Factor Xa Inhibitory Profile of Apixaban, Betrixaban, Edoxaban, and Rivaroxaban Does Not Fully Reflect Their Biologic Spectrum

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Abstract
The currently available oral anti-Xa agents are claimed to produce their anticoagulant and antithrombotic effects solely by the inhibition of factor Xa. This study profiled various anti-Xa drugs in routinely used laboratory assays to demonstrate that their effects are not solely related to the anti-Xa activities. Apixaban, betrixaban, edoxaban, and rivaroxaban were obtained commercially. Native and citrated whole blood was used for the activated clotting time (ACT) and thromboelastography (TEG). Citrated plasma was used for monitoring the prothrombin time (PT), activated partial thromboplastin time (aPTT), Heptest, and prothrombinase-induced clotting time (PiCT) tests. An amidolytic method was used for the determination of anti-Xa effects. Thrombin-induced fibrinokinetics was monitored optically. Thrombin generation studies were carried out using the calibrated automated thrombogram. All of the anti-Xa agents produced concentration- and assay-dependent effects. In the ACT at 2.5 μg/mL and TEG at 1.0 μg/mL, edoxaban exhibited the strongest anticoagulation effect. In the PiCT, PT, and aPTT assay at 1 μg/mL, edoxaban showed stronger effects than other agents. The half maximal inhibitory concentration of these agents for the inhibition of factor Xa ranged from 340 to >1000 ng/mL. In the thrombin generation inhibition assay, apixaban showed the strongest activity. In the fibrinokinetics, different anti-Xa agents produced varying degrees of inhibition. These results demonstrate that the measured anti-Xa activity alone does not fully reflect the overall biologic spectrum of these agents.

Keywords
anti-Xa agents, laboratory monitoring, anticoagulant

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Introduction
The introduction of direct oral anticoagulants (DOACs) has added a new dimension to the management of thrombotic and cardiovascular disorders.1 Owing to better safety and efficacy, these drugs are widely used in agent-specific indications, which include venous thromboembolism and nonvalvular atrial fibrillation.2,3 These drugs are currently being developed for other indications, including acute coronary syndrome, peripheral arterial disease, and cancer-associated thrombosis. Currently, there is one oral antithrombin agent, namely, dabigatran, and four anti-Xa agents, namely, apixaban, betrixaban, edoxaban, and rivaroxaban, approved for specific indications. Although these drugs are orally administered, their dosages for different indications vary widely. The anti-IIa drug dabigatran can be readily differentiated from the currently available anti-Xa agents. Differences in the currently available anti-Xa agents are also notable in terms of their inhibitory profile in factor Xa and thrombin generations assays.

Both the chemical and the enzymatic methods are used to synthesize new oral anticoagulant agents. Minor microchemical differences in their structure contribute to their pharmacological identity.4 Table 1 lists the currently available oral anti-Xa drugs. All of these exhibit similar characteristics in term of molecular weight (400-600 Da). All these agents mediate their antithrombotic actions via inhibiting factor Xa. The relative inhibitory potency of each of these agents differs. The dosage of these agents also varies widely and ranges from 2.5 to 160 mg/d with...
certain adjustments. Apixaban is used at a relatively lower dosage, whereas betrixaban is used at a relatively higher dosage. Moreover, the pharmacokinetic and pharmacodynamic profiles of these agents widely differ. Each of these agents is approved for specific indications at a fixed dosing regimen. A direct comparison of these agents in terms of their biochemical and pharmacological properties is not available. The therapeutic effects of these agents are mainly attributed to their inhibitory effects on factor Xa. As these drugs represent synthetic heterocyclic agents, it may be possible that besides inhibiting factor Xa, these agents may have additional effects on the coagulation network. In this study, the effects of these drugs on various laboratory assays used to assess the anticoagulant effects of drugs were studied. It is hypothesized that the overall anticoagulant and antiprotease effects of these drugs may not be directly proportional to their anti-Xa potency.

## Materials and Method

### Whole Blood Analysis

Thromboelastographic (TEG) studies were carried out using TEG 5000 Hemostasis System (Haemonetics Corp, Massachusetts). Blood samples were drawn from healthy donors \((n = 5-7)\) and placed into tubes containing 3.2% sodium citrate. To the TEG cup, CaCl₂, the Xa inhibitor at a final concentration of 1.0 \(\mu\)g/mL, and citrated human whole blood were added. The TEG profile was monitored for 45 minutes. The parameters such as \(r\)-time, \(k\)-time, maximum amplitude (MA), and angle were measured.

Whole blood activated clotting time (ACT) was measured by using Hemochron, Whole Blood Coagulation System (Accriva Diagnostics, Inc, California). Whole blood was supplemented with the factor Xa inhibitors at a final concentration of 2.5 \(\mu\)g/mL and transferred into celite ACT tubes. Results were recorded in seconds.

### Clot-Based Assays

**Whole blood supplementation studies.** Citrated whole blood and retrieved plasma studies were carried out. Citrated whole blood from healthy donors was supplemented with the factor Xa inhibitors in a concentration range of 1.0 to 0.61 \(\mu\)g/mL. Samples were analyzed using 1-stage prothrombinase-induced clotting time (PiCT), activated-partial thromboplastin time (aPTT), and prothrombin time (PT) assays. All reagents were reconstituted according to the manufacturer’s instructions. The PiCT assay was obtained from PentaPharm (Basel, Switzerland). The TriniCLOT aPTT reagent was obtained from Diagnostica Stago (Parsippany, New Jersey). The PT, HemosIL reagent was obtained from Instrumentation Laboratory (Bedford, Massachusetts). All assays were run on the ST4 from Diagnostica.

### Table 1. Comparison of the Direct Anti-Xa Agents: Apixaban, Betrixaban, Edoxaban, and Rivaroxaban.

| Name of Drug | Apixaban | Betrixaban | Edoxaban | Rivaroxaban |
|--------------|----------|------------|----------|-------------|
| Commercial name | Eliquis | Bevyxxa | Savaysa | Xarelto |
| Manufacturer | Bristol Myers Squibb Co and Pfizer Partnership | Portola Pharmaceuticals | Daichi Sankyo Inc | Bayer and Johnson and Johnson Partnership |
| Structure | \(C_{25}H_{25}N_5O_4\) | \(C_{23}H_{22}ClN_5O_3\) | \(C_{24}H_{30}ClN_7O_4S\) | \(C_{19}H_{18}ClN_3O_5S\) |
| Molecular weight | 459.4971 | 451.91 | 548.06 | 435.88 |
| Mechanism of action | Inhibits factor Xa activity in a direct, reversible, competitive, and specific manner. | Inhibits factor Xa activity in a direct, reversible, competitive, and specific manner. | Inhibits factor Xa activity in a direct, reversible, competitive, and specific manner. | Inhibits factor Xa activity in a direct, reversible, competitive, and specific manner. |
| Dosage (US) recommendation | 2.5 mg twice daily for the prophylaxis of DVT and PE and hip or knee replacement surgery | Initial dose of 160 mg and then a dose of 80 mg once daily for the period of 35 to 42 days for the prophylaxis of VTE | 60 mg once daily for patients with CrCl levels >50 mL/min. | 15 mg twice daily for treatment of DVT and PE. |
| Indications | DVT | VTE | DVT | DVT |
| | Stroke | | Stroke | Stroke |
| | Systemic embolism | | Systemic embolism | Systemic embolism |
| | PE | | NVAF | PE |
| | Atrial fibrillation | | Bleeding | Atrial fibrillation |
| | Acute Hemorrhage | | Bleeding | Anaphylactic reactions |
| Contraindications | Heart valve surgery | | Severe allergic reactions | Severe bleeding |
| | | | | Renal impairment |
| | | | | Discontinuation may result in increased risk of ischemic events |

**Abbreviations:** CrCl, creatinine clearance; DVT, deep vein thrombosis; NVAF, nonvalvular atrial fibrillation; PE, pulmonary embolism; VTE, venous thromboembolism.
Stago. Whole blood supplemented with the factor Xa inhibitors was centrifuged at 3000 × g for 20 minutes to obtain retrieved plasma. The plasma samples were further analyzed for PiCT, aPTT, and PT using the same methodologies. Results were compiled in terms of mean (standard deviation; n = 3).

**Citrated plasma studies.** Factor Xa inhibitors were supplemented in citrated plasma in a concentration range of 1.0 to 0.062 mg/mL. The PT, aPTT, and Heptest (Haemachem, St. Louis, Missouri) were measured using an ACL-Elite (Instrumentation Laboratory, Bedford, Massachusetts). The PiCT was measured on the ST4. Results were compiled in terms of mean (SD; n = 5).

**Chromogenic assay**

Anti-factor Xa activity was measured by a kinetic amidolytic method using ACL-Elite instrument. The factor Xa inhibitors were supplemented in normal human plasma/blood bank plasma in a concentration range of 1.0 to 0.62 μg/mL. Bovine factor Xa (Enzyme Research Laboratories, South Bend, Indiana) and factor Xa substrate (BioMedica Diagnostics, Connecticut) were used in this assay. The inhibitory potency was calculated in terms of half maximal inhibitory concentration (IC50) as ng/mL or μM.

**Inhibition of Thrombin Generation**

Inhibition of thrombin generation was measured by fluoroskan ascent fluorimeter, calibrated automated thrombogram (CAT; Diagnostica Stago). Drugs were supplemented in normal pooled plasma to obtain concentrations of 1.0 to 0.062 μg/mL. Reagents used in this assay included the Fluo-Substrate and tissue factor high reagent (mixture of tissue factor and phospholipids) and a thrombin calibrator were obtained from Diagnostica Stago. The thrombin generation potential was measured by using the peak thrombin, lag time, endogenous thrombin potential /area under the curve (AUC), % peak thrombin, and % endogenous thrombin potential. The potency was measured in terms of IC50 in ng/mL and μM. Results were compiled in terms of mean (SD; n = 5).

**Fibrinokinetic Measurements**

Fibrinokinetics was carried out using an optical kinetic method, where thrombin was used to trigger clot formation. Fibrinokinetics profile was measured in a microtiter plate using SpectraMax Plus 384 microplate reader (Molecular Devices, San Jose, California). The factor Xa inhibitors were supplemented into normal human plasma in a concentration range of 1.0 to 0.062 μg/mL. Thrombin (5U) and 0.025 M CaCl2 were added to the plasma supplemented with the inhibitor, and the clot density was measured in terms of increased in the absorbance, over 15 minutes. All results were compiled in terms of mean (SD; n = 3-5).

**Results**

Figure 1 shows the composite thromboelastogram of various factor Xa agents compared at 1 μg/mL. Apixaban, edoxaban, and rivaroxaban exhibited similar effects on the TEG. However, betrixaban produced a stronger anticoagulant effect in this assay system. The TEG parameters including r-time, k-time, angle, and MA were compiled from three individual donors for each of these agents as shown in Table 2. Betrixaban produced the strongest effects and the r-time values (32.7 [10.9]) were higher than with other inhibitors. Edoxaban was marginally lower than betrixaban (31.7 [8.6]) followed by rivaroxaban (28.2 [3.5]), whereas apixaban showed relatively weaker effects (23.2 [0.5]) than the other factor Xa agents. The rank order for the k-time followed a slightly different pattern, betrixaban > rivaroxaban > edoxaban > apixaban. The ranked order in the angle was found to be apixaban > betrixaban > edoxaban > rivaroxaban. The MA values for all four agents were comparable and ranged from 51.5 to 54.4 mm. Figure 2 shows a comparison of the ACT results with the different factor Xa inhibitors. In the ACT test, at 2.5 μg/mL, apixaban produced a marginal effect on the ACT (150 seconds) in comparison to saline control (144 seconds). The other agents produced stronger effects prolonging the ACT in the range of 169 to 254 seconds. The difference between edoxaban and rivaroxaban was minimal.

![Figure 1](image-url)
A comparison between factor Xa agents in whole blood and retrieved plasma is shown in Figures 3 and 4, respectively. In the whole blood PiCT test, edoxaban showed maximum response followed by betrixaban and edoxaban, which demonstrated comparable effects. Apixaban showed a relatively weaker response. The effects of drugs in aPTT assay for whole blood and retrieved plasma were similar: Betrixaban and edoxaban showed stronger and comparable anticoagulant effects followed by rivaroxaban and apixaban which showed lesser effects. The PT test for whole blood revealed that edoxaban was the stronger anti-Xa inhibitor, followed by betrixaban, rivaroxaban, and apixaban. In contrast to the whole blood, results obtained in retrieved plasma showed slightly different trends (Figure 4). Edoxaban showed stronger anticoagulant effects, followed by rivaroxaban, betrixaban, and apixaban. In the retrieved plasma, edoxaban showed stronger anticoagulant effects whereas betrixaban and rivaroxaban showed comparable effects, which were slightly weaker than edoxaban. Apixaban produced relatively weaker effects in this test.

Table 2. Comparison of the Direct Anti-Xa Agents: Apixaban, Betrixaban, Edoxaban, and Rivaroxaban on TEG Profile.

| Drug             | $r$-Time, Minutes | $k$-Time, Minutes | Angle, ° | MA, mm |
|------------------|-------------------|-------------------|----------|--------|
| Apixaban, FC = 1 μg/mL | 23.2 (10.5)       | 8.8 (4.97)        | 23.9 (12.4) | 51.5 (3.4) |
| Betrixaban, FC = 1 μg/mL | 32.7 (10.9)       | 12.3 (6.6)        | 21.9 (8.6)  | 53.8 (3.9)  |
| Edoxaban, FC = 1 μg/mL | 31.7 (8.6)        | 9.8 (1.6)         | 19.1 (7.8)  | 54.4 (6.6)  |
| Rivaroxaban, FC = 1 μg/mL | 28.2 (3.5)        | 10.1 (3.2)        | 18.6 (5.78) | 52.0 (4.1)  |

Abbreviations: FC, fold change; TEG, thromboelastography.

Figure 2. A comparison of the direct anti-Xa agents: apixaban, betrixaban, edoxaban, and rivaroxaban in whole blood using ACT system. ACT indicates activated clotting time.

Figure 3. A comparison of the direct anti-Xa agents: apixaban, betrixaban, edoxaban, and rivaroxaban in whole blood. (A) Prothrombinase-induced clotting time, (B) activated-partial thromboplastin time, and (C) prothrombin time.
with the exception of betrixaban, which did not have any effects on Heptest. Assay-dependent variations in the anticoagulant activities of these agents were noted. In the PiCT test, rank order of the anticoagulant activity was edoxaban > rivaroxaban > apixaban = betrixaban. In Heptest, apixaban, rivaroxaban, and edoxaban produced comparable anticoagulant effects with minor variations. Betrixaban did not produce any anticoagulant effect on this assay. In the aPTT assay, the rank order followed betrixaban > edoxaban > rivaroxaban > apixaban. In the PT test, the rank order was rivaroxaban > edoxaban > betrixaban > apixaban.

Figure 6 shows the effect of various anti-Xa inhibitors in the amidolytic anti-Xa assay in citrated normal plasma. All agents produced a concentration-dependent inhibition of factor Xa. The IC_{50} values followed the ranked order edoxaban < apixaban < rivaroxaban < betrixaban. The anti-Xa activity of edoxaban and apixaban was comparable.

Figure 7A to D shows the thrombokinetograms obtained in the thrombin generation (CAT) assay. All of the factor Xa inhibitors produced a concentration-dependent inhibition of thrombin generation. Apixaban, betrixaban, and edoxaban exhibited comparable inhibitory effects; however, rivaroxaban produced relatively weaker effects. At a 1.0 μg/mL concentration, the peak thrombin values were 9.5 (4.4) nM for apixaban, 10.3 (4.2) nM for betrixaban, and 11.1 (6.2) nM for edoxaban, whereas rivaroxaban showed relatively higher generation of thrombin 16.0 (6.7) nM in comparison to the control values 104.6 (19.3) nM.

Figure 8 and Table 3 shows the composite of the individual thrombin generation parameters for the four anti-Xa agents as measured by peak thrombin, AUC, and lag time. As shown in Figure 8A, the peak thrombin values show comparable inhibitory patterns with apixaban, betrixaban, and edoxaban, whereas rivaroxaban produced weaker effects. Figure 8B shows endogenous thrombin potential in terms of AUC. Apixaban showed relatively stronger inhibition in this parameter, followed by betrixaban and edoxaban that were comparable. Rivaroxaban produced weaker values at all concentrations in comparison to the other agents. Figure 8C shows the effects of these agents on lag time values. Edoxaban and betrixaban showed delayed lag time in a comparable manner, which was different when compared to apixaban and rivaroxaban. Rivaroxaban produced the most pronounced effects on the lag time followed by apixaban.

Figure 9 shows the effect of various anti-Xa drugs on thrombin-induced fibrin formation as measured by the fibrinokinetics method. At a 1 μg/mL concentration, rivaroxaban produced the most pronounced inhibitory effects followed by edoxaban. Apixaban also inhibited fibrin formation though not as strongly as rivaroxaban and edoxaban. Betrixaban produced relatively weaker effects on this assay. The clot density at 15 minutes range from 0.28 to 0.7 in the presence of these agents. Saline-supplemented system showed stronger clot formation with the higher rate of the formation of a clot in 15 minutes. Clot density was much higher than those observed with anti-Xa agents.

**Discussion**

The relative potencies of the anticoagulant drugs of different classes are usually expressed in terms of their effects on a laboratory assay. The potency of unfractionated heparin is expressed in terms of units per milligram. The low-molecular-weight heparins are potency standardized in terms of anti-Xa units. However, a reliable study relating the anti-Xa potency to their biological activity is not available. The newly developed parenteral and oral anti-IIa and anti-Xa
drugs are usually characterized in terms of their global anticoagulant effects and their inhibitory effects toward thrombin and factor Xa.5-9 Several studies have compared the anticoagulant effects of these DOACs in global anticoagulant and other assays; however, none of these studies have shown any relevance of the antiprotease potency (Xa and IIa) with other tests. The in vitro inhibitory profile and antiprotease effects of these agents may not be proportional to their pharmacological and clinical effects. This is primarily due to the differences in their pharmacodynamic profiles. The currently developed anti-Xa agents include apixaban, betrixaban, edoxaban, and rivaroxaban. These drugs are approved for specific indications, which are largely based on the clinical trial data.10,11

The structures of these drugs are similar to molecular weights ranging from 435 to 548 kDa.3 All of these drugs inhibit factor Xa in a competitive reversible manner. All of these drugs are used orally, and their dosages vary widely. Other pharmacological differences are also known and are product specific. These differences may be responsible for the clinical profiles of these newer drugs.10-12 A study to compare these four agents in standardized biochemical and pharmacological assays is not available. This study represents the first report where all four agents are compared in standardized assay systems to relate their activities with the determined anti-Xa actions.

The use of TEG for the detection of the newer oral anticoagulants has been reported previously.13 In the whole blood clotting assay, the anticoagulant behavior of these drugs was assay dependent. In the TEG studies, all of these drugs produced mild and concentration-dependent anticoagulant effects.
At a 1 μg/mL concentration, betrixaban exhibited relatively stronger anticoagulant effects as measured by r-time, k-time, angle, and MA. The other three drugs exhibited a comparable anticoagulant profile. Once again, the TEG profile in terms of anticoagulant parameters was not proportional to the anti-Xa potency of these agents. In the ACT assay, all of these drugs were capable of producing varying degrees of anticoagulation in whole blood. In these studies, edoxaban produced the strongest anticoagulant effects followed by rivaroxaban. Betrixaban and apixaban exhibited relatively milder anticoagulant effects. The relative anticoagulant effects followed the rank order: edoxaban > rivaroxaban > betrixaban > apixaban. Apixaban exhibited much weaker anticoagulant effects than other drugs. The anticoagulant potency as measured by this assay was not proportional to anti-Xa effects. In addition, in the whole blood assays, the relative anticoagulant effect produced in the TEG was different from the rank order in the ACT. These results are consistent with the previously reported study. This may be due to the differences in the mechanisms of thrombogenesis involved in the two systems.

In the current studies, the anticoagulant profile of all of these drugs was also studied in the whole blood obtained from normal human volunteers. This study was designed to investigate the relative anticoagulant effects in the whole blood and the retrieved plasma from the same systems to differentiate the effects of cells on the anticoagulant profile of the four anti-Xa agents. The PiCT, aPTT, and PT assays were used. In these assays, all four drugs produced a concentration-dependent anticoagulant effect. In whole blood, PiCT was found to be the most sensitive to demonstrate the anticoagulant effects of these drugs. The aPTT was more sensitive compared to the PT test. Apixaban consistently produced milder anticoagulant coagulant effects compared to the other agents in all assays. In the PiCT test, edoxaban produced the strongest anticoagulant effects, whereas betrixaban and rivaroxaban produced comparable anticoagulant effects. In the aPTT assay, edoxaban and betrixaban were almost comparable, whereas rivaroxaban was much weaker than these two agents. In the PT assay, edoxaban produced the strongest effect followed by betrixaban, whereas rivaroxaban showed much weaker effects.

The same assays were performed on the plasma retrieved from the whole blood samples. In the retrieved plasma, PiCT test once again was found to be much more sensitive than aPTT and PT. Apixaban consistently produced relatively weaker anticoagulant effects in the retrieved plasma systems. Consistent with the results of the whole assay, edoxaban produced relatively stronger anticoagulant effects in the PiCT assay in the retrieved plasma. Although betrixaban and rivaroxaban showed comparable anticoagulant effects in the whole blood PiCT assay, in the retrieved plasma, rivaroxaban produced stronger effects than betrixaban. In the aPTT assay, edoxaban and betrixaban produced similar effects to those observed in the whole blood. Rivaroxaban produced milder anticoagulant effects in comparison to betrixaban and edoxaban. In the PT test, in the retrieved
plasma, edoxaban produced the strongest anticoagulant effects while betrixaban and rivaroxaban were comparable, unlike the whole blood results where these two drugs produced differential effects. Interestingly, the anticoagulant effects of these drugs were found to be stronger in the PiCT test as measured in the plasma in comparison to the retrieved plasma; however, in the aPTT and the PT tests, the anticoagulant profiles were stronger in the whole blood in comparison to the retrieved plasma.

These studies underscore the effect of cellular components on the anticoagulant responses as measured by different assays. Moreover, these observations also underscore the multiple mechanisms that may be involved in mediating the anticoagulant responses of the anti-Xa agents. On the basis of these collective results as shown in Table 4, once again the anti-Xa potency is found not to be relevant to the in vitro anticoagulant effects as measured in the whole blood and plasma systems.

The relative anticoagulant activities of apixaban, betrixaban, edoxaban, and rivaroxaban were also studied by directly supplementing these drugs to citrated normal plasma in order to compare their anticoagulant effects. These studies were carried out in the concentration range of 0 to 1 μg/mL using PiCT, aPTT, Heptest, and PT tests. These assays showed varying sensitivities to the anticoagulant effects of these drugs. The

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**Table 3. Comparison of the Direct Anti-Xa Agents: Apixaban, Betrixaban, Edoxaban, and Rivaroxaban on Thrombin Generation.**

| Drug      | Apixaban | Betrixaban | Edoxaban | Rivaroxaban |
|-----------|----------|------------|----------|-------------|
| Peak thrombin |          |            |          |             |
| 0         | 104.6 (19.3) | 104.6 (19.3) | 104.6 (19.3) | 104.6 (19.3) |
| 0.062     | 45.2 (16.4)   | 49.5 (14.4)   | 49.1 (19.4)   | 60.7 (24.1)   |
| 0.125     | 33.9 (12.8)   | 36.4 (11.5)   | 35.9 (14.9)   | 47.9 (17.7)   |
| 0.25      | 23.5 (8.7)    | 25.5 (8.6)    | 25.4 (12.2)   | 32.8 (14.8)   |
| 0.5       | 14.3 (5.0)    | 17.3 (6.1)    | 17.3 (9.1)    | 23.0 (9.2)    |
| 1.0       | 9.5 (4.4)     | 10.3 (4.3)    | 11.1 (6.2)    | 16.0 (6.7)    |
| Area under the curve (AUC) |          |            |          |             |
| 0         | 658.6 (76.4)  | 658.6 (76.4)  | 658.6 (76.4)  | 658.6 (76.4)  |
| 0.062     | 519.9 (72.4)  | 542.0 (72.4)  | 519.5 (85.6)  | 531.5 (71.7)  |
| 0.125     | 499.9 (81.1)  | 503.3 (73.6)  | 477.8 (109.5) | 519.4 (56.2)  |
| 0.25      | 454.0 (76.9)  | 435.5 (72.1)  | 407.3 (112.0) | 475.9 (116.8) |
| 0.5       | 276.0 (150.6) | 377.0 (63.2)  | 342.2 (113.9) | 430.9 (81.3)  |
| 1.0       | 98.1 (126.9)  | 211.1 (120.4) | 242.4 (155.5) | 292.1 (162.2) |
| Lag time  |          |            |          |             |
| 0         | 3.0      | 3.0 (0.4)   | 3.0 (0.4)   | 3.0 (0.4)    |
| 0.062     | 4.60 (0.8) | 5.3        | 5.3 (0.9)   | 4.0          |
| 0.125     | 5.0 (0.7)  | 6.1 (0.9)   | 6.3 (1.1)   | 4.6          |
| 0.25      | 6.0       | 7.3 (1.0)   | 7.6 (1.3)   | 5.3 (1.2)    |
| 0.5       | 7.3       | 9.4 (1.1)   | 9.7 (1.8)   | 6.6 (1.8)    |
| 1.0       | 9.6 (1.3)  | 12.5 (1.1)  | 13.6        | 8.1 (2.1)    |

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**Figure 8.** A comparison of the direct anti-Xa agents: apixaban, betrixaban, edoxaban, and rivaroxaban in thrombin generation. (A) Peak thrombin, (B) area under the curve, and (C) lag time.

**Figure 9.** A comparison of the direct anti-Xa agents: apixaban, betrixaban, edoxaban, and rivaroxaban on thrombin-induced clot formation.
PiCT test was found to be the most sensitive test, while aPTT, Heptest, and PT showed relatively weaker responses. In the PiCT assay, edoxaban produced stronger anticoagulant effects than rivaroxaban, edoxaban, and betrixaban. At 1 μg/mL, all inhibitors showed >100 seconds clotting time. In the aPTT assay, apixaban produced marginal anticoagulant effects. The other three agents produced relatively weaker anticoagulant effects in comparison to PiCT test. The anticoagulant effects ranked betrixaban followed by edoxaban, rivaroxaban, and apixaban. In the PT assay, all Xa inhibitors produced relatively weaker anticoagulant effects, with apixaban showing the weakest effect on PT. At concentrations up to 0.5 μg/mL, betrixaban, edoxaban, and rivaroxaban produced comparable anticoagulation; however, at 1 μg/mL, rivaroxaban produced relatively stronger anticoagulation followed by edoxaban and betrixaban. In the Heptest assay, betrixaban did not produce any anticoagulant effect; however, the other three agents showed a concentration-dependent effect. Interestingly, in this assay, apixaban was relatively stronger in producing anticoagulant effects.

The observed difference in the anticoagulant profile in the PiCT, aPTT, PT, and Heptest assays may be due to the differences in the activation mechanisms triggering the clot formation in these assays. The PiCT test seems to be the most sensitive in comparison to all of the other methods and may be useful in the monitoring of global anticoagulant effects of these drugs. The results obtained with Heptest assay are somewhat paradoxical, in particular, the lack of response with betrixaban. Moreover, these agents cannot be differentiated based on the anticoagulant activity measured in this assay. Once again, results of these supplementation studies underline the nonrelevance of the anticoagulant activities of these agents with the anti-Xa effects.

Standardized coagulation and amidolytic assays are used to assess the potency of the anti-Xa effects of these agents.15-18,6 In the anti-Xa studies carried out, the IC50 of the currently available anti-Xa agents varied widely. Betrixaban was found to have a relatively higher value for the IC50 at 1300 ng/mL (2.88 nM) and rivaroxaban exhibited an IC50 value of 670 ng (1.5 nM), whereas apixaban exhibited a value of 490 ng (1.06 nM) and edoxaban was relatively stronger than the other 3 drugs exhibiting an IC50 of 430 ng (0.78 nM). Therefore, a large variation in the in vitro potency for inhibiting factor Xa is evident. The current dosages for edoxaban are not proportional to the observed IC50 for the anti-Xa effects. Apixaban is used at relatively lower dosages in comparison to other drugs, which are considerably higher for various indications. Thus, the in vitro potency and the clinically effective dosing for these drugs are not proportional.

The comparative thrombin generation inhibitory profile of these agents was investigated in normal plasma samples in a concentration range of 0 to 1 μg/mL, which represents the therapeutic range of these agents. The thrombin generation profile was measured in terms of peak thrombin, AUC, and the lag time. In terms of peak thrombin, all agents produced comparable inhibitory effects with the exception of rivaroxaban, which showed somewhat weaker inhibition of thrombin at equivalent concentrations. Although IC50 values for apixaban, betrixaban, and edoxaban were in the range of 50 to 60 ng/mL, rivaroxaban showed a relatively higher value of 100 ng/mL. On a molar basis, this is approximately twice as high as for the other agents. In terms of AUC, all agents produced concentration-dependent decreases in the AUC, with apixaban exhibiting the strongest effect, and minor differences in AUC values noted with other agents. In terms of lag time, all agents produced a concentration-dependent increase. Apixaban and rivaroxaban produced relatively milder prolongation of the lag time, whereas edoxaban and betrixaban produced more pronounced effects on lag time. A previous report has compared the effects of apixaban and rivaroxaban on the thrombin generation inhibition assay.19 These thrombin generation parameters were product specific and did not relate to their anti-Xa activities.

The mechanistic basis for the differential effects of rivaroxaban and apixaban on global anticoagulation tests has been reported recently.20 A complex mathematical analysis on the associated rate constant and projected pharmacodynamics of apixaban and rivaroxaban provides theoretical rationale without any relevance to biologic outcomes.21 These complex studies may not have any bearing on the observed anticoagulant effects.
effects of the anti-Xa agents. A more plausible way is to develop logistic regression models incorporating PT, aPTT, and the PiCT test along with the anti-Xa and thrombin generation assays. The cumulative result then can be compared with the measured factor Xa inhibitory activities of these drugs.

The fibrinokinetic study represents an approach where thrombin-induced fibrin formation is measured in citrated plasma by monitoring clot density over time. All of these agents were found to produce an inhibition of fibrin formation at a concentration of 1 μg/mL in this system. While rivaroxaban produced the strongest inhibition of fibrin formation, edoxaban and apixaban produced moderate effects for the formation of fibrin and betrixaban only produced a mild effect. These studies show that the anti-Xa agents are capable of inhibiting fibrin formation in a varied manner. Although thrombin generation inhibition is a key factor in this process, interestingly the thrombin generation inhibitory potency doesn’t correlate with their fibrinokinetic inhibitory effects. For example, although rivaroxaban is the strongest inhibitor in this assay, its potency in the thrombin generation inhibition assay is weaker than that of other agents. In regard to the relevance of fibrinokinetic inhibition by these agents with anti-Xa activity once again, a direct correlation is not observed; therefore, additional factors may contribute to the fibrinokinetics inhibitory potential of these agents.

Evidently, the factor Xa inhibitory profile as measured utilizing the standardized amidolytic assay to designate the potency of these drugs doesn’t reflect their biologic effects in the in vitro studies carried out in whole blood, plasma, and defined biochemical systems. Each agent has a distinct clotting and biochemical profile that cannot be predicted on the basis of their anti-Xa potency. The studies reported on the anti-Xa profiling were carried out in a bovine factor Xa system, which is commonly used to characterize their anti-Xa spectrum. It is likely that similar outcomes will be observed if human factor Xa is used. Based on these findings, each of the factor Xa agents should be considered as a distinct drug whose biochemical and pharmacological actions are independent of its measured anti-Xa activity. Furthermore, beside the reported activities, these drugs may produce their biologic effects through other mechanisms that are not completely understood at this time. This includes their effects on blood cells, endothelial cells, and platelets. Finally, it must be underscored that the pharmacodynamic profile of these drugs may also be dependent on the differential nature of the pharmcophores in these molecules. Despite their simple structure, these drugs may exhibit complex interactions with enzymes and other binding sites to exert their effects.

An antidote, andexanet alfa representing a decoy protein, as recently, becomes available to neutralize the anti-Xa effects of apixaban and rivaroxaban. This antidote is likely to neutralize other anti-Xa drugs, such as betrixaban and edoxaban. However, it remains unclear that solely neutralizing the anti-Xa activities will antagonize many of the other pharmacodynamic effects of the anti-Xa agents. The recently observed thrombogenicity and other adverse events along with the high mortality rate associated with the use of andexanet may be due to residual actions of anti-Xa agents and other undetermined effects of andexanet.

In summary, the reported studies in this manuscript clearly suggest that despite their central action targeting factor Xa inhibition, the oral anti-Xa drugs exhibit markedly different anticoagulant properties as measured by different assay systems. Since these assays are designed to measured distinct target sites in the coagulation cascade, the cumulative biologic effects of each of these agents may not be directly related to their observed anti-Xa potencies. Therefore, the relevance of their clinical spectrum and potential neutralization by specific antidotes such as andexanet alfa may not be proportional to their anti-Xa effects. These observations warrant further investigations in both the laboratory and clinical setting to generate data on each of the individual anti-Xa agents for developing paradigms for their optimal clinical use.

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