

Standardization of Protein C in Plasma: Establishment of an International Standard

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Key words

Protein C – Plasma – International Standard

Summary

An international collaborative study, involving 18 laboratories, was carried out to establish an international standard for protein C in plasma. The proposed standard, which consisted of a freeze-dried ampouled plasma preparation coded 86/622, was assayed against fresh normal plasma and the participants' local standards. Protein C activity assays were placed in four groups, depending on the method of activation and detection of protein C. The combined potencies (units per ampoule) for the proposed international standard were: thrombin activation/clotting assays, 0.86; thrombin activation/chromogenic assays, 0.81; snake venom activation/clotting assays, 0.81 and snake venom activation/chromogenic assays, 0.82. Measurement of protein C antigen gave potency estimates of 0.81 and 0.82 units per ampoule for the Laurell electroimmunoassay and ELISA techniques, respectively. The good agreement in potency estimates between the different methods indicates that the overall combined figure (226 assays) for the international standard of 0.82 international units per ampoule should serve for all methods. Accelerated degradation studies have indicated that the standard should be suitably stable when stored at -20°C .

The freeze-dried plasma 86/622 has been established by the WHO Expert Committee on Biological Standardization as the 1st International Standard for Protein C in Plasma, with an assigned unitage of 0.82 international units per ampoule.

Introduction

Protein C is a vitamin K-dependent serine protease which, following activation, inhibits blood coagulation by the proteolytic degradation of factors Va and VIIIa (1, 2, 3). Hereditary deficiency of protein C has been linked with recurrent thrombotic disease in numerous families (4, 5, 6). In the past, the measurement of protein C in plasma has involved the use of locally collected pooled plasmas as reference material. However, the normal range of protein C activity in plasma is fairly wide (0.61–1.32 units/ml) (7) and this could lead to variation between local pools. The introduction of an International Standard for Protein C in plasma should overcome this variability by defining the International Unit and bring about an improvement in inter-laboratory agreement of potency estimates. In this paper, the results are presented from an international collaborative study designed to calibrate an ampouled freeze-dried plasma preparation for protein C by measurement of both activity and antigen.

Materials and Methods

1. *The proposed International Standard, P (coded 86/622)*, was prepared from a pool of normal plasma collected from 15 donors, kindly supplied by the North London Blood Transfusion Centre, Edgware, Middlesex, UK. Blood was collected into citrate-phosphate-dextrose-adenine anticoagulant. Each donation was tested for the presence of Hepatitis B surface antigen and anti-HIV antibodies and was found to be negative. The donations were centrifuged twice to remove cells and the plasma was also buffered by the addition of N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) at a final concentration of 0.04 M. Approximately 4,000 glass ampoules each received 1 ml of the pooled plasma (mean weight 1.0049 g; range 1.0022–1.0061 g), which was subsequently freeze-dried, secondary desiccated and finally sealed under nitrogen as described by Campbell (8). The ampouled plasma was reconstituted by the addition of 1 ml distilled water.

2. *Fresh, normal, pooled plasma, N*, was collected and prepared by each participating laboratory according to Appendix 1. A total of 242 donors was used by the participants to produce the normal pools.

3. *Local standards, L*, were included in the assays. Of the 18 laboratories, 14 used a frozen pool and four used a freeze-dried pool of normal plasma.

4. *Design of the collaborative study*. The names of the 18 participants in the study are listed alphabetically in Appendix 2. The participants were assigned code numbers for the study, which do not relate to the order in which they appear in the Appendix.

Each participant was requested to carry out six assays by each method they used. It was recommended that the six assays be performed in three sessions, each of two assays, against three different fresh pools of normal plasma. Participants were asked to use their normal methodology but to follow balanced designs in which three dilutions of the materials P, N and L were assayed, preferably in replicate. It was also recommended that activity assays should be carried out against fresh pooled normal plasma, whereas antigen assays could be carried out against frozen aliquots of the fresh pools. Most participants carried out assays using one activity and one antigen method. The raw data from the assays were returned to NIBSC for analysis.

5. *Assay methods: Protein C activity*. The different methods currently used to measure protein C activity were divided into four groups, depending on the method of activating protein C (either thrombin or snake venom) and the method of detecting the activated protein C (either chromogenic or clotting).

a. *Thrombin activation/clotting assays*. Four laboratories used methods very similar to that of Francis and Patch (9), except that one laboratory used a colorimetric PTT instead of a conventional clotting end-point and another included thrombomodulin, which is an optional cofactor in the activation of protein C in vitro.

b. *Thrombin activation/chromogenic assays*. Five laboratories used the method of Bertina et al. (7), two of which used this in the form of the Coa-Set Protein C kit (KabiVitrum, Sweden) and two included thrombomodulin in the activation step. One additional laboratory used the method of Sala et al. (10).

c. *Snake venom activation/clotting assays*. Five out of seven laboratories used commercial kits for this assay. The methodology included that given in three published sources (11, 12, 13). One laboratory performed a colorimetric PTT instead of a conventional clotting time.

d. *Snake venom activation/chromogenic assays*. Three out of eight laboratories used commercial kits for this assay and the methods used included those of Lobermann et al. (11), Vinazzer and Pangraz (12) and an adaptation of the method of Martinoli and Stocker (13).

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Protein C antigen. Ten participants used either the Laurell electroimmunoassay technique (14) or variations of this method (5, 11, 15). Five laboratories used ELISA techniques which included those of Boyer et al. (16) and Amiral et al. (17).

6. Statistical methods. All assays were analysed using parallel line methods generally relating log-response to log dose (18). Validity of the assays was based on the linearity and parallelism of the dose/response lines. The potency of the proposed standard (P) relative to fresh normal plasma (N) was calculated as a function of the horizontal distance between the dose/response lines. Potencies from individual assays were combined to give geometric means and 95% confidence limits. Combinations of all potencies from a particular method and for the overall mean potency were calculated from the individual assays (total of 226 assays).

Intra-laboratory variability of potencies (geometric coefficient of variation, %) was calculated from estimates of the local standards, L, relative to the proposed standard, P since, within each laboratory, P and L were consistent for all assays, whereas the fresh normal plasma, N, usually consisted of three separate pools.

7. Definition of the unit of protein C. One unit of protein C was defined as that found in 1 ml of fresh pooled normal plasma, N.

8. Stability studies. Samples of the proposed standard, P, were stored at elevated temperatures (+4, +20 and +37°C) for 11 or 13 months before being assayed against samples stored at -20°C. The results were analysed according to the method of Kirkwood and Tydeman (19), which used a fitted Arrhenius equation to predict the degradation rates.

Results

A total of 235 assays was submitted, of which 226 were accepted as statistically valid.

Table 1 Potency estimates (units per ampoule) of the proposed standard (P), relative to fresh normal pooled plasma (N), as measured using protein C activity methods, together with estimates of intra-laboratory assay variability (gcv %)

Laboratory No.	Mean potency (+ 95% confidence limits)	GCV%
a) Thrombin activation/clotting assays		
7*	0.81 (0.74–0.91)	8.2
9	0.92 (0.84–1.00)	7.2
10	0.82 (0.76–0.87)	9.0
18	0.88 (0.82–0.94)	3.9
b) Thrombin activation/chromogenic assays		
4	0.88 (0.80–0.97)	4.6
6	0.81 (0.74–0.90)	6.9
10	0.78 (0.69–0.88)	5.8
12	0.76 (0.55–1.05)	5.9
13	0.78 (0.75–0.81)	4.6
16	0.80 (0.77–0.83)	6.0
c) Snake venom activation/clotting assays		
1	0.87 (0.84–0.91)	5.1
2	0.79 (0.71–0.89)	6.4
3	0.76 (0.71–0.81)	6.2
5	0.76 (0.72–0.80)	3.8
7*	0.78 (0.72–0.84)	8.5
7	0.85 (0.81–0.89)	7.3
11	0.91 (0.88–0.95)	3.4
d) Snake venom activation/chromogenic assays		
1	0.70 (0.69–0.71)	0.9
2	0.80 (0.79–0.82)	0.8
3	0.76 (0.73–0.79)	6.4
8	0.96 (0.93–0.98)	4.0
11	0.96 (0.92–0.99)	3.2
15	0.80 (0.68–0.94)	2.9
16	0.82 (0.80–0.84)	4.3
17	0.83 (0.72–0.94)	5.6

* Colorimetric PTT using Thromboquant reagent

1. Comparison of the Proposed Standard (P) with Fresh, Normal Plasma (N)

Potency estimates for the proposed standard (P) relative to fresh, normal plasma (N), together with estimates of intra-laboratory variability (gcv %), are given in Tables 1 and 2, according to the type of method used.

a. Protein C activity. The potency estimates for all the activity assays ranged from 0.70 to 0.96 units per ampoule. The combined potency estimates for the thrombin activation/chromogenic assays and for the snake venom activation clotting and chromogenic assays were very similar, being 0.81, 0.81 and 0.82 units per ampoule, respectively (Table 3). The thrombin activation/clotting assays gave a slightly higher combined potency of 0.86 units per ampoule.

b. Protein C antigen. The estimates for all the antigen assays ranged from 0.67 to 0.94 units per ampoule. The combined potency estimates for the electroimmunoassay and ELISA techniques were 0.81 and 0.82 units per ampoule, respectively (Table 3).

The overall combined potency from all activity and antigen estimates (226 assays) was 0.82 units per ampoule with 95% confidence limits of 0.80 to 0.83 units per ampoule.

2. Intra-Laboratory Assay Precision

Estimates of intra-laboratory precision in the form of geometric coefficients of variation (gcv %) are given in Tables 1 and 2. These are based on estimates of local standards, L, relative to the proposed standard, P. Although overall the gcv's ranged from 0.8 to 9%, the average gcv's for each method were similar: thrombin activation/clotting (7.0%), thrombin activation chromogenic (5.6%), snake venom/clotting (5.8%), snake venom/chromogenic (3.5%), electroimmunoassay (5.0%) and ELISA (5.4%).

3. Variability of Normal Pools (N)

Estimates of the protein C content of the normal pools (N), relative to the overall mean value of the proposed standard, P, were calculated by taking the reciprocal of each laboratory's estimate of the proposed standard (P), measured against the normal pool, N, and multiplying this by 0.82. The estimates ranged from 0.85 to 1.22 units per ml (Table 4).

Table 2 Potency estimates (units per ampoule) of the proposed standard (P), relative to normal pooled plasma (N) as measured using protein C antigen assays, together with estimates of intra-laboratory assay variability (gcv %)

Laboratory No.	Mean potency (+ 95% confidence limits)	GCV %
a) Laurell "rocket" electroimmunoassay		
2	0.76 (0.72–0.79)	7.1
4	0.87 (0.83–0.90)	5.1
5	0.80 (0.76–0.83)	2.5
6	0.76 (0.68–0.84)	5.0
9	0.81 (0.71–0.94)	5.8
11	0.94 (0.94–0.95)	2.4
12	0.82 (0.60–1.11)	7.5
13	0.67 (0.65–0.70)	7.1
14	0.86 (0.79–0.93)	3.5
18	0.82 (0.74–0.90)	4.4
b) ELISA methods		
3	0.79 (0.74–0.84)	2.9
7	0.78 (0.71–0.86)	3.6
8	0.88 (0.86–0.90)	7.8
10	0.83 (0.75–0.92)	5.3
15	0.78 (0.69–0.87)	7.4

Table 3 Combined potency estimates (units per ampoule) of the proposed standard (P) relative to fresh normal pooled plasma (N) as measured by different methods

Method	Mean potency (+ 95% conf. limits)	No. of assays
<i>Protein C activity</i>		
<i>Activator</i>	<i>Detection</i>	
Thrombin	Clotting	0.86 (0.82–0.89) 24
Thrombin	Chromogenic	0.81 (0.78–0.83) 33
Snake venom	Clotting	0.81 (0.78–0.83) 44
Snake venom	Chromogenic	0.82 (0.79–0.86) 43
<i>Protein C antigen</i>		
Laurell electroimmunoassay		0.81 (0.79–0.84) 54
ELISA		0.82 (0.79–0.85) 28
OVERALL COMBINATION		0.82 (0.80–0.83) 226

Table 4 Range of estimates of protein C in normal pooled plasma (N) relative to the overall mean value for the proposed standard (P), expressed as units per ml for each method

expressed as units per ml for each method		
Method		Range
<i>Protein C activity</i>		
<i>Activator</i>	<i>Detection</i>	
Thrombin	Clotting	0.89–1.01
Thrombin	Chromogenic	0.93–1.08
Snake venom	Clotting	0.90–1.08
Snake venom	Chromogenic	0.85–1.17
<i>Protein C antigen</i>		
Laurell electroimmunoassay		0.87–1.22
ELISA		0.93–1.05

4. Local Standards (L)

Estimates of the protein C content of the local standards (L) were calculated as units per ml, relative to the overall mean value of the proposed standard (P). The estimates ranged from 0.84 to 1.15 units per ml (Table 5).

5. Stability Studies

Samples of the proposed standard, P, were stored at elevated temperature (+4, +20, +37°C) for 11 or 13 months before being assayed for protein C by three different methods in three different laboratories. The potencies of the stored samples as a percentage of the –20°C samples are given in Table 6, together with the predicted loss per year (%).

Discussion

One of the main reasons for establishing an International Standard for Protein C is to overcome the variability in the currently-used local reference preparations and so improve the inter-laboratory agreement in potency estimation. Most laboratories in the study used a locally collected frozen pooled plasma which, relative to the proposed standard, ranged from a protein C concentration of 0.84 to 1.15 units per ml. A similar range was found when the protein C content of the normal pooled plasma (N) was expressed relative to the proposed standard (P), where values of N ranged from 0.85 to 1.22 units per ml. This wide range of values for local reference materials emphasises the need for an International Standard for protein C in plasma.

The intra-laboratory assay precision, as measured in terms of a geometric coefficient of variation, covered a fairly wide range (0.8 to 9%). However, the mean gcvs for each method (range 3.5 to

Table 5 Range of estimates for protein C in the local standards (L), relative to the overall mean value for the proposed standard (P), expressed as units per ml, for each method

Method	Range	
<i>Protein C activity</i>		
<i>Activator</i>	<i>Detection</i>	
Thrombin	Clotting	0.88–1.10
Thrombin	Chromogenic	0.84–1.15
Snake venom	Clotting	0.95–1.09
Snake venom	Chromogenic	0.87–1.08
<i>Protein C antigen</i>		
Laurell electroimmunoassay		0.91–1.15
ELISA		0.92–0.98

Table 6 Stability of the proposed standard, P. Potency estimates by three methods for protein C in samples stored at elevated temperatures for 11 or 13* months as a percentage of samples stored at –20°C, together with predicted loss per year (%)

Storage temp. °C	Snake venom/ chromogenic Mean potency (% of –20°C sample)	Thrombin/ chromogenic	*Electro- immunoassay
+ 4	99	100	101
+20	99	94	104
+37	85	79	–
Predicted loss per year (%)			
–20	0.000	0.126	*
+ 4	0.032	1.539	–
+20	0.828	6.359	–
+37	16.764	22.788	–

* No detectable loss of antigen

7.0%) were all lower than those found in the international collaborative study on the performance of protein C antigen assays (20). Stability studies carried out on samples stored at elevated temperatures by three laboratories using three different techniques indicated that the proposed standard should be suitably stable when stored at –20°C.

The different methods of measuring protein C were placed into six groups for this collaborative study: four types of activity method and two types of antigen method. The combined potency of the proposed standard (P), relative to the normal pooled plasma (N), was found to be very similar by all the methods used. The thrombin activation/clotting assays gave a slightly higher mean potency of 0.86 units per ampoule; however, only four laboratories used this method. The overall good agreement between the different methods agrees with the findings of Bertina (20) and Mannucci et al. (21) for normal plasma and indicates that one combined figure of 0.82 units per ampoule would be acceptable for all methods used to assay protein C.

The findings of the collaborative study were presented to the Protein C and Protein S Subcommittee of the International Committee on Thrombosis and Haemostasis in Brussels, July 1987. The standard was established by the World Health Organization Expert Committee on Biological Standardization in Geneva, December 1987.

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References

- Kisiel W, Canfield W M, Ericsson L H, Davie E W. Anticoagulant properties of bovine plasma protein C following activation by thrombin. *Biochemistry* 1977; 16: 5824-31.
- Walker F J, Sexton P W, Esmon C T. The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. *Biochim Biophys Acta* 1979; 571: 333-42.
- Marlar R A, Kleiss A J, Griffin J H. Mechanism of action of human activated protein C, a thrombin-dependent anticoagulant enzyme. *Blood* 1982; 59: 1067-72.
- Griffin J H, Evatt B, Zimmerman T S, Kleiss A J, Wideman C. Deficiency of protein C in congenital thrombotic disease. *J Clin Invest* 1981; 68: 1370-3.
- Bertina R M, Broekmans A W, van der Linden I K, Mertens K. Protein C deficiency in a Dutch family with thrombotic disease. *Thromb Haemostas* 1982; 48: 1-5.
- Broekmans A W. Hereditary protein C deficiency. *Haemostasis* 1985; 15: 233-40.
- Bertina R M, Broekmans A W, Krommenhoek-van Es C, van Wijngaarden A. The use of a functional and immunologic assay for plasma protein C in the study of the heterogeneity of congenital protein C deficiency. *Thromb Haemostas* 1984; 51: 1-5.
- Campbell P J. International biological standards and reference preparations. II: Procedures used for the production of biological standards and reference preparations. *J Biol Stand* 1974; 2: 259-67.
- Francis R B, Patch M J. A functional assay for protein C in human plasma. *Thromb Res* 1983; 32: 605-13.
- Sala N, Owen W G, Collén D. A functional assay of protein C in human plasma. *Blood* 1984; 63: 671-5.
- Lobermann H, Kolde H-J, Deubel R, Peter R, Tourte E, Becker U. Determination of protein C in plasma. *Behring Inst Mitt* 1986; 79: 112-20.
- Vinazzer H, Pangraz U. Protein C: comparison of different assays in normal and abnormal plasma samples. *Thromb Res* 1987; 46: 1-8.
- Martinoli J L, Stocker K. Fast functional protein C assay using Protac, a novel protein C activator. *Thromb Res* 1986; 43: 253-64.
- Laurell C B. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal Biochem* 1966; 15: 45-52.
- Marlar R A, Endres-Brooks J, Miller C. Serial studies of protein C and its plasma inhibitor in patients with disseminated intravascular coagulation. *Blood* 1985; 66: 59-63.
- Boyer C, Rothschild C, Wolf M, Amiral J, Meyer D, Larrieu M J. A new method for estimation of protein C by ELISA. *Thromb Res* 1984; 36: 579-89.
- Amiral J, Adalbert B, Adam M. Application of enzyme immunoassays to coagulation testing. *Clin Chem* 1984; 30: 1512-6.
- Finney D J. *Statistical Method in Biological Assay*. Griffin & Co Ltd, London 1978.
- Kirkwood T B L, Tydeman M S. Design and analysis of accelerated degradation tests for the stability of biological standards. II: A flexible computer program for data analysis. *J Biol Stand* 1984; 12: 207-14.
- Bertina R M. An international collaborative study on the performance of protein C antigen assays. *Thromb Haemostas* 1987; 57: 112-7.
- Mannucci R M, Boyer C, Tripodi A, Viganò-D'Angelo S, Wolf M, Valsecchi C, D'Angelo A, Meyer D, Larrieu M J. Multicenter comparison of five functional and two immunological assays for protein C. *Thromb Haemostas* 1987; 57: 44-8.

Appendix 1

Preparation of Fresh Normal Plasma

The assays were designed to be carried out in three sessions, each requiring a fresh pool of normal plasma derived from at least four, preferably different, volunteers each time. Participants were asked to prepare fresh normal plasma according to the following specifications:

- Donors*. Normal healthy volunteers, excluding women taking oral contraceptives.
- Anticoagulant*. 0.109 Molar Tri-sodium citrate. Mix 9 vols. of blood with 1 vol. of anticoagulant.

- Centrifugation*. Blood should be centrifuged at 4°C as soon as possible after collection. If a high speed centrifuge is available, spin at 50,000 g for 5 min; otherwise, spin at 2,000 g for 20 min.
- Storage*. During assays, keep the plasma in a stoppered tube at 4°C. Aliquots of each pool should be snap-frozen for assays of protein C antigen.

Appendix 2

Participants in the Collaborative Study

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