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Unveiling the complex effects of direct oral anticoagulants on dilute Russell's viper venom time assays

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Running title

Effects of DOACs on integrated dRVVT assays.

Essentials

- There is a great variability of the DOAC effects on dRVVT assays
- There are also large between-assay differences in sensitivities towards the different DOACs
- The dRVVT ratio can be false high or low depending on the effects on the screen and confirm tests
- The phospholipid composition may explain the observed differences

Keywords

dilute Russell's viper venom time, direct thrombin inhibitor, direct Xa inhibitor, lupus anticoagulant, phosphatidylserine

Summary

Introduction: Dilute Russell's viper venom time (dRVVT) assays can be affected by direct oral anticoagulants (DOACs), which may cause false-positive results. However, there are conflicting results indicating significant differences between different reagents and DOACs.

Objectives: To evaluate the effect of DOACs on dRVVT assays.

Material and Methods: Samples were prepared by adding DOAC (dabigatran, rivaroxaban, apixaban or edoxaban) to pooled normal plasma in the concentration range 0 – 800 µg/L. Six integrated dRVVT reagents were used, all composed of a screen assay (low phospholipid content) and a confirm assay (high phospholipid content). The screen/confirm dRVVT results were expressed as normalized ratios. To further evaluate the observed differences between tests and DOACs, addition of synthetic phospholipids was used.

Results: The dRVVT ratios increased dose-dependently for all DOACs, with four of the six tests and the DOAC rivaroxaban having the greatest effect. With one test the ratios were almost unaffected with increasing DOAC concentration while another test revealed a negative dose-dependency for all DOACs. Variable DOAC effects can be explained by different effects on dRVVT screen and confirm clotting time. Adding synthetic phospholipids to samples containing rivaroxaban resulted in greatly reduced screen clotting times and thereby lower calculated dRVVT ratios.

Conclusions: There is a great variability in the dRVVT test result with different DOACs. The dRVVT ratios are unaffected for some reagents and this can be explained by an equal dose-dependent effect on both screen and confirm assays. The phospholipid type and content of the different reagents may contribute to the observed differences.

Introduction

The direct oral anticoagulants (DOAC) represent a new class of drugs that are increasingly replacing vitamin K antagonists in indications such as the prevention of stroke in patients with atrial fibrillation and the treatment and secondary prevention of venous thromboembolism (*1-4).* During the last decade four different DOACs have been introduced: the thrombin inhibitor dabigatran and three factor Xa inhibitors named rivaroxaban, apixaban and edoxaban. It is well known that the DOACs may interfere with common coagulation assays (5-8) making it difficult to evaluate patient coagulation status during anticoagulant therapy. This interference also includes laboratory testing for lupus anticoagulant (LA) antibodies, a specific class of antiphospholipid antibodies that are prothrombotic in nature. LA testing is part of a laboratory panel for thrombophilia investigations as patients with LA have an increased risk for both arterial and venous thrombosis (*9-11*).

The most commonly used LA test is the dilute Russell's viper venom time (dRVVT). The reagent contains a factor X activating enzyme, the resultant FXa then forms the phospholipid-dependent prothrombinase assembly to generate thrombin, and the clotting time is registered (12). As antibodies of the LA-type are dependent on phospholipids it is possible to probe this activity by varying the phospholipid content; with low phospholipid content LA prolongs the dRVVT more compared to a reagent with higher phospholipid content. Thus, the LA test is often performed as an integrated test based on two steps: one dRVVT screen test with low phospholipid content that is sensitive for LA and one dRVVT confirmation test with high phospholipid content that is not. In clinical practice, it is recommended that the confirmation step is done only if the screen test is prolonged. Often is also a mixing step included after a prolonged screen test in the test algorithm, where the patient sample is mixed with equal volume of normal plasma, in order to increase the specificity for LA. If the sample contains true LA antibodies the screen test clotting time would be prolonged and much longer than the confirmation test clotting time. The result is often expressed as a normalized dRVVT screen/confirm ratio (or LA ratio).

The ratio is close to 1 if there are no LA antibodies and increases with LA antibodies present.

As the dRVVT test is dependent on both factor Xa and thrombin the test may be influenced by all DOACs in a dose-dependent manner. Indeed, there are now several interesting reports on DOAC interferences on different LA tests that warrant a cautious interpretation of the results when DOACs are present (*13-34*). The reports mainly emphasize the risk of false-positive LA test results during DOAC therapy (although the effects vary with the type of DOAC as well as the type of dRVVT reagent), indicating that a local validation of the test system might be necessary to avoid diagnostic problems. There are also reports of opposite effects with apixaban which may cause false-negative dRVVT results (*22*, 34*).* There is evidence in the literature that rivaroxaban has the greatest effect while apixaban has the least effect (or even the opposite effect) on the dRVVT ratio, explained by different effects on the underlying dRVVT screen and confirm tests. However, the causes of these differences have not been elucidated. In this study, we have in a more systematic way tried to explore the variable effects by a direct head-to-head comparison of all four DOACs with six different integrated dRVVT tests.

Material and methods

Material

Dabigatran, rivaroxaban, and edoxaban were purchased from Selleckchem (Munich, Germany). Apixaban was from Adooq Bioscience (Irvine, CA, USA). Dimethyl sulfoxide (DMSO) was from Merck (Darmstadt, Germany. A stabilized phospholipid emulsion, with defined content, was a kind gift from Dr. Steffen Rosén, Rossix (Mölndal, Sweden). The emulsion, denoted TGT, contained 0.5 mmol/L phospholipids based on a mixture of 28 mol% phosphatidylserine, 30 mol% sphingomyeline and 42 mol% phosphatidylcholine. Platelet poor (<10 x 10^9/L) pooled normal plasma (PNP) was obtained from our local blood bank by mixing citrated plasma from 20 different healthy blood donors. The plasma was aliquoted and stored in -80 ºC freezer until use. A LA positive control plasma was obtained from Precision BioLogic (Dartmouth, Canada).

Preparation of plasma samples

Stock preparations of 0.1 mg/mL of each DOAC were made by dissolving the drug in 100 % DMSO. The concentrations of the stock preparations were calculated using the molecular weight of the drugs and the use of a Sartorius QT6100 analytical scale (Goettingen, Germany) to precisely determine the amount of drug dissolved in each solution. The stock solutions were further diluted 1:125 with PNP to obtain a concentration of 800 µg/L of each DOAC and then further diluted with PNP to obtain 7 concentrations between 0 – 800 µg/L. The samples were stored at -80˚C until they were transported on dry ice to the participating laboratories for analysis.

Dilute Russell's viper venom time (dRVVT) assays

Six different integrated dRVVT reagents were used: (LA1/LA2 from Siemens Healthcare Diagnostics (Deerfield, IL, US); LA Screen/Confirm from Technoclone (Vienna, Austria); HemosIL dRVVT Screen/Confirm from Instrumentation Laboratory SpA (Milano, Italy); StaClot DRVV Screen/Confirm from Stago (Asnières sur Seine, France); dRVV

Screen/Confirm from Sekisui Diagnostics (Stamford, CT, USA); Hemoclot LA-S and LA-C from Hyphen BioMed (Neuville-sur-Oise, France). The assay from Siemens was run on a Siemens instrument model BCS-XP, the assays from Instrumentation Laboratory, Sekisui and Hyphen were all run on the ACL Top instrument from Instrumentation Laboratory and the assays from Technoclone and Stago were run on a STAR MAX instrument from Stago. All tests were run according to the manufacturer's recommendations regarding pipetting volumes and incubation times. The screen/confirm ratio results were expressed as normalized ratios based on PNP and a value >1.2 was considered being positive as recommended by the manufacturers. DOACs are commonly reported to elevate screening test results more than confirmatory tests results, meaning that not only can screening test b false positive but also the interpretation for the presence on an LA can be false positive. In this study we disregarded from current testing algorithms as we would like to systematically investigate the DOAC effects on the different reagents with all samples. Thus, the dRVVT screen and dRVVT confirmation tests were performed irrespective if the dRVVT screen test was prolonged or not.

Tests with additional phospholipids

In order to explore the nature of the variable effect on the dRVVT screen and confirmation tests samples with DOAC were analyzed after addition of increasing amounts of phospholipids. The DOACs with the most pronounced and the least pronounced effects were chosen, rivaroxaban and apixaban, respectively. Both DOACs were tested at a fixed concentration of 400 µg/L in PNP. To these samples increasing amounts of TGT were added to obtain phospholipid concentrations between 0 – 100 µmol/L. The samples were analyzed with five of the six dRVVT reagents on the ACL Top instrument. The StaClot DRVV Screen/Confirm from Stago could not be included in this exercise due to unavailability through our local supplier during the time of experiments.

Statistics

All samples were run in duplicate and results are presented as mean. Graphs were constructed using the Sigma Plot 10.0 (Systat Software Inc., San Jose, CA, USA).

Normalized ratios (NR) for dRVVT screen/confirm coagulation times were calculated as follows:

NR = (clotting time patient screen/clotting time PNP screen)/ (clotting time patient confirm/clotting time PNP confirm)

Results

Integrated dRVVT reagents were variably and dose-dependently affected by plasma samples spiked with DOACs. Rivaroxaban had the greatest effect in four of the six investigated dRVVT reagents (the assays from Stago, Siemens, Technoclone and IL) and the normalized ratios were invariably positive (>1.2) at concentrations above 50 µg/L rivaroxaban (Figure 1). One reagent (Sekisui assay) displayed negative dose dependency with all four DOACs whereas the reagent from Hyphen showed a weakly dose dependent increase of the dRVVT ratio with dabigatran but all the Xa-inhibitors gave unaffected or slightly negative ratios with increasing amount of anticoagulant drugs in the samples.

The variable effects, as well as common features, of the six different dRVVT reagents are further illustrated in figure 2, where all test results are compared for each DOAC and reagent. The reagents from Technoclone and Siemens were the most DOAC sensitive assays, followed by the reagent from Stago, although the inter-DOAC effects vary to a great extent. The reagents from IL and Hyphen showed mixed results with both positive and negative effects on the dRVVT ratios depending on the type of DOAC. The reagent from Sekisui was negatively affected by all four DOACs and resulted in dose dependent reduction of the normalized dRVVT ratios.

As the dRVVT ratios are calculated from the underlying dRVVT screen and confirmation times it follows that the screen test is more affected compared to the confirmation test, and vice versa, when the spiked DOAC samples display a positive or negative dose dependency, respectively (not shown). Thus, when the DRVVT ratio is influenced by the DOACs it is likely that the effects are in some way dependent on the phospholipid content

in the screen and confirmation tests. This hypothesis was tested by spiking phospholipid to the samples containing 400 µg/L rivaroxaban or apixaban prior to the testing, which resulted in a phospholipid concentration-dependent reduction of the ratios for the dRVVT reagents that were most DOAC sensitive (Fig. 3). This was explained by a selective effect on the underlying dRVVT screen test whereas the dRVVT confirm test was almost left unaffected by the addition of extra phospholipids. The three investigated assays that resulted in false positive ratios due to addition of 400 µg/L rivaroxaban (Technoclone, Siemens and HemosIL) displayed a greater effect of the phospholipid on the dRVVT screen test compared to the dRVVT confirmation test. With two assays (Hemoclot and Sekisui) the underlying screen and confirmation tests were less affected by the addition of phospholipid in the samples and the normalized ratios were similarly unaffected by the phospholipid. Similar patterns were obtained with the samples containing 400 µg/L apixaban, although the effects were less accentuated (not shown).

Discussion

The potential problems with testing for LA in patients on DOAC therapy have been illustrated in many reports (13-34). The main issue is that DOACs may cause false positive LA results, but the effects seem to be dependent on both the type of DOAC and the LA reagent making it difficult to compare results from published studies. In this investigation, we have tried to systematically evaluate the effects of four DOACs on six different commercially available integrated dRVVT tests. Our results clearly illustrate the heterogeneity in the DOAC effects. The differences between the DOACs, when each assay is looked at independently, are illustrated in figure 1. All four DOACs display a dose dependent increase of the dRVVT NR in three of the six assays. These results are in accordance with previous reports on the risk of false positive results under certain conditions. With one of the reagents (HemosIL), a dose-dependent increase of the NR was only shown for rivaroxaban (up to a concentration of 400 µg/L) but not for the other DOACs. For the remaining two assays (Hyphen and Sekisui) the effects were less pronounced or opposite. Thus, we can confirm that the Hemoclot assay that was recently developed by Hyphen as a dRVVT reagent with improved specificity (35) indeed is less sensitive to interferences by rivaroxaban as well as the other DOACs. When we display the results of each individual DOAC, as shown in figure 2, it becomes clear that the reagents are not the same. All four DOACs display positive or negative dose dependent curves (higher and lower NR) depending on the type of reagent. The ratios are explained by the effects on the underlying dRVVT screen and dRVVT confirmation clotting times. The most extreme DOAC-effect in this sense is caused by rivaroxaban with a much greater effect on the dRVVT screen test compared to the dRVVT confirmation test in four of the six reagents. The DOAC with the least positive effect on the dRVVT results, apixaban, showed a similar pattern although less pronounced. The most interesting result is perhaps that all DOACs displayed a similar negative dose response effect with one of the dRVVT reagents (Sekisui) that is explained by greater effects on the dRVVT confirmation test instead of the dRVVT screen test. This phenomenon could also be seen for the FXa-inhibitors apixaban and edoxaban with the Hemoclot dRVVT reagent whereas the NR for rivaroxaban was almost unaffected by increasing concentrations. On

the other hand, the FIIa- inhibitor dabigatran displayed a weak, but positive, dose dependent effect also with the Hemoclot assay.

When all results from this direct comparison are taken together it's clear that the effects of DOACs are not the same. With several assays the effects can be ranked with rivaroxaban having the greatest influence on the NR and the other DOACs to a lesser extent. However, there are also surprisingly large differences between different reagents as illustrated in figure 2, a phenomenon without an obvious explanation. The dRVVT test is a rather straight-forward assay principle, where a defined plasma volume is mixed with Russell's viper venom and phospholipids, thus it can be anticipated that the variability among commercially available tests should be low. However, the different reagents are not equivalent concerning their sensitivities towards LA as well as interfering substances, such as DOACs, shown in this investigation. This is probably due to variation in the reagent composition; source and amount of venom and/or phospholipids (12, 35, 36). As the content and composition of lipids in the reagents is not declared by the manufacturers' we studied the effects of adding defined phospholipid emulsions to the samples before testing. By this approach we found that the apparent differences between DOACs are, at least in part, explained by the phospholipid content. With increasing proportion of phospholipids in the reaction mixture it was possible to reduce the selective prolongation of the dRVVT screen clotting time whereas the dRVVT confirmation test was almost unaffected (Fig. 3) which thereby reduced the NR.

Our study has limitations that need to be discussed. One obvious limitation is the use of spiked plasma samples instead of plasma from patients on DOAC therapy. However, there are now many studies that have reported DOAC interference on LA tests (and other coagulation tests) using different approaches and the results based on both *in vitro* and *ex vivo* samples are consistent. Based on our study and the literature we believe that our results are clinically applicable in the sense that it is possible to predict the relative DOAC sensitivities for the investigated dRVVT reagents. However, we cannot predict decision levels, i.e. at what DOAC concentration we can anticipate a false (high or low) LA ratio in a given patient sample. For classification in tentatively positive results we have used the manufacturer-recommended cut-off for normalized ratio. These recommended ratios are usually only valid for a certain analytical platform and in this investigation we had to run

three of the six reagents on other instruments for logistical reasons. Nevertheless, all manufacturers recommended that the cut-off, expressed as NR, is >1.2 and was therefore chosen as the cut-off in our study. Another limitation is that the experiments with synthetic phospholipids only aimed at explaining the risk of false high dRVVT ratios using rivaroxaban. Some reagents led to false low dRVVT ratios and, while the reason for this effect was not examined in our study, this may also be explained by the phospholipid content but needs to be further explored in new studies. We would also need to include true LA positive patient samples in order to investigate if DOACs interferences with these reagents could lead to false negative LA results.

Recent guidelines about LA testing recommend not testing for LA while patients are taking DOACs (37, 38). To circumvent the problem of DOAC interference alternative tests which are not affected by rivaroxaban (14, 31), (and possibly other DOACs), such as the Taipan venom time have been suggested. However, the clinical value of this assay in LA testing is less well documented as compared to the dRVVT and is not widely available. In our opinion, a practical solution to avoiding interferences by DOACs is to adsorb the drug by active charcoal, i.e. through use of DOAC-adsorbent additives in the sample, before testing. We cannot recommend to abstain from the anticoagulant treatment in order to reduce the drug level prior testing, mainly because this will leave the patient without anticoagulant protection, but also because a low level of DOAC is not a guarantee for correct interpretation of the dRVVT assay (39-43). A problem for the laboratory and the interpretation of test results is quite often lack of clinical information, for example the presence or absence of DOAC treatment. One way forward is to retest all samples positive for lupus anticoagulant with the use of DOAC-adsorbent in order to avoid false positive results. However, it is unknown if this approach will reduce the risk of false negative results and such a procedure will also pose financial and logistical restraints. Increasing the phospholipid content, in order to reduce the DOAC effect, is not feasible as this would reduce the LA sensitivity of the test as well. We believe that this study may contribute to the development of new dRVVT reagents with an improved phospholipid formula that is less sensitive to the effects of DOACs.

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Author contribution

All authors contributed to the design of the study, interpretation of data, revision of manuscript and final approval of manuscript. A Hillarp and TL Lindahl wrote the manuscript. KM Gustafsson drew the figures and performed experiments.

Disclosures

None of the authors have any disclosures.

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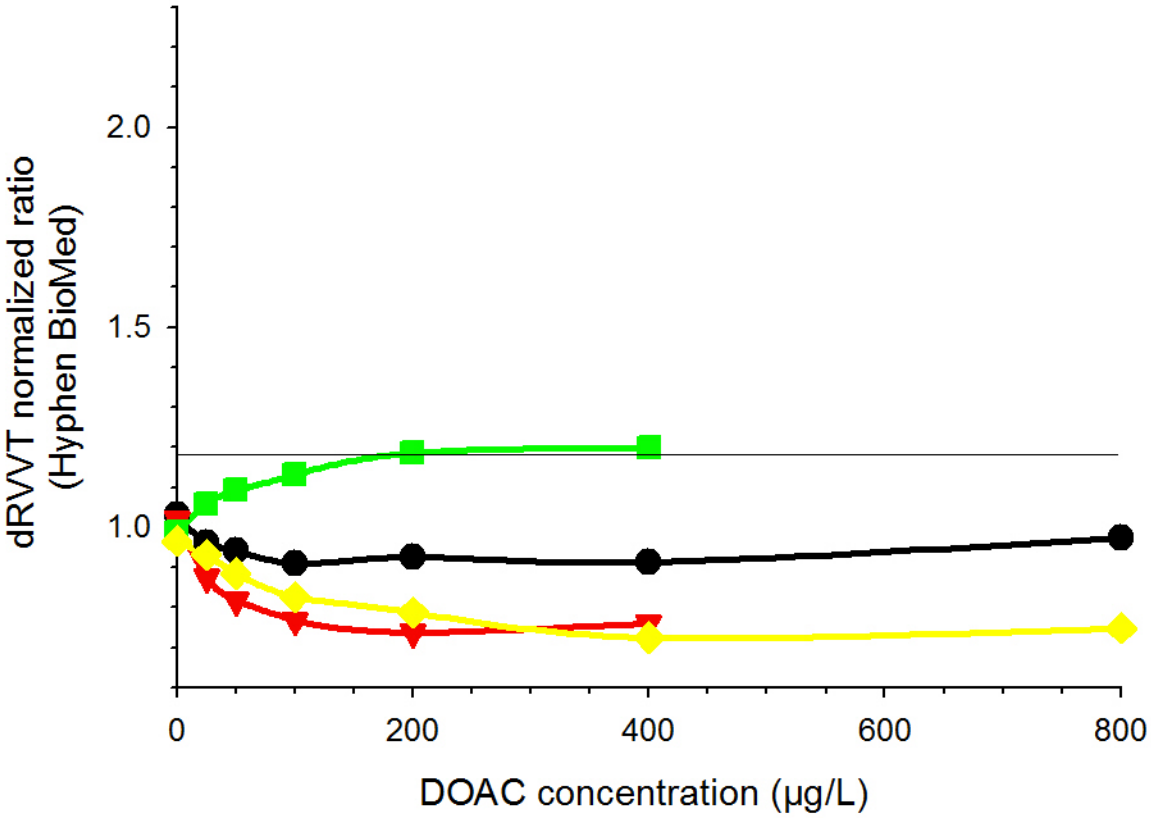
Figure legends

Figure 1. Effect of DOACs on six different LA tests. Each graph (a-f) represents a separate dRVVT test where the calculated mean normalized LA ratios are plotted against the concentrations of four different DOACs. ■, dabigatran; ●, rivaroxaban; ◆, apixaban; ▼, edoxaban. Missing values indicate that the dRVVT ratios were not possible to calculate due to unmeasurable dRVVT screen or confirmation tests.

Figure 2. The variable effect on different LA reagents. Each graph (a-d) illustrates the calculated mean normalized LA ratios for the six different dRVVT tests plotted against the separate DOAC concentrations. Assays are defined as: ■, Siemens; ●, IL; ◆, Technoclone; ▼, Sekisui; ▲, Stago; ⬢Hyphen. Missing values indicate that the dRVVT ratios were not possible to calculate due to unmeasurable dRVVT screen or confirmation tests.

Figure 3. Impact of phospholipid emulsions on the dRVVT screen and dRVVT confirm tests. A plasma sample with 400 µg/L rivaroxaban was spiked with phospholipid at concentrations between 0 – 100 µM/L and analyzed with different LA reagents. The graphs represents a) in the dRVVT screen results b) the dRVVT confirmation results and c) the dRVVT normalized ratios. Assays are defined as: ■, Siemens; ◆, IL; ▼, Technoclone; ●, Sekisui; ▲Hyphen.



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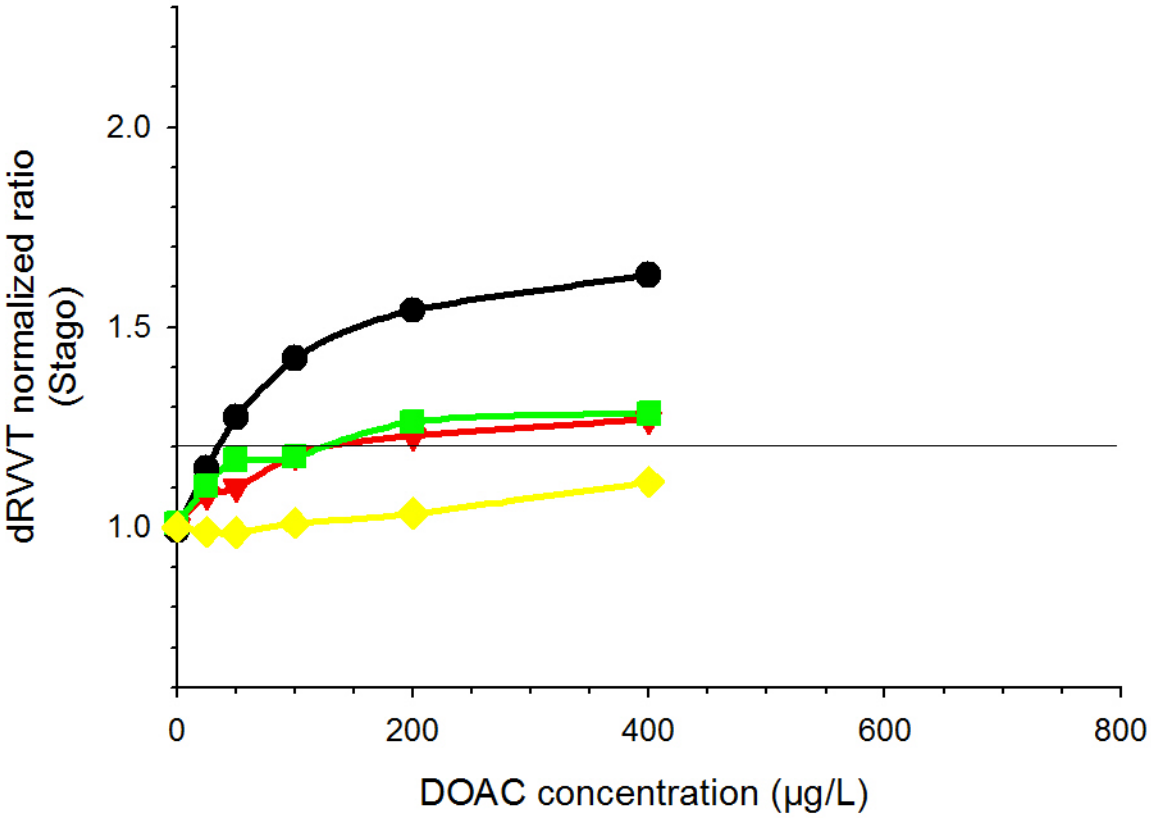

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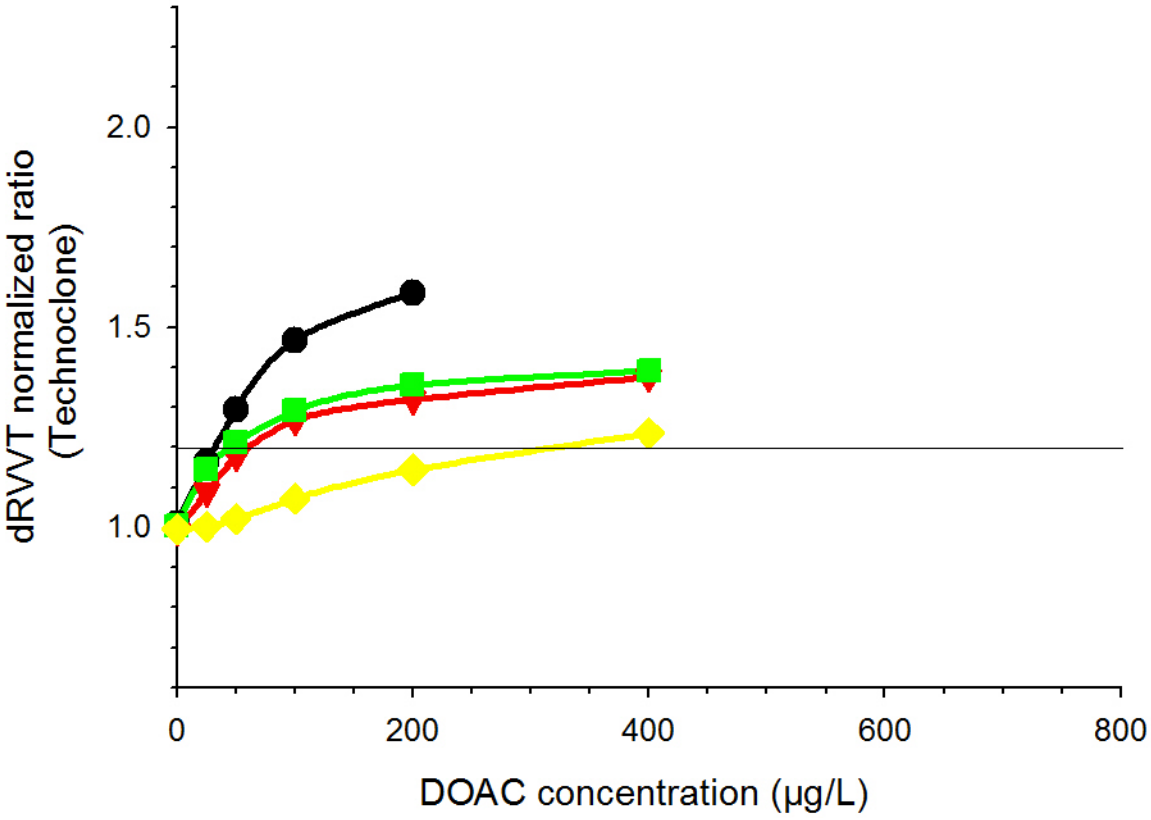

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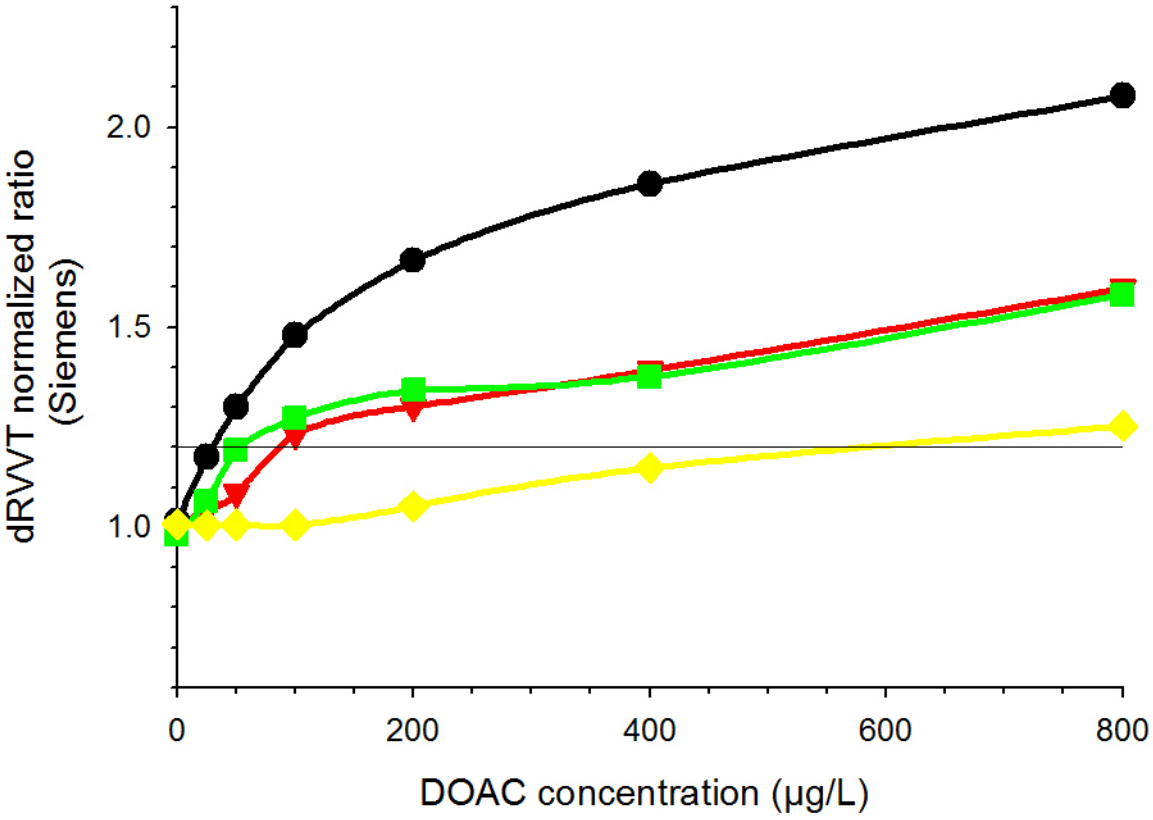

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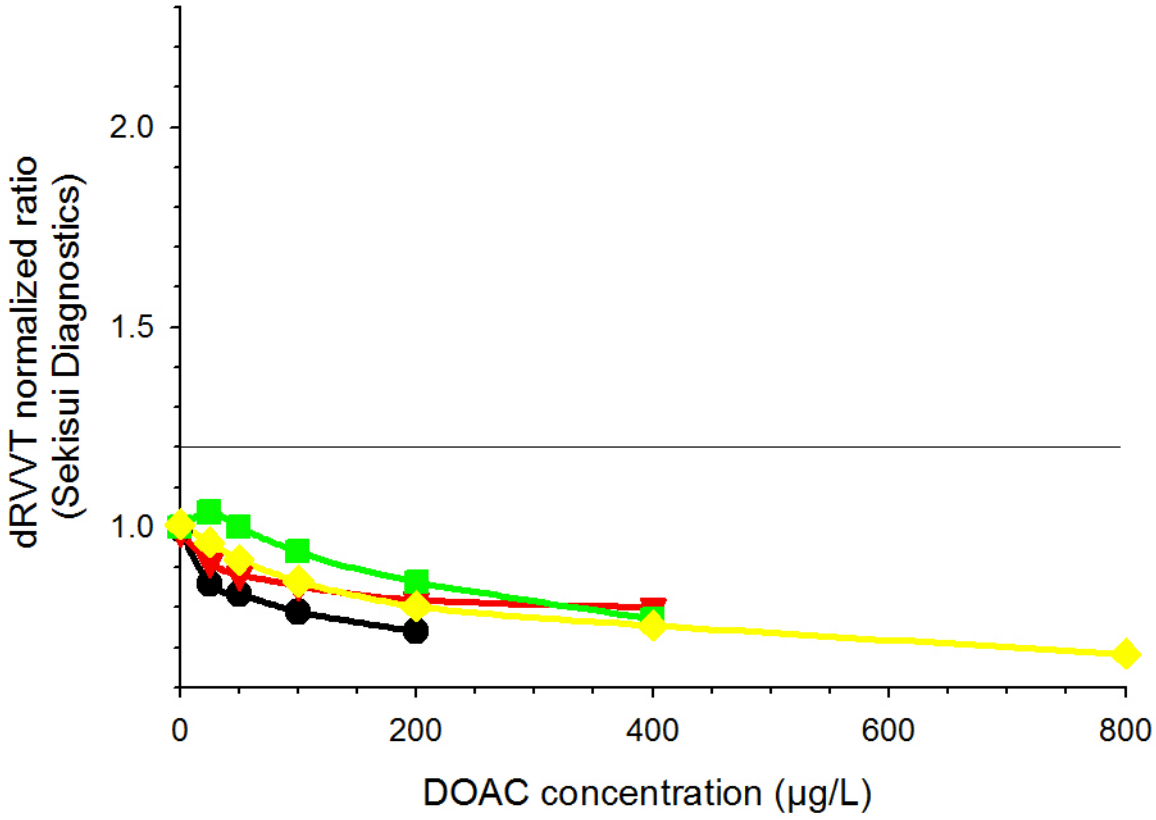

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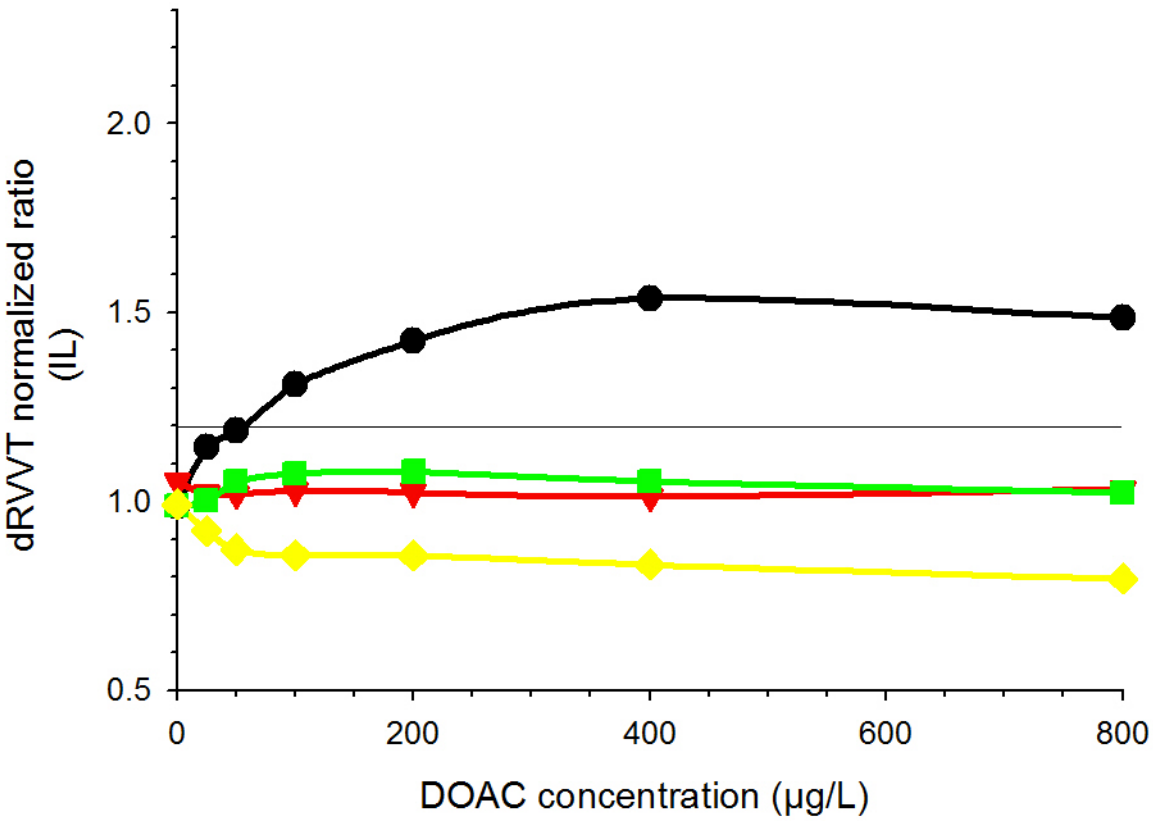

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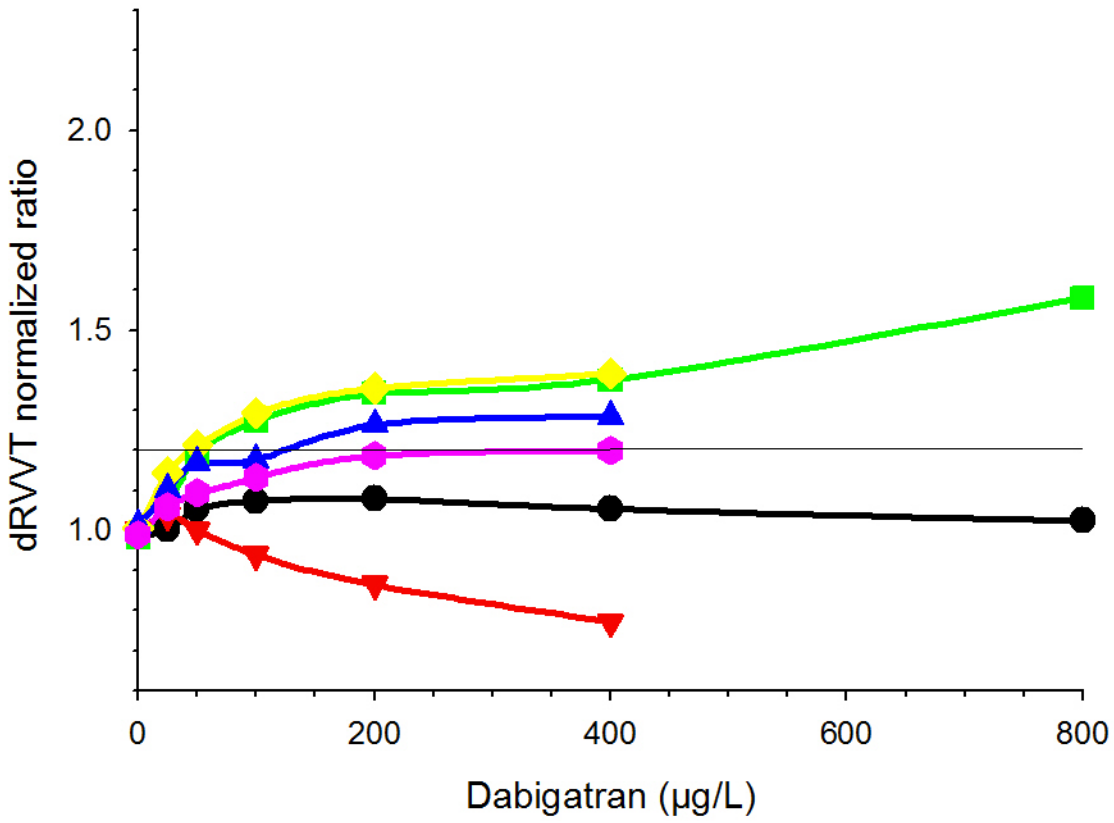

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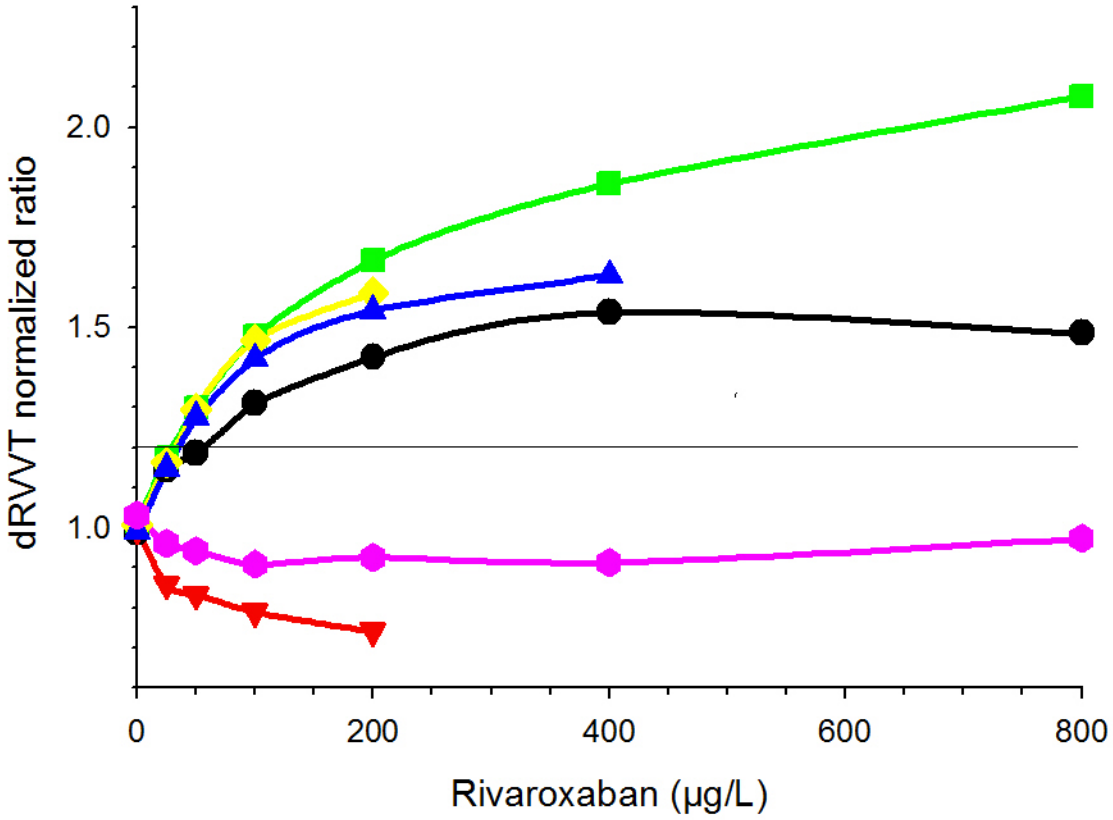

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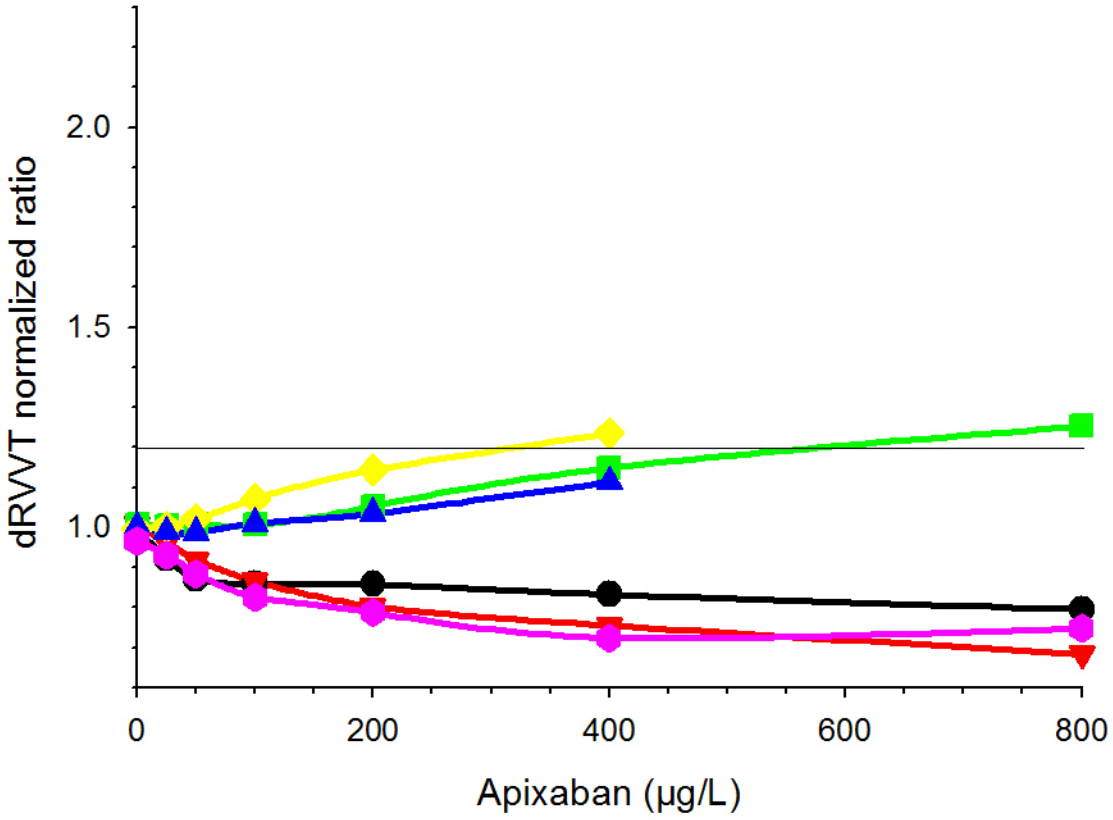

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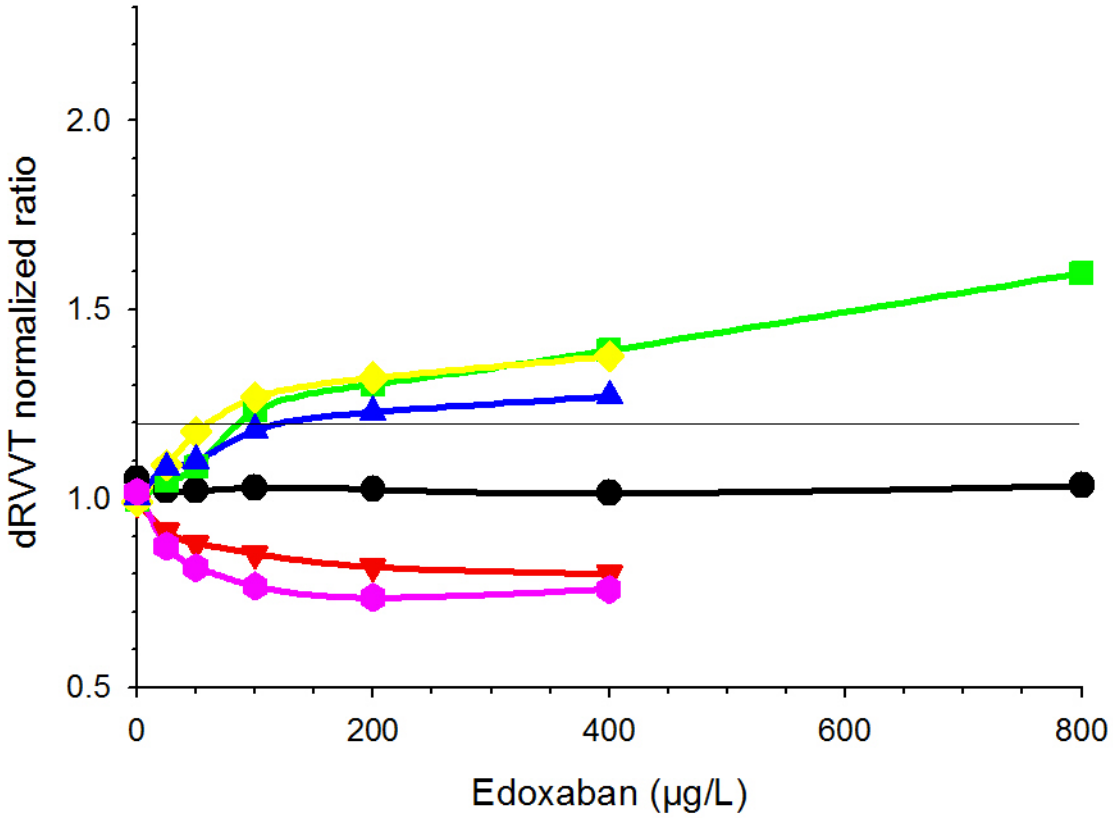

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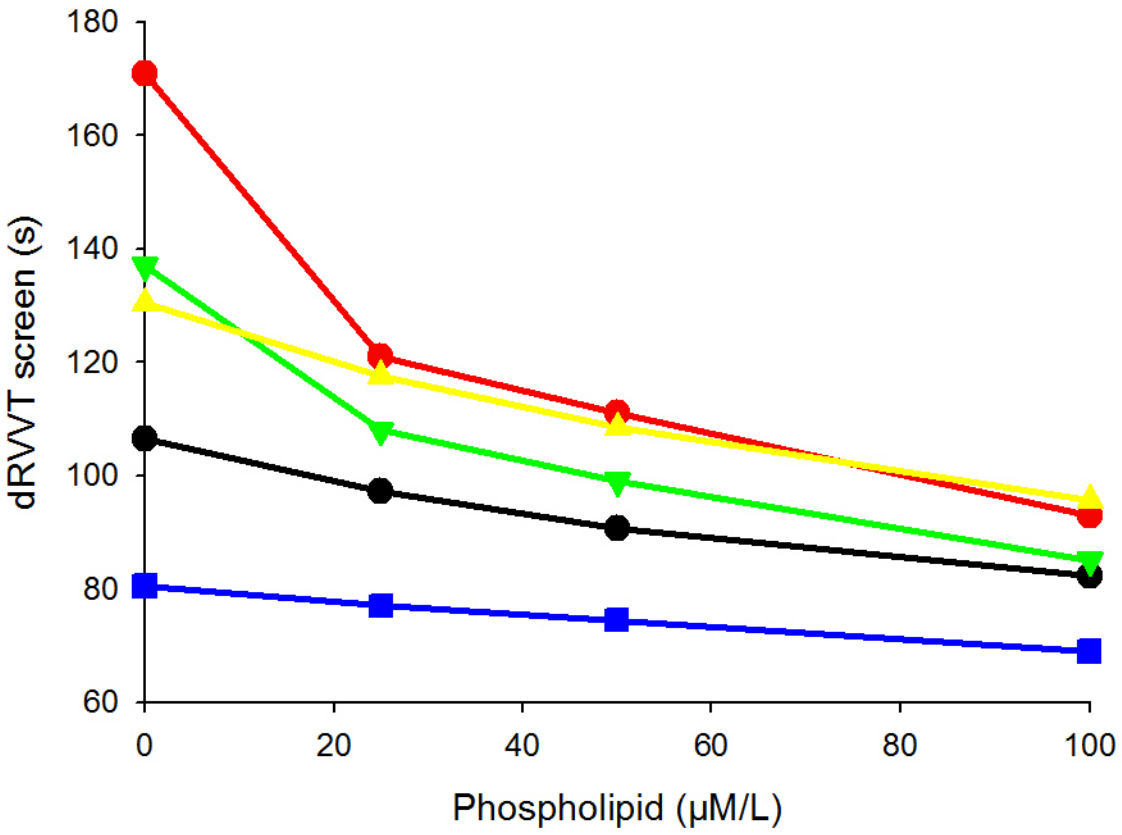

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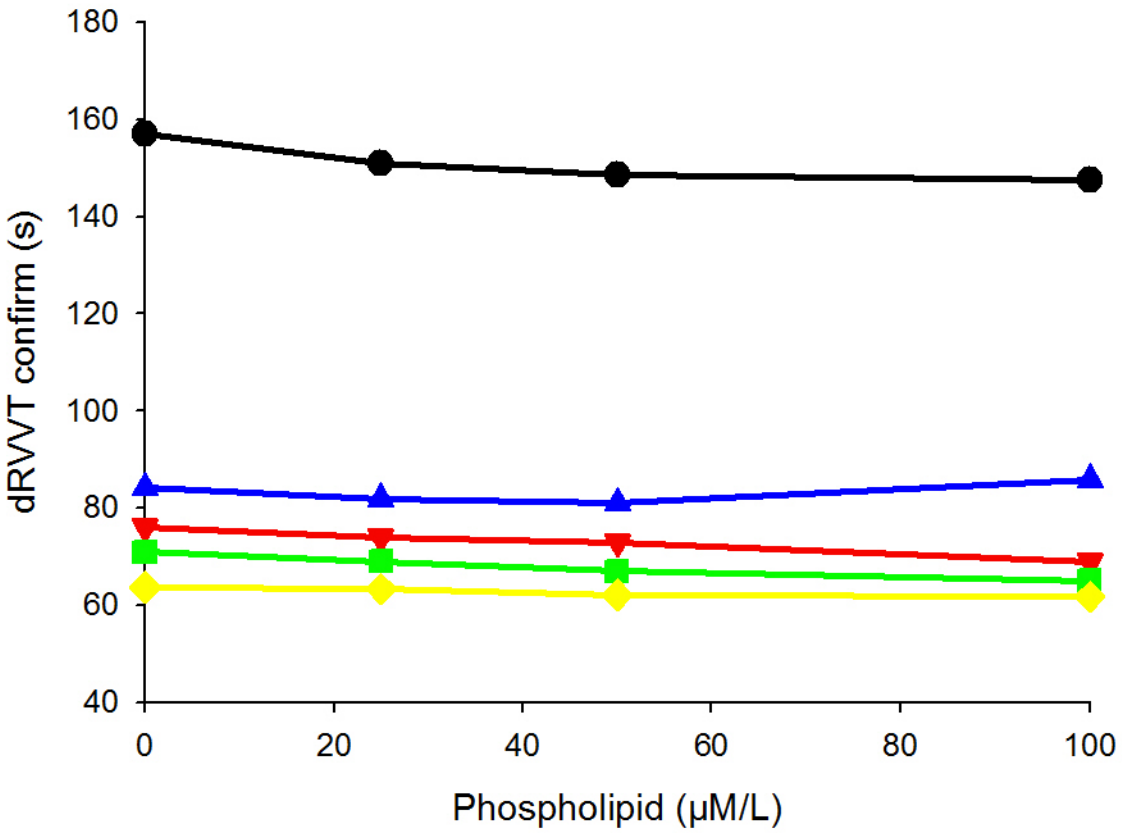

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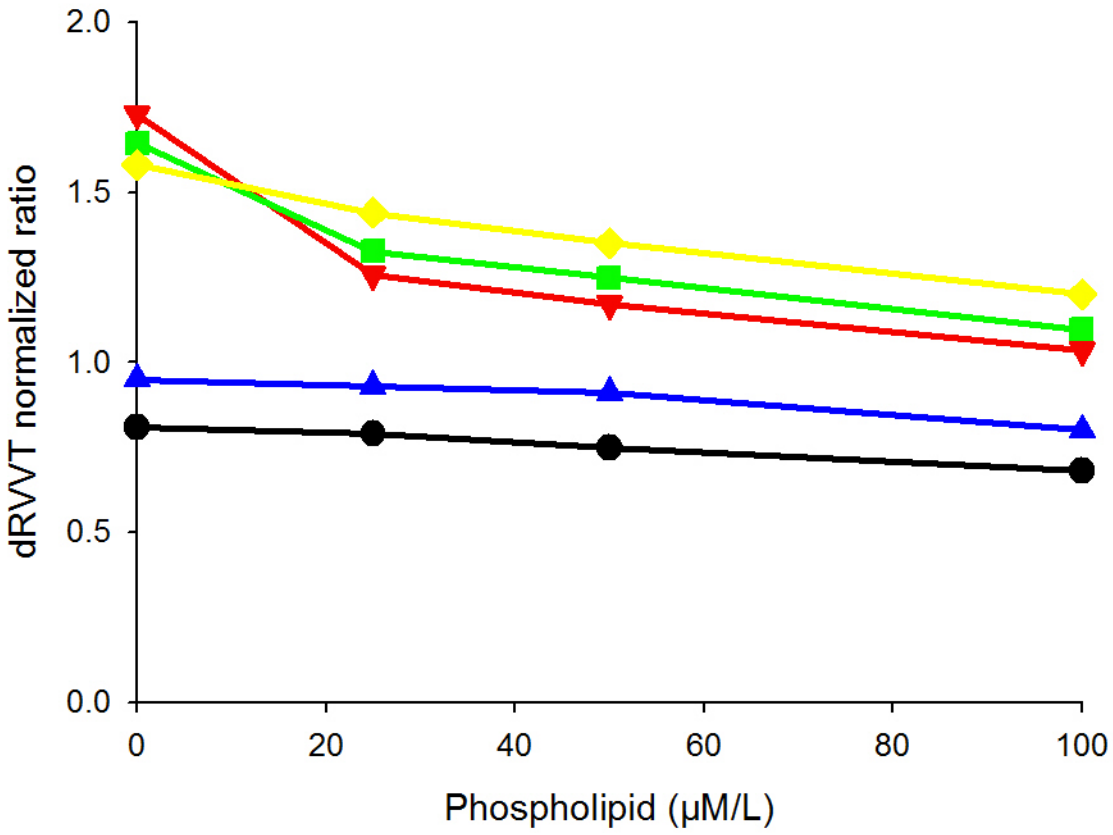

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