Criteria for the specific measurement of Plasmin Inhibitor activity using an enzymatic procedure.

Piet Meijer Gaubius Laboratory, TNO-PG, Zernikedreef 9, 2333CK Leiden, The Netherlands. Fax. +31-71-5181496, E-mail: P.Meijer@pg.tno.nl

Michel Hanss Laboratoire d’Hematologie, Hopital Cardiologique, Lyon Cedex, France

Ulla Christensen Kemisk laboratorium 4, H.C. Orsted Institutet, Copenhagen, Denmark

Bjorn Wiman Department of Clinical Chemistry and Blood Coagulation, Karolinska Hospital, Stockholm, Sweden

Kees Kluft Gaubius Laboratory, TNO-PG, Zernikedreef 9, 2333CK Leiden, The Netherlands

Correspondence to: Piet Meijer, Gaubius Laboratory, TNO-PG, Zernikedreef 9, 2333CK Leiden, The Netherlands. Fax. +31-71-5181496, E-mail: P.Meijer@pg.tno.nl

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Specific measurement of Plasmin Inhibitor activity

Summary
There is a lack of well-established criteria for the specific measurement of fibrinolytic parameters. On behalf of the SSC, the subcommittee on Fibrinolysis started a process to develop criteria for the specific measurement of fibrinolytic variables. This report describes the criteria for the specific measurement of plasmin inhibitor activity. In summary, a plasma deficient in plasmin inhibitor should show an activity close to 0%. Plasma containing only the non-plasminogen binding form of plasmin inhibitor should show an activity nearby the activity of a plasma deficient for plasmin inhibitor. Other inhibitors of plasmin, like a 2 -macroglobulin, antithrombin in the presence of heparin, and C1-esterase inhibitor should not interfere in the assay at the level usually found in pathological conditions or at the higher normal level.

Introduction
Plasmin Inhibitor, previously known as a 2 -plasmin inhibitor or a 2 -antiplasmin (1), occurs in blood partially as a very fast-acting inhibitor of plasmin and therefore is an important regulator of the fibrinolytic system (2). The glycoprotein, plasmin
inhibitor, is a serine protease inhibitor of molecular weight 65-70 kD, present in plasma at a concentration of approximately 1 mM/l (3). The circulating glycoprotein is mainly synthesized by the liver and has a catabolism corresponding to a plasma half-life of about 2.5 days (2). The human gene is constituted of 16 Kb, 10 exons, 9 introns, and is located on chromosome 17 (4,5).

Reduced plasma levels of plasmin inhibitor can occur due to congenital deficiencies I and II (6). These deficiencies can be associated with bleeding occurring some hours after the initial injury. Clotting and wound healing are usually normal, but the haemostatic plug breaks down prematurely (7). Decreased concentrations are known for thrombotic therapy, severe chronic liver diseases, nephrotic syndrome, disseminated intravascular coagulation, amyloidosis, leukaemia (specially acute promyelocytic leukaemia), L-asparaginase therapy, the postoperative period and extracorporeal circulation (6,8-11). Elevated levels of plasmin inhibitor have been observed in some cases with thrombotic complications and in cases with type II hyperlipoproteinemia and progressive renal failure (12-17).

The plasmin inhibitor occurs in blood mainly in two molecular forms: a plasminogen-binding (PB) and a non-plasminogen binding (NPB) form (18). On average the ratio PB:NPB is 2:1 (19). The PB form is a very fast-acting plasmin inhibitor; NPB reacts at least 20 times more slowly (20-25). The PB form of plasmin inhibitor is responsible for the rapid plasmin inactivation observed in plasma. The inhibitor further can lose its N-terminal 12 amino acid peptide in the circulation (26,27) and acquires the capacity to cross-link to fibrin catalysed by coagulation factor XII (26,28). In blood other molecules can also complex with plasmin, e.g. a 2-macroglobulin, antithrombin and C1-esterase inhibitor (29).

For the quantitative analysis of the fast-acting PB form of plasmin inhibitor in plasma, several chromogenic methods have been developed (30-34). Recently it has been shown that some of the commercially available activity methods have restricted specificity, especially at low concentrations. This effect results in values of 10-30% in the analysis of plasmin inhibitor deficient plasmas (35-37). It was shown that the apparent plasmin inhibitor values for deficient plasmas will increase with increased levels of added plasmin (37). The reason could be an increased effect of a 2-macroglobulin. It is reported that at low plasmin concentrations a 2-macroglobulin can play an important role in the inhibition of plasmin (25,38). Using selected low plasmin concentrations (37) a new commercially available method has been developed with enhanced specificity (39).

This report describes the criteria for a specific method of the functional measurement of the fast-acting form of plasmin inhibitor in plasma.

**Kinetics**

In the measurement of plasmin inhibitor three reactions of plasmin are of importance. First is the reaction with the PB-form of the plasmin inhibitor. Second is the reaction with the NPB-form of the plasmin inhibitor and third the reaction with a 2-macroglobulin. Using a small excess of plasmin (~1 mM), all three reactions take place as a second order reaction ([plasmin inhibitor] total approx. [plasmin] total). At a plasmin inhibitor concentration of 1 mM gives this a velocity for the plasmin-PB-plasmin inhibitor complex formation of 27 s⁻¹. For the plasmin-NPB-plasmin inhibitor complex formation this is 0.2 s⁻¹ and for the plasmin-a 2-macroglobulin complex formation this is 1.0 s⁻¹. Expressed in ratios of the complexes this is 95.7% for the plasmin-PB-plasmin inhibitor complex, 0.7% for the plasmin-NPB-plasmin inhibitor complex and 3.6% for the plasmin-a 2-macroglobulin complex.

At a level of 0.1 mM of plasmin inhibitor the ratios of the complexes will be 72.6% for the plasmin-PB-plasmin inhibitor complex, 0.8% for the plasmin-NPB-plasmin inhibitor complex and 26.9% for the plasmin-a 2-macroglobulin complex (25,38).

The increased importance of the influence of a 2-macroglobulin at low plasmin inhibitor concentrations indicates the need for inhibition of this effect. The effect of a 2-macroglobulin could be abolished by the addition of methylamine to the assay system (40,41).

**Principle of the assay procedure**

The assay of the fast-acting form of plasmin inhibitor involves two reaction steps illustrated in fig 1.

The rate of pNA release is compared to similar data of a calibration curve constructed by using different dilutions of pooled plasma standard. The content of the pooled plasma standard is set at 1 arbitrary unit/ml (~100%).
The amount of plasma used in the test and in relation to that the concentration of the added plasmin and the incubation time is important for the specificity of the method (37).

Since plasmin and plasmin inhibitor forms a 1:1 molar complex the excess of plasmin added to the test should be minimised to avoid the influence of other inhibitors.

The preincubation time should be as short as possible (< 60 seconds) to avoid the participation of slow-acting inhibitors in the reaction.

Manufacturers of commercially available tests should have stated these variables in their kit insert, expressing the final plasmin concentration (nKat) and the time of the different reaction steps (preincubation and reading).

The measurement of plasmin inhibitor can be influenced by a 2-macroglobulin. This effect can be abolished by the addition of methylamine to the dilution buffer (40).

In a specific test for plasmin inhibitor no interference was observed for _-amino caproic acid (4 mmol/l), Heparin (2 IU/ml), Fragmin (2 IU/ml) and Lysine (2000 m mol/l) (43).

**Manuals**

The determination of plasmin inhibitor is described in a laboratory manual.

ECAT assay procedures: Plasma Plasmin Inhibitor activity by C. Kluft and P. Meijer (34)

**Criteria for specificity**

1. Plasma deficient in plasmin inhibitor, both for the PB and NPB form, should show an activity close to 0%.

2. Plasma containing only the NPB form of plasmin inhibitor should show an activity near to the activity of a plasma deficient in plasmin inhibitor.

3. A 2 -macroglobulin, antithrombin in the presence of heparin, and C1-esterase inhibitor should not interfere in the assay at the level usually found in pathological conditions or at the higher normal level.

**Test method for criteria**

A method for the detection of plasmin inhibitor should be tested for specificity by measuring the following samples:

a. Plasma naturally deficient in or immuno-depleted for plasmin inhibitor should show a residual activity less then 5%.

b. The plasmin inhibitor in plasma containing only the NPB form of plasmin inhibitor should be equal to plasma deficient in plasmin inhibitor (residual activity less then 5%, see point a).
c. Plasma charged with 3 times higher level of a 2-macroglobulin, C1-esterase inhibitor or antithrombin in the presence of heparin (2 IU/ml), in normal plasma should have the same plasmin inhibitor as the plasma without the addition of an excess of these inhibitors.

**Standardisation and quality assurance**

No reference material is available at present. Calibrator plasma should be obtained by pooling plasma of apparently healthy volunteers (at least 20 donors), using a sex ratio of approximately 1. Users of oral contraceptives or hormone replacement therapy should be excluded. The value of this calibrator plasma is set at 1 arbitrary unit (AU) (= 100%). A calibration curve should cover the whole reference range and exists of minimal 5 points.

Two control plasmas should be included in each set of measurements, including a normal range value (0.80-1.00 AU) and a low range value (0.20-0.40 AU). Repeatability and reproducibility should allow preferably less than 6% of variation coefficient on 10 consecutive determinations.

**Remarks**

1. **Collection of the blood sample**

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

To avoid variability in haematocrit, select either the sitting or lying position of the patient during venepuncture (42).

2. **Processing of the blood sample.**

Storage of blood for a longer time and at higher temperatures promotes the conversion of the PB-form to the NPB-form (19).

3. **Instrumentation**

The test for the determination could be done by a manual method as well as with automated analysers with the possibility of 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.

**4. Reference ranges**

In 25 apparently healthy volunteers, aged between 20 and 50 years and sex ratio approximately 1, plasmin inhibitor, assayed with a method fulfilling the criteria described above, showed a narrow range: 83 - 108%.

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