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Validation of Kinetic Method for the PKA Assay in Plasma-Derived Products

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Abstract – A kinetic assay was carried out in order to compare the ability of detection for prekallikrein activator(PKA) in plasma-derived products with that of an endpoint assay and a commercial method. Using these methods, 9 human albumin preparations were assayed and compared to each other. The coefficient of variation between the kinetic assay and the end point assay was found within 6.6% and this result showed that two methods were highly correlative and the end point assay could act as a replacement of the kinetic assay. Another important goal of this study was to investigate the reproducibility among laboratories on the kinetic assay. A collaborative study was performed to validate the kinetic method with intra and inter assays. The coefficient of variation for the intra assay of each laboratory was less than 4% and that for between individuals in the inter assay was 4.1%. These results revealed that the kinetic assay showed good reproducibility. The contents of PKA in plasma-derived products were also determined by the kinetic assay. As a result, it was found that trace amounts of PKA were present in 32 human immunoglobulin preparations, however the average concentration of PKA in 171 albumin preparations was 5.8 IU/mL.

Keywords □ PKA, Plasma derived products, Collaborative study, Validation

The prekallikrein activator(PKA) is the active form of factor XII(factor XIIa), which initiates the cascade of enzymatic reactions leading to blood coagulation(Kaplan *et al.*, 2002; Kaplan 1978; Fossum *et al.*, 1999; Regoli *et al.*, 1997; Revak *et al.*, 1974). In plasma, PKA converts prekallikrein to kallikrein that in turn liberates the vasoactive peptide bradykinin from the high molecular weight kininogen and kallikrein activates production of the PKA. This amplification pathway makes PKA as the potential vasodilating agent in intravenous plasma-derived products. Actually, the occurrence of hypotension in patients receiving the therapeutic dose of plasma derivatives with high levels of PKA had been sporadically reported(Alving *et al.*, 1980, 1982; Heinonen *et al.*; 1980). Although there was another report that PKA is not related to the hypotensive effect during or after the intravenous infusion of albumin solution(Turner *et al.*, 1987), PKA is considered as one of the important factors determining the safety of plasma-derived products, such as human albumin and intravenous immunoglobulin preparations. Therefore in the previous study we established PKA kinetic assay for lot release test(Shin *et al* 2002). The national authorities of the European Union already regulated the level of PKA present in plasma-derived products, and WHO has also issued a guideline on PKA and recommended that the test should be performed in some plasma-derived products. The purpose of this study is to compare to the kinetic assay with other methods and confirm the reliability using the collaborative study. Additionally, the levels of PKA in plasma-derived products have also been determined by the kinetic assay.

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MATERIALS AND METHODS

Reagents

Bicinchoninic acid(BCA) and bovine serum albumin(BSA) were purchased from Sigma Chemical Co(St. Louis, MO, USA). The chromogenic substrate, S-2302 (H-D-Pro-Phe-Arg-ρNA·2HCl, mw 611.6), was purchased(Chromogenix, Italy). 25 mg of S-2302 was dissolved in 6.8 mL of distilled water to make a 6 mmol/L stock solution and should be kept at 4°C until use for assay. The first international standard of PKA(82/530, 85IU/mL) was obtained from the National Institute of Biological Standards and Control(NIBSC, UK).

Human albumin and immunoglobulin preparation

203 human plasma-derived products were obtained from two local manufacturers. Samples from 171 lots of human albumin and 32 lots of human immunoglobulin preparations were tested.

Protein assay in prekallikrein(PK) fraction

The protein concentration in PK fraction was determined by the BCA assay. BSA standard solution was diluted as 1.5, 1.0, 0.75, 0.5, 0.25 and 0.125 mg/mL with saline for the dose-response curve. The PK fraction was diluted 1:9 with saline and the reaction solution with 20 μl of each diluted BSA solution or PK fraction and 200 ml of BCA working solution was incubated at 37°C for 30 min in 96 plate well(Falcon, USA). The absorbance of mixture was measured at 562 nm using microplate reader(VERSAmaxTM Tunable Microplate Reader, Molecular Devices, USA).

Participants

Total 4 laboratories participated in this study for the purpose of assay validation. There are three manufacturers and one national control laboratory. Laboratories were referred to their code numbers allocated at random.

Kinetic assay for PKA

In the first stage of the assay, 25 μL of various concentrations of the PKA-containing samples or standard dilution in 0.05 mol/L Tris buffer(pH 8.0) including 0.15 mol/L NaCl(dilution buffer) was added to 100 μL of PK substrate. Then the mixture was incubated at 37°C for 45 min. Before this step, the NaCl concentration of the PK substrate was adjusted to 150 mmol/L, then diluted 1:2 or 1:3 using the dilution buffer. In the second stage, 1 ml of 0.6 mmol/L S-2302 substrate solution was warmed to 37°C and added to the PKA-PK substrate mixture. In order to measure the kinetic signal, the change of optical density at 405 nm was monitored after 10 seconds lag time for 5 minutes. The measurements were carried out using a programmed 6-channel UV/VIS array spectrophotometer(DU 7500, Beckman, USA).

End point assay for PKA

All procedures of the assay were performed exactly the same way as the kinetic assay but at the last stage, the chromogenic reaction was stopped using 50% acetic acid after reaction at 37°C for 10 min exactly. Background amidase activity was measured by a method described by Shin *et. al*(2002). The commercial assay for PKA was performed using the Chromogenix method.

Reproducibility tests

The reproducibility tests were carried out using an intra assay(day to day) and inter assays(for between individuals and between laboratories). All participants performed 5 independent assays on the 7 lots of albumin in order to evaluate the reproducibility of method.

RESULTS

Comparative study of end-point assay and kinetic assay

The PKA assays were carried out in albumin preparations in order to compare the end-point assay with kinetic assay. A total of 9 lots were tested. The reproducibility is characterized by the mean coefficient of variation(CV) of ±6.6% between assays and the correlation factor was 0.978(Table I). No significant difference was found in the results of CV between assays.

Comparative study of commercial assay and kinetic assay

The kinetic assay and commercial assay, the Chromogenix assay, were compared with 20% albumin preparation and two kinds of immunoglobulin preparations for intravenous use. The commercial assay was slightly modified in order to minimize problem due to the ionic strength and pH. The Tris-HCl buffer(pH 7.8) containing 12 mmol/L NaCl was used in dilution of S-2302, samples and standards. PKA standard as the final concentration of 28 IU/mL was spiked into samples including albumin in order to check the recovery rate for each assay. The linearity($r^2 > 0.99$) was observed in each assay and the average coefficient of variation was ±1.8% in kinetic assay

Table I. Comparison of assay methods for PKA

Sample	PKA concentration (IU/mL)			CV (%)[b]
	Kinetic[a]	End-point[a]	Average	
A	6.1±0.52	6.3±0.82	6.2	2.3
B	5.7±0.83	5.9±0.99	5.8	2.4
C	6.9±0.45	7.8±1.21	7.35	8.7
D	12.3±0.12	12.1±0.82	12.2	1.2
E	13.2±0.44	15.4±0.98	14.3	10.9
F	15.7±0.98	17.0±0.43	16.35	5.6
G	13.2±0.31	16.4±1.33	14.8	15.3
H	12.9±0.27	14.1±0.21	13.5	6.3
I	10.4±0.72	11.4±0.53	10.9	6.5

[a]Each data represents as a mean±SD of four independent examinations.
[b]Coefficient of variation(CV) was obtained between assays.

and ±2.0% in commercial assay. The recovery rate of kinetic assay and commercial assay was 101.9% and 82.1% respectively, and that of of commercial assay was increased according to the dilution rate(Fig. 1).

Reproducibility

The studies were carried out using the intra assay(day to day) and inter assays(between individuals, between laboratories) on 7 lots of human albumin in order to evaluate the reproducibility. In the intra assay, participants performed 5 independent assays to calculate the variability between days. The average standard deviation of each laboratories was ±3.6, ±1.2, ±0.5 and ±1.6 IU/mL(Fig. 2). Four laboratories submitted results of assays in order to check the reproducibility between individuals. The average coefficient of variation was ±4.1% and mean correlation factor was 0.992(Table II). The assays were performed more than 10 times at each laboratories as 5 independent assays in order to confirm reproducibility of the inter assay between laboratories. The average standard deviation was determined as ±1.7 IU/mL and the correlation factor was 0.991.

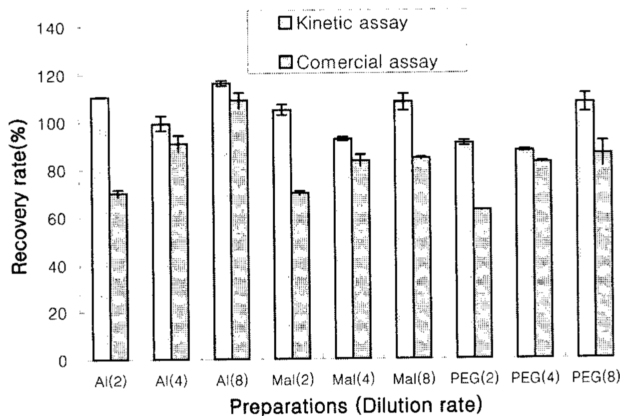

Fig. 1. Comparison of the recovery rate(N=4) with prekallikrein activator international standard to human albumin and intravenous immunoglobulin(IVIG) preparations between kinetic assay (new assay) and commercial assay. Abbreviated terms are as follows : Al(Albumin preparation), Mal(Maltose treated immunoglobulin preparation), PEG(Polyethylene glycol treated immunoglobulin preparation).

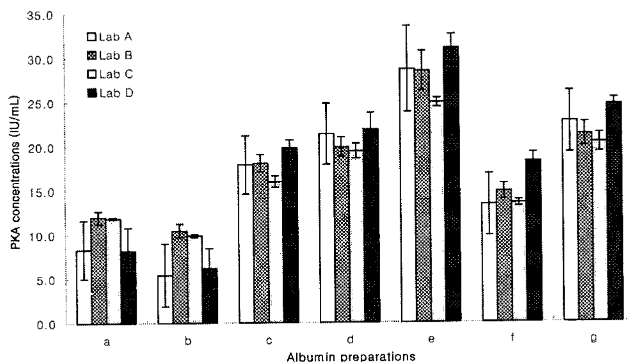

Fig. 2. Reproducibility of albumin panel(a~g) using kinetic assay in 4 laboratories. A~D and a~g represent the participating laboratories and albumin lots, respectively. Sample size(N) is more than 10.

PKA levels in plasma derived products

The study was carried out on 171 lots of albumin preparations derived from domestic and abroad plasma. It was found that amounts of PKA showed the relatively high level in the early stage of this study although it was not out of the specification, and the level of PKA was gradually decreased and stabilized. The trend of PKA level was observed as the mean value in albumin(Fig. 3) and we could not find out the significant differences between plasma sources and manufacturers. The average concentrations of PKA in domestic plasma were 6.4 IU/mL, and that in abroad plasma was 8.0 IU/mL. The contents of PKA in intravenous immunoglobulin, maltose and polyethylene glycol treated immunoglobulin preparations, were also determined on 32 lots produced in Korea. It was found that little amounts of PKA are present in these preparations.

DISCUSSION

In this study, we described the comparative study, the validation of method using collaborative study and potency of PKA

Table II. Reproducibility of results between individuals in each laboratory

	PKA concentration[a] (IU/mL)							
	Sample	a	b	c	d	e	f	g
A	P1	9.2	5.3	19.1	22.1	31.5	14.3	23.7
	P2	8.3	5.4	17.9	21.4	28.7	13.3	22.7
	CV%	7.3	1.6	4.6	2.4	6.7	5.0	3.0
B	P1	13.0	11.0	17.1	18.8	27.2	15.1	20.9
	P2	12.0	10.4	18.1	19.9	28.5	14.8	21.3
	CV%	5.9	3.7	3.9	3.9	3.4	1.0	1.3
C	P1	12.9	11.0	17.5	18.0	27.7	15.1	20.5
	P2	11.8	9.8	16.0	19.4	24.9	13.5	20.3
	CV%	6.2	7.9	6.4	5.3	7.5	8.0	0.6
D	P1	7.7	5.8	19.7	21.6	29.8	19.3	25.6
	P2	8.2	6.2	19.8	21.8	31.0	18.2	24.7
	CV%	4.1	4.5	0.4	0.8	2.8	4.1	2.6

[a]Each data represents as a mean of four independent examinations. A~D represents the laboratory code and P means person of experiment.

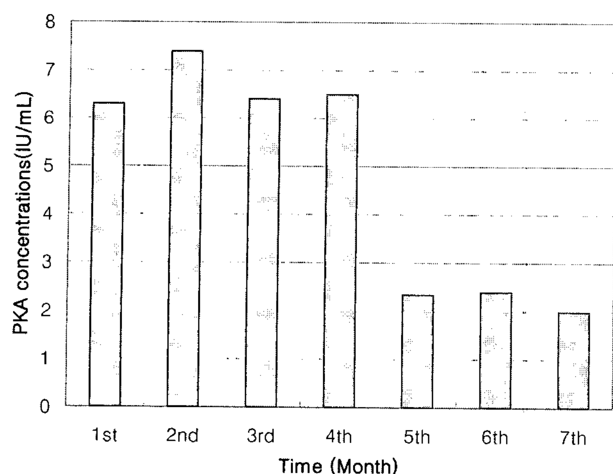

Fig. 3. Trends of PKA concentrations in albumin preparations. The concentrations of PKA were assayed for 7 months.

in plasma-derived products. The comparative study was based on the kinetic assay, and the end point assay has been found to be sensitive, simple and reproducible method. Because WHO and EP recommend kinetic assay for the lot release test which is performed by national authority before the biologics released in market, we do the some tests with only commercial and official methods like recovery test but in the preliminary test the accuracy for end point and kinetic assay was compatible to each other. And the end point assay was found sufficiently correlative to be performed as an official method. In the commercial method, we could not obtain the good correlation with the kinetic assay because the recovery rate was poor due to the interference of ionic strength in sample, so we concluded that this assay would not be adopted for official method.

In order to validate the method, we carried out the collaborative study for checking the reproducibility between days, individuals and laboratories. One of reported results was rather higher than those of the others but this might be due to the technical errors. The standard deviations of other laboratories were less than 3% of the European and Korean specification in blood derived products. However, due to some albumin panel have low PKA concentration near the detection limit(4.3 IU/mL), the total inter laboratory standard deviation was 2.06 IU/mL and CV% was 14.8% which is for acceptable inter-laboratory assay, but except those albumins the standard deviation was 1.84 IU/mL and CV% was reduced to 9.2%. Therefore, these results showed that the reproducibility of the method between laboratories was good.

The level of PKA in plasma derived products for intravenous use was determined by the kinetic method. In the early stage of this study, PKA contents were detected as the relatively high concentrations in human albumin preparations. However, it was gradually decreased to trace amounts in the timing of the late stage of this study. These phenomena indicate that the removal of PKA in the manufacturing process for albumin preparations is properly established and the quality control of PKA on each step also is adequately achieved nowadays in Korea.

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REFERENCES

Alving, B. M., Tankersley, D. L., Mason, B. L., Rossi, F., Aronson, D. L. and Finlayson, J. S., Contact-activated Factors: Contaminants of immunoglobulin preparations with coagulant and vasoactive properties. *J. Lab. Clin. Med.,* 96, 334-346 (1980).

Clements, J., Mukhtar, A., Yan, S. and Holland, A., Kallikreins and kinins in inflammatory-like events in the reproductive tract. *Pharm. Res.,* **35**(6), 537-540(1997).

Fossum, S., Hoem, N., Gjonnass, H. and Briseid, K., Contact activation factors in plasma from women on estrogen replacement therapy after ovariohysterectomy. *Thromb. Res.,* **93**, 161-70(1999).

Griffin, J.H., Role of surface in surface-dependent activation of Hageman factor (blood coagulation factor XII). *Proc. Natl. Acad. Sci,.* **75**(4), 1998-2002(1978).

Heinonen, J., Peltola, K., Himberg, J.J. and Suomela, H., Hypotensive Effect of Prekallkirein activator (PKA) in plasma protein fraction (PPF). *Dev. Biol. Stand.,* **48**, 129-130(1980).

Heinonen, J., Peltola, K., Himberg, J. J. and Suomela, H., Correlation of hypotensive effect of plasma protein fraction with prekallikrein activator activity: a clinical study in patients having open-heart surgery. *Ann. Thorac. Surg.,* **33**(3), 244-249 (1982).

Kaplan, A. P., Joseph, K. and Silverberg, M., Pathways for bradykinin formation and inflammatory disease. *J. Allerg. Clin. Immunol.,* **109**(2), 195-209(2002).

Kaplan, A. P., Initiation of the intrinsic coagulation and fibrinolytic pathways of man: The role of surfaces, Hageman factor, prekallikrein, high molecular weight kininogen, and factor XI. *Prog. Hemost. Thromb.,* 4, 127-175(1978).

Regoli, D., Rizzi, A. and Calo, G., Pharmacology of the kallikrein-kinin system. *Pharma. Res.,* **35**(6), 513-515(1997).

Revak, S. D., Cochrane, C. G., Johnston, A. R. and Hugli, T. E., Structural changes accompanying enzymatic activation of human Hageman factor. *J. Clin. Invest.,* **54**(3), 619-627(1974).

Rob F., Van Rosevelt, R. F., Bakker, J. C., Sinclair, D. M., Damen, J. and Van Mourik, J. A., Bradykinin-mediated hypotension after infusion of plasma-protein fraction. *J. Lab. Clin. Med.,* **100**,

288-295(1982).

Shin, I. S., Shim, Y. B., Hong, C. M., Koh, H. C., Lee, S. H. and Hong, S. H., An improved, reliable and practical kinetic assay for the detection of prekallikrein activator in blood products. *Arch. Pharm. Res.,* **25**(4), 505-510(2002).

Snape, T. J., Griffith, D., Vallet, L. and Wesley, E. D., The assay of prekallikrein activator in human blood products. *Dev. Biol. Stand.,* **44**, 115-120(1979).

Tankersley, D. L., Fournel, M. A. and Schroeder, D. D., Kinetics of activation of prekallikrein by prekallikrein activator. *Biochemistry,* **19**(14), 3121-3127(1980).

Turner, P. J., Young, I. F., Marley, P. B., Herrington, R. W. and Schiff, P., Albumin solutions: Their production and quality control. *Dev. Biol.. Stand.,* **67**, 119-127(1987).

Yasda, J., Ueno, G., Kuratsuka, K., Homma, R., Hara, S., Kazama, M. and Ito, H., Japanese minimum requirements for albumin preparations recent amendments and current problems. *Dev. Biol. Stand.,* **48**, 143-52(1981).