°C for several minutes, preferably 10 minutes, a chromogenic plasmin substrate is added, preferably together with a sodium chloride solution (100 - 800 mM NaCl in the reaction mixture) and/or CsCl solution (30 - 300 mM in the reaction mixture) in order to obtain linear reaction kinetics, and the rate of extinction change (delta E/t) is determined.

Alternatively, the same quantity of chromogenic plasmin substrate (without NaCl and/or CsCl) can be added in the first incubation, preferably with the plasminogen activator reagent, which, however, results in hyperbolic reaction kinetics. The reaction is preferably terminated after approximatley 5 minutes by addition of an acid, e.g. 100 μl of 8.5 M acetic acid.

The invention is further defined in the claims and illustrated by examples in the following text.

Example 1

Implementation of the method according to the invention on different pathological plasmas

100 μl of human citrated plasma, a) standard human plasma, b) A2-AP-deficient plasma (i.e. no A2-AP in the plasma; prepared by immunoadsorption), c) 50 % strength plasminogen-deficient plasma (i.e. plasminogen content in the plasma = 50 % of normal; prepared by immuno-adsorption), d) PAI-rich plasma with 4.2 u-PA-inhibiting units/ml, were incubated with 200 μl of u-PA reagent 5, 7.5 and 10 IU of u-PA/ml in 150 mM Tris, 3 mM tranexamic acid, 1 % polygeline, 0.01 % Triton X 100, pH 8.4), for 10 minutes at 37°C. 500 μl of chromogenic substrate HD-norvalylcyclohexylalanyl-lysyl paranitroanilide (HD-Nva-CHA-Lys-pNA), 0.6 mM in 480 mM NaCl, 100 mM CsCl were then added, the substrate reaction was terminated after 3 min. (37°C) by addition of 200 μl of 3.4 M acetic acid and the resulting extinction was determined at 405 nm.

Table 1

	Addition of		
	1 IU u-PA	1.5 IU u-PA	2 IU u-PA
	delta A/3 min (x 1000)		
a) Normal plasma	290	402	536
b) A2-AP-deficient plasma	696	863	929
c) 50 % Plasminogen-deficient plasma	154	216	275
d) PAI-rich plasma (4.2 U/ml)	234	329	428

It can be seen that, compared with normal plasma, antiplasmin deficiency leads to an increase in fibrinolysis which results in increased delta E values and that PAI-rich and plasminogen-deficient plasma cause a decrease in fibrinolysis, expressed by lower delta E values. The test according to the invention therefore provides information about changes in the activity of the key components, i.e. PAI, A2-AP and plasminogen. The maximum extinction yields coupled with high sensitivity to variations in the plasma concentrations of the fibrinolysis components were obtained on addition of 2 IU of u-PA to 100 μl of human plasma.

Example 2

Effect of pH on the test according to the invention

Example 1 was carried out using normal plasma as the sample at different pH values.

Table 2

pH 7	pH 7.5	pH 8	pH 8.5	pH 9	pH 9.5
delta E/3 min (x 1000)					
309	413	522	536	414	337

A pH optimum was obtained at pH 8.5.

Example 3

Effect of different tranexamic acid concentrations on the test according to the invention

Example 1 was carried out using normal plasma as sample at different concentrations of tranexamic acid or chloramine T.

Table 3

	mM	delta E/min
	0	74
Conc. of tranexamic acid in 300 µl test volume	0.75	443
	1.5	600
	2.25	553
	3	501
Conc. of chloramine T in 300 µl test volume	0	74
	1.25	103
	5	234
	10	178

An optimum is obtained on use of approximately 1.5 mM tranexamic acid in the reaction mixture. Use of chloramine T also leads to improved extinction yields with an optimum at approximately 5 mM in the test mixture.

Example 4

Fibrinolysis test via contact phase activation of endogenous (intrinsic) plasminogen activator

100 µl of human citrated plasma, a) standard human plasma, b) A2-AP-deficient plasma, c) plasma supplemented with 10 µg/ml prourokinase (sc-u-PA), d) plasma supplemented with 20 ng/ml prourokinase, e) plasma supplemented with 40 IU/ml single-chain tissue plasminogen activator (sc-t-PA), f) plasma supplemented with 80 IU/ml sc-t-PA, g) h) i) plasma from patients with a history of hyperfibrinolysis (bleeding without defective coagulation) were mixed with 100 µl of reagent I. Reagent I comprised 1 part by volume of ®Neothromtin (Behringwerke, Marburg), dissolved in 36 ml of 150 mM Tris, 50 mM NaCl, 0.02 % Triton, 1 % polygeline, 0.01 % sodium azide, pH 8.4, and 1 part by volume of tranexamic acid (12 mM).

For zero adjustment, 400 μl of stop solution (480 mM NaCl, 100 mM CsCl, 30 mM arginine, 50 mM Tris, pH 8.4) were added to the sample before addition of reagent I.

5 After incubation at 37°C for 10 minutes, 500 μl of 0.6 mM substrate solution (HD-Nva-CHA-Lys-pNA), dissolved in distilled water/stop solution in the ratio 1:4 or, for the zero value, 100 μl of 3 mM substrate solution in distilled water, were added, incubated for 60 min at 30°C, and the reaction was terminated by addition of
10 250 μl of 3.4 M acetic acid, and the resulting extinction was determined at 405 nm.

Table 4

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Plasma	Generated plasmin activity (A x 1000)
a) Standard human plasma	284 ± 1
b) A2-AP-deficient plasma	453 ± 0
c) a) + 10 ng/ml sc-u-PA	376 ± 1
d) a) + 20 ng/ml sc-u-PA	440 ± 5
e) a) + 40 IU/ml sc-t-PA	374 ± 5
f) a) + 80 IU/ml sc-t-PA	437 ± 3
g) Patient 1	205 ± 10
h) Patient 2	551 ± 17
i) Patient 3	502 ± 6

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25 It can be seen that the test mixture according to the invention measures the fibrinolysis system including the components sc-u-PA and sc-t-PA: increased plasma levels of sc-u-PA or free sc-t-PA are associated with increased fibrinolytic activity. The result of the fibrinolysis
30 test showed a pathological increase in two of the three patients having a history of a tendency to bleeding.

Example 5

Fibrinolysis test by use of chelating agents and chlor-

amine for measuring exogenous plasminogen activator (predominantly of the tissue type).

100 μl of human citrated plasma a) - e), see Example 4, were incubated as in Example 4 with the difference that reagent I was replaced by 25 mM EDTA, 10 mM chloramine T, 150 mM Tris (Tris buffer could also be replaced by BICIN buffer), 50 mM NaCl, 0.02 % ®Triton X 100, pH 8.4.

Table 5

Plasma	Generated plasmin activity (E x 1000)
a) Normal plasma	134 ± 6
b) A2-AP deficient plasma	523 ± 2
c) a) + 10 ng/ml sc-u-PA	118 ± 1
d) a) + 20 ng/ml sc-u-PA	114 ± 1
e) a) + 40 IU/ml sc-t-PA	167 ± 4
f) a) + 80 IU/ml sc-t-PA	227 ± 5

The result shows that this variation of a fibrinolysis test is independent of the sc-u-PA content (intrinsic system of fibrinolysis), but that it responds well to different amounts of (extrinsic) tissue plasminogen activator.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for the global measurement of the components of the fibrinolysis system in a sample which comprises:
   a) adding (i) a plasminogen activator and (ii) an omega-amino carboxylic acid or a methionine-specific oxidant to the sample;
   b) determining the plasmin activity of the sample; and
   c) comparing the plasmin activity of the sample with a control sample to determine the global function of the components of the fibrinolysis system in the sample, wherein the components of the fibrinolysis system include plasminogen, α-2 antiplasmin and plasminogen activator inhibitor.

2. The method as claimed in claim 1, wherein urokinase is used as plasminogen activator.

3. The method as claimed in claim 1, wherein tissue plasminogen activator is used as plasminogen activator.

4. The method as claimed in claim 1, wherein the plasminogen activator is generated in the sample using a plasminogen activator-activating substance.

5. The method as claimed in claim 4, wherein a surface activator is used as the plasminogen activator-activating substance.

6. The method according to claim 5, wherein the surface activator is ellagic acid or a sulfatide.

7. The method as claimed in claim 1, wherein an N-chloramine or another singlet oxygen-liberating substance is added.

8. The method as claimed in claim 1, wherein the omega-amino carboxylic acid is tranexamic acid.

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9. The method as claimed in claim 8, wherein the tranexamic acid is added in a concentration of 0.5 to 5 mM.

10. The method as claimed in any one of claims 1 to 9, further including a t-PA-stimulating substance.

11. The method as claimed in any one of claims 1 to 10, further including a chelating agent.

12. The method according to claim 11, wherein the chelating agent is EDTA.

13. The method as claimed in any one of claims 1 to 12, wherein the method is a one-step method.

14. A method according to any one of claims 1 to 13, wherein the sample is plasma.

15. A method according to any one of claims 1 to 13, wherein the sample is biological fluid.