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ORIGINAL ARTICLE

An assay to measure levels of factor Xa inhibitors in blood and plasma

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Abstract

Background: Rivaroxaban and apixaban are the most commonly used anti-factor (F) Xa direct oral anticoagulants (DOAC), with indications for prevention of stroke in nonvalvular atrial fibrillation as well as treatment and prevention of venous thromboembolism. However, lacking is accessibility to a detection method that is able to quantify low levels of anti-FXa DOACs.

Objective: We report a new assay that measures anti-FXa DOAC levels in plasma and whole blood.

Methods: This is achieved by the use of a prothrombin derivative that is labeled with a fluorescent probe (Flu-II), which then acts as the macromolecular substrate to measure residual FXa activity. The Flu-II cleavage is then initiated by the addition of a solution containing FXa, FVa, and phospholipid vesicles composed of 75% PC and 25% PS (PCPS) vesicles with calcium, in the presence of hirudin to prevent feedback activity by the native thrombin generated. The Flu-II cleavage is monitored by fluorescence in real time where the initial rate of fluorescence change is inversely proportional to DOAC levels.

Results: In plasma systems, the assay demonstrates dose-response between 0 and 5 nmol/L rivaroxaban and between 0 and 10 nmol/L apixaban. Corn trypsin inhibitor did not affect this assay. With individual plasma samples, the assay showed excellent consistency and reproducibility. From 2 μL of whole blood, the assay showed dose-response between 0 and 2 nmol/L of DOACs in the final mixture of 100 μL, thus representing up to 100 nmol/L in circulating blood.

Conclusion: The assay is ideal for rapidly and accurately measuring DOAC levels in plasma and blood, demonstrating its potential for point-of-care applications.

KEYWORDS
blood, DOACs, inhibition, plasma, quantification

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1 | INTRODUCTION

A new class of anticoagulant drugs often referred to as direct oral anticoagulants (DOAC) are being used more frequently in the prevention and treatment of arterial and venous thrombosis.[1] The DOACs are classified as targeting either thrombin or factor (F) Xa. As such, with the increased use of anti-FXa DOACs, it is becoming more important to be able, at certain times, to measure the levels of these agents in the blood of patients efficiently and accurately.[1–3] Rivaroxaban and apixaban are two of the more commonly used anti-FXa DOACs.

A distinct advantage of DOACs is that, unlike warfarin, ongoing monitoring of anticoagulation is not as necessary because of their predictable pharmacokinetics and bioavailability; thus they have been adopted despite limited ability to measure their levels. However, in some situations accurate quantitation of anti-FXa DOACs is needed, for instance, when a patient stops anticoagulation in preparation for surgery or when a patient is experiencing a bleeding or thrombotic event.[1,4] Current guidelines by different organizations offer conflicting recommendations of when patients should stop their anti-FXa DOAC, which result in miscommunication that often leads to delayed patient care that wastes time, money, and health care resources.[5–8]

The inability to correlate the levels of anti-FXa DOACs with bleeding risk remains the largest reason for disparate recommendations of fixed time post cessation of anticoagulation prior to high-bleed risk procedures, including epidural anesthesia.[4,6,7] The inability to measure very low levels of these anti-FXa DOACs[9–11] accurately contributes to this problem. Situations where DOACs may not have predictable pharmacokinetics such as extremes in body weight, liver or renal impairment, and drug interactions further complicate the problem.[1,5,6] Currently there are two major methods used to quantify levels of anti-FXa DOAC levels accurately in the blood of patients: a chromogenic substrate enzyme assay[12] and a method that utilizes high-performance liquid chromatography to isolate and quantify DOACs.[13] There are two major drawbacks on these methods. The high-performance liquid chromatography assay is only done in research facilities, thus not practical for use as a diagnostic tool in a clinical setting; however, it is accurate to very low levels. The chromogenic assay requires a skilled technician in a specialty laboratory to perform it and lacks sensitivity, as the assay cannot accurately detect DOACs <20 ng/mL (~45 nmol/L).[2,9,10,13]

Here, we describe a simple assay that is sensitive to levels of anti-FXa DOACs below the current limit of detection of the chromogenic assays. It demonstrates dose-response between 0 and 5 nmol/L for rivaroxaban and between 0 and 10 nmol/L for apixaban when measured in plasma. In addition, levels of DOACs can be measured in whole blood, suggesting its potential for use as a point-of-care assay.

Essentials

- Direct oral anticoagulants (DOAC) are used for stroke and venous thromboembolism prevention.
- We report a new assay that measures anti-factor Xa DOAC levels in plasma and whole blood.
- Rivaroxaban and apixaban can be accurately quantified below trough levels.
- The ease and accuracy of the assay demonstrate its potential for point-of-care applications.

2 | EXPERIMENTAL PROCEDURES

2.1 | Materials

Human FXa and FVa were purchased from Haematologic Technologies Inc. (Essex Junction, VT). The QuikChange Lightening Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA). The QIAprep® Spin Miniprep Kit and Plasmid Maxi Kit were purchased from Qiagen (Hilden, Germany). The Lipofectamine® 3000 Transfection Kit was purchased from Life Technologies-Invitrogen (Carlsbad, CA). Baby hamster kidney cells and the pNUT vector, used for mammalian expression, were kindly provided by Dr. Ross MacGillivray (University of British Columbia). Methotrexate (Mayne Pharma Inc., Montreal, Quebec Canada) and vitamin K_1 were purchased from Hamilton General Hospital. Q-Sepharose Fast Flow anion-exchange resin and Mono-Q HR 5/5 column were obtained from GE Healthcare (Burlington, Ontario Canada). Gibco® D-MEM/F-12 media, newborn calf serum and Opti-MEM I media, 0.5% Trypsin-EDTA, and Antibiotic-Antimycotic solution were purchased from Thermo Fisher Scientific (Carlsbad, CA). Phosphatidyl-L-serine, phosphatidyl-L-choline, and XAD-2 resin were obtained from Sigma. The Matched-Pair Antibody Set for ELISA of Human Prothrombin Antigen was purchased from Affinity Biologicals Inc. (Ancaster, Ontario Canada). 5-Iodoacetamidofluorescein (5-IAF) was purchased from Marker Gene Technologies Inc. (Eugene, OR). The recombinant prothrombin with a single mutation of the latent active site serine to a cysteine (S525C) was isolated and labeled with 5IAF (Flu-II) as described by Brufatto and Nesheim.[15] The PCPS was prepared as previously described.[16] Bio-Rad Protein Assay reagent was purchased from Bio-Rad (Mississauga, Ontario Canada). Corn trypsin inhibitor was from Enzyme Research Labs (South Bend, IN). Rivaroxaban and apixaban were purchased from Suzhou Howsine Biological Technology Company (Suzhou, China). Hirudin was purchased from EMD Chemicals, Inc. (Gibbstown, NJ).

2.2 | Rate of Flu-II cleavage by residual FXa with increasing levels of DOACs

To determine residual FXa activity upon inhibition by DOACs, Flu-II cleavage was carried out in plasma or whole blood. A mixture (50 μL) containing 0.4 μmol/L Flu-II, 2 μmol/L hirudin, 33 μL of normal human pooled plasma (NHP), and either rivaroxaban or apixaban at varying concentrations (0 to 30 nmol/L) was monitored continuously with a FlexStation 3 (Molecular Devices, Sunnyvale, CA) fluorescent plate reader at 37°C at 3-s intervals, with excitation and emission wavelengths at 495 and 540 nm, respectively, with a 530-nm

emission cut-off filter. After 1 min of equilibration, 50 μL of a mixture containing 0.1 nmol/L FXa, 10 nmol/L FVa, 50 μmol/L PCPS, and 20 mmol/L $CaCl_2$ was added and mixed by instrument trituration. The initial rates of fluorescence change were then measured by determining the slope of the first 15% to 20% of the reaction that is linear and plotted with respect to the final DOAC concentration in the reaction mixture. To examine whether the assay would be sensitive to individual differences, the DOAC dose-response experiment was repeated using four individual citrated plasma samples without generating a pool.

Next it was tested whether Flu-II cleavage by residual FXa can be measured in whole blood. Rivaroxaban or apixaban was added to citrated whole blood to mimic blood samples collected from patients taking these drugs. A mixture (50 μL) containing 2 μL of whole blood containing varying levels of the DOACs (0 to 100 nmol/L), 0.4 μmol/L Flu-II, and 2 μmol/L hirudin was monitored with FlexStation 3 fluorescent plate reader as described previously. Again after 1 min of equilibration, 50 μL of a mixture containing 0.1 nmol/L FXa, 10 nmol/L FVa, 50 μmol/L PCPS, and 20 mmol/L $CaCl_2$ was added and mixed by instrument trituration. The initial rate of fluorescence change was then measured and plotted with respect to the final DOAC concentration in the reaction mixture, which results in a 1:50 dilution.

2.3 | Intraassay and interassay variability

The NHP was prepared with known amounts of DOACs (2 nmol/L rivaroxaban or apixaban), aliquoted and frozen at −80°C. These samples subsequently were thawed, and each was measured three times on three different days using a different standard curve each day. To obtain the intraassay variability, the standard deviations for each day was determined. The mean of these averages for all three days was then determined to be the intraassay variability. The interassay variability was calculated by determining the average of the measurements over three days. The average of the standard deviations as percentages of the means was then taken as the interassay variability.

2.4 | Measurement of rivaroxaban or apixaban levels in patient plasma

To ensure that the assay is able to quantify anti-FXa DOAC levels in plasma of patients who are prescribed rivaroxaban or apixaban, random selected samples from the Perioperative Anticoagulant Use for Surgery Evaluation (PAUSE) study were measured for rivaroxaban or apixaban levels.[17] The experiment was performed as described earlier whereby the known standard in plasma was replaced with the patient plasma to achieve a final dilution of 1:3 in the reaction. To ensure that the measurement levels would be quantifiable within reason, all samples were additionally subjected to a 1:5 dilution to achieve a final dilution of 1:15 in the reaction. Once the DOAC levels were quantified using this assay, the coagulation lab was contacted for the values determined using the chromogenic assay for comparison.

2.5 | Statistical analyses

The initial rates of Flu-II cleavage determined in the presence of rivaroxaban or apixaban at varying concentrations were tested for normality and subsequently compared using 1-way analysis of variance (SigmaPlot v11, SPSS Inc.). In addition, where applicable, Tukey's post hoc analyses were performed for individual comparisons between groups. P values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Effect of DOAC concentration on the rate of Flu-II cleavage

The initial rates of Flu-II cleavage by prothrombinase in the absence or presence of DOACs at varying concentrations were measured by fluorescence signal change. Using NHP as the background, the initial rate of fluorescence change decreased with increasing levels of DOAC. The initial rates of Flu-II cleavage were dependent on rivaroxaban concentrations between 0 and 5 nmol/L, with the overall inhibition approaching ~85% of total activity (Figure 1A). The initial rates were also dependent on apixaban concentrations between 0 and 10 nmol/L, with the overall inhibition approaching ~60% (Figure 1B). The reproducibility of the standard curve is shown in the insets. There were no observable differences in the dose-response curves by the presence of corn trypsin inhibitor, a commonly used FXIIa inhibitor and anticoagulant.[18] One-way analysis of variance showed significant dose-responses of both rivaroxaban (P < 0.001) and apixaban (P < 0.001). Post hoc Tukey's test indicated that all rivaroxaban groups were significantly different from each other. For apixaban, all groups were significantly different from each other except for 0 nmol/L versus 2 nmol/L (P = 0.277) and 2 nmol/L versus 5 nmol/L (P = 0.143).

3.2 | Flu-II cleavage in individual plasma samples

Similar trends to those with NHP were observed when the reactions were carried out using plasma samples isolated from different individuals (Figure 2). Overall trends were similar to those observed with NHP (Figure 1), whereby rivaroxaban showed greater sensitivity compared with apixaban. In addition, rivaroxaban again demonstrated sensitivity between 0 and 5 nmol/L with the maximal inhibition of ~80% of total FXa activity with great reproducibility between individuals. The assay also showed good reproducibility with apixaban concentrations ranging between 0 and 10 nmol/L, with the maximal inhibition approaching ~50%.

3.3 | Rate of Flu-II cleavage in whole blood

The initial rate of Flu-II cleavage demonstrated a dose-dependence with concentrations of the DOACs (Figure 3). Because the reaction results in a 50-fold dilution, the highest dose of the DOAC investigated (2 nmol/L) represents 100 nmol/L circulating in the blood.

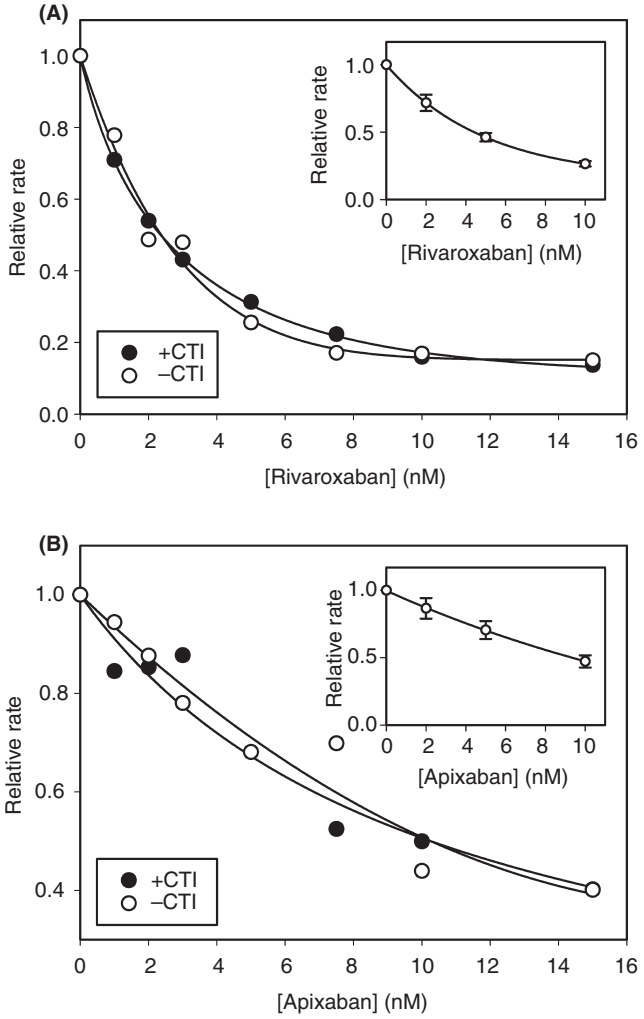

FIGURE 1 Residual FXa activity as a function of increasing concentration of A, rivaroxaban or B, apixaban, with (*closed*) or without (*open*) corn trypsin inhibitor. To determine residual FXa activity upon inhibition by DOACs, Flu-II cleavage was carried out in plasma. A mixture (50 μL) containing 0.4 μmol/L Flu-II, 2 μmol/L hirudin, 33 μL of NHP, and either rivaroxaban or apixaban at varying concentrations (0 to 30 nmol/L) was placed in a clear-bottom 96-well microtiter plate. Fluorescence was monitored using excitation and emission wavelengths at 495 nm and 540 nm, respectively, with a 530-nm emission cut-off filter. After 1 min of equilibration, 50 μL of a mixture containing 0.1 nmol/L FXa, 10 nmol/L FVa, 50 μmol/L PCPS, and 20 mmol/L $CaCl_2$ was added and mixed by FlexStation 3 instrument trituration. The initial rate of fluorescence change was then measured and plotted with respect to the final DOAC concentration in the reaction mixture, which results in a 1:2 dilution. *Inset* shows the reproducibility of the standard curves generated in the absence of corn trypsin inhibitor (n = 6). DOAC, direct oral anticoagulant; FXa, factor Xa; PCPS, phospholipid vesicles composed of 75% PC and 25% PS

Unlike with plasma, the dose-response was similar between rivaroxaban and apixaban, with maximal inhibition approaching ~50%. One-way analysis of variance showed significance in the dose-response of both rivaroxaban ($P < 0.001$) and apixaban ($P = 0.007$). Post hoc Tukey's test indicated that all rivaroxaban groups were significantly

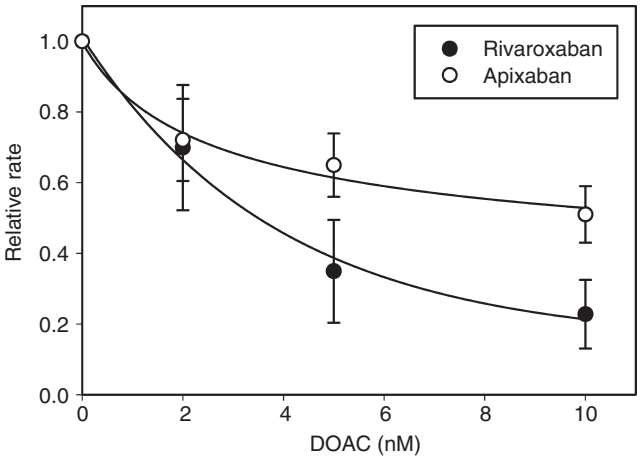

FIGURE 2 Dose-response of the assay to anti-FXa DOACs in individual plasma samples. Four different individuals were tested with the assay. Either rivaroxaban (*closed*) or apixaban (*open*) was added into the isolated plasma samples to a final concentration ranging between 0 and 10 nmol/L. The rates were normalized to no DOACs added. Symbols indicate mean ± SD (n = 4). DOAC, direct oral anticoagulant; FXa, factor Xa

different from each other except the comparison between 25 and 50 nmol/L ($P = 0.136$). For apixaban, only the comparison against the control showed significance.

3.4 | Determination of the intraassay and interassay variability

To determine the reproducibility of the DOAC assay, the intraassay variability and interassay variability were determined.[19] They were determined by subjecting DOACs at a known concentration (2 nmol/L) in NHP to the current DOAC assay. Each sample was measured four different times on three different days using a different standard curve each day. The mean concentration ± SD for four measurements were then calculated. The intraassay and interassay variability for the rivaroxaban assay were determined to be 25.7% and 31.5%, respectively. The intraassay and interassay variability for the apixaban assay were 60.3% and 58.0%, respectively.

3.5 | Measurement of DOAC levels of selected PAUSE study patient samples

To determine whether the assay can be applied to patient samples, we measured patient plasma samples for rivaroxaban and apixaban. Furthermore, to mimic easy point-of-care analyses, the plasma samples were applied directly without any normalization or a singular 10-fold dilution. Of the rivaroxaban samples quantified using our assay, 86% of the samples were deemed unmeasurable using the chromogenic assay (i.e. <45 nmol/L or 20 ng/mL). For apixaban-containing samples, however, 55% of the samples that we quantified were deemed unmeasurable with the chromogenic assay (Figure 4). The DOAC levels reported by the chromogenic assay for the samples that were deemed measurable with both assays were 71.4 ± 11.5

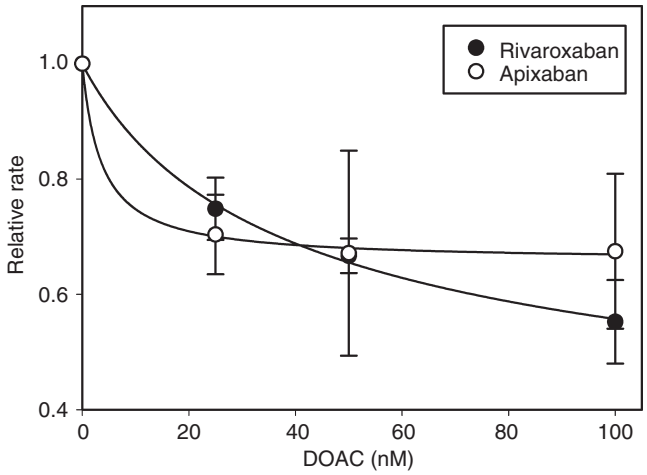

FIGURE 3 Dose-response of the assay to anti-FXa DOACs in whole blood. Whole blood was collected in citrate to which rivaroxaban (*closed*) or apixaban (*open*) was added to achieve an in-blood concentration ranging between 0 nmol/L and 100 nmol/L. Similarly to earlier, a mixture (50 μL) containing 0.4 μmol/L Flu-II, 2 μmol/L hirudin, 2 μL whole blood containing rivaroxaban or apixaban was placed in a clear-bottom 96-well microtiter plate. Fluorescence was monitored using excitation and emission wavelengths at 495 nm and 540 nm, respectively, with a 530-nm emission cut-off filter. After 1 min of equilibration, 50 μL of a mixture containing 0.1 nmol/L FXa, 10 nmol/L FVa, 50 μmol/L PCPS, and 20 mmol/L $CaCl_2$ was added and mixed by FlexStation 3 instrument trituration. The initial rate of fluorescence change was then measured and plotted with respect to the final DOAC concentration in the reaction mixture, which results in a 1:50 dilution (n ≥ 3). DOAC, direct oral anticoagulant; FXa, factor Xa; PCPS, phospholipid vesicles composed of 75% PC and 25% PS

nmol/L and 66.9 ± 10.6 nmol/L for rivaroxaban and apixaban, respectively (Table 1).

4 | DISCUSSION

We report a novel assay that is sensitive to levels of DOACs that are well below the current limit of detection at specialized coagulation laboratories (20 ng/mL or 45 nmol/L). Furthermore, the assay utilizes a prothrombin derivative S525C, which is advantageous for a number of reasons. First, because the active site serine is mutated to a cysteine, the resulting thrombin does not possess any proteolytic activity that would otherwise result in a positive feedback and propagation of thrombin generation. To ensure that no endogenous thrombin remains active, a thrombin-specific inhibitor, hirudin, is added into the assay. Therefore, any prothrombin cleavage observed is as a direct result of FXa that is provided by the assay and not endogenous FXa generated. The second advantage of S525C-prothrombin is labeling of the only free-thiol group on the cysteine with a fluorescent probe such as 5-IAF, so that any fluorescent change observed is simply due to the cleavage of Flu-II by the remaining exogenous FXa. This simplifies the complicated analyses of the entire coagulation cascade, where the initial rate of Flu-II cleavage is simply

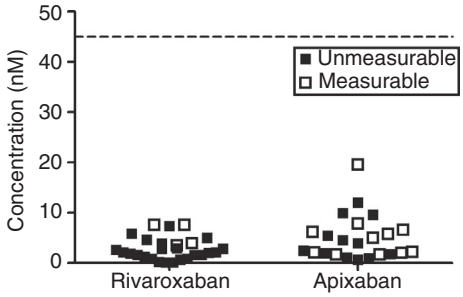

FIGURE 4 Quantification of the PAUSE study patient plasma samples containing rivaroxaban or apixaban. Random patient samples were quantified using the generated standard curve. The majority of the samples that were quantifiable with the Flu-II assay were deemed unmeasurable using the chromogenic assay (*closed*) while the remainder were measurable with both assays (*open*). The values of the samples that were quantified by both assays are shown in Table 1. The dotted line represents the detection limit of the chromogenic FXa activity assay

TABLE 1 Comparison of the DOAC values for each sample that was able to be measured by both Flu-II and chromogenic assays

DOAC	Sample ID	Flu-II Assay (nmol/L)	Chromogenic (nmol/L)
Rivaroxaban	Sample 2333	3.6	83.3
	Sample 1456	7.6	58.5
	Sample 3420	7.6	78.8
	Sample 1423	4.0	65.3
	Average	5.7 ± 2.2	71.4 ± 11.5
Apixaban	Patient 3152	1.7	56.3
	Patient 3190	7.9	47.3
	Patient 3193	2.2	78.8
	Patient 1425	5.8	72.0
	Patient 3147	2.1	65.3
	Patient 3248	6.2	60.8
	Patient 2914	1.7	81.0
	Patient 3266	6.6	58.5
	Patient 3155	5.1	67.5
	Patient 3261	2.0	78.8
	Patient 3169	19.6	63.0
	Average	8.3 ± 5.2	66.9 ± 10.6

Abbreviation: DOAC, direct oral anticoagulant.

inversely proportional to DOAC concentrations without needing the resulting thrombin activity to quantify the kinetics of prothrombin activation. The third advantage of using Flu-II is that the assay no longer becomes dependent on the intrinsic coagulation factor composition of the individuals since the output simply depends on the cleavage of Flu-II by the exogenous FXa that is also provided by the assay reagent. This assay is not sensitive to the presence of other coagulation inhibitors such as corn trypsin inhibitor or vitamin K antagonists such as warfarin (since all the necessary coagulation

factors are provided by the assay and have normally γ-carboxylated GLA domains). The assay is, however, sensitive to the levels of low-molecular-weight heparin (such as dalteparin) and antithrombin in the plasma sample (not shown), whereby dalteparin enhances the inhibitory properties of antithrombin for FXa.[20] The sensitivity to antithrombin could, however, be ameliorated by supplementing the assay with additional antithrombin, whereby physiologic and higher concentrations (2 μmol/L) of antithrombin do not appear to influence the rate of Flu-II cleavage. By doing so, the assay also has the potential to measure low-molecular-weight heparin levels in patients.

Because the fluorescence change of Flu-II by FXa is reflective of the activation cleavage at Arg320 of prothrombin to generate the intermediate meizothrombin, the substrate could also be a mutant variant of prothrombin that can only generate meizothrombin as the final product (e.g. R271A/Q and/or R155A/Q and/or R284A/Q) to produce similar signal changes.[19] Furthermore, the assay is not necessarily limited to using 5-IAF as the probe to provide a signal change, although the labeling efficiency appears to be better for 5-IAF (~35%) compared with similar probes such as Alexa-488 (~20%).

The range of sensitivity to detect the DOACs can be varied by two methods. The first is to dilute the patient plasma samples containing high DOAC levels using a standard reference DOAC-free plasma so that the residual FXa activity falls within the appropriate range. The second method would be to alter the concentration of the prothrombinase/activating solution; with a higher activator concentration for samples from patients undergoing active therapy or a lower activator concentration for measuring residual DOAC levels upon cessation of anticoagulation. This flexibility and versatility of the assay allow for tailoring and customization to meet various needs.

While prothrombin activation can be triggered by a number of different reagents such as tissue factor or polyphosphates,[21,22] these pathways are more upstream of where prothrombin activation would take place and thus become dependent on the individual procoagulant potential to activate FX. Therefore, activating the assay system with purified FXa along with FVa, PCPS, and calcium (prothrombinase) makes the assay independent of these individual differences of the upstream processes needed to generate FXa.

The ability of this assay to measure rivaroxaban or apixaban in whole blood certainly hints at its potential to be used in a point-of-care setting, where a small amount of blood is sufficient to measure the drug levels during active therapy. These methods would be ideal for testing for anticoagulation on an ongoing basis (a) to ensure that patients are taking the drugs as recommended to be in therapeutic range and (b) to identify/monitor anticoagulation in patients with altered drug metabolism or clearance (liver or kidney failure, drug-drug interactions or extremes in body weight). This assay as a point-of-care strategy would certainly provide the means for personalized treatment and care during anticoagulation.

Clinical trials are under way to investigate how measurable levels of DOACs at the time of surgical intervention are correlated with bleeding complications.[17] Since readily available methods of measurements cannot detect below 20 ng/mL, this assay can be implemented into such a study comparing residual anti-FXa DOAC levels with bleeding complications, specifically investigating whether bleeding can be correlated with DOAC levels below the current detection limit of clinically used assays.

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CONFLICT OF INTEREST

P.L.G. has received honoraria from Bayer, Bristol Myers Squibb, Pfizer, Leo Pharma, and Servier. J.D.D. has received honoraria from AstraZeneca, Bayer, Biotie, Boehringer-Ingelheim, Bristol Myers Squibb, Daiichi Sankyo, Pfizer, Portola, Sanofi, and the Medicines Company. The other authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

P.Y.K. conceptualized and designed experiments and wrote the manuscript. P.L.G. designed experiments and wrote the manuscript. C.W. performed experiments to develop the assay (Figure 1 through Figure 3). L.R.D. performed the assay on clinical samples (Figure 4) and edited the manuscript. J.D.D. provided the PAUSE study samples along with their measured properties and edited the manuscript.

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