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(CONVENTION. By one or more persons and/or a Company) 647730 Form 4.

COMMONWEALTH OF AUSTRALIA

Patents Act 1952-1969

CONVENTION APPLICATION FOR A PATENT

(1) Here insert (in full) Name or Names of Applicant or Applicants, followed by Address(es).

I/We BEHRINGWERKE AKTIENGESELLSCHAFT
of D-3550 Marburg, Federal Republic of Germany

(2) Here insert Title of Invention.

hereby apply for the grant of a Patent for an invention entitled:(2)
A METHOD FOR THE DETERMINATION OF THE PLASMINOGEN ACTIVATOR ACTIVITY IN SAMPLES CONTAINING ALPHA-2-ANTIPLASMIN

which is described in the accompanying complete specification. This applications is a Covention application and is based on the application numbered(3)

(3) Here insert number(s) of basic application(s).

P38 43 422.9

(4) Here insert Name of basic Country or Countries, and basic date or dates.

for a patent or similar protection made in(4) Federal Republic of Germany on 23rd December 1988

My/Our address for service is WATERMARK PATENT & TRADEMARK ATTORNEYS 290 Burwood Road, Hawthorn, Victoria, Australia.

DATED this 21st day of December 19 89

(5) Signature(s) of Applicant(s) or Seal of Company and Signatures of its Officers as prescribed by its Articles of Association.

(5) BEHRINGWERKE AKTIENGESELLSCHAFT
by
D. B. Mischlewski
Registered Patent Attorney

To: THE COMMISSIONER OF PATENTS.

Form 7

COMMONWEALTH OF AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION UNDER PART XVI. FOR A PATENT.

In support of the Convention application made under Part XVI. of the Patents Act 1952 by BEHRINGWERKE AKTIENGESELLSCHAFT of D-3550 Marburg, Federal Republic of Germany for a patent for an invention entitled:

A method for the determination of the plasminogen activator activity in samples containing alpha-2-antiplasmin

We, Philipp Stein, Höhenweg 28, D-3550 Marburg
Heribert Bug, Amselweg 7, D-3551 Niederweimar
Federal Republic of Germany

do solemnly and sincerely declare as follows:

1. We are authorized by BEHRINGWERKE AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.

2. The basic application(s) as defined by Section 141 of the Act was (were) made at München in the Federal Republic of Germany under No. P 38 43 422.9

on December 23, 1989

by BEHRINGWERKE AKTIENGESELLSCHAFT

3. 1) Thomas Stief, Avda. Kansas City, 28. Bloque, 1. Apt. 225, E-41007 Sevilla, Spain
   2) Norbert Heimburger, Sonnenhang 10, D-3550 Marburg, Federal Republic of Germany

is/are the actual inventor(s) of the invention and the facts upon which BEHRINGWERKE AKTIENGESELLSCHAFT is entitled to make the application are as follows:

The said BEHRINGWERKE AKTIENGESELLSCHAFT is the assignee of the said Thomas Stief, Norbert Heimburger

4. The basic application referred to in paragraph 2 of this Declaration was (were) the first application(s) made in a Convention country in respect of the invention the subject of the application.

DECLARED at Marburg, Federal Republic of Germany

this 15th day of November, 1989

To the Commissioner of Patents

BEHRINGWERKE AKTIENGESELLSCHAFT

Prokurist (Dr. Stein)

Prokurist (Dr. Bug)

AU8947207

(12) PATENT ABRIDGMENT (11) Document No. AU-B-47207/89
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 647730

(54) Title
A METHOD FOR THE DETERMINATION OF THE PLASMINOGEN ACTIVATOR ACTIVITY IN SAMPLES CONTAINING ALPHA-2-ANTIPLASMIN

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(57) Claim

1. A method for the determination of the plasminogen activator activity in a biological fluid containing alpha-2-antiplasmin, which comprises addition of proteolytically degraded plasminogen to this fluid, and determination of the resulting plasmin by means of a chromogenic substrate specific for plasmin.

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PATENTS ACT 1952-69

COMPLETE SPECIFICATION

(ORIGINAL)

Class Int. Class

Application Number:
Lodged:

Complete Specification Lodged:
Accepted:
Published:

Priority :

Related Art :

Name of Applicant : BEHRINGWERKE AKTIENGESELLSCHAFT

Address of Applicant : D-3550 Marburg, Federal Republic of Germany

Actual Inventor : THOMAS STIEF and NORBERT HEIMBURGER

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Complete Specification for the invention entitled:

A METHOD FOR THE DETERMINATION OF THE PLASMINOGEN ACTIVATOR ACTIVITY IN SAMPLES CONTAINING ALPHA-2-ANTIPLASMIN

The following statement is a full description of this invention, including the best method of performing it known to :- us

BEHRINGWERKE AKTIENGESELLSCHAFT

88/B 046 - Ma 710
Dr. Ha/Sd

A method for the determination of the plasminogen activator activity in samples containing alpha-2-antiplasmin

The invention relates to a method for the determination of the activity of plasminogen activators in plasma or other biological fluids.

The key enzymes in the physiological fibrinolysis in mammalian blood are the two plasminogen activators a) tissue plasminogen activator (t-PA) and b) urinary plasminogen activator (u-PA). Plasminogen activators convert the inactive zymogen plasminogen into the protease plasmin. Plasmin degrades insoluble fibrin into soluble fibrin split products.

The activity of these PAs in human blood is of great diagnostic relevance for measuring the fibrinolytic potential of a patient. The fibrinolytic potential provides information on, inter alia, possible thrombo-embolisms which are to be expected. However, PAs occur only in small amounts (ng/ml) in plasma. This makes great demands on the sensitivity and specificity of the detection method.

The functional determination of PAs according to the state of the art entails their natural substrate, plasminogen , and a chromogenic plasmin substrate being added to the sample. In this case, the measured plasmin activity represents PA activity which is enhanced in direct proportion.

However, the plasmin which is produced is immediately inhibited, almost quantitatively, by alpha-2-antiplasmin which is likewise contained in the sample, so that only low plasmin activity can be measured.

The accuracy of functional PA determination thus depends on eliminating this antiplasmin effect. The methods for the functional determination of PAs described in the state of the art therefore either provide for additional plasma separation steps such as euglobulin precipitation or require acidification and subsequent neutralization of a highly diluted sample. Both methods are relatively time-consuming and methodologically demanding.

Hence the object of the present invention was to provide a method with which the activity of PA in plasma, serum or other biological fluids can be determined rapidly and reliably. This object is achieved according to the invention by treating the plasminogen which is necessary for the determination of the PAs with a protease, preferably elastase, or with a serine protease, preferably plasmin or trypsin, particularly preferably elastase at pH 7 - pH 8, or plasmin or trypsin at pH 10 - 13, until a molecule is produced which, after conversion into plasmin, is no longer inhibited by alpha-2-antiplasmin . An assay suitable for monitoring this reaction is, for example, "Berichrom® alpha-2-antiplasmin" from Behringwerke AG, Marburg, FRG.
The exposure to these proteases can be carried out in such a way that they are present in immobilized form (for example coupled to ᴿSepharose) and thus do not interfere in the activity assay which is to be carried out subsequently.

It has emerged, surprisingly, that in a method for the determination of PA the addition of plasminogen treated in this way allows the activity of PA to be determined without interference from alpha-2-antiplasmin.

The invention relates to a method for the determination of the plasminogen activator activity in a biological fluid containing alpha-2-antiplasmin, which comprises addition of proteolytically degraded plasminogen to this fluid, and determination of the resulting plasmin by

means of a chromogenic substrate specific for plasmin. The invention is furthermore defined in the claims and explained in the examples which follow, without restricting the inventive concept.

Example 1

Pretreatment of plasminogen with elastase-^{R}Sepharose and use in the detection of plasma PA activity.

3 × 80 CTA-U (Committee of Thrombolytic Agents Units) of plasminogen (Behringwerke assay plasminogen, Marburg, FRG) were dissolved in 7 ml of 50 mM tris/HCl, pH 8.4, and a) adjusted to pH 8.0, b) adjusted to pH 12.0 and c) left unchanged. One a) and one b) solution were passed at a flow rate of 30 ml/h through 1 ml of elastase-^{R}Sepharose (2.5 mg of protein/ml of gel bed, coupled by the method of Axen et al., Nature 214, 1302-1304, 1967). The flow-throughs were adjusted to pH 8.4 (solutions a) to c)), made up to 8 ml with 50 mM tris, pH 8.4, and used in a PA assay as follows:

50 µl of human citrated plasma which contained various amounts of alpha-2-antiplasmin, prepared by mixing normal plasma with plasma deficient in alpha-2-antiplasmin (prepared by immunoadsorption), were incubated with 200 µl of the above plasminogen samples and 200 µl of 5 IU/ml u-PA in 100 mM tris, 100 mM NaCl, 1 % polygeline, 0.1 % ^{R}Triton X 100 at 37°C for 5 min. 500 µl of 0.6 mM chromogenic plasmin substrate HD-norvalyl-cyclohexyl-alanyl-lysyl-para-nitroanilide in 480 mM NaCl were then added, the substrate conversion was stopped after 5 min at 37°C by addition of 100 µl of 8.5 M acetic acid, and the resulting extinction at 405 nm was determined. The same assay mixture with addition of only tris buffer without u-PA content was used for blank correction.

Table 1

Content of alpha-2-antiplasmin	Addition of plasminogen degraded with elastase-®Sepharose pH 8	pH 12	untreated plasminogen (control)
%	A_{405nm}/5 min (E × 1000)*		
100	910 ± 4	648 ± 5	107 ± 0
75	962 ± 1	676 ± 5	198 ± 17
50	1037 ± 3	771 ± 3	280 ± 1
25	1152 ± 2	860 ± 5	496 ± 1
0	1309 ± 3	1061 ± 4	749 ± 1

* Means of duplicate determinations

It is evident that when plasminogen which has not been pretreated is used the extinction (PA activity measurement) greatly depends on the alpha-2-antiplasmin content of the sample. This dependence is reduced by proteolytic pretreatment of plasminogen with elastase, preferably at pH 8.

When plasminogen which had been degraded at pH 12 was added, the extinction was 860 ± 5 at an antiplasmin content of 25% and 648 ± 5 at an antiplasmin content of 100 %, that is to say only 25 % less.
The corresponding extinctions for the control are 496 ± 1 and 107. This is a decrease of almost 80 %.

Example 2

Pretreatment of plasminogen with plasmin-®Sepharose and use in the detection of plasma PA activity

Plasminogen was dissolved as in Example 1, then a) and b) preparations were passed through 1 ml of plasmin-Sepharose (2.5 mg of plasmin/ml of gel), and

flow-throughs and c) solution were made up to 8 ml (pH 8.4) and investigated in the PA assay as in Example 1.

Table 2

Plasma content of alpha-2-antiplasmin	Addition of plasminogen degraded with plasmin-^{R}Sepharose	
	pH 8	pH 12
%	A_{405nm}/5 min (E × 1000)*	
100	114 ± 2	554 ± 9
75	113 ± 1	591 ± 1
50	168 ± 3	644 ± 2
25	346 ± 4	749 ± 3
0	603 ± 0	937 ± 5

* Means of duplicate determinations

See Table 1 for control, because test mixture the same.

It is again evident that the dependence of the PA activity measurement on the antiplasmin concentration is greatly reduced by comparison with the control (Tab. 1) especially on pretreatment of plasminogen at pH 12 with plasmin.

HOE 88/B 046

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:
~~Patent Claims~~

1. A method for the determination of the plasminogen activator activity in a biological fluid containing alpha-2-antiplasmin, which comprises addition of proteolytically degraded plasminogen to this fluid, and determination of the resulting plasmin by means of a chromogenic substrate specific for plasmin.

2. The method as claimed in claim 1, wherein the plasminogen is degraded using elastase, plasmin or trypsin.

3. The method as claimed in claim 1, wherein the plasminogen is degraded using elastase.

4. The method as claimed in claim 3, wherein degradation is carried out at pH 7 - 8.

5. The method as claimed in claim 1, wherein the plasminogen is degraded using plasmin or trypsin.

6. The method as claimed in claim 5, wherein degradation is carried out at pH 10 - 13.

7. The method as claimed in claim 1, wherein the proteases are immobilized.

8. The use of proteolytically degraded plasminogen in a method for the determination of plasminogen activators.

DATED this 21st day of December 1989.
BEHRINGWERKE AKTIENGESELLSCHAFT

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