

Effect on Plasma Protein S Activity in Patients Receiving the Factor Xa Inhibitors

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Aims: Measurement of protein S (PS) activity in patients taking direct oral anticoagulants (DOACs) using reagents based on a clotting assay results in falsely high PS activity, thus masking inherited PS deficiency, which is most frequently seen in the Japanese population. In this study, we investigated the effect of factor Xa (FXa) inhibitors on PS activity using the reagent on the basis of the chromogenic assay, which was recently developed in Japan.

Methods: The study enrolled 152 patients (82 males and 70 females; the average age: 68.5 ± 14.0 years) receiving three FXa inhibitors (rivaroxaban, edoxaban, and apixaban). PS activity was measured using the reagents on the basis of the clotting and chromogenic assays.

Results: PS activity measured by the clotting assay reagents exhibited falsely high values depending on the plasma concentrations of FXa inhibitors in patients taking either rivaroxaban or edoxaban. However, none of the three FXa inhibitors affected PS activity when measured using the chromogenic assay.

Conclusion: In patients taking rivaroxaban or edoxaban, inherited PS deficiency is likely missed because the levels of PS activity measured using the reagents based on the clotting assay are falsely high. However, we report that three FXa inhibitors do not affect PS activity measured by the chromogenic assay. When measuring the levels of PS activity in patients undergoing DOACs, the principles of each reagent should be understood. Furthermore, plasma samples must be collected at the time when plasma concentrations of DOACs are lowest or the DOAC-Stop reagent should be used.

Key words: Factor Xa inhibitor, Inherited protein S deficiency, Protein S Tokushima, Clotting assay, Chromogenic assay

Introduction

Direct oral anticoagulants (DOACs) have been available since 2014 for the prevention of stroke caused by nonvalvular atrial fibrillation (AF) and for the treatment of venous thromboembolism (VTE),

and their frequency of use continues to increase yearly¹⁻⁵. DOACs are classified as thrombin inhibitor (dabigatran) or factor Xa (FXa) inhibitors (rivaroxaban, edoxaban, and apixaban) based on the target of inhibition. Compared with vitamin K antagonists such as warfarin, DOACs exhibit more

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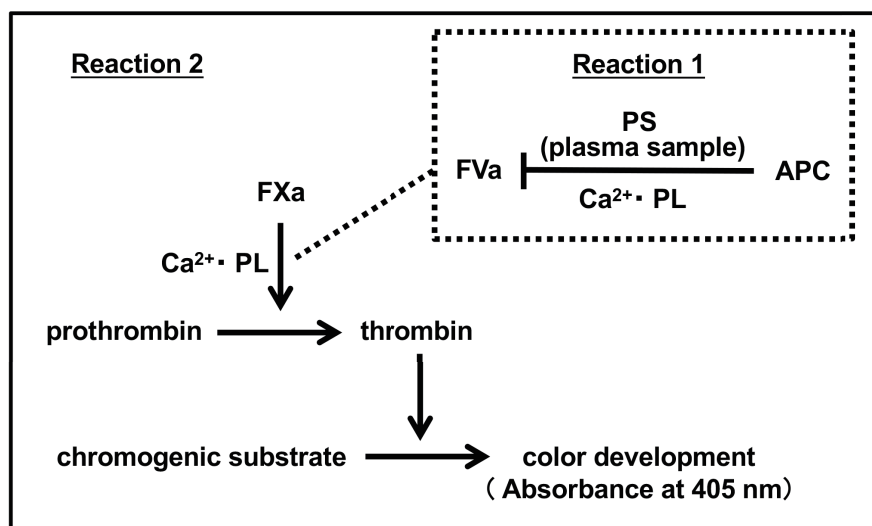


Fig. 1. Principle of total PS activity measurement using a chromogenic assay

PS in the plasma sample is reacted with APC and FVa for a certain period of time, and FVa is inactivated by PS (reaction 1). Residual FVa forms a prothrombinase complex with FXa and phospholipid and then activates prothrombin to thrombin. Since the generated thrombin degrades the chromogenic substrate, PS activity is indirectly detected by colorimetric quantification of its coloration (reaction 2).

predictable pharmacokinetic and pharmacodynamics profiles and therefore do not require routine coagulation monitoring⁶).

Thrombosis is caused by a combination of various environmental and genetic factors. Inherited thrombophilia is associated with a high risk of VTE and involves deficiencies in the natural anticoagulants: protein S (PS), antithrombin (AT), and protein C (PC)^{7, 8}. Thus, the assays that measure the levels of PS, AT, and PC activities are essential screening tests. In Japan, inherited PS deficiency is the most common inherited thrombophilia, with an incidence of 1%–2% in the Japanese population, approximately 5–10 times higher than that (0.16%–0.21%) in Europe and the United States^{9–12}. This higher incidence in Japan is associated with the presence of the PS Tokushima variant (PS p.Lys196Glu: PS K196E). The reported frequency of the PS K196E allele in the Japanese population is 0.9%^{9–11}, and PS K196E is considered a pathogenic variant in the Japanese population^{13, 14}.

Inherited PS deficiency is classified internationally into three types and diagnosed primarily based on the antigen and activity levels of PS. PS K196E is classified as a type II PS deficiency, and the average PS activity in PS K196E heterozygotes is approximately 16% lower than that of wild-type individuals (PS K196), but this is not associated with a decrease in PS antigen levels. Furthermore, since the decrease in PS activity of PS K196E is mild (16% decrease), even if PS activity is accurately measured,

PS K196E is likely to be missed¹⁵.

Previous studies reported that patients taking DOACs exhibit falsely high PC and PS activity levels when measured using the reagents based on the clotting assay^{16–20}. We reported that this effect of FXa inhibitors can be avoided by measuring PC activity using the reagents based on the chromogenic assay²¹. However, global guidelines recommended measuring the free PS antigen levels, not PS activity, in patients taking DOACs because there is no other method available for measuring total PS activity other than the clotting assay reagents²². Hence, a novel chromogenic assay for measuring total PS activity was recently developed in Japan^{23, 24}.

Fig. 1 shows the principle of the chromogenic assay. In the first reaction, PS in plasma reacts with activated protein C (APC) and factor Va (FVa) in the reagent for a certain period of time, and then, APC inactivates FVa by using PS as a cofactor. Since the reagent contains a certain excess amount of FVa, FVa remains after the inactivation of FVa in the first reaction. In the second reaction, the residual FVa, together with FXa and phospholipid, activates prothrombin to thrombin, and the generated thrombin degrades the synthetic substrate, resulting in coloration. The PS activity is measured indirectly by colorimetric determination of the coloration. Since this method uses the degradation of the synthetic substrate, it is not affected by clotting time. Our data show that the reagent for measuring PS activity based

on the chromogenic assay is not affected by FXa inhibitors. Furthermore, we report the results of the effect of FXa inhibitors on AT activity using several reagents.

Aim

In this study, we investigated the effect of FXa inhibitors on PS activity in patients receiving them measured using a chromogenic assay reagent. We also measured PS activity using several reagents based on the conventional clotting assay and compared the results with those of the chromogenic assay to determine how the different measurement principles of the reagents affect the analyses.

Materials and Methods

Study Subjects

DOACs targeted in this study included the FXa inhibitors: rivaroxaban, edoxaban, and apixaban. The subjects included two groups of 152 patients who attended outpatient clinics of the Departments of Cardiology or Hematology and were diagnosed with AF or VTE: (i) patients already undergoing anticoagulant treatment with FXa inhibitors at the time of study initiation and (ii) patients newly prescribed FXa inhibitor therapy. The study was approved by the University of Kanazawa Ethics Committee (approval no. 530-2). Written informed consent was obtained from all patients before enrollment. Patients with creatinine concentration > 2.0 mg/dL and estimated glomerular filtration rate < 30 mL/min/1.73 m² (according to the Cockcroft–Gault equation) were excluded. Patients were also excluded if they had been diagnosed with inherited PS, AT, PC deficiency or antiphospholipid syndrome, acute thrombosis, severe liver dysfunction, or nephrotic syndrome.

Sample Collection and Evaluation of Patients on FXa Inhibitor Therapy

Blood samples were collected in tubes containing 3.2% sodium citrate within 2 h after oral administration of FXa inhibitor in patients already undergoing FXa inhibitor therapy. Samples were then immediately centrifuged to prepare platelet-poor plasma and the plasma samples were stored at -80°C . Plasma concentration of FXa inhibitor, prothrombin time (PT), activated partial thromboplastin time (APTT), PS activity (clotting assay and chromogenic assay), the total amount of PS antigen, and AT activity were measured for all samples.

Sample Collection and Evaluation of Patients Newly Prescribed Rivaroxaban

Blood samples were collected from seven patients newly started on rivaroxaban before as well as 2 and 4–8 weeks after initiation of treatment. Within 2 h after oral administration, the samples were collected and immediately centrifuged. Plasma samples were stored at -80°C and evaluated the changes in plasma rivaroxaban concentration, PT, APTT, and PS activity (clotting assay and chromogenic assay) before and after rivaroxaban administration.

Coagulation Tests

a) Plasma Concentrations of FXa Inhibitor (Rivaroxaban, Edoxaban, and Apixaban)

For all samples collected from patients treated with FXa inhibitors, drug concentrations were estimated using the Biophen DiXal assay (Hyphen BioMed, Neuville-sur-Oise, France) using appropriate FXa inhibitor calibrators. Assays were performed on the CS-5100 hemostasis system (Sysmex, Kobe, Japan).

b) PT and APTT

Laboratory-based PT and APTT were measured using each reagent (Thromborel S, Siemens, Marburg, Germany, and Actin FSL, Siemens, respectively) on the CS-5100 hemostasis system. The normal ranges for PT and APTT are 10.6–13.0 and 27.3–40.3 s.

c) PS Activity (Clotting Assay)

Stago STA Staclot Protein S (Fujirebio Inc., Tokyo, Japan), Protein S Ac (Siemens), and HEMOCLOT Protein S (Hyphen BioMed) were used to evaluate PS activity before and after newly introduction of rivaroxaban. Additionally, Hemos IL PS-Clot (IL Japan, Tokyo, Japan) was used to evaluate PS activity in patients undergoing FXa inhibitor therapy. Assays were performed on the CS-5100 hemostasis system.

d) PS Activity (Chromogenic Assay)

Total PS activity was measured using the Total Protein S activity “Shino-Test” (Shino-Test Corp., Kanagawa, Japan). **Fig. 1** illustrates the measurement principle of the chromogenic assay.

e) PS Antigen

PS antigen level was measured using Cygnus Auto Total Protein S mass (Shino-Test Corp.) on the basis of a latex photometric immunoassay.

f) AT Activity

AT activity was measured by the chromogenic

assay reagents using Berichrom AT III (Thrombin-based assay, Siemens) and Testzym S AT III (Xa-based assay, Sekisui Medical, Tokyo, Japan).

Statistical Analyses

Statistical analyses were performed using the STAT VIEW program (Abacus Concepts, Berkeley, CA, USA). The Pearson product–moment correlation coefficient was used to evaluate the correlation between the levels of activity and antigen of natural anticoagulants assessed with various reagents. $P < 0.05$ was considered indicative of statistical significance.

Results

Study Subjects (Patients Undergoing FXa Inhibitor Therapy)

A total of 152 patients (82 males and 70 females) with AF or VTE were on anticoagulant therapy with FXa inhibitors. The average age (\pm SD) of patients was 68.5 ± 14.0 years. **Table 1** summarizes the distribution of diseases among the patients. Of 123 patients with AF, 69 had AF alone, and 38 had AF with chronic heart failure (CHF). Of the 38 patients with CHF, 22 also had other diseases (14 patients had sick sinus syndrome, and eight patients had atherothrombotic cerebral infarction), and the remaining 16 patients also had hypertrophic cardiomyopathy (five also with deep vein thrombosis). The diseases for which there were three or fewer patients are omitted from **Table 1**.

Effect of Rivaroxaban, Edoxaban, and Apixaban on PT and APTT

We also examined the effects of plasma FXa inhibitors on PT and APTT. Both PT and APTT were significantly prolonged in a dose-dependent manner in patients taking rivaroxaban or edoxaban (**Fig. 2A, 2B, 2D, and 2E**). The greatest prolongation of PT was observed with edoxaban as compared with other drugs, and it prolonged PT to a greater degree than APTT (**Fig. 2B**; $r = 0.838$, $P < 0.001$). Similarly, the greatest prolongation of APTT was observed with rivaroxaban as compared with other drugs, and it prolonged APTT to a greater degree than PT (**Fig. 2D**; $r = 0.851$, $P < 0.001$). By contrast, apixaban had no effect on either PT (**Fig. 2C**; $r = 0.158$, $P = 0.396$) or APTT (**Fig. 2F**; $r = 0.404$, $P = 0.062$) at any plasma concentration examined.

Effect of Rivaroxaban, Edoxaban, and Apixaban on PS Activity

a) Clotting Assay (**Fig. 3, Open Circles**)

PS activity was measured using the clotting assay

Table 1. Characteristics of patients' diseases and complications

Disease	Cases
Atrial fibrillation (AF)	Total 123
AF	69
AF & CHF	16
AF & Hypertrophic cardiomyopathy	16
AF, CHF & Sick sinus syndrome	14
AF, CHF & Atherothrombotic cerebral infarction	8
VTE	Total 34
DVT	28
DVT & AF	5
Retinal vein thrombosis	1

Of the 123 patients with atrial fibrillation, if the number of patients with other comorbidities was less than 3, they were not listed.

reagents. Both rivaroxaban and edoxaban increased PS activity in a dose-dependent manner (rivaroxaban: $r = 0.792$, $P = 0.0001$; edoxaban: $r = 0.616$, $P = 0.002$) (**Fig. 3A, 3B**). By contrast, apixaban had no effect on PS activity ($r = 0.037$, $P = 0.909$) (**Fig. 3C**).

b) Chromogenic Assay (**Fig. 3, Closed Circles**)

None of the three FXa inhibitors affected PS activity when measured using the chromogenic assay reagent (**Fig. 3A–C**).

Effect of Rivaroxaban, Edoxaban, and Apixaban on PS Antigen (**Fig. 3, Closed Triangles**)

None of the three FXa inhibitors had any effect on the amount of PS antigen based on the principle of the latex photometric immunoassay (**Fig. 3D–F**).

Changes in PT, APTT, PS Activity, and Plasma Rivaroxaban Concentration before and after Rivaroxaban Introduction

Blood samples were collected before, as well as 2 and 4–8 weeks after rivaroxaban introduction in seven sequential patients. As shown in **Fig. 4**, at each blood collection point, the plasma concentration of rivaroxaban was approximately 50–450 ng/mL 2 h after drug administration (**Fig. 4A**). PT and APTT were clearly prolonged after administration compared with baseline before rivaroxaban administration (**Fig. 4B, 4C**).

Additionally, PS activity was significantly higher compared with baseline, as determined using the clotting assay reagents (**Fig. 4D**). Interference was more pronounced with Protein S Ac (Siemens) and HEMOCLOT Protein S (Hyphen BioMed). By contrast, little effect on PS activity was observed after rivaroxaban administration when measured using the chromogenic assay reagent (**Fig. 4D**).

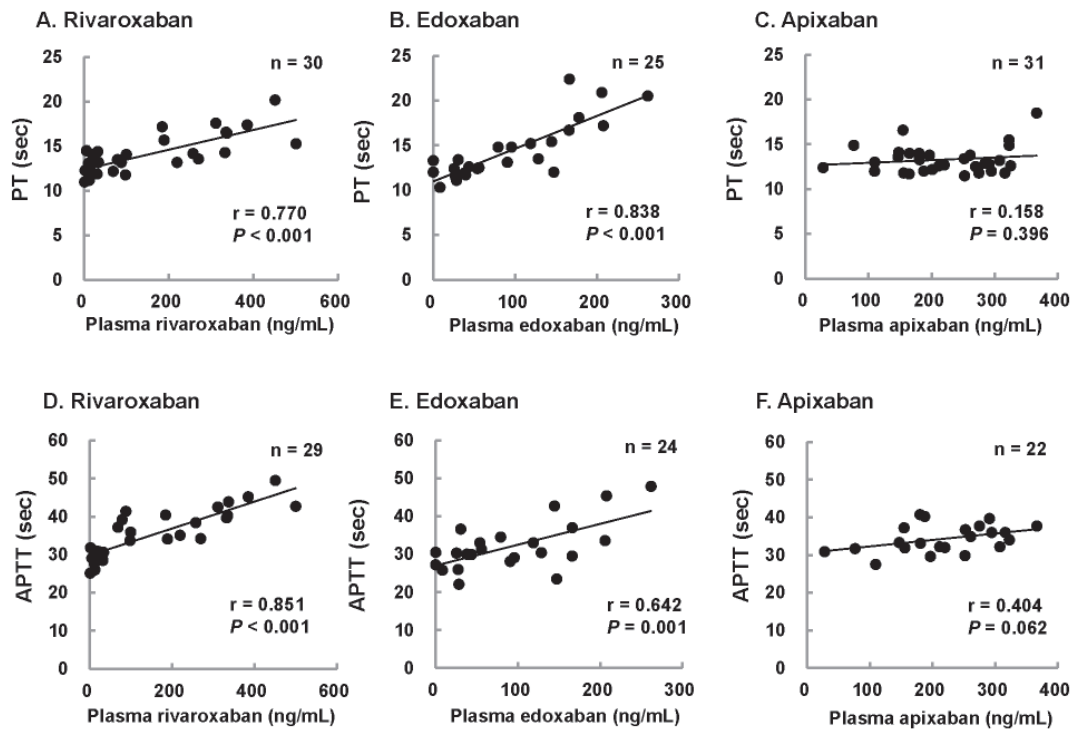


Fig. 2. Effect of rivaroxaban, edoxaban, and apixaban on PT and APTT

A, D: Rivaroxaban, B, E: Edoxaban, C, F: Apixaban

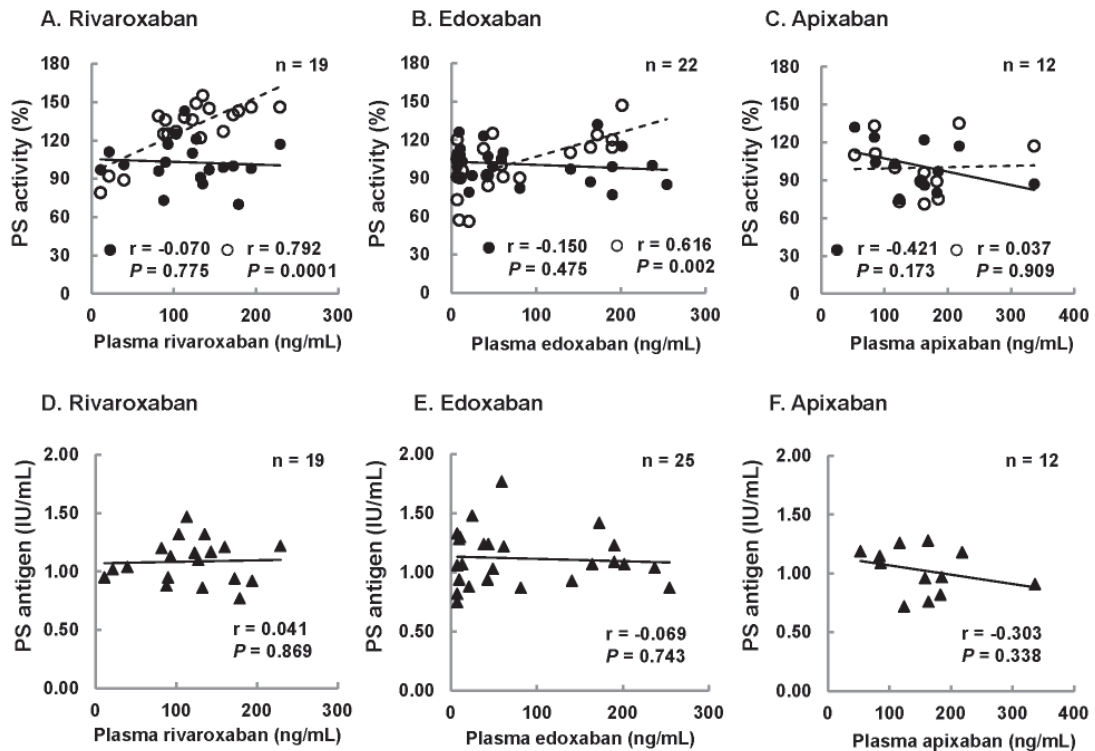


Fig. 3. Effect of rivaroxaban, edoxaban, and apixaban on the levels of PS activity and PS antigen

The graphs indicated PS activity measured using Chromogenic assay (●) and Clotting assay (○), and PS antigen (▲). A, D: Rivaroxaban, B, E: Edoxaban, C, F: Apixaban.

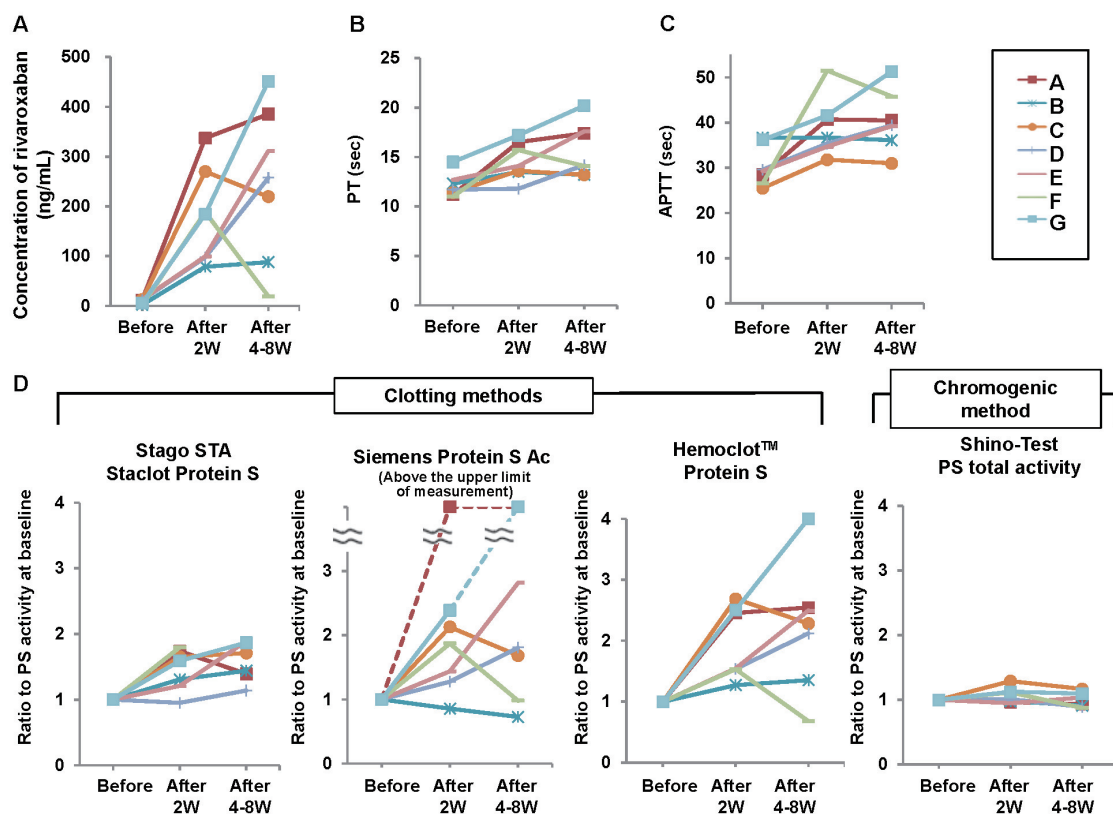


Fig. 4. Plasma concentration of rivaroxaban and the changes in PT, APTT, and PS activity before and after rivaroxaban introduction

The graphs indicated the concentration of rivaroxaban (A), the change of PT (B) and APTT (C), and the changes in PS activity values by clotting assays or chromogenic assay before and after rivaroxaban introduction (D). “Stago STA Staclot Protein S,” “Siemens Protein S Ac,” and “Hemoclot™ Protein S” are the clotting assay reagents, and “Shino-Test PS total activity” is the reagent based on the chromogenic assay.

Effect of Rivaroxaban, Edoxaban, and Apixaban on AT Activity

a) Thrombin-Based Assay (Fig. 5, Closed Squares)

None of the three FXa inhibitors affected AT activity when measured using the thrombin-based assay with a chromogenic method (Fig. 5A–C)^{19, 25–27}.

b) Xa-Based Assay (Fig. 5, Open Squares)

All three FXa inhibitors increased AT activity in a dose-dependent manner, as measured by the Xa-based assay using a chromogenic method (Fig. 5A–C; rivaroxaban, $r=0.844$, $P<0.0001$; edoxaban, $r=0.581$, $P=0.002$; apixaban, $r=0.596$, $P=0.0003$). Rivaroxaban had a particularly marked effect on AT activity^{19, 25–27}.

Discussion

The frequency of using DOACs has increased along with the increase in the number of patients with AF and VTE. DOACs have also been reported to

affect the measurement of the activity of natural anticoagulants as well as the common coagulation tests, PT and APTT, which can lead to diagnostic errors.

The present study investigated the effect of FXa inhibitor administration on PS activity using two different methods, the clotting assay, and the chromogenic assay. In measurements of PS activity using the clotting assay, inactivation of FVa by APC in the reagent is detected as a prolongation of PT, with PS in the sample serving as a cofactor. Thus, DOAC-associated prolongation of PT can lead to overestimation of plasma PS activity, resulting in falsely high values^{18, 19, 28}. By comparison, the chromogenic assay is not affected by clotting time because the chromogenic substrate is degraded, resulting in a color change that corresponds to the final thrombin activity produced; PS activity is subsequently calculated by colorimetric determination^{23, 24} (Fig. 1). In samples from patients taking FXa inhibitors, significant prolongations of both PT and APTT were observed with increasing plasma concentrations of rivaroxaban

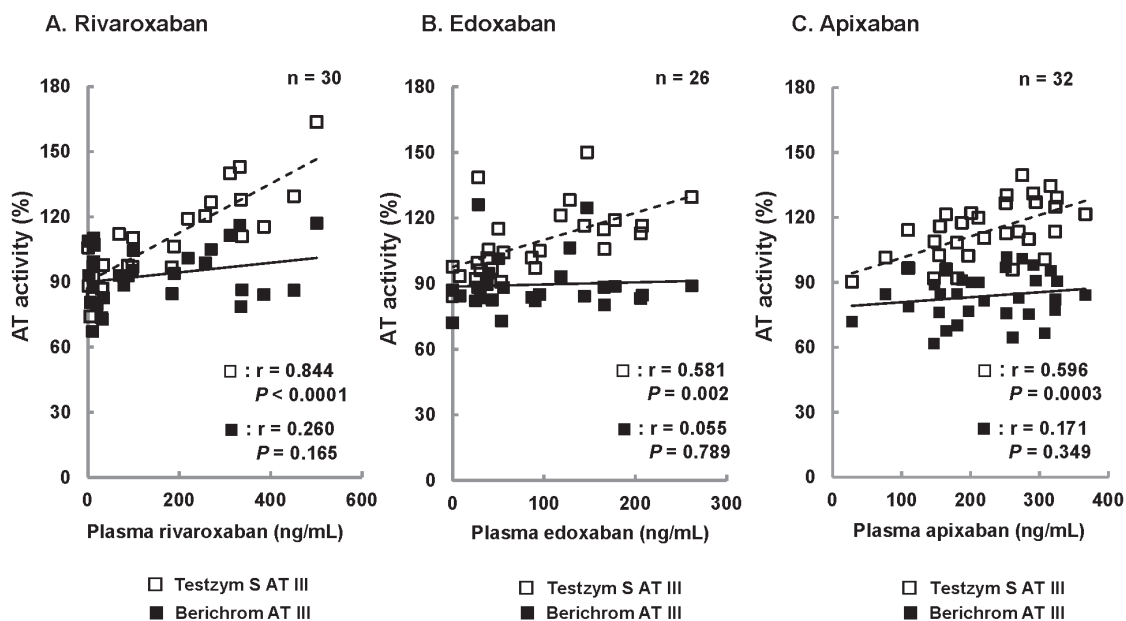


Fig. 5. Effect of rivaroxaban, edoxaban, and apixaban on AT activity

A: Rivaroxaban, B: Edoxaban, C: Apixaban, □: Xa-based assay, ■: Thrombin-based assay

and edoxaban. Apixaban had no such effect, however (Fig. 2). Before our study, Kuma *et al.* reported that rivaroxaban and edoxaban prolonged PT and APTT in a drug concentration-dependent manner, whereas apixaban had no such effect *in vitro* experiments, and these results were similar to ours²⁹). However, the study by Kuma *et al.* differs from our study because they did not use patients' plasma samples. The prolongation of clotting time observed in this study could be due to physical or chemical interactions between the individual FXa inhibitors and phosphatidylserine present in the reagents. Additionally, it has been reported that the anti-FXa activity of apixaban at the peak plasma concentrations was only approximately 60% of that of rivaroxaban^{30,31}), so the difference in anti-FXa activity at the time of blood collection may have appeared as a difference in PT prolongation effect.

PS activity measured using the clotting assay depended on the concentrations of rivaroxaban and edoxaban and exhibited a significant positive correlation (Fig. 3A, 3B). Again, however, apixaban exhibited no such effect (Fig. 3C). This result was consistent with the observation that apixaban has little effect on the measurement of PS activity by clotting assay, as PT was not prolonged in patients taking apixaban. In a previous study, FXa inhibitors caused falsely high PS activity when measured using the clotting assay¹⁶⁻¹⁹). However, in the present study, only two drugs, rivaroxaban and edoxaban, increased PS

activity in a drug concentration-dependent manner, perhaps because of the differences in reactivity with phosphatidylserine in the reagents used and in anti-FXa activity in blood at the time of blood collection. By contrast, none of the three FXa inhibitors affected PS activity as measured using the chromogenic assay (Fig. 3A–C, closed circles). Similarly, none of the three FXa inhibitors affected the amount of total PS antigen as determined using the latex photometric immunoassay (Fig. 3D–F).

We also evaluated the PT, APTT, and PS activity in patients before and after rivaroxaban introduction to confirm that the changes in clotting time and PS activity were associated with the introduction of rivaroxaban. These analyses showed that PT and APTT were significantly prolonged after the introduction of rivaroxaban (Fig. 4B, 4C). Additionally, PS activity measured using the clotting assay correlated well with these prolongations of clotting time and exhibited falsely high values (Fig. 4D). However, PS activity measured using the chromogenic assay did not otherwise change before and after rivaroxaban introduction. Hence, FXa inhibitors had no effect on PS activity measured using the reagent based on the chromogenic assay newly developed in Japan. Thus, PS activity should be measured using the chromogenic assay to accurate measurement during FXa inhibitor treatment.

AT activity is measured using the reagents on the basis of the chromogenic assay, of which there are two

Table 2. Effect of DOACs on coagulation testing for inherited thrombophilia

Methods		Thrombin inhibitor	Factor Xa inhibitors			
		Dabigatran	Rivaroxaban	Edoxaban	Apixaban	
PS activity	Chromogenic assay	no effect*	no effect	no effect	no effect	
	Clotting assay	false high*	false high	false high	no effect	
PS antigen	Latex photometric immunoassay	no effect*	no effect	no effect	no effect	
AT activity	Chromogenic assay	Thrombin-based assay	false high*	no effect	no effect	no effect
		Xa-based assay	no effect*	false high	false high	false high

*We have not investigated thrombin inhibitor. The effects of thrombin inhibitor have already been reported^{16, 18, 19, 26, 36}, and the parameters for thrombin inhibitor is marked with an asterisk to indicate that they are taken from the literature.

types: thrombin-based assay and Xa-based assay. AT activity measured using the thrombin-based assay was not significantly correlated with plasma concentration for any of the FXa inhibitors examined. By contrast, AT activity measured using the Xa-based assay was positively correlated with plasma concentration for all three FXa inhibitors, with rivaroxaban having the greatest effect on AT activity (Fig. 5). In the Xa-based assay, the FXa inhibitor present in the blood inhibits FXa contained in the reagent, which is thought to reduce the residual FXa activity, resulting in falsely high AT activity^{16-19, 25-27}. The different effects of FXa inhibitors on AT activity could be related to differences in reactivity regarding the FXa inhibitory mechanism of each drug. In the present study, similar to the previous reports^{16, 18, 19, 25-27}, AT activity in patients taking FXa inhibitors was unaffected by the FXa inhibitors when measured using the thrombin-based assay reagents.

The anticoagulant effects of various DOACs interfere with tests that measure coagulation activity in patient plasma samples. Thus, the screening tests for inherited thrombophilia in patients undergoing DOAC therapy may show falsely high PS, AT, and PC activity. It is desirable to stop DOAC therapy before coagulation testing, but in practice, it is difficult to stop administration once it has been started for the sole purpose of testing. Collecting blood samples during the trough period, when the plasma concentration of DOAC is as low as possible, is one way to minimize interference^{19, 32}. Presently, coagulation testing can be performed without DOAC interference using the “DOAC Stop” reagent, which neutralizes the anticoagulant effect of DOACs in blood^{33, 34}, but the high cost of this reagent can be prohibitive.

Table 2 summarizes the results of this study. The chromogenic assay developed in Japan was shown to

be suitable for measuring PS activity, which can lead to the identification of inherited PS deficiency. Although the effect of thrombin inhibitor was not investigated in this study, PS and PC activity measured using the clotting assay may be falsely high, as the anticoagulant effect of dabigatran is reflected in the APTT^{16, 18, 19, 32, 35, 37}. By contrast, thrombin inhibitor as well as FXa inhibitors is presumed to have no effect on PS and PC activity measured using the chromogenic assay^{16, 18, 19}. The differences of DOAC-specific reactivity in the reagents of natural anticoagulant activity could be due to characteristics of DOAC, such as their pharmacologic activity, mechanism of FXa or thrombin inhibition, and reactivity to assay reagents, but this is not clear and should be investigated in further studies^{19-21, 32, 38, 39}.

Conclusion

Measurements of the activity of various natural anticoagulants (PS, AT, and PC) in patients taking FXa inhibitors are affected by the type of DOAC and the principle of the assay reagent. Additionally, reagents with the same measurement principle can exert different effects on the measured values. In this study, we found that a newly developed reagent for measuring PS activity based on a chromogenic method in Japan was not affected by FXa inhibitors. In the future, this chromogenic method will be used to measure PS activity for rapid and accurate diagnosis of inherited PS deficiencies such as the PS K196E allele variant. Screening for inherited thrombophilia in patients undergoing DOAC therapy requires familiarity with the principles and characteristics of the assay reagents as well as parameters specific to DOACs. Furthermore, it is important to control the effect on coagulation tests of these drugs by collecting blood at the appropriate time, such as during the

trough phase or by using the DOAC Stop reagent.

Conflicts of Interest

All authors have no conflicts of interest to disclose.

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