

# The effect of direct oral anticoagulants on blood protein C activity

Takako Terakami<sup>1)</sup>, Akiko Sekiya<sup>2) \*\*</sup>, Kenshi Hayashi<sup>1)</sup>, Takeshi Suzuki<sup>3)</sup>, Hiroshi Furusho<sup>2)</sup>,  
Hidesaku Asakura<sup>4)</sup>, Eriko Morishita<sup>2) 4) \*</sup>, Takashi Wada<sup>5)</sup>

## Abstract

**[Aim]** In this study, the effect of direct oral anticoagulants (DOACs) on protein C (PC) activity was examined using several measuring reagents.

**[Materials and Methods]** In total, 90 patients (60 male and 30 female) with nonvalvular atrial fibrillation or venous thromboembolism (VTE) who were on anticoagulation therapy with DOACs (rivaroxaban, apixaban, or edoxaban) were studied. The plasma levels of PC activity were measured by means of a clotting assay and chromogenic substrate assay, using three reagents for each type of assay.

**[Result]** Prothrombin time (PT) and activated partial thromboplastin time (APTT) were significantly prolonged in a dose-dependent manner in patients who were taking rivaroxaban or edoxaban. PC activity, as measured by all three reagents using the clotting assay, was influenced only by rivaroxaban, indicating an increase in PC activity in a dose-dependent manner. Apixaban did not have any influence on the measurements made using all three reagents in the clotting assay. On the other hand, none of the three FXa inhibitors had any influence on PC activity when it was measured using the three reagents in the chromogenic substrate assay. Plasma samples were collected before, as well as two and four to eight weeks after rivaroxaban administration in seven patients with AF or VTE sequentially. In all three reagents using the clotting assay, plasma levels of PC activity had increased after the administration of rivaroxaban. On the other hand, all three reagents using the chromogenic assay had very little influence on PC activity after the administration of rivaroxaban.

**[Conclusion]** The inhibitory effects of the different types of DOACs on clotting activity interfere with clotting test measurement systems in patients receiving DOAC therapy. When measuring PC activity while the patient is taking rivaroxaban or edoxaban, it is necessary to use the chromogenic substrate assay to avoid false highs. Moreover, collecting specimens when blood levels of drugs are low, e.g. during the trough phase, whenever possible, would be one way to minimize interference.

## KEY WORDS

direct oral anticoagulant, protein C, clotting assay, chromogenic assay

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1) Department of Clinical Laboratory, Kanazawa University Hospital, Ishikawa, Japan

2) Department of Clinical Laboratory Science, Faculty of Health Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Ishikawa, Japan

3) Hemostasis Business Development, Sysmex, Kobe, Japan

4) Department of Hematology, Kanazawa University, Ishikawa, Japan

5) Department of Nephrology and Laboratory Medicine, Kanazawa University, Ishikawa, Japan

\* Corresponding author

\*\* Equally contributed author

## Introduction

Thrombosis is caused by a combination of various environmental factors and genetic factors. Thrombophilia is defined as a predisposition condition towards thrombosis, and is classified into inherited and acquired<sup>1)</sup>. Inherited thrombophilia includes physiologic coagulation inhibitors deficiency: antithrombin (AT), protein C (PC), and protein S (PS).

AT, PC and PS play a central role in control of the coagulation system. PC is the zymogen of a vitamin K-dependent serine protease and is rapidly activated by the thrombin-thrombomodulin complex, becoming activated PC (APC). With PS as a cofactor, APC inactivates activated factor V (FVa) and activated factor VIII (VIIIa) in the presence of phosphatidylserine and  $\text{Ca}^{2+}$ <sup>2)</sup>. Inherited PC deficiency is, along with AT deficiency and PS deficiency, a key risk factor for venous thromboembolism (VTE)<sup>3, 4)</sup>. Diagnosis of PC deficiency is mainly made by measurements of PC activity and antigen levels. Inherited PC deficiency involves quantitative and qualitative abnormalities in PC caused by a mutation in *PROC*<sup>5)</sup>. Inherited PC deficiency is classified into type I deficiency, in which both the PC antigen level and PC activity level are decreased, and type II deficiency, a molecular abnormality in which the antigen level is normal but activity is decreased<sup>6)</sup>. Type I deficiency is the most common (75-80%). Thus, in the diagnosis of PC deficiency, measurement of PC activity is a very important test.

Treatment of VTE is represented by anti-vitamin K antagonists (VKA) or direct oral anticoagulants (DOACs). DOACs are indicated for the prevention of cerebral infarction due to nonvalvular atrial fibrillation (AF), treatment and prevention of recurrent VTE, and prevention of VTE following orthopedic surgeries, such as joint arthroplasty<sup>7-11)</sup>. DOACs are classified as thrombin inhibitors (dabigatran) and factor Xa inhibitors (rivaroxaban, apixaban, edoxaban) according to their site of action. Currently, Xa inhibitors are indicated for the treatment and prevention of recurrent VTE. The DOACs exhibit more predictable pharmacokinetic and pharmacodynamic profiles than VKAs; consequently, routine coagulation monitoring is not required<sup>12)</sup>. So, in recent years, it is estimated that the use of direct FXa inhibitors for VTE has been increasing.

When conducting screening tests for thrombotic diathesis, including inherited thrombophilia, measurements of the activity of protein S (PS), antithrombin (AT), and protein C (PC) are essential to determine the cause of VTE. When taking warfarin, PC, a VK-dependent protein, has reduced activity. On the other hand, in recent years, there have been reports of false high values of PC activity in patients on DOACs, depending on the measurement method used<sup>13-16)</sup>. Since it has already been clarified that the degree of prolongation of APTT and PT differs depending on the DOACs, we thought that PC activity value with the reagent using a clotting assay would cause a difference depending on the drugs. We investigated the effects of the three Xa inhibitors on PC activity measured using a clotting assay and chromogenic substrate assay.

## Materials and Methods

### 1. Study subjects

Patients diagnosed with nonvalvular atrial fibrillation (AF) or venous thromboembolism (VTE) who attended out-patient clinics in the departments of cardiology or hematology and were already receiving anticoagulation treatment (rivaroxaban, apixaban, or edoxaban) were invited to participate in this study. The study was approved by the University of Kanazawa Ethics Committee (Approval No. 530-2), and was performed in accordance with the ethical principles of the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients before enrollment. Patients with creatinine concentrations  $>2.0$  mg/dL and estimated glomerular filtration rates  $<30$  mL/min/1.73 m<sup>2</sup> (according to Cockcroft-Gault equation) were excluded. Patients were also excluded if they had hereditary thrombophilia or antiphospholipid antibody syndrome, acute thrombosis, severe liver dysfunction, or nephrotic syndrome.

#### 1) Sample collection

Samples were collected in 3.2% sodium-citrate tubes and instantly centrifuged to acquire platelet-poor plasma. Plasma samples were used in the screening tests on the day of collection and were stored at  $-80^{\circ}\text{C}$  until their use in specific coagulation assays.

#### 2) Coagulation tests

a) Prothrombin time (PT) and activated partial

thromboplastin time (APTT)

In all samples, laboratory-based PT and APTT were measured using a single reagent (Thromborel S; Siemens, Marburg, Germany, and Acitin FSL; Siemens, respectively) and the CS-5100 hemostasis system (Sysmex, Kobe, Japan). The normal ranges were PT 10.6-13.0 seconds and APTT 27.3-40.3 seconds.

b) DOAC concentrations

In all samples containing factor Xa (FXa) inhibitors, these drug concentrations were estimated using Biophen DiXaI (Hyphen BioMed, Neuville-sur-Oise, France) with each applied DOAC calibrator and were performed using the CS5100 hemostasis system.

c) PC activity

The level of PC activity was measured by a clotting assay and chromogenic substrate assay using three reagents for each type of assay. The three reagents used for the clotting assay included STA-Staclot Protein C (Fujirebio Inc., Tokyo, Japan), Protein C Reagent (Siemens), and Hemoclot Protein C (Hyphen BioMed); all these tests were performed using the CS5100 hemostasis system. Figure 1 shows measurement principle of the chromogenic substrate assay. In the measurement by the chromogenic substrate assay, the colorimetric determination by decomposition of chromogenic substrate due to APC activated by PC activator is performed.

The three reagents used for the chromogenic substrate assay included Test Team S PC (Sekisui Medical, Tokyo, Japan), Berichrom Protein C (Siemens), and Biophen Protein C (LRT) (Hyphen BioMed); these tests were performed using a Coapresta 2000 coagulation analyzer (Sekisui Medical).

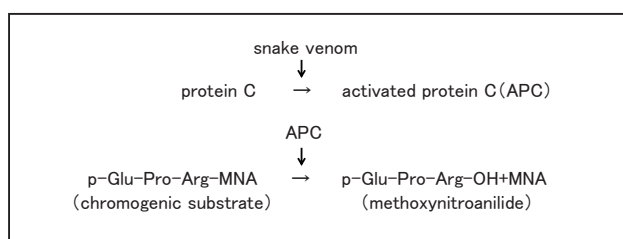


Figure.1. Measurement principle of a chromogenic substrate assay

d) PC antigen

The level of PC antigen was measured using LPIA-S PCII (LSI Medience, Tokyo, Japan) based on a latex photometric immunoassay system (STACIA, LSI

Medience).

3) Statistical analysis

Statistical analysis was performed using the STAT VIEW program (Abacus Concepts, Berkeley, CA, USA). Pearson product-moment correlation coefficient was used to assess the correlation between the distribution of PC activity and antigen, as assessed with the different PC reagents. P <0.05 was considered statistically significant.

Results

1. Study subjects

A total of 90 patients (60 male and 30 female) with AF or VTE who were on anticoagulation therapy with DOACs. The mean age of patients was 66 ± 14.8 years (±SD). Table 1 shows disease breakdown of patients. The breakdown 74 patients with af is as follows; 43 alone, 26 with chronic heart failure (CHF), of 26 patients with CHF 16 other diseases (11 with sick sinus syndrome, 5 with atherothrombotic cerebral infarction), 5 with hypertrophic cardiomyopathy, 5 with deep vein thrombosis. Table 1 omits the diseases of three or less cases.

Table 1. Characteristics of patients' diseases and complications

Disease	Cases
Atrial fibrillation	Total 74
Af	43
Af & CHF	10
Af, CHF & Sick sinus syndrome	11
Af, CHF & Atherothrombotic cerebral infarction	5
Af & Hypertrophic cardiomyopathy	5
VTE	Total 21
DVT	16
DVT & Af	5

2. Effect of rivaroxaban, apixaban and edoxaban on PT and APTT (Fig. 2)

First, we examined whether PT and APTT were affected by DOACs at different concentrations. We demonstrated that PT and APTT were significantly prolonged in a dose-dependent manner in patients who were taking rivaroxaban or edoxaban. Edoxaban resulted in the greatest prolongation of PT as compared to the other drugs, and it prolonged PT more than APTT (Fig. 2C, r = 0.838, p < 0.001). Similarly,

rivaroxaban resulted in the greatest prolongation of APTT as compared to the other drugs, and it prolonged APTT more than PT (Fig.2D,  $r = 0.851$ ,  $p < 0.001$ ). On the other hand, apixaban did not influence either PT (Fig. 2B,  $r = 0.158$ ,  $p = 0.396$ ) or APTT (Fig. 2E,  $r = 0.404$ ,  $p = 0.062$ ) regardless of drug concentration.

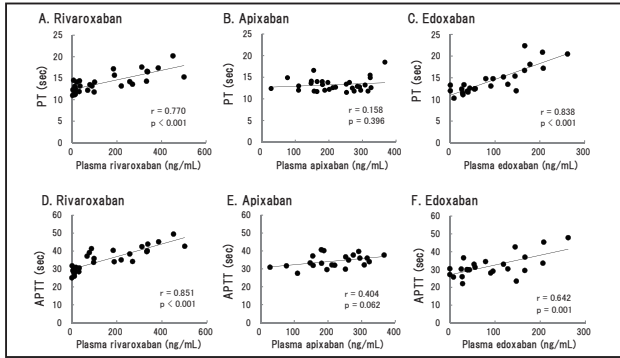


Figure.2. Impact of rivaroxaban, apixaban and edoxaban on PT and APTT

### 3. Effect of rivaroxaban, apixaban and edoxaban on PC activity (Fig. 3, Fig. 4, Table 2)

PC activity was measured by a clotting assay using three reagents. The results showed that PC activity, as measured by all three reagents, was influenced only by rivaroxaban, indicating an increase in PC activity in a dose-dependent manner (Fig. 3A). In particular, the PC activity measured using the STA-Sta clot Protein C reagent was most affected by rivaroxaban ( $r = 0.695$ ,  $p < 0.001$ ), and that measured using the Hemoclot Protein C reagent was least affected by rivaroxaban ( $r = 0.378$ ,  $p = 0.039$ ). PC activity assessments performed using Protein C Reagent were slightly, but not significantly, affected by the use of edoxaban ( $r = 0.341$ ,  $p = 0.076$ ) (Fig. 3C). On the other hand, apixaban did not have any influence on the measurements made using Hemoclot Protein C and Protein C Reagent (Fig. 3B).

None of the three FXa inhibitors had any influence

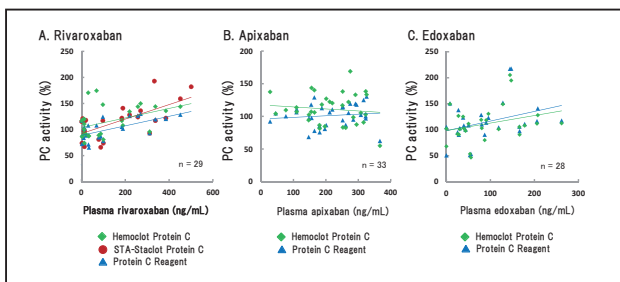


Figure.3. Impact of rivaroxaban, apixaban and edoxaban on PC activity (Clotting assays)

on PC activity when it was measured using the three reagents in the chromogenic substrate assay (Fig. 4, Table 2).

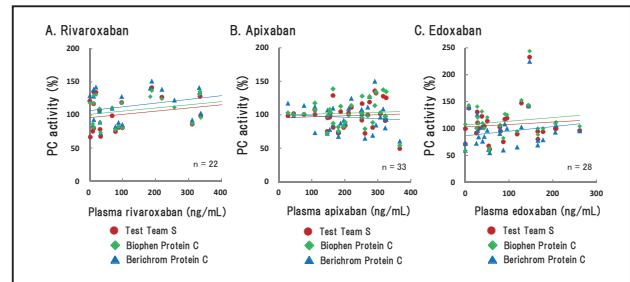


Figure.4. Impact of rivaroxaban, apixaban and edoxaban on PC activity (Chromogenic assays)

Table 2. Relationships between plasma concentrations of DOACs and plasma levels of PC activity and antigen

	Methods	DOACs				
		Rivaroxaban	Apixaban	Edoxaban		
PC activity	Clotting assay	STA-Sta clot Protein C	$r = 0.695$ $p < 0.001$	-	-	-
		Protein C Reagent	$r = 0.551$ $p = 0.002$	$r = 0.124$ NS $p = 0.493$	$r = 0.341$ NS $p = 0.076$	
		Hemoclot Protein C	$r = 0.378$ $p = 0.039$	$r = -0.113$ NS $p = 0.529$	$r = 0.297$ NS $p = 0.125$	
	Chromogenic assay	Biophen Protein C	$r = 0.212$ NS $p = 0.260$	$r = 0.062$ NS $p = 0.731$	$r = 0.137$ NS $p = 0.489$	
		Test Team S PC	$r = 0.163$ NS $p = 0.388$	$r = 0.068$ NS $p = 0.708$	$r = 0.098$ NS $p = 0.619$	
		Berichrom Protein C	$r = 0.218$ NS $p = 0.246$	$r = -0.093$ NS $p = 0.608$	$r = 0.177$ NS $p = 0.368$	
PC antigen	Latex photometric immunoassay	$r = 0.410$ NS $p = 0.146$	$r = -0.103$ NS $p = 0.576$	$r = -0.039$ NS $p = 0.846$		

### 4. Effect of rivaroxaban, apixaban and edoxaban on PC antigen (Fig. 5, Table 2)

As with the previous assays, none of the three FXa inhibitors had any influence on the amount of PC antigen measured in assays based on the principles of the latex agglutination assay (rivaroxaban:  $r = 0.410$ ,  $p = 0.146$ , apixaban:  $r = -0.103$ ,  $p = 0.576$ , edoxaban:  $r = -0.039$ ,  $p = 0.846$ ).

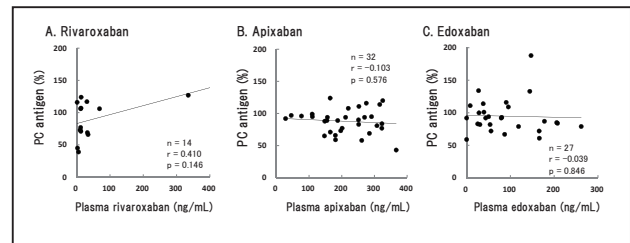


Figure.5. Impact of rivaroxaban, apixaban and edoxaban on PC antigen

### 5. The changes in PC activity and plasma rivaroxaban concentrations before and after rivaroxaban administration

Plasma samples were collected before, as well

as two and four to eight weeks after rivaroxaban administration in seven patients with AF or VTE sequentially. As shown in Fig. 6, the plasma concentration of rivaroxaban was 20-500 ng/mL after the drug administration. Compared to baseline, both PT and APTT were significantly prolonged after the administration of rivaroxaban.

In all three reagents using the clotting assay (Fig. 7A), plasma levels of PC activity were increased after the administration of rivaroxaban. The Interference was more pronounced in the STA Staclot Protein C as compared to Protein C Regent. On the other hand, all three reagents using the chromogenic assay had very little influence on PC activity after the administration of rivaroxaban (Fig. 7B).

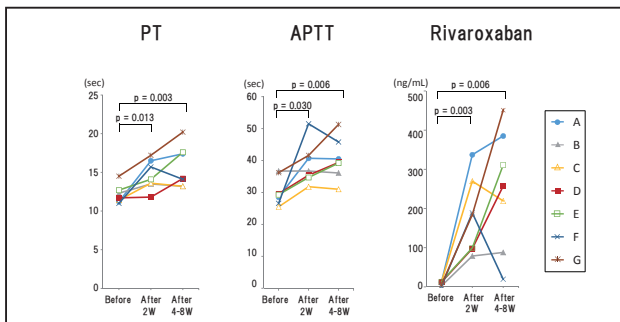


Figure.6. The changes in PT, APTT and plasma concentrations of rivaroxaban before and after rivaroxaban administration

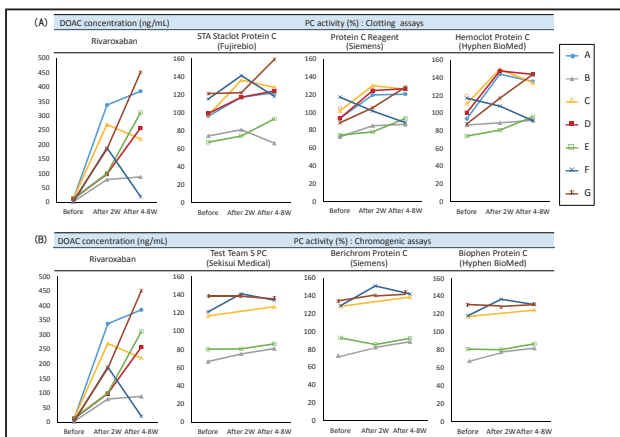


Figure.7. The changes in PC activity and plasma concentrations of rivaroxaban before and after rivaroxaban administration (A): Clotting assay, (B): Chromogenic assay

Discussion

DOACs affect commonly used global coagulation assays (PT and APTT) <sup>15,17</sup> and therefore influence coagulation function assays such as thrombophilia or lupus anticoagulant. On the basis of the current

literature for the DOACs' effects on the coagulation times, the PT may be relatively sensitive (as compared with the APTT) to FXa inhibitors, and the APTT may be relatively sensitive (as compared with the PT) to dabigatran concentration <sup>13,18,19</sup>. Our study shows that PT and APTT were significantly prolonged by edoxaban and rivaroxaban. On the other hand, apixaban did not influence PT or APTT regardless of the concentration. As with previous reports, increasing concentrations of apixaban and rivaroxaban prolong PT or APTT incrementally <sup>20,21</sup>. The variation may be due to the physical and/or chemical interactions between individual FXa inhibitors and phosphatidylserine found within these thromboplastin reagents. Also, the PT and APTT prolongation of apixaban were milder than those of the other two drugs. It might be considered that the difference in Ki value is involved.

Two different methods were used to measure PC activity, namely, clotting assays and chromogenic substrate assays. In commercial assays, both clotting and chromogenic types of assays use a snake venom activator to convert the plasma PC to APC. The clotting-based assays utilize mainly the APTT, and PC activity is detected using the prolongation in clotting time resulting from inactivation of FVa and FVIII by APC (APTT prolongation effect). Therefore, APTT prolongation overestimate the PC value in plasma <sup>8,15,18</sup>. In contrast, the chromogenic assay is based on the addition of chromogenic substrate to the generated APC in the diluted plasma sample. The formation of chromogenic color is proportional to the amount of APC in the plasma sample. Therefore, the chromogenic assays are not influenced by prolongation of the clotting time <sup>22</sup>.

PC activity values with the three reagents used in the clotting assays in this study showed significantly positive correlations with plasma concentrations of rivaroxaban (Fig. 3A). Among them, PC activity was affected most by rivaroxaban when the STA-Staclot Protein C reagent was used (Fig. 3A). With edoxaban, PC activity tended to show a positive correlation with plasma concentrations of the drug when Protein C Reagent was used, although the difference was not significant (Fig. 3C). With apixaban, on the other hand, almost no effect on PC activity was seen with two of the reagents (Hemoclot Protein C and Protein

C Reagent) used in the clotting assays (Fig. 3B). With rivaroxaban, prolongation of APTT in particular was conspicuous, as shown in Fig. 2D, suggesting that PC activity showed greater false high values with rivaroxaban as compared to edoxaban and apixaban. As shown in Fig. 2E, APTT did not increase at all with apixaban, suggesting that this drug has almost no effect on PC activity measurements in the clotting assay. In patients on FXa inhibitor therapy, PC activity levels are reported to be falsely elevated with all reagents when using clotting assay<sup>22)</sup>, although only rivaroxaban showed dose-dependent false high values in our study. Almost no effect was seen with apixaban. One reason for the differences in measured PC activity according to the type of reagent used for the assay is thought to be that the APTT prolongation effect differs depending on the composition of the reagent. The APTT-based assays are influenced by the phosphatidylserine source and concentration.

Although we did not investigate the thrombin inhibitor dabigatran in this study, the antithrombin effect of dabigatran is better reflected by APTT than PT<sup>23-25)</sup>, due to greater false high values of PC activity with clotting assays.

In contrast, PC activity measurements were not affected by any of the three direct FXa inhibitors (rivaroxaban, apixaban, and edoxaban) in the chromogenic substrate assays using the three reagents. Similarly, PC antigen levels measured using a latex photometric immunoassay system were not shown to be affected by the three FXa inhibitors (Fig. 5). Thus, in patients receiving these FXa inhibitors, measurements of PC activity with the chromogenic substrate assay and antigen levels are thought to be unaffected by the FXa inhibitor being used.

There are two types of congenital PC deficiency: type I, in which both PC activity and antigen levels are decreased (quantitative defect), and type II, in which PC activity is decreased but antigen levels are normal (qualitative defect)<sup>6)</sup>. The role of the PC antigen assay is to distinguish between type I and type II deficiencies when a defect in PC function is found. Comparison of the ratio of PC activity and PC antigen values can differentiate type I from type II. Thus, type II PC deficiency might be missed by measurements of antigen levels alone. Hence, when screening for

congenital thrombophilia, PC activity always needs to be measured. Further, our results suggest that in screening tests for congenital thrombophilia in patients on DOACs, PC activity should probably be measured using chromogenic substrate analysis.

As shown above, the inhibitory effects of the different types of DOACs on clotting activity interfere with clotting test measurement systems in patients on DOAC therapy. Therefore, when measuring the activity of the various anticoagulant factors in screening tests for congenital thrombophilia, it is possible that results that should show low values instead show high values, preventing accurate diagnosis. While it would be better to perform the tests after discontinuing DOACs, it is often difficult to discontinue DOAC treatment. Collecting specimens when blood levels of drugs are low, e.g. during the trough phase, whenever possible, would be one way to minimize interference<sup>18,25)</sup>. In addition, a reagent called “DOAC Stop”, which neutralizes anticoagulant activity in specimens, has recently become available<sup>26,27)</sup>, allowing clotting assays to be performed while excluding the effects of DOACs.

In conclusion, plasma levels of PC activity are affected by the type of DOACs and the measurement principles. Moreover, even when reagents having the same measurement principle are used, the effect on measured levels differs for each reagent. When screening for congenital thrombophilia in patients on DOAC therapy, a good understanding of not only the principles and characteristics of reagents, but also parameters specific to DOACs (pharmacological activity, inhibitory mechanism, inhibition constant  $K_i$  value, factors affecting reactivity to reagents)<sup>21,25,28-30)</sup> and other factors is necessary, and steps should be taken to limit the effects of these drugs to the extent possible.

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## 直接経口抗凝固薬が血中プロテイン C 活性測定値に与える影響

寺上 貴子<sup>1)</sup>, 關谷 暁子<sup>2)\*\*</sup>, 林 研至<sup>1)</sup>, 鈴木 健史<sup>3)</sup>, 古莊 浩司<sup>2)</sup>,  
朝倉 英策<sup>4)</sup>, 森下 英理子<sup>2)4)\*</sup>, 和田 隆志<sup>5)</sup>

### 要 旨

**【目的】** 本研究では、直接経口抗凝固薬 (DOAC) がプロテイン C (PC) 活性値に与える影響について、複数の測定試薬を用いて検討した。

**【方法】** 対象は、DOAC (リバーロキサバン, エドキサバン, アピキサバン) 内服中の非弁膜症性心房細動あるいは静脈血栓塞栓症の患者 90 例とした。それぞれの薬剤血中濃度、ならびに PT・APTT, PC 活性 (凝固時間法を用いた 3 試薬ならびに合成基質法を用いた 3 試薬), PC 抗原量をそれぞれ測定した。リバーロキサバン治療前後で検体を採取できた 7 症例についても、同様に薬剤血中濃度ならびに PC 活性を測定し、薬剤の影響を検討した。

**【結果】** DOAC の中でも特にリバーロキサバンは、凝固時間法で測定した PC 活性値に影響を及ぼし、偽高値となった。一方、アピキサバンは全く PC 活性値に影響しなかった。合成基質法による PC 活性値ならびに PC 抗原量は、3 剤とも DOAC の影響をうけなかった。また、リバーロキサバンの治療前後で PC 活性を測定したところ、凝固時間法を用いた測定法では薬剤血中濃度の増加に伴い 1.2 ~ 1.5 倍程度偽高値となった。一方、合成基質法を用いた測定法では、PC 活性値はほとんど治療前後で変化しなかった。

**【考察】** 以上の結果から、リバーロキサバンとエドキサバン内服中に PC 活性を測定する場合は、偽高値をさけるために、合成基質法を用いる必要がある。また、薬剤血中濃度が低下したタイミングで検体を採取し、薬剤の影響を極力除くことが重要である。