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FUNCTIONAL ASSAY FOR DETERMINING THE PROTEIN S ACTIVITY

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(56) Prior Art Documents
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(57) Claim

1. A method for the functional determination of protein S in a sample of a biological fluid, said method comprising the steps of:

a) adding activated protein C to the sample

b) starting the coagulation reaction by adding a reagent containing a defined amount of an activator of clotting to the sample

c) determining the clotting time,

said amount of an activator being such that a sample without protein S will give a clotting time of at least 50 seconds.

2. The method of claim 1, further comprising the step of adding an excess of protein S-deficient plasma to the sample.

8. A reagent when used in a method for the functional determination of protein S in a sample of biological fluid, said method comprising the steps of:

a) adding activated protein C to the sample

b) starting the coagulation reaction by adding a reagent containing a defined amount of an activator of clotting to the sample

c) determining the clotting time,

said amount of an activator being such that a sample without protein S will give a clotting time of at least 50 seconds.

Form 10

COMMONWEALTH OF AUSTRALIA 652737

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Complete Specification for the invention entitled:

FUNCTIONAL ASSAY FOR DETERMINING THE PROTEIN S ACTIVITY

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

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Functional assay for determining the protein S activity

The invention relates to a method for the functional determination of protein S in liquids, in particular in plasma, and to a reagent suitable for this.

Protein S is an inhibitor of blood clotting and acts as a cofactor of activated protein C in its proteolytic degradation of the clotting factors Va and VIIIa.

An inborn or acquired deficiency in protein S can lead to thromboembolic complications.

Protein S is synthesized in the liver (Stern, D. et al. (1986), J. Cell Biol. 102, 1971-1978). The biosynthesis is vitamin K-dependent and the concentration in the blood is therefore reduced on treatment with vitamin K antagonists. The start of the treatment can, in the presence of a protein S deficiency, lead to serious impairment of the health of the patient (Grimaudo, V. et al. (1989) BMJ 298, 233-234).

The activity may also be reduced in the case of disseminated intravasal clotting or thromboembolic diseases.

It is clear from the abovementioned findings that protein S has a decisive influence on the capability of the protein C/thrombomodulin inhibitor system, and that a reliable diagnostic system is correspondingly required. In plasma, only about 40% of the protein S is present in the free form. The rest of about 60% is in a complex with the C4b-binding protein (Dahlbäck, B. (1981), Proc. Natl. Acad. Sci. 78, 2512-2516).

This bound protein S is not available as cofactor for activated protein C and therefore has no anticoagulatory

action. An excessive proportion of bound protein S can therefore also be found as a functional deficiency, while the antigen is found to be normal or nearly normal (Comp, P.C. et al. (1986), Blood 67, 504-508; Girolami, S. et al. (1989), Thromb. Haemost. 61, 144-147).

On the other hand, the activity can be normal in the case of reduced protein S antigen if only a very small proportion is present in the inactive bound form, as is normally the case in newborns (Schwarz, H.P. et al. (1988), Blood 71, 562-565).

It is known to determine protein S immunologically. However, these methods do not provide any information on the activity of the protein and are not a subject of the invention.

Methods for determining the activity of protein S have also been described.

DE 3724443 A1 describes a method for determining the activity of protein S. This assay requires relatively complex reagents, such as a synthetic substrate plasma, purified factor Xa and prothrombin, and is inconvenient to carry out due to the incubation times. Also, it is interfered with by, e.g., heparin in the sample.

DE 3607559 A1 mentions a functional determination of protein S in combination with protein C. It is necessary to determine a reference value without protein C activator for each sample. The use of an activator of F VII or F II is also claimed but no further evidence is shown. Data on the measurements to be expected or interfering influences for all the test systems are not given.

A functional assay is described by Comp, P.C. et al. in J. Clin. Invest. 74, 2082-2088 (1984). Here the prolongation of the clotting time by activated protein C is measured in the 1-step factor Xa assay. This assay is

relatively insensitive having a prolongation of only 24 sec for the maximum concentration of 50% which can be measured with this method. It was not possible to measure plasmas from patients under vitamin K antagonist treatment or having liver damage.

Bertina et al. (Thromb. Haemost. 1985, 53 (2), 268-272) indicate a possibility of determining functional protein S in human plasma. However, the test cannot be evaluated quantitatively for protein S insofar as there is no possible control of the endogenous factor VIII or factor V in the plasma to be measured and the prolongations depend decisively on these two factors. If these factors are present in the sample in an increased or reduced concentration, findings which are correspondingly reduced or increased result for protein S.

Van de Waart et al. (1987, Thromb. Res. 48, 427-437) use a system comprising adsorbed substrate plasma, added prothrombin, activated protein C, phospholipids and calcium chloride. A 100% difference in the protein S content of the sample only cause about 20 seconds of prolongation, i.e. the assay is likewise relatively insensitive.

Suzuki K. and Nishioka J. (1988, Thromb. Res. 49, 241-251) describe a further assay system. They use Protac®, an activator for protein C from snake venom. This method is also time-consuming and inconvenient. The sensitivity is very low at a prolongation of 13 sec for a 100% difference in activity.

Kobayashi I. et al. (1989, Clin. Chem. 35, 1644-1648) also propose a protein S assay which likewise represents a determination via a modified aPTT determination.

The test methods known hitherto have in common that, in addition to an inconvenient procedure because of the multiplicity of reagents used, they only have a low

sensitivity which is measured by the prolongation of the reaction time in relation to the degree of protein S activity.

It was therefore an object of the invention to develop a method and a reagent which allows the determination of the activity of protein S in plasma in both a simple and a reliable, sensitive and specific way.

According to the invention, a method for determining protein S by measuring the clotting time of a biological sample is now disclosed, wherein the amount of added activators is adjusted in such a way that the clotting time is prolonged beyond the normal clotting time.

Prolonging the clotting time made it possible in a surprisingly simple way to achieve a very great increase in sensitivity which can, moreover, be adapted to the requirements.

Methods for determining the clotting time are known per se to those skilled in the art. They can, inter alia, be methods which determine the liberation of thrombin from prothrombin via the formation of a clot or the conversion of a chromogenic substrate. The preferred method in this connection is the chromogenic one, with a very particular preference for the use of a chromogenic thrombin substrate, in particular of Tos-Gly-Pro-Arg-ANBA-IPA.

In a preferred embodiment of the method according to the invention, an excess of protein S-deficient plasma is added to the undiluted sample and the clotting time is then determined by admixing a reagent comprising activated protein C, an activator of the exogenous or endogenous path of clotting, phospholipids, Ca^{++}, a chromogenic substrate for thrombin and a heparin neutralizing substance. The activated protein C can also be added to the sample separately a short time before the rest of the reagent.

In a particularly preferred embodiment of the method according to the invention, the undiluted sample is admixed with 4 to 10 times the volume of protein S-deficient plasma. The clotting reaction is started by admixing (5 to 10 times the sample volume of) a reagent comprising activated protein C (1 to 50 pmol/ml), an activator of the clotting system, preferably a thromboplastin or a snake venom protease, phospholipids (5-300 ppm (w/v)), e.g. cephalin, calcium ions (2-10 mmol/l), preferably $CaCl_2$, a heparin neutralizing substance, such as e.g. Polybren (0.1-10 µg/ml) and a chromogene thrombin substrate, such as e.g. Tos-Gly-Pro-Arg-ANBA-IPA. The time from adding the reagent to reaching a certain extinction (e.g. 0.1) at the absorption optimum (e.g. 405 nm) of the liberated chromophor is measured.

The activator concentration which guarantees a clotting time (without protein S) of at least 50 sec under the given reaction conditions, can in each particular case be determined by simple experiments.

Very particularly preferred methods and reagents are those described in the examples.

The clotting time is, when using a chromogenic substrate, preferably determined by determining the time from adding the reagent to reaching a certain extinction at the absorption optimum of the liberated chromophor. For the evaluation, a calibration curve is advantageously prepared by using dilutions of a pool plasma (e.g. 100%, 75%, 50%, 25%, 12.5%, 10%) in the assay and determining the clotting times.

The biological sample can preferably be plasma of human origin, the use of undiluted samples being particularly preferred in this case.

A suitable protein S-deficient plasma can be obtained by

methods known per se to those skilled in the art, e.g. by immunoadsorption. Factor VIII is, if necessary, adjusted by adding purified factor VIII. The factor V can, if necessary, be adjusted by adding a protein S-deficient rabbit plasma which contains large amounts of factor V. The volume ratio of sample to protein S-deficient plasma is preferably 1:4 to 1:10.

The deficient plasma preferably has a factor V content of about 20 to 100%, particularly preferably of 50-80%.

Protein C can be purified from plasma by various processes which have been described (e.g. Bajaj S.P. et al. (1983) Preparative Biochemistry 13(3) 191-214) or can be prepared by biotechnological processes. The purified protein C can be activated by means of PROTAC® or thrombin which have been coupled to a support material such as Sepharose (R) or activated protein C obtained directly by genetic engineering processes (Ehrlich H.J. et al., J. Biol. Chem. 264 (24) 14298-14309) can be employed. The concentration of activated protein C in the assay is advantageously between 1 and 50 pmol/ml.

A protein C activated by a snake venom protease from the venom of Agkistrodon contortrix can preferably be employed.

Activators of the clotting system are known per se to those skilled in the art. Activators for the purpose of this invention can also be snake venom proteases and activated factors of the clotting cascade, such as e.g. factor VIIa, factor IXa and factor Xa. Preferably, proteases from the venom of Vipera russellii, sulfatides, ellagic acid, thromboplastins and/or silica particles can be employed (Shimada T. et al. (1985) J. Biochem. 97, 429-439). The optimum concentration of the particular activators can be determined by simple experiments.

Phospholipids are a known class of substances which can

be prepared by methods known per se to those skilled in the art or are commercially available. Concentrations of 5-300 ppm (w/v) in the test mixture are preferred. Ca^{2+} ions can advantageously be generated by adding $CaCl_2$. Concentrations of 2-10 mM in the test mixture are preferred.

Heparin-neutralizing substances are a class of compounds known to those skilled in the art, such as e.g. Polybren, protamine chloride, protamine sulfate.

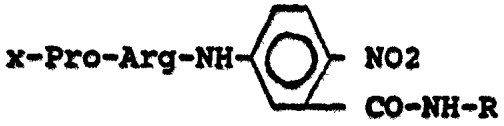
The reaction can be carried out at 15-40°C, preferably at 20-40°C, very preferably at 37°C.

It has been found that the protein S present in the plasma can be measured in a sensitive and specific way in the assay described (Table 1). Changes in concentration of factor VIII between 50 and 150%, of the vitamin K-dependent clotting factors, such as prothrombin or protein C (50-150%), except for protein S itself, or the presence of up to 0.4 U/ml heparin have no influence. The factor V content of the sample, as expected, shows a certain influence, since variations in the sample cannot be completely balanced out due to the content in the protein S-deficient plasma and the inhibition of this factor is a measurement of the activity of the protein S.

The invention furthermore relates to a reagent for determining protein S by a method according to the invention, the clotting time of a sample without protein S being at least 50 seconds.

Preferred reagents are, in this connection, those which contain a protease from the venom of Vipera russellii as activator, and those which contain a thromboplastin as activator.

Preferred reagents are also those which contain a chromogenic thrombin substrate of the formula I



(I)

where R is C_{1-5}-alkyl or $-CH[CH(CH_3)_2]COOCH_3$ and
X is H-D-Phe-, Boc-Gly or tosyl-Gly.

Table 1: Specificity of the assay for the functional determination of protein S

Changed parameter	Concentration	Protein S activity found
Control		100,0 %
Heparin	0.1 U/ml	102 %
Heparin	0.2 U/ml	97 %
Heparin	0.3 U/ml	109 %
Heparin	0.4 U/ml	109 %
Heparin	0.5 U/ml	132 %
Heparin	1.0 U/ml	> 150 %
F V	50 %	114 %
F V	75 %	111 %
F V	100 %	100 %
F V	125 %	96 %
F V	150 %	91 %
F VIII	50 %	106 %
F VIII	75 %	99 %
F VIII	100 %	98 %
F VIII	125 %	103 %
F VIII	150 %	104 %
Protein C	50 %	96 %
Protein C	100 %	100 %
Protein C	150 %	103 %

The following examples are intended to illustrate the invention and shall not restrict it in any way.

Example 1

Preparation of a ready-to-use reagent based on an aPTT reagent

0.5 units of activated protein C and 10 µg polybrene were added to an aPTT reagent (Partochrom®, Behringwerke AG, D-3550 Marburg), comprising phospholipid, sulfatide, Polybren, a chromogenic thrombin substrate (Tos-Gly-Pro-Arg-ANBA-isopropylamide) and Hepes, pH 7.6, and the mixture is heated at 37°C. The reagent is then ready to use.

Example 2

Preparation of a ready-to-use reagent based on a PT reagent

0.5 units of activated protein C are added to a PT reagent (Behringwerke AG, D-3550 Marburg) comprising phospholipid, thromboplastin at a low concentration, a chromogenic thrombin substrate (Tos-Gly-Pro-Arg-ANBA-isopropylamide) and Hepes, pH 7.4, and the mixture is heated at 37°C. The reagent is then ready to use.

Example 3

Preparation of a ready-to-use reagent based on an activator from a snake venom

40 ng/ml of a snake venom from Vipera russellii are added to a buffer comprising phospholipid, a chromogenic thrombin substrate (Tos-Gly-Pro-Arg-ANBA-isopropylamide), a heparin antagonist (Polybren), sodium chloride and Hepes, pH 7.0, and the reaction mixture is heated at 37°C. The reagent is then ready to use.

Example 4

Determination of the protein S content in plasma

For the calibration, 100%, 75%, 50%, 25%, 10% and 0% dilutions of a healthy-donor plasma are prepared in phosphate-buffered isotonic saline. The measurement of the standards and the samples is carried out as described below:

10 µl of sample
50 µl of protein S-deficient plasma
500 µl of reagent according to a) Example 1
b) Example 2

are pipetted into a cuvette.

When adding the reagent, a clock is started and the extinction at 405 nm is monitored until an increase in the absorption by a certain value (e.g. of 0.1) has been reached. The prolongation of the clotting time beyond the value for 0% protein S is proportional to the concentration of protein S in the sample (Fig. 1).

The data for the determination according to the method described in DE 37 24 443 have been taken from German Offenlegungsschrift DE 37 24 443 A1, Figure 1.

Example 5

Determination of the protein S content in plasma when separately adding activated protein C

For the calibration, 100%, 75%, 50%, 25%, 10% and 0% dilutions of a healthy-donor plasma are prepared in saline. The measurement of the standards and the samples is carried out as described below:

10 µl of sample
50 µl of protein S-deficient plasma
25 µl of activated protein C
500 µl of reagent according to Example 3

are pipetted into a cuvette.

On adding the reagent, a clock is started and either the extinction at 405 nm is monitored until an increase in absorption by a certain value (e.g. of 0.1) has been reached, or the occurrence of clot formation is measured. The prolongation of the clotting time beyond the value for 0% protein S is proportional to the concentration of protein S in the sample (Fig. 2).

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for the functional determination of protein S in a sample of a biological fluid, said method comprising the steps of:

a) adding activated protein C to the sample

b) starting the coagulation reaction by adding a reagent containing a defined amount of an activator of clotting to the sample

c) determining the clotting time,

said amount of an activator being such that a sample without protein S will give a clotting time of at least 50 seconds.

2. The method of claim 1, further comprising the step of adding an excess of protein S-deficient plasma to the sample.

3. The method of claim 1 or 2, wherein the clotting time is determined by adding a chromogenic substrate for thrombin and measuring the time until a defined increase in optical density is reached.

4. The method of any one of claims 1 to 3, wherein the biological fluid is plasma deficient in protein S and has a factor V content of 20 to 100%.

5. The method of any one of claims 1 to 4, wherein the protein C is activated by a snake venom protease from venom of Akgistrodon contortrix.

6. The method of claim 1, wherein said reagent containing an activator of clotting further contains calcium ions, phospholipids, activated protein C and an inhibitor of heparin.

7. The method of claim 1, wherein an undiluted sample is admixed with 4 to 10 times the volume of protein S-deficient plasma, the clotting reaction is started by admixing 5 to 10 times the sample volume of a reagent comprising 1 to 50 pmol/ml activated protein, an activator of the clotting system, 5-300 ppm by w/v

phospholipids, 2-10 mmol/l calcium ions, a heparin-neutralizing substance and a chromogenic thrombin substrate, and the time from adding the reagent to reaching a certain extinction at the adsorption optimum of the liberated chromophor is measured.

8. A reagent when used in a method for the functional determination of protein S in a sample of biological fluid, said method comprising the steps of:

a) adding activated protein C to the sample
b) starting the coagulation reaction by adding a reagent containing a defined amount of an activator of clotting to the sample
c) determining the clotting time,

said amount of an activator being such that a sample without protein S will give a clotting time of at least 50 seconds.

9. The reagent as claimed in claim 8 which contains activated protein C, an activator of the exogenous or endogenous path of clotting, phospholipids, calcium ions, if appropriate, a chromogenic substrate for thrombin, and a heparin-neutralizing substance.

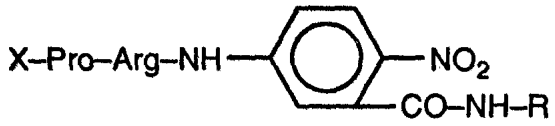
10. The reagent as claimed in claim 8, wherein the activator is a protease from the venom of a snake or the refractioned snake venom.

11. The reagent as claimed in claim 8, wherein a thromboplastin is used as activator.

12. The reagent as claimed in claim 8, wherein the activator is a sulfatide or a mixture of sulfatides.

13. The reagent as claimed in claim 8, wherein the heparin inhibitor is Polybren.

14. The reagent as claimed in claim 8, wherein chromogenic thrombin substrate used is a compound of the formula I



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where R is C_{1-5}-alkyl or -CH [CH $(CH_3)_2$]$COOCH_3$ and
X is H-D-Phe-, Boc-Gly or Tosyl-Gly.

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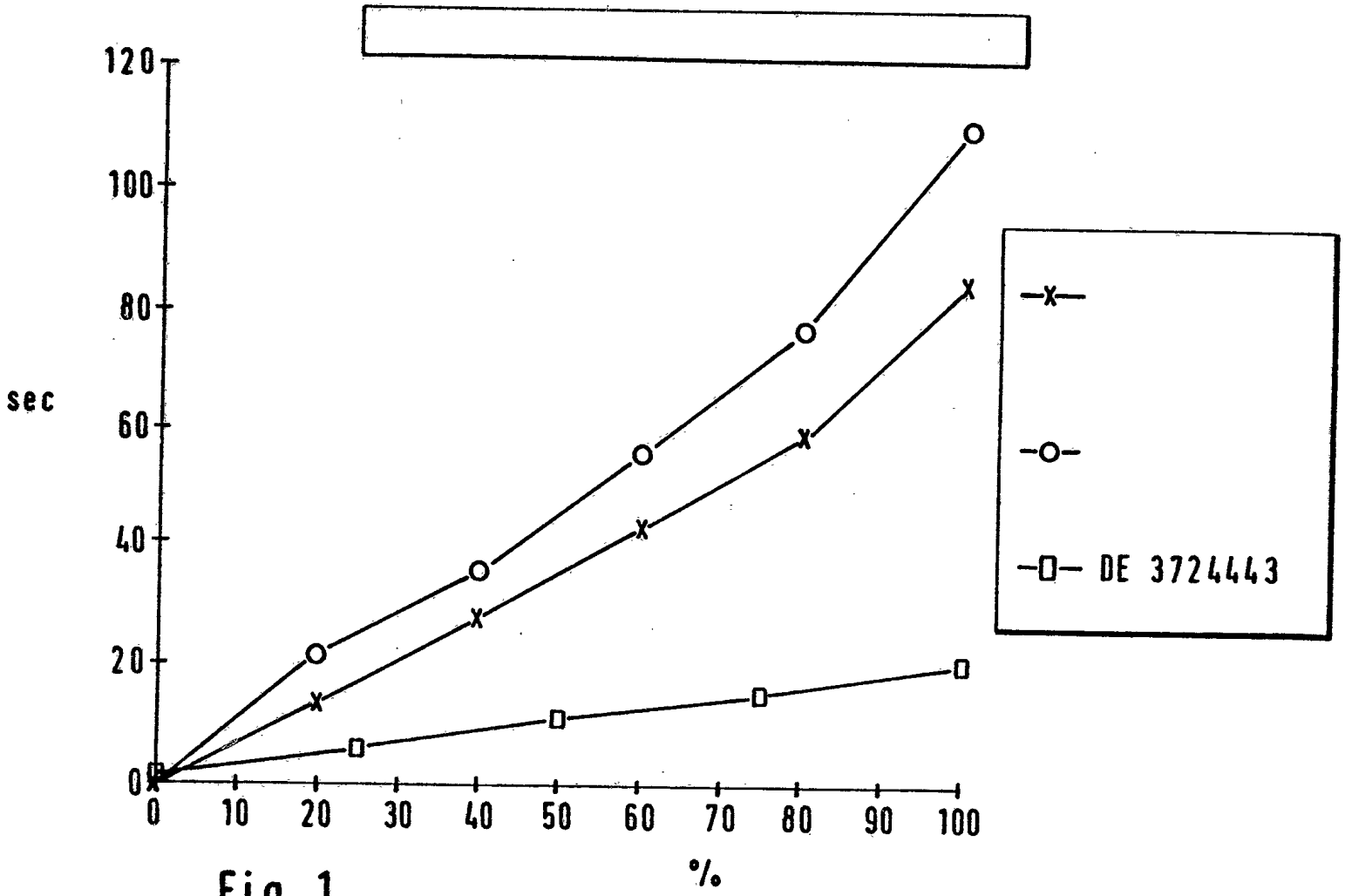
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Fig. 1

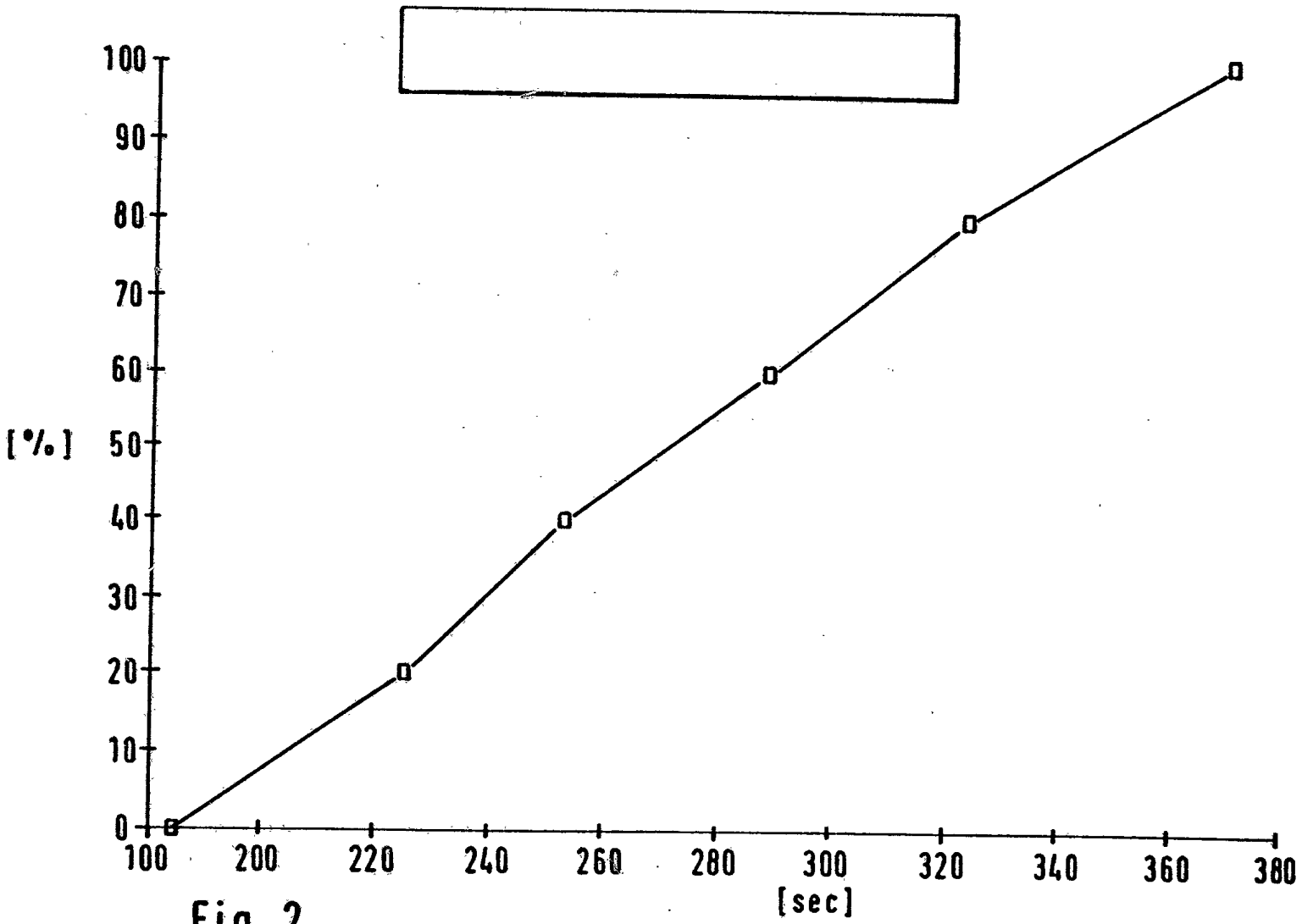
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Fig. 2