

# Comparison of Anti-Xa and Dilute Russell Viper Venom Time Assays in Quantifying Drug Levels in Patients on Therapeutic Doses of Rivaroxaban

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• **Context.**—Rivaroxaban is a new oral anticoagulant that functions as a direct anti-Xa inhibitor. Although routine monitoring is not required, measurement of plasma concentrations may be necessary in certain clinical situations. Routine coagulation assays, such as the prothrombin time and, to a lesser degree, activated partial thromboplastin time, correlate with drug concentration, but because of reagent variability, these methods are not reliable for determining rivaroxaban anticoagulation.

**Objective.**—To compare different methods and calibrators for measuring rivaroxaban, including the chromogenic anti-Xa assay, which, when calibrated with a rivaroxaban standard, may be more appropriate for determining anticoagulation.

**Design.**—We compared measured rivaroxaban concentrations with the same anti-Xa kit but used different calibrators, with different anti-Xa kits but the same calibrators, with antithrombin-supplemented anti-Xa kit versus nonsupplemented kits, and with 2 methods based on

rivaroxaban-calibrated, high-phospholipid, dilute Russell viper venom time. Regression and paired *t* test statistics were used to determine correlation and significant differences among methods and calibrator sources.

**Results.**—Although there was strong correlation, statistically significant biases existed among methods that report rivaroxaban levels. A single-source calibrator did not alleviate those differences among methods. High-phospholipid Russell viper venom reagents correlated with rivaroxaban concentration but were not better than chromogenic anti-Xa methods.

**Conclusions.**—Rivaroxaban-calibrated, anti-Xa measurements correlate well, but the clinical significance of the variation with rivaroxaban measurements is uncertain. The antithrombin-supplemented, anti-Xa method should be avoided for measuring rivaroxaban.

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Rivaroxaban (Xarelto, Janssen Pharmaceuticals, Titusville, New Jersey) is a novel, oral anticoagulant that directly inhibits activated factor X (FXa). The drug has been used for stroke prevention in nonvalvular atrial fibrillation,<sup>1</sup> as treatment for pulmonary embolism<sup>2</sup> and symptomatic deep vein thrombosis,<sup>3</sup> for anticoagulation in recent ST-segment elevation myocardial infarction,<sup>4</sup> as thromboprophylaxis in

patients after knee or hip replacement,<sup>5</sup> and in medically ill patients.<sup>6</sup> Rivaroxaban has predictable pharmacokinetics, is not affected by diet, is given at fixed doses, and does not require routine coagulation monitoring. In emergent situations, for example, in cases of bleeding, an assessment of the anticoagulation effect of rivaroxaban may be desirable. Rivaroxaban has been shown to prolong the prothrombin time,<sup>7,8</sup> activated partial thromboplastin time,<sup>9</sup> and dilute Russell viper venom time (DRVVT)<sup>10</sup> in a concentration-dependent manner, but measuring drug activity by chromogenic anti-Xa assay may be preferred.<sup>11,12</sup> Most published studies that have provided an estimation of the effect of rivaroxaban on coagulation testing have used pooled normal plasma spiked with various concentrations of the drug, and that may not reflect real-world patient testing.<sup>7,8,10–12</sup> In this study, we sought to determine and compare various anti-Xa methods, as well as DRVVT-based methods, for quantifying rivaroxaban levels in patients receiving rivaroxaban as part of their routine care. To that end, we compared measured rivaroxaban concentrations with the same anti-Xa kit but employing different calibrators, with different anti-Xa kits but the same calibrators, with antithrombin (AT)-supplemented anti-Xa kit versus nonsupplemented kits, and with 2 DRVVT-based methods.

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Comparisons of Different Methods and Calibrators for Measuring Rivaroxaban<sup>a,b</sup>

| Reference (Calibration) Methods | Coamatic (Biophen) | Coamatic (TECHNOVIEW) | Berichrom (Biophen) | Rotachrome (Biophen) | DiXal (Biophen) |
|---------------------------------|--------------------|-----------------------|---------------------|----------------------|-----------------|
| LC-MS/MS, n = 60                |                    |                       |                     |                      |                 |
| R                               | 0.988              | 0.948                 | 0.975               | 0.978                | 0.985           |
| m                               | 0.89               | 0.85                  | 0.78                | 0.86                 | 0.76            |
| P                               | <.001              | <.001                 | <.001               | <.001                | <.01            |
| Coamatic (Biophen), n = 60      |                    |                       |                     |                      |                 |
| R                               | ...                | 0.965                 | 0.978               | 0.988                | 0.994           |
| m                               |                    | 0.95                  | 0.88                | 0.97                 | 0.85            |
| P                               |                    | .05                   | <.001               | .04                  | .06             |
| Coamatic (TECHNOVIEW), n = 60   |                    |                       |                     |                      |                 |
| R                               | 0.965              | ...                   | 0.946               | 0.958                | 0.961           |
| m                               | 1.02               |                       | 0.91                | 1.05                 | 0.91            |
| P                               | .05                |                       | .40                 | .19                  | .16             |
| Berichrom (Biophen), n = 60     |                    |                       |                     |                      |                 |
| R                               | 0.978              | 0.946                 | ...                 | 0.972                | 0.973           |
| m                               | 1.11               | 1.04                  |                     | 1.07                 | 0.95            |
| P                               | <.001              | .40                   |                     | .03                  | .03             |
| Rotachrome (Biophen), n = 60    |                    |                       |                     |                      |                 |
| R                               | 0.988              | 0.958                 | 0.972               | ...                  | 0.994           |
| m                               | 1.02               | 0.92                  | 0.91                |                      | 0.88            |
| P                               | .04                | .19                   | .03                 |                      | .59             |
| DiXal (Biophen), n = 60         |                    |                       |                     |                      |                 |
| R                               | 0.994              | 0.961                 | 0.973               | 0.994                | ...             |
| m                               | 1.16               | 1.06                  | 1.03                | 1.13                 |                 |
| P                               | .06                | .16                   | .03                 | .59                  |                 |

Abbreviation: LC-MS/MS, tandem liquid chromatography-mass spectrophotometry.

<sup>a</sup> Analysis of correlation (R), slope (m), and P values by Student paired t test for methods measuring rivaroxaban levels using anti-Xa methods with respective calibration methods in parentheses. The vertical, stub column represents the reference method, whereas the horizontal, top row represents the evaluation method.

<sup>b</sup> Biophen, Aniaira (West Chester, Ohio); Coamatic, DiaPharma (West Chester, Ohio); Berichrom, Siemens Healthcare Diagnostics (Newark, Delaware); Rotachrome, Diagnostica Stago (Parsippany, New Jersey); Biophen DiXal, Aniaira (West Chester, Ohio).

## MATERIALS AND METHODS

After approval by the institutional review board on human subject testing, 30 patients taking either 15 mg (n = 1; 3%) or 20 mg (n = 29; 97%) of rivaroxaban daily for at least 4 days as part of their routine care consented to participate in this study. Details of this study have been described elsewhere,<sup>13</sup> but briefly, blood was collected by standard phlebotomy techniques at trough (just before the next dose) and peak (2.5 hours after the dose) periods and anticoagulated with 3.2% sodium citrate. After collection, the citrated blood was processed to obtain platelet-poor plasma (<10 × 10<sup>3</sup> platelets/μL), aliquoted into cryovials, and frozen at -70°C. Samples were coded with study subject identification numbers and the time of blood draw (trough or peak) and distributed on dry ice to 4 external laboratories to perform rivaroxaban testing. Each laboratory performed rivaroxaban anti-Xa testing according to their laboratory protocol and provided information about reagent method, instrumentation, and calibration method. Two sites also evaluated a rivaroxaban-calibrated, high-phospholipid content DRVVT test as an alternate approach to quantifying rivaroxaban, as previously described.<sup>10</sup>

Laboratory A measured rivaroxaban concentration in plasma using 2 different chromogenic anti-Xa methods—the Coamatic heparin assay (DiaPharma, West Chester, Ohio) and the Berichrom heparin assay (Siemens Healthcare Diagnostics, Newark, Delaware)—as well as testing with a high-phospholipid content DRVVT (LA2 Confirmation Reagent, Siemens) on the BCS XP System coagulation analyzer (Siemens). Of note, the Berichrom method was the only anti-Xa method used that included AT as a reagent component. Laboratory B used the STA Rotachrom heparin kit (Diagnostica Stago, Parsippany, New Jersey) on the STA compact analyzer (Diagnostica Stago) and the high-phospholipid content DRVVT reagent (LA Sure, Precision BioLogic, Dartmouth, Nova Scotia, Canada) on the STA-R Evolution (Diagnostica Stago). Laboratory C

used a chromogenic anti-Xa method optimized for rivaroxaban, the Biophen DiXal kit (Aniaira, West Chester, Ohio) on the STA-R Evolution analyzer. Laboratory site D measured rivaroxaban levels using tandem high-performance liquid chromatography/mass spectrophotometry (LC-MS/MS) as previously described.<sup>14</sup>

All anti-Xa assays were performed using rivaroxaban calibrators. For each anti-Xa kit studied, a single source (Biophen) rivaroxaban calibrator was used, consisting of 3 levels with drug concentrations from 0 to approximately 500 ng/mL. Laboratory A also performed calibration on the anti-Xa Coamatic heparin assay and the high-phospholipid DRVVT (LA2 Confirmation Reagent) using TECHNOVIEW rivaroxaban calibrator sets (Technoclon, Vienna, Austria). TECHNOVIEW rivaroxaban calibrator sets (high and low) use a 5-point calibration ranging from 0 to 433 ng/mL.

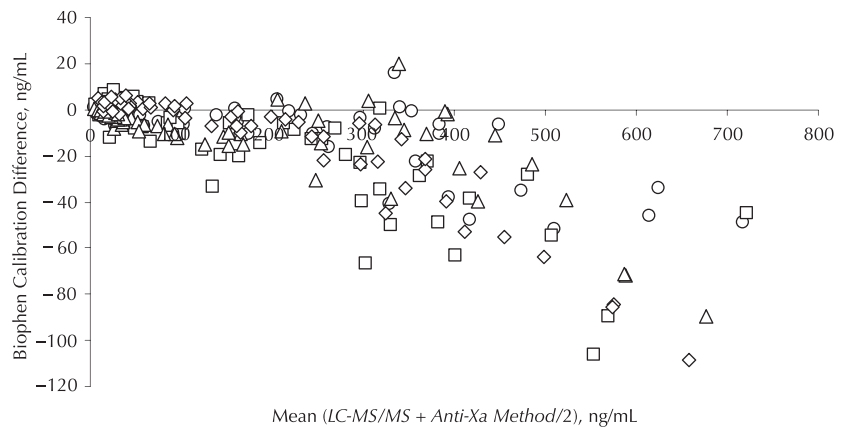
To determine whether the anti-Xa methods were correlated, all anti-Xa and DRVVT methods were compared with the LC-MS/MS method and among reagent systems, using regression analysis, with a desired correlation coefficient (R) of greater than 0.95. Furthermore, to determine whether differences existed among methods, each anti-Xa method was compared with LC-MS/MS and among reagent systems using the Student paired t test, with a P > .05 indicating no statistical difference among methods. Areas of bias were determined using Bland-Altman graphs demonstrating the differences between anti-Xa and LC-MS/MS rivaroxaban levels.

## RESULTS

### Anti-Xa Assays

In this data set, using LC-MS/MS trough rivaroxaban levels varied from 4 to 176 ng/mL (median, 34 ng/mL), with peak drug levels from 17 to 766 ng/mL (median, 320 ng/mL). All anti-Xa methods calibrated with the Biophen

**Figure 1.** Overlaid Bland-Altman bias plots demonstrating the differences noted among anti-Xa methods that were calibrated using a single-source calibrator (Biophen [Aniara]) and tandem liquid chromatography-mass spectrophotometry (LC-MS/MS). Circles, Coamatic (DiaPharma); squares, Berichrom (Siemens); triangles, Rotachrome (Diagnostica Stago); diamonds, DiXalt (Aniara).



calibrator were strongly correlated ( $R > 0.97$ ) with the LC-MS/MS method, but on a concentration basis, all were significantly different from the rivaroxaban levels measured by LC-MS/MS (Table). The Bland-Altman bias plots for all anti-Xa measurements demonstrated a negative bias, indicating that lower levels were detected by chromogenic methods, as compared with those obtained using LC-MS/MS (Figure 1). When calibrated with the TECHNOVIEW rivaroxaban calibrator set, the correlation between the Coamatic method and the LC-MS/MS method (Table) was lower than the predetermined acceptable limit ( $R = 0.948$ ).

In comparing the rivaroxaban levels measured with the same kit (Coamatic) but with different calibrators, there was strong correlation between results ( $R = 0.965$ ), with no significant differences ( $P = .05$ ) between Biophen and TECHNOVIEW rivaroxaban calibrators and reported drug levels.

In comparing rivaroxaban levels measured with the same calibrator (Biophen) but with different commercial anti-Xa kits (Coamatic, Berichrom, Rotachrome, and Biophen DiXalt), there was a strong correlation ( $R > 0.95$ ) among all methods. Rivaroxaban measurements obtained using the same Biophen calibrator demonstrated no significant differences between the Coamatic or Rotachrome anti-Xa methods and the DiXalt methods ( $P = .06$  and  $P = .59$ , respectively) (Table). TECHNOVIEW-calibrated rivaroxaban levels using the Coamatic method were not significantly different from all other Biophen-calibrated anti-Xa methods. There were significant ( $P = .04$ ) differences between Biophen-calibrated Coamatic and Biophen-calibrated rivaroxaban measurements. The least favorable correlation among different calibrators or methods occurred between Coamatic calibrated with TECHNOVIEW and Berichrom calibrated with Biophen ( $R = 0.946$ ), which did not meet the predetermined criteria for acceptability.

#### DRVVT Assays

The clotting time (in seconds) determined by the uncalibrated, high-phospholipid content DRVVT correlated well with the LC-MS/MS methods ( $R = 0.87$  and  $R = 0.80$ ) for Siemens LA2 and Precision Biologics DRVVT reagents, respectively (Figure 2, A). The correlation between high-phospholipid content DRVVT methods and rivaroxaban levels, as determined by LC-MS/MS, improved when rivaroxaban calibrators were used for the DRVVT methods. TECHNOVIEW and Biophen calibrators provided correlations of  $R = 0.85$  and  $R = 0.88$ , respectively (Figure 2, B). There was no significant difference between TECHNOVIEW-

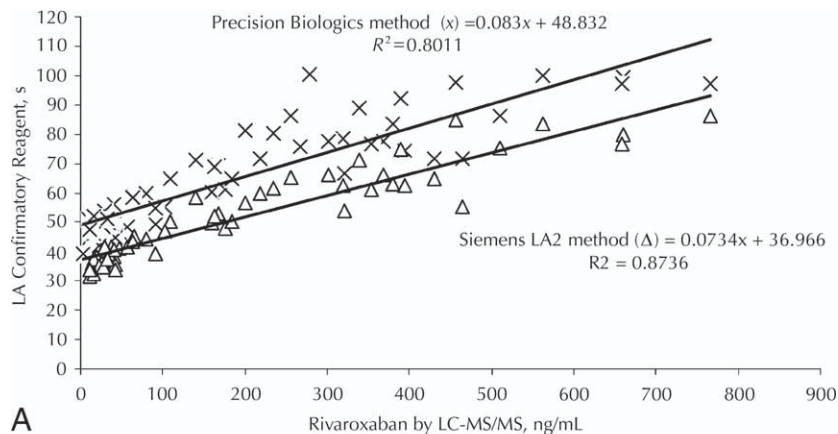
calibrated DRVVT rivaroxaban levels and LC-MS/MS results ( $P = .30$ ), but there were statistically significant differences and a negative bias with the Biophen-calibrated DRVVT method for determining rivaroxaban levels (Figure 2, C).

#### COMMENT

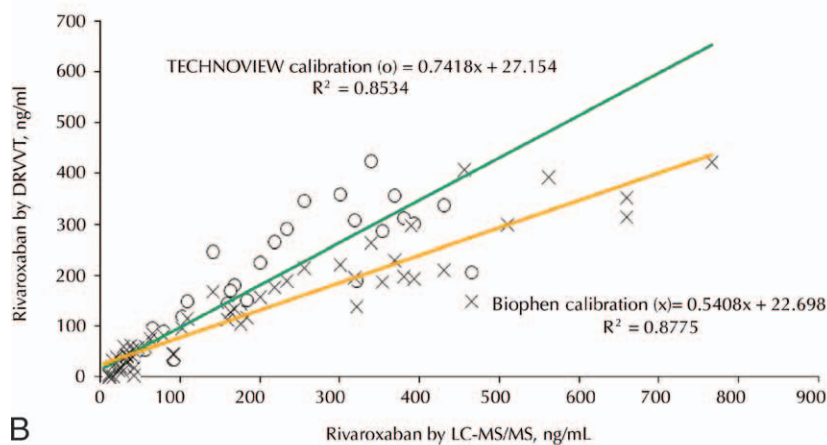
Although routine laboratory monitoring is not required when patients are administered rivaroxaban, there are several clinical situations where determination of the level of anticoagulation is of value. There have been recent reports evaluating the use of routine screening tests for assessing the relative anticoagulant effect of rivaroxaban,<sup>7-12</sup> but most of those studies used drug spiked into pooled normal plasma. Other studies that used samples from patients treated with rivaroxaban were somewhat limited by the testing platforms (reagents/instruments) evaluated or the studies did not compare results of clot-based assays to measures of drug concentration using high-performance liquid chromatography or LC-MS/MS.<sup>7,8,10,12</sup> Overall, those studies showed the use of routine activated partial thromboplastin time and prothrombin time reagents is limited by the variability in responsiveness to rivaroxaban among reagents and because those global assays cannot reliably provide drug concentration, especially throughout the expected broad range of concentrations observed when collected at peak or trough times.

#### Anti-Xa Assays

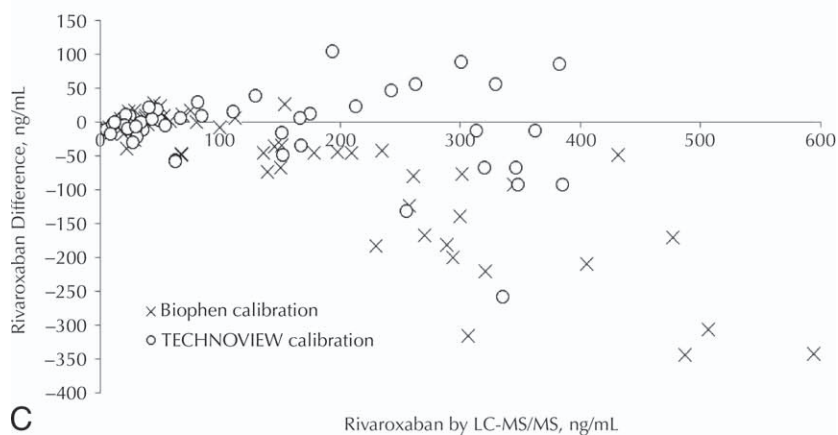
Currently, the chromogenic anti-Xa assay is the preferred assay for measuring plasma rivaroxaban concentrations in the clinical laboratory. In that assay, the reagent provides excess FXa, and the inhibition of FXa is inversely proportional to the concentration of Xa-inhibitor drug present. Studies have shown that, when calibrated against a rivaroxaban calibrator, rivaroxaban concentrations can be measured throughout a wide range using that method. Such studies have been performed using normal plasma samples spiked with drug as well as samples from patients treated with rivaroxaban. Similar to our study, Douxfils et al<sup>15</sup> found good correlation between the commercial chromogenic DiXalt kit and LC-MS/MS ( $R = 0.95$ ) using both commercial and internally prepared calibrators. In their study, it was unclear whether the patient samples represented trough or peak levels. Both Mani et al<sup>9</sup> and Douxfils et al<sup>7</sup> demonstrated poorer correlation with samples containing low levels of the drug (eg,  $<100$  ng/mL of drug,  $R = 0.83$ ). Given this, Mani and colleagues<sup>9</sup> suggest that calibration using a low and high curve may be necessary to measure



A



B



C

**Figure 2.** A, Correlation between Biophen (Aniara)-calibrated rivaroxaban levels and tandem liquid chromatography-mass spectrophotometry (LC-MS/MS) using 2 different high-phospholipid Russell viper venom confirmatory reagents: Precision Biologics method and Siemens LA2 method. Refer to text for additional methods and calibrator descriptions. B, Correlation between Biophen and TECHNOVIEW rivaroxaban calibration of Siemens LA2 high-phospholipid Russell viper venom reagent and predicated LC-MS/MS. C, Overlaid Bland-Altman bias plots demonstrating areas of bias for Biophen and TECHNOVIEW rivaroxaban calibration of Siemens LA2 high-phospholipid Russell viper venom reagent and true drug levels measured by LC-MS/MS. Abbreviation: LA, lupus anticoagulant.

rivaroxaban concentrations from less than 25 ng/mL to more than 300 ng/mL. In our study, we noted that low (<30 ng/ml) and high (>350 ng/ml) drug levels were more likely to have higher biases (>20%) when compared with rivaroxaban levels measured by LC-MS/MS. For samples with less than 30 ng/mL of drug (12 of 60; 20%), as measured by LC-MS/MS, the number of samples with less than 20% absolute difference ranged from 33% (Rotachrome) to 75% (DiXaI), whereas within the range of 30 ng/mL to 350 ng/mL (35 of 60; 58%), the number of samples with greater than 20% absolute difference from the LC-MS/MS measurement ranged from 1% (Coamatic with Biophen calibrator) to 37% (Berichrom and Rotachrome). For those samples that were greater than 350 ng/mL (13 of 60; 22%),

the number of samples with greater than 20% difference ranged from 15% (Coamatic with Biophen calibrator) to 46% (Berichrom and Biophen DiXaI).

In our study, we demonstrated that a single source calibrator (Biophen) used on different commercial anti-Xa kits provided good correlation among methods, albeit with significantly different measured concentrations. The variation evident among different manufacturers' anti-Xa kits, given a common calibrator, points to inherent differences in the methods. Such variation among manufacturer anti-Xa kits has been reported when measuring unfractionated heparin.<sup>16</sup> TECHNOVIEW rivaroxaban calibrators were significantly correlated with Biophen-calibrated rivaroxaban results, regardless of the kit used. Although there were statistically

significant differences noted in the measured concentrations between LC-MS/MS and different anti-Xa kits, it seems unlikely that those differences would be clinically significant given the drug's wide plasma concentration range when patients are administered therapeutic doses. The correlation data and paired *t*-test analysis obtained in this study may assist laboratories considering evaluating rivaroxaban-calibrated anti-Xa methods. Method validation requires accuracy assessment (comparing to a predicate laboratory reporting rivaroxaban levels), and knowledge of the biases among methods may be useful in the analysis of comparison data.

Previous reports have indicated that anti-Xa methods that incorporate AT as a kit component (commonly referred to as *2-step methods*) have a higher coefficient of variation<sup>17</sup> and are also reported to have measurable rivaroxaban level in their normal controls,<sup>18</sup> suggesting that chromogenic anti-Xa kits with added AT should not be employed for measuring rivaroxaban. Our study also found the greatest increased method bias with samples containing less than 100 ng/mL of rivaroxaban using the AT-supplemented anti-Xa method (Berichrom). Isolating the data from the samples containing less than 100 ng/mL of drug, the regression was not as favorable for the AT-supplemented method ( $R = 0.83$ ) as it was for the other chromogenic anti-Xa methods, regardless of calibration source (all  $R > 0.97$ ).

### DRVVT Assays

In our study, we also sought to determine the performance of 2 commercial high-concentration phospholipid DRVVT reagents as a means of measuring rivaroxaban's anti-Xa effect, as suggested by Exner and colleagues.<sup>10</sup> In addition, the effect of using different commercial calibration materials on that method was evaluated. In our assessment, although there was a linear correlation with DRVVT and increasing drug concentration, the poor slopes evident for each reagent type indicated inadequate sensitivity to increasing drug concentrations. The DRVVT assays performed with calibration from 2 different commercial sources and using extrapolation of the patient drug concentration from that calibration curve did not perform as well as the anti-Xa methods for assessing rivaroxaban levels, with  $R = 0.85$  and  $R = 0.88$  for TECHNOVIEW- and Biophen-calibrated results, respectively. The TECHNOVIEW calibration method with a combination of low- and high-curve calibrator sets achieved a better measurement range than a single calibrator set alone. However, neither the low or the high calibrator set (or both in combination) nor the Biophen calibrator set improved the overall correlation of quantifying rivaroxaban using DRVVT methods, especially at lower (<100 ng/mL) and upper (>300ng/mL) rivaroxaban levels, as determined by LC-MS/MS.

There are some potential limitations to our study. First, we did not evaluate the effect of different lots of calibrator material, which may introduce an additional bias. Additionally, baseline (pretreatment) prothrombin time and activated partial thromboplastin time results were not available to the investigators, so it is unclear whether those patients tested had lupus anticoagulant or other abnormalities that may have affected clot-based testing, such as the DRVVT. As such, it is possible that some patients may have had abnormal coagulation test results, which may have contributed to the relatively poorer correlation seen with DRVVT confirmation reagent testing, as compared with chromogenic anti-Xa methods. Lastly, it is unclear what affect samples with hemolysis, lipemia, or icterus had on the accuracy of chromogenic-based anti-Xa testing. Samples

with those pre-examination conditions would require careful result interpretation.

In conclusion, we demonstrated that statistically significant biases exist among methods that report rivaroxaban levels and that those biases are associated with kit method differences because a single-source calibrator did not alleviate result differences. Whether this variability will be of clinical importance needs to be investigated. The AT-supplemented anti-Xa method was the least favorable anti-Xa method used, and use of AT-supplemented kits should be avoided for measuring rivaroxaban. High-phospholipid Russell viper venom reagents were correlated with rivaroxaban concentration but did not correlate better than chromogenic anti-Xa methods. Lastly, given the wide range of peak levels in patients taking therapeutic doses of rivaroxaban, it may be preferable to measure trough levels when assessing a patient taking this drug.

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