Evaluation of Home-Made ELISA's for Protein C and Protein S Antigenic Assays

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Abstracts : Home-made enzyme-linked immunosorbent assays (ELISA) for protein C (PC) and protein S (PS) antigenic assays, using commercial antibodies, were set up in our laboratory. The latter can be used for the measurement of total PS and also free PS, after the precipitation of bound form. Here we describe the procedure for both PC and PS ELISA's, their quality evaluation and cost.

Intra- and inter-assay variation (n = 20) were calculated to be 7.3% and 8.1% for the PC ELISA and 10.2% and 10.1% for free PS ELISA. The accuracy of the tests assessed by external quality assurance of WHO International External Quality Assessment Scheme in Blood Coagulation (IEQAS) was satisfactory. The level of PC antigen in 50 healthy volunteers was 89 ± 18% and that of free protein S was 94 ± 16%.

In conclusion the quality of the home-made ELISA's was acceptable while the cost was much cheaper than that of commercial ELISA kits.

เรื่องย่อ : การประเมินการทดสอบหาระดับแอนติเจนของโปรตีนซีและโปรตีนเอส โดยใช้วิธี ELISA ที่จัดทำขึ้นเองในห้องปฏิบัติการ

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คณะผู้รายงานได้ผลิตการทดสอบเพื่อหาระดับ แอนติเจนของโปรตีนซี และโปรตีนเอส ด้วยวิธี ELISA โดยใช้แอนติบอดีย์ที่ซื้อมา สำหรับวิธี ELISA ของการตรวจหาระดับโปรตีนเอส สามารถตรวจได้ทั้งปริมาณโดยรวมหรือเฉพาะโปรตีนเอสที่อยู่เป็นอิสระ ซึ่งทำได้โดยตกตะกอนโปรตีนเอสที่จับกับโปรตีนอื่นก่อน รายงานนี้

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บอกถึงวิธีทำของการทดสอบดังกล่าว รวมถึงผลการประเมินประสิทธิภาพของการทดสอบและค่าใช้จ่ายในการทำ

ค่าความแปรปรวนของผลการทดสอบที่ทำในครั้งเดียวกัน และระหว่างการทดสอบของการหาระดับโปรตีนซี เป็น 7.3 % และ 8.1 % ส่วนของการหาระดับโปรตีนเอส มีค่าเป็น 10.2 % และ 10.1 % ตามลำดับ ความถูกต้องของการทดสอบประเมินจากระบบการประกันคุณภาพจากภายนอกขององค์การอนามัยโลกได้ผลเป็นที่น่าพอใจ ผลการตรวจเลือดจากอาสาสมัครที่ปกติ 50 คน ได้ระดับโปรตีนซี 89 ± 18% ระดับโปรตีนเอสอิสระ 94 ± 16%

โดยสรุป คุณภาพของการทดสอบด้วยวิธี ELISA ที่ได้จัดทำขึ้นเองในห้องปฏิบัติการอยู่ในเกณฑ์ที่ยอมรับได้ และมีราคาถูกกว่าชุดการทดสอบที่ซื้อสำเร็จรูป

INTRODUCTION

Protein C (PC) and its cofactor, protein S (PS) are vitamin K dependent proteins that act as natural coagulation inhibitors by inactivating factor Va and VIIIa, and facilitates fibrinolysis by neutralizing plasminogen activator inhibitor.[1,2] Hereditary deficiencies of these proteins are associated with thrombotic disease.[2-6] Their genetic transmission are usually autosomal dominant.[6,7]

PC deficiency is divided into two types. Type I deficiency (quantitative defect), which is the most common, is characterized by concomitant reduction of PC antigen and functional levels due to a reduction of normal PC synthesis. Type II deficiency (qualitative defect) is characterized by normal antigen level and reduced functional level. Dysfunctional PC deficiencies are categorized in type IIa, which is characterized by a reduction of PC activity regardless of the functional assay methods used, and type IIb, which exhibits low activity only in clotting-based method, not in chromogenic assay.[7]

In plasma, 60% of PS binds with C4b-binding protein (C4b-BP), a regulatory protein of the classical pathway of the complement system. The remaining 40% circulates in free form which is the functional part.[8,9] Three types of PS deficiency have been defined according to the International Society on Thrombosis and Haemostasis Standardization subcommittee.[10] Type I deficiency, which corresponds to typical quantitative deficiency, is characterized by parallel reduction in both total and free PS. In type II deficiency, PS activity is decreased while total and free PS levels are normal, which is characteristic of classical qualitative deficiency. Type III deficiency exhibits normal concentration of total PS but low free PS level.

Assays for PC and PS are recommended as components of the laboratory investigation of patients suspected of having hereditary thrombotic disease.[11-13] Thus, we set up ELISA's for antigenic determinants of PC and PS in 1996, established normal ranges, and had external quality assurance with WHO International External Quality Assessment Scheme in Blood Coagulation (IEQAS).

In this report, we describe the procedures of these two ELISA's, their quality evaluation and cost.

MATERIALS AND METHODS

Blood collection and preparation of plasma

Blood samples from 50 healthy volunteers were collected in 3.8% sodium citrate, the anticoagulant ratio was 1:10, and centrifuged at 1,500 g for 15 minutes to obtain platelet poor plasma for the measurement of normal values. Pooled normal plasma was prepared from platelet poor plasma of 30 healthy volunteers and stored at –70°C.

ELISA procedure for determination of PC antigen

Polystyrene microtiter plates (Maxisorp, Nunc, Demmark) were coated with 100 µl/well of rabbit anti PC (Dako, Denmark), diluted to 10 µg/ml in coating buffer (20mmol/l $NaCO_3$, 30mmol/l $NaHCO_3$, 3 mmol/l NaN_3, pH 9.6). The plates were kept in a moisture closed chamber at 4°C overnight.

Before use, the plates were washed three times with wash buffer (10 mmol/l Na_2HPO_4, 1 mmol/l KH_2PO_4, 2 mmol/l KCL, 140 mmol/l NaCl 0.05% Tween–20, pH 7.4). Subsequently, 100 µl of diluted test sample and standard were added to the wells and incubated for 2 hours at room temperature. The assay plates were then washed three times with wash buffer. After that, 100 µl/well of rabbit anti PC peroxidase–conjugate (1.3 g/L Dako, Denmark), diluted to 1:1000 (the dilution was recommended in leaflet) in wash buffer was added, and then incubated for 1 hr at room temperature.

After the incubation with enzyme-labeled antibody, the assay plates were washed three times and then added to 100 µl/well of substrate solution (2 mg of ortho-phenylenediamine tablet (Dako, Denmark), 2.5 µl of 35% H_2O_2 in 5 ml of 0.1 M citrate 0.2 M phosphate buffer, pH 5.0), and incubated for 30 minutes. The reaction was then stopped by 100 µl/well of 2M H_2SO_4, after which the absorbance was read at 492 nm using ELISA reader.

To calibrate the standard curve, pooled normal plasma was diluted with dilution buffer (1mM Na_2 EDTA in wash buffer) to concentrations of 1:80 (200%), 1:160 (100%), 1:620 (50%), 1:640 (25%) and 1:1280 (12.5%). Test plasma was diluted with dilution buffer at 1: 160. The absorbance was plotted against the standard concentration in double-logarithmic scale.

ELISA procedure for determination of PS antigen

The procedure of ELISA for PS was similar to that of PC, except that the microtiter plates were coated with rabbit anti PS (Dako, Denmark) and anti PS peroxidase-conjugate (1.3 g/L, Dako, Denmark), diluted to 1:5000, was used as enzyme-labeled antibody. The incubation time for the substrate was 15 minutes. This method can be used for measurement of total PS and also free PS, after the precipitation of bound form.

Free PS separation

To separate free PS, the PS complexed with C4b-BP was precipitated with polyethyleneglycol (PEG 6000).[14] Therefore, 85 µl of plasma was added to 15 µl of 25 % PEG6000 in an Eppendorf tube. After vigorous mixing for 3 minutes on a vortex mixer, the tube was immersed in melting ice for 30 minutes, and then centrifuged for 10 minutes in an Eppendorf centrifuge at room temperature. The supernatant was used to measure free PS.

RESULTS

The calibration curves of ELISA for PC and free PS antigen developed in our laboratory are shown in figure 1 and 2, respectively. The curve of free PS between 100% and 200% shows an altered slope which has a slightly decreased gradient.

Precision

Intra-assay variation of ELISA's for PC and free PS tested on a plasma sample with normal value and plasma from a patient receiving oral anticoagulant, resulted in low levels of PC and free PS. Inter-assay variations were calculated from the results of commercial Control Plasma N and Control Plasma P (Dade Behring). The reproducibility of PC and PS assay are shown in table 1 and 2, respectively.

Accuracy

Our laboratory has been enrolled in WHO IEQAS since 1991. Lyophilized plasma samples are sent to our laboratory every 3 months. Samples for PC and PS assays are enclosed in some surveys. The results are shown in table 3. The central reference point is taken as the overall United Kingdom National External Quality Assessment Schemes (UK NEQAS) consensus median. Participants' results are compared with this figure by calculating percentage deviations. The performance is based on ranked grading analysis where individual results are ranked into 5 unequal quantiles above and below the median. A is designated for the nearest 25% of results (ie. 50% of results). B, C, D, E are represented for the next 10% (ie. 20% of results), 5% (10 % results), 5% (10% of results), and the 5% of results furthest from the median respectively.

Measurements of free PS and PC antigen in healthy volunteers

Healthy volunteers comprised 27 males and 23 females, not taking oral contraceptives. The mean age was 32 ± 15 years (range 15-61). The level of PC antigen in this group was 89 ± 18% and that of free protein S was 94 ± 16%.

Table 1. The reproducibility of ELISA for PC Antigen (n = 20), showing mean CV of intra-assay =7.3%, inter assay = 8.1%.

Assay	Mean (%)	SD (%)	CV (%)
Intra	72	5.3	7.4
	48	3.5	7.2
Inter	98	7.2	7.3
	32	2.9	8.8

Table 2. The reproducibility of ELISA for free PS antigen (n = 20), showing mean CV of intra-assay =10.2%, inter assay =10.1%.

Assay	Mean (%)	SD (%)	CV (%)
Intra	94	10.0	10.6
	61	6.0	9.8
Inter	97	8.4	8.7
	41	4.7	11.4

Table 3. The results of home-made ELISA's for PC and PS antigenic assays in the surveys of WHO IEQAS.

3a. PC antigen

No. of survey	Date	Our result	Median result	% deviation	Performance
23	Feb-97	49	43	14	B
28	Oct-98	89.2	102	12.5	B
32	Jan-00	102	92	4.1	A

3b. PS antigen

No. of survey	Date	Our total PS	Median Total PS	% deviation	Perfor-mance	Our Free PS	Median Free PS	% deviation	Perfor-mance
25	Nov-97	46	31	48.4	C	13	*	*	A
27	May-98	84.1	90	6.6	A	89.3	79.6	12	A
31	Oct-99	90	87	3.4	A	79	73	2.6	A

*Data was not given by WHO IEQAS.

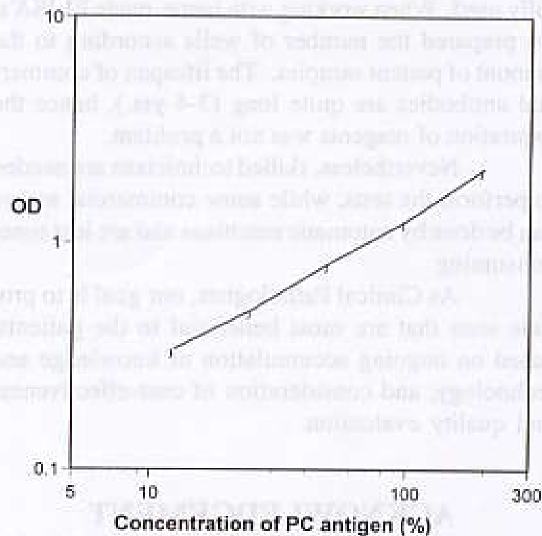

Figure 1. A calibration curve of the ELISA for CP antigen.

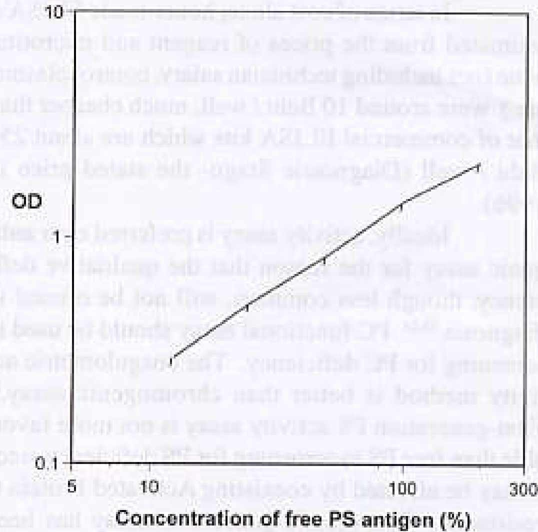

Figure 2. A calibration curve of the ELISA for PS antigen.

DISCUSSION

In our laboratory, patient samples requested for investigation of hereditary thrombotic disease were analyzed for the level of antithrombin III activity, PC antigen and free PS antigen. The latter two were measured by home-made ELISA "sandwich" methods, using commercial antibody and enzyme-labeled antibody. Assay for total PS was done in case of low free PS level to differentiate the type of deficiency.

For free PS assay, slight deviations from linearity in the calibration curve plotted on log-log paper (Figure 2) were also observed by a majority of laboratories using a commercial ELISA for PS, Asserachrom-PS (Diagnostic stago).[15]

The precision of our home-made ELISA for PC assay was acceptable. Our intra-and inter-assay variation were approximately 7.3% and 8.1%, respectively, compared to 2-6% and 4-8% in another report.[16] In a multicenter evaluation of PS antigen assay, the average CV of within-laboratory reproducibility for total PS using Asserachrom-PS was 11.9 ± 4.1%,[15] while our CV for free PS (Table 2) was in this range, as well.

For accuracy of the tests, we used commercial controls, both normal and low-level values as internal quality controls for each measurement. Most results of the external quality assurance assessed by WHO IEQAS were satisfactory (Table 3).

Free PS levels in our normal subjects were agreeable with another report[3], 94 ± 16% and 99 ± 17%, in the order mentioned. Nevertheless, that of PC in this study was 89 ± 18% which seemed to be lower than others, 99 ± 22%[16] and 99 ± 17%.[17] Plasma PC levels are variable in the normal populations, influenced by age, sex and serum lipids.[18-20] PC levels are higher in males than in females and increase with age in both sexes.[18,19] A rise in total cholesterol or triglyceride is strongly associated with increased PC antigen levels and activity.[20] The question remains

whether the finding of different PC levels could be explained by distinct populations.

In terms of cost alone, home-made ELISA's, estimated from the prices of reagent and microtiter plate (not including technician salary, control plasma, etc.) were around 10 Baht / well, much cheaper than that of commercial ELISA kits which are about 250 Baht / well (Diagnostic Stago- the stated price in 1996).

Ideally, activity assay is preferred over antigenic assay for the reason that the qualitative deficiency, though less common, will not be missed in diagnosis.[13,21] PC functional assay should be used in screening for PC deficiency. The coagulometric activity method is better than chromogenic assay.[21] First-generation PS activity assay is not more favorable than free PS in screening for PS deficiency since it may be affected by coexisting Activated Protein C resistance. The second-generation assay has been developed to eliminate this interference.[21] However, the cost–effectiveness should be taken into account. Considering commercial activity assays, the stability of the reconstituted reagents determines the cost per test, as well. In laboratories that have a few numbers of tests, the reagents can expire before being fully used. When working with home-made ELISA's, we prepared the number of wells according to the amount of patient samples. The lifespan of commercial antibodies are quite long (3-4 yrs.), hence the expiration of reagents was not a problem.

Nevertheless, skilled technicians are needed to perform the tests, while some commercial assays can be done by automatic machines and are less time-consuming.

As Clinical Pathologists, our goal is to provide tests that are most beneficial to the patients, based on ongoing accumulation of knowledge and technology, and consideration of cost-effectiveness and quality evaluation.

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