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Criteria for specific measurement of plasminogen (enzymatic; procedure) in human plasma

Johannes J. Sidelmann [1], Nuala A. Booth [2], Johannes Hoffmann [3], Michael E. Nesheim [4] and Steffen Rosén [5].

Abstract

There is a lack of well-established criteria for the specific measurement of fibrinolytic variables. On behalf of the SSC the Subcommittee on Fibrinolysis started a process to develop such criteria. This report describes the criteria for the measurement of plasminogen (enzymatic; procedure) in human plasma.

Apparently, the most specific methods for determination of plasminogen (enzymatic; procedure) adhere to the principle of streptokinase induced activation of plasminogen and recording of activity using a chromogenic substrate. Incorporation of fibrinogen attenuates the potential effect of elevated fibrinogen or fibrin(ogen) fragments in the plasma sample. The criteria for specific measurement of plasminogen(enzymatic; procedure) are based on this analytical principle.

The kinetics and principles of the assay procedure are described, and criteria as well as test methods for criteria are detailed. Guidelines for standardization, quality assurance, analytical sensitivity and establishment of reference intervals are given. The pre-analytical conditions regarding preparation of the patient and the specimen are delineated.

Nonstandard abbreviations: SSC, Scientific and Standardization Committee ; ISTH, The International Society on Thrombosis and Haemostasis; IFCC, The International Federation of Clinical Chemistry; NCCLS, National Center for Clinical Laboratory Standards; HRG, histidine-rich glycoprotein; Lp(a), lipoprotein (a); SK, streptokinase

Introduction Human plasminogen is a single-chain glycoprotein and the molecular mass is 92,000 g/mol. The molecule consists of 5 kringle domains and a catalytic domain. The human gene is constituted of 55 kb, 19 exons, 18 introns (1), and is located on chromosome 6 (2). The plasma concentration is about 2 $\mu\text{mol/l}$. Plasminogen is mainly synthesized in the liver, and the native form with a N-terminal glutamic acid (Glu-plasminogen) has a catabolism corresponding to a plasma half-life of about 2.2 days. The slightly degraded Lys-plasminogen has a half-life of 0.8 days (3). Plasminogen circulates in human blood in two different forms. Approximately half of the plasminogen is bound to histidine-rich

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1 Department for Thrombosis Research, University of Southern Denmark, Esbjerg, Denmark.

2 Department of Molecular & Cell Biology, Institute of Medical Sciences, University of Aberdeen, Scotland.

3 Clinical Laboratories, Catharina Hospital, Eindhoven, the Netherlands.

4 Department of Biochemistry, Queens University, Kingston, Ontario, Canada.

5 Chromogenix AB, Mölndal, Sweden.

Correspondence to: Johannes J. Sidelmann, Department for Thrombosis

glycoprotein (HRG) while the other half circulates in free form (4). The binding of plasminogen to HRG may cause an antifibrinolytic effect, but the role of HRG in fibrinolysis is so far not convincingly established (5).

Plasminogen is the precursor of plasmin - the ultimate fibrinolytic enzyme. The conversion of plasminogen to plasmin requires proteolytic cleavage by one of the plasminogen activators, e.g. by plasminogen activator, tissue type or plasminogen activator, urokinase type. Plasminogen has the capacity to bind to fibrin through its kringle structures and thus direct the fibrinolytic process to its target, fibrin. Plasminogen shows a high degree of homology with lipoprotein(a) (Lp(a)), which inhibits the binding of plasminogen both to fibrin and to a cellular plasminogen receptor (6), a process that can interfere with the fibrinolytic process.

Reduced plasma levels of plasminogen can occur due to congenital deficiencies, of which two types are known, type I and II respectively. Whether heterozygous plasminogen deficiency is a thrombotic risk factor is currently under debate. Some studies have failed to demonstrate an association between reduced plasma concentrations of plasminogen and thrombosis (7, 8), while other studies have shown an increased risk (9). Recently, homozygous type I plasminogen deficiency has been reported in a family, and these patients suffer from ligneous conjunctivitis, but none of the reported patients had experienced any episodes of thrombosis (10). Acquired plasminogen deficiency can be found in patients receiving thrombolytic therapy (11), among patients suffering from liver cirrhosis (12) and renal disease (13). Elevated plasminogen concentrations are observed among women receiving oral contraceptives (14), and among subjects using anabolic steroids (15 -18).

Several chromogenic methods are currently available for the determination of plasminogen (enzymatic; procedure) in human plasma, and most of these methods adhere to the principle of streptokinase induced activation of plasminogen. One commercially available method for plasminogen activity includes addition of fibrinogen to the reaction mixture in order to attenuate the overestimation of plasminogen in pathological plasma samples containing elevated levels of fibrinogen or fibrin fragments.

This report describes the criteria for a specific method for determination of plasminogen (enzymatic; procedure) in human plasma. The criteria are restricted to glu-plasminogen, but other forms of plasminogen such as lys- and mini-plasminogen are not expected to be found in human plasma. However, in any case when these forms are to be analysed a separate evaluation of the efficiency of recording should be undertaken.

Kinetics Streptokinase mediated activation of human plasminogen is a multistep reaction (19). First, plasminogen reversibly binds streptokinase and forms a plasminogen*strep-kinase complex intermediate, which has no proteolytic activity. The complex is then converted to an intermediate with proteolytic activity belonging to the plasminogen moiety, and this intermediate is then rapidly converted to the final proteolytic plasmin*streptokinase complex. Excess of streptokinase is mandatory in order to convert all plasminogen into the active plasminogen*strep-

tokinase complex. Furthermore, it is documented that addition of human fibrinogen to the reaction mixture has a potentiating effect on the activity of the plasminogen*streptokinase complex (20), and that addition of fibrinogen attenuates the overestimation of plasminogen induced by elevated concentrations of fibrinogen or fibrin fragments in the sample (21).

Principles of the assay procedure

The assay of plasminogen (enzymatic; procedure) by addition of strepto-

Fibrinogen

Plasminogen + SK (excess) → plasminogen*SK-complex + SK (residual)

- b. Determination of the amidolytic activity of the plasminogen*SK-complex on a synthetic tripeptide chromogenic substrate (p-nitro-aniline (pNA) release at 405 nm)

Plasminogen*SK-complex + chromogenic substrate → pNA

kinase (SK) and fibrinogen to diluted human plasma involves two reaction steps:

The rate of pNA release is compared with similar data of a calibration curve constructed by using different dilutions of a pooled plasma calibrator. The plasminogen content of the pooled plasma calibrator is set at 100% or 1 arbitrary unit/ml.

The plasminogen*SK-complex is assumably poorly inhibited by most protease inhibitors (22-24). Lp(a) has been shown to interfere with the streptokinase induced activation of plasminogen (25). Other potential interfering proteins might be HRG, plasmin inhibitor, fibrinogen and fibrin(ogen) fragments. Various drugs such as aprotinin, heparin, tranexamic acid and hirudin might influence the assay .

Manuals The determination of plasminogen (enzymatic; procedure) in human plasma should be described in detail in a laboratory manual.

Criteria for specificity

Analytical recovery studies should show a recovery of the added plasminogen ±5%.

Dose-response curves of the calibrator and patient samples should be parallel when plotted in a double-logarithmic graph.

Plasma deficient in plasminogen should show a concentration below the detection limit of the assay procedure.

Lp(a), HRG and plasmin inhibitor, at the level usually found in pathological conditions or at the higher normal level, should not interfere with the assay.

Fibrinogen and fibrin(ogen) fragments, at the level usually found in pathological conditions, should not interfere with the assay.

Test methods for criteria Measuring the following samples should test a method for the determination of plasminogen for specificity:

A plasminogen activity corresponding to 100% of normal plasma spiked to plasmino-gen deficient plasma should be recovered in the range of 95-105% ($100 \pm 5\%$).

Five point dose-response curves of the calibrator and the sample should be parallel in a double-logarithmic plot. When subjected to linear regression analysis the slope of the dose-response curve of the sample should be within $\pm 5\%$ of the slope for the calibration curve.

Plasma naturally deficient or immunosorbed for plasminogen should show a plasminogen concentration below the analytical detection limit.

Plasma charged with up to 800 mg/l of Lp(a)[\[2\]](#) , up to 3,6 $\mu\text{mol/l}$ of HRGb , up to 2,5 $\mu\text{mol/l}$ of plasmin inhibitorb and up to 30 $\mu\text{mol/l}$ of fibrinogen[\[3\]](#) should display the same plasminogen concentration as plasma without excess of these components. (Spiking of plasma with fibrin(ogen) fragments [\[4\]](#) (If one or more substances show interference which cannot be prevented, specimens containing such substances are unsuitable for analysis[\[5\]](#) .

Spiking of plasma with fibrin(ogen) fragments?[\[3\]](#)

If one or more substances show interference which cannot be prevented, specimens containing such substances are unsuitable for analysis.[\[4\]](#)

Standardisation, quality assurance and analytical detection limit Standardisation An International Standard prepared from human plasma is not available. A pool of plasma from at least 30 apparently healthy volunteers not taking oral contraceptives, hormonal replacement therapy or anabolic steroids should therefore be used as calibrator. The calibration curve should cover the whole reference range and should include at least 5 different dilutions. Data for calibration curves and linearity should be subjected to linear regression analysis, and should include the slope, intercept, standard error of estimate (standard deviation about the regression line), and the standard deviations of the slope and the intercept. The goals with respect to these estimates should be defined.

Analytical imprecision Studies must include estimates of intra-assay and inter-assay coefficients of variation (CV). Each should be determined at low and normal concentrations with the use of specimens that are in an appropriate matrix. Commercially available plasma specimens can be

used to control the assay procedure.

Two control specimens should be included in each set of measurement, including a normal range value (e.g.100%) and a low range value (e.g. 20%). Both the inter-assay and intra-assay CV should be lower than 6%. The intra-assay CV should be based on at least 20 single determinations on each control specimen analysed in one analytical run. The inter-assay CV should be based on determination of each control specimen in at least 10 individual runs.

Detection limit of the analytical procedure The analytical detection limit should be defined as the concentration of plasminogen corresponding to a signal 3 SD above the mean for a calibrator that is free of plasminogen.

Remarks Preparation of the patient (pre-analytical conditions)

Since no diurnal rhythm for plasminogen is known, blood sampling can take place any time of the day.

To avoid variation in haematocrit, select between the sitting and supine position of the patient during blood collection. General guidelines for preparation of the patient before collection of fibrinolytic variables are recently published (27) and should be followed. Further information can be obtained from the guidelines given by the IFCC (28).

Preparation of the specimen (pre-analytical conditions) The NCCLS guidelines given for collection, transport, and processing of blood specimens for coagulation testing and general performance of coagulation assays should be followed in detail (29).

Instrumentation The assay procedure can be done by a manual method as well as with automated analysers with the possibility for photometric measurements (405 nm). Since analysers from different suppliers have their own specifications and limitations, the criteria for specificity should be tested for all type of equipments separately, or made available from the reagent manufacturers.

Reference interval in healthy adult subjects The reference interval should be established using samples from at least 240 healthy individuals; 120 women and 120 men (30) covering all ages between 20 and 80 years. Individuals receiving oral contraceptives, hormonal replacement therapy or anabolic steroids must not be included in the reference population.

The reference interval should be established by statistical treatment of the results obtained. The reference interval should preferably be given as the 2.5-97.5 inter-percentile range including the uncertainty of the percentile estimates according to the recommendations given by the IFCC (30).

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[1]

In a clinical study no significant correlation between the plasma concentrations of plasminogen (enzymatic; procedure) and Lp(a) (immunological; procedure), HRG (immunological; procedure) and plasmin inhibitor (enzymatic; procedure) was observed (JJ Sidelmann, unpublished data). Plasminogen (enzymatic; procedure) was determined as described by Gram & Jespersen (21). Plasma samples from 89 patients showed plasminogen concentrations between 75 - 168%. The Lp(a) concentrations were up to 1,134 mg/l, HRG concentrations were between 67 - 198% and plasmin inhibitor concentrations were between 56 - 164%. Correlation analysis revealed no statistically significant association between plasminogen and Lp(a), HRG, or plasmin inhibitor. $P=0.90$, $P=0.20$, $P=0.09$, respectively. This indicates that Lp(a), HRG and plasmin inhibitor are without influence on the determination of plasminogen (enzymatic; procedure).

[2]

Identical standard curves were obtained when two different levels of fibrinogen were added to the streptokinase reagent, 167 mg/ml and 333 mg/ml, and there was no (further) influence on the plasminogen activity at the higher fibrinogen level. Also, five different samples (normal/abnormal controls, normal plasma samples, samples containing high concentration of fibrin fragments and fibrinogen, respectively) were

evaluated at these two fibrinogen levels. There was no significant difference in the assigned values (S. Rosén, unpublished data).

[3]

Fibrin(ogen) fragments represent a particular problem because a variety of fibrin(ogen) fragments may be present in plasma. Thus, a standardised preparation of fibrin- and fibrinogen fragments is not available. Ideally the influence of all the various forms of fragments should be studied, but obtaining pure preparations might be very difficult. Furthermore, the outcome of quantification of fibrin(ogen) fragments depends on the method used, and international standards are not presently available. Therefore it is not possible to add an exact amount of fibrin(ogen) fragments to a normal plasma sample, and subsequently study the influence on the determination of plasminogen (enzymatic; procedure). One way to overcome this problem could be to study normal plasma mixed with pathological samples containing elevated concentrations of fibrin(ogen) fragments.

The correlation between plasminogen assays performed in the presence and absence of added fibrinogen was studied using plasmas from normal healthy individuals and plasmas from patients where a majority had elevated D-Dimer levels. As expected the correlation was strong for the normal samples; however, the analysis of patient plasmas showed a more diverse picture. In most cases considerably lower values were (correctly) obtained in the presence of added fibrinogen, but there were a few with similar high plasminogen activities in both methods. The most straightforward explanation is that also in these cases correct assignments were obtained, and that thus the plasminogen activity indeed was elevated possibly as an acute phase response. Importantly all these patients had significantly elevated D-Dimer and they might well have been in an acute phase response state (S. Rosén, unpublished data).

In the work of Gram and Jespersen (21) it was demonstrated that addition of fibrinogen to the assay resulted in a good correlation ($y = 1.0x - 1.1$, $r = 0.98$) between plasminogen (immunological; procedure) and plasminogen (enzymatic; procedure) in plasma samples from patients with elevated concentrations of fibrin fragments. Thus, addition of fibrinogen results in an apparent complete activation of plasminogen. Additional studies might be necessary.

[4]

Plasma from patients undergoing thrombolytic therapy is not recommended to be assayed by this procedure (22). Plasma containing protease inhibitors such as aprotinin (26) is unsuitable to be assayed by this procedure. Other potentially interfering components might be heparin, tranexamic acid and hirudin. The influence of these components need to be studied in detail before it can be decided whether plasma samples containing these components are suitable for analysis.

IFCC Technical Secretariat,
30 Rue Lionnois, F-54000 Nancy,
France

Phone: +33 3 83 35 26 16
Fax: +33 3 83 32 13 22
Email: Chantal.Thirion@ifccs.u-nancy.fr

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