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Effects of the oral, direct thrombin inhibitor dabigatran on five common coagulation assays

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Running title

Effects of dabigatran on coagulation assays

Keywords

Dabigatran, thrombin inhibitor, interference, anticoagulant, coagulation assays

Previous presentation of this paper

None

Abstract

BACKGROUND

Dabigatran is an oral, reversible thrombin inhibitor that has shown promising results in large clinical trials. Laboratory monitoring is not needed but the effects on common coagulation assays are incompletely known.

METHODS

Dabigatran was added to plasma from healthy subjects in the concentration range 0-1000 µg/L and analyzed using several reagents for activated thromboplastin time (APTT), prothrombin time (PT), fibrinogen, antithrombin, and activated protein C resistance.

RESULTS

Typical trough concentrations are about 50 µg/L, peak concentrations 100-300 µg/L. At 100 µg/L all APTT-results were prolonged. The concentration required to double APTT ranged between 227 and 286 µg/L, the responses for all five reagents were similar. PT-reagents were much less affected with almost no samples above INR 1.2 at 100 µg/L. The effect was sample dilution dependent with PT Quick type more sensitive than PT Owren type methods. If a patient on dabigatran has prolonged APTT, >90 sec, and Quick PT INR>2 or Owren PT INR>1.5 over-dosing or accumulation of dabigatran should be considered.

Two of four fibrinogen reagents underestimated the fibrinogen concentration considerably at expected peak concentration. Methods based on inhibition of thrombin over-estimated the antithrombin concentration, but not Xa-based. The APC-resistance methods over-estimated the APC-ratio, which may lead to miss-classification of factor V Leiden patients as being normal.

CONCLUSION

Different coagulation assays, and even different reagents within an assay group, display variable effects at therapeutic concentrations of dabigatran. Some of these assay variations are of clinical importance, thus knowledge is needed for a correct interpretation of results.

Introduction

Oral anticoagulants are used world-wide to prevent thrombosis, for example in patients with atrial fibrillation, venous thrombosis and prosthetic heart valves. Despite their first use on humans in 1940, coumarins (Vitamin K antagonists, VKA) still are the only oral anticoagulants approved for antithrombotic treatment in atrial fibrillation and venous thrombosis. Millions of patients have been treated and the knowledge of pros and cons of this very efficient drug is well established, including effects on coagulation assays. One of the draw backs is the need for monitoring by measuring prothrombin time to achieve appropriate degree of anticoagulation. The inter-individual variation in weekly dosage is ten-fold, depending on many causes, for example intake of vitamin K with the food, polymorphisms in the liver enzymes degrading the drug and in the target enzyme VKORC1 (1-2). In recent years oral, reversible direct thrombin inhibitors have been developed. Practical advantages with these drugs are that the patients are given a fixed dose and that no monitoring is required. The first to reach phase III clinical trials, ximelagatran, was not approved for clinical routine use due to side effects on the liver (3-5). Ximelagatran is a prodrug, metabolised to the active inhibitor melagatran, in the patient. During the clinical trials some physicians were worried about increased INR found when measuring prothrombin time (outside the study protocol). Therefore effects on prothrombin time assays by melagatran were studied and large differences between assays were found (6). There are many reports on effects on coagulation assays by other thrombin inhibitors such as argatroban and lepirudin (7-12).

The new oral, reversible direct thrombin inhibitor dabigatran etexilate from Boehringer-Ingelheim is likewise a prodrug which is metabolised to its active form dabigatran by esterases. The drug is eliminated by the kidneys and a glomerular filtration rate below 30 mL/min is a contra-indication.

The drug is already approved for prevention of thrombosis in elective orthopaedic surgery based on successful clinical trials (13-14). The results from phase III clinical trials, RE-LY for prevention of thrombosis in atrial fibrillation and RE-COVER for treatment of venous thrombosis are very promising (15-16). We expect a wide-spread use of the drug in the near future, when approved by the medical agencies for treatment of patients with atrial fibrillation and venous thrombosis. Therefore it is important to know the effects of dabigatran on common coagulation assays not only in therapeutic concentration range but also above; since some patients with renal failure may accumulate the drug and non prescribed over-dosing may occur. Although there are few reports on effects on coagulation assays (17-18), we believe the coagulation laboratories need a more thorough knowledge, i.e. more coagulation analyses and different method principles and reagents, to avoid erroneous interpretation of results affected by analytical interferences. The aim of the present inter-laboratory study was to systematically investigate the effects on the most commonly used coagulation assays over a broad concentration range, including expected trough and maximum concentrations in patients on dabigatran 110 or 150 mg twice daily and in situations of over-dosage or accumulation. For practical and ethical reasons the study has been done utilizing plasma samples spiked with dabigatran.

Materials and Methods

Dabigatran (the active metabolite) was a gift from the manufacturer Boehringer-Ingelheim (Ingelheim, Germany). Blood collection tubes were from Becton Dickison (Franklin Lakes, NJ, USA).

Blood collection and addition of study drug

Human blood from 10 adult healthy volunteers, equal numbers of men and women, was collected after permission in evacuated plastic tubes containing 1:10 volume of 0.129 mol/L tri-sodium citrate as anticoagulant. Ten tubes, in total 50 mL citrated blood, were obtained from each individual and marked with a code not coupled to the identity of the donor. The tubes were centrifuged 2000 x g for 20 min and the platelet poor plasma was pooled for each donor separately and stored frozen at -70°C. The plasma from each donor was thawed in 37°C water-bath for 15 min and divided into 20 aliquots of 1.0 mL each and “spiked” with dabigatran to the following final concentrations 0.0, 10, 25, 50, 100, 200, 400, 600, 800 and 1000 µg/L and then stored at -70°C until analysis. The dabigatran was dissolved in DMSO; all dilutions in plasma were 1:100, resulting in a final DMSO concentration of 1 % (v/v) in all samples including control samples, i.e. irrespective of the dabigatran concentrations.

Coagulation analyses

The following coagulation analyses were studied; Activated thromboplastin time (APTT), prothrombin time (PT), antithrombin (AT), fibrinogen and activated protein C resistance (APCR).

APTT was assayed in the Malmö laboratory using 5 different reagents: Actin FSL from Siemens Healthcare Diagnostics (Deerfield, IL, USA); PTT-Automate from Stago; APTT-SP IL test liquid from Instrumentation Laboratory (Milan, Italy); TriniCLOT aPTT HS from Trinity Biotech (Bray, Ireland); and DG-APTT from Grifols (Barcelona, Spain). The APTT assays were performed on a BCS-XP instrument from Siemens Healthcare Diagnostics according to the manufacturer’s instructions.

PT was performed with combined thromboplastin reagents and final plasma sample dilution of 1:21 (the Owren type PT assay), which is the type used in almost all laboratories in the

Nordic countries. For this investigation we tested all currently used Owren type PT assays: Stago prothrombin complex assay, SPA+, from Stago; Nycotest PT from Axis-Shield plc (Dundee, Scotland); PT Owren from Medirox AB (Nyköping, Sweden); and Simple Simon PT from Zafena AB (Borensberg, Sweden). The Owren PT assays were all calibrated using the national INR calibrators certified by the Swedish external quality assessment organization EQUALIS (Uppsala, Sweden) (19). One commonly used plain recombinant thromboplastin reagent internationally giving a final plasma dilution of 1:3 (the Quick PT assay), was also used (Dade Innovin from Siemens Healthcare Diagnostics). The Dade Innovin PT assay was calibrated using a locally determined ISI of 1.151 and a mean normal PT (9.9 sec). All PT assays were performed at the Linköping laboratory on an ACL Top (Instrumentation Laboratory SpA) coagulation instrument according to standard procedures.

Three different chromogenic reagents for determination of antithrombin activity were evaluated in the Gothenburg laboratory. Two were based on the inhibition of thrombin, Berichrom ATIII from Siemens Healthcare Diagnostics, which utilizes bovine thrombin and Stachrom ATIII from Stago that contain human thrombin. One reagent was based on inhibition of factor Xa, Coamatic LR from Instrumentation Laboratory (Milan, Italy). The Stachrom ATIII test was run on the STA-R analyzer from Stago whereas the two other antithrombin assays were performed on the BCS-XP instrument (Siemens).

Fibrinogen was evaluated at the Stockholm laboratory using four Clauss type clotting assays: Fibri-Prest from Stago; Fibrinogen C from Instrumentation Laboratory, Dade thrombin and Multifibren U from Siemens Healthcare Diagnostics utilizing the Sysmex CA-1500 instrument from Siemens Healthcare Diagnostics.

Coagulation based test for the determination of APC resistance was performed in Gothenburg with two methods based on different principles: Coatest APC Resistance V from Chromogenix and Pefakit APC resistance Factor V Leiden from Pentapharm (Basel, Switzerland) utilizing the STA-R analyzer from Stago according to the manufacturer's instructions.

Statistics

Results are presented as mean \pm standard deviation (SD). Concentrations required for doubling the clotting times (CT_2) for APTT were calculated using Excel 97 software (Microsoft®). A 2nd order polynomial function was used for the APTT assays, one for each donor, in all cases r^2 was >0.99 . Linear regression was used for antithrombin to calculate average effects of dabigatran.

Results

All APTT-reagents responded similarly to addition of dabigatran despite differences in contact activating material and phospholipids (Figure 1). The dose-response was non-linear with a relatively steep increase at low concentrations transforming to an almost linear increase from 200 $\mu\text{g/L}$. First at 100 $\mu\text{g/L}$ all APTT-reagents gave a result above the reference interval, i.e. >40 sec. For all reagents many samples had APTT >40 sec at 50 $\mu\text{g/L}$, for DG-APTT from Grifols all samples. Actin FSL from Siemens Healthcare Diagnostics was slightly less sensitive than the other reagents. Mean APTTs and ranges at baseline, 50 $\mu\text{g/L}$ and 200 $\mu\text{g/L}$

are given in Table 1. The effect on the APTT, as expressed as the concentration required to double the APTT (CT_2), was calculated to be between 227 – 286 $\mu\text{g/L}$ for the five different reagents (Table 1), which is a relative difference of only 1.26-fold.

All PT-reagents were much less influenced than APTT, almost no samples were above the reference interval, i.e. $> \text{INR } 1.2$, at 100 $\mu\text{g/L}$ (Figure 2). However, the variation in sensitivities was substantial with SPA+ and Nycotest PT least sensitive and the only studied Quick PT reagent Innovin most sensitive (Table 2). Even at the highest tested concentration, 1000 $\mu\text{g/L}$, only one spiked plasma had an INR above 2 for SPA+ and Nycotest PT, but for Innovin all plasmas had $\text{INR} > 3$.

The antithrombin assays based on inhibition of factor Xa (Chromogenix AT) were not affected, but the assays based on thrombin inhibition slightly overestimated the antithrombin concentration (Figure 3). For the latter linear regression analysis resulted in a slope that corresponded to an increase in average of 0.12 IU/ml per 200 $\mu\text{g/L}$ dabigatran.

The difference in interference on fibrinogen reagents was considerable, with almost no effects on two reagents Fibri-Prest and Dade thrombin but great underestimation with Fibrinogen C and Multifibren U from 200 $\mu\text{g/L}$ dabigatran (Figure 4).

For APC-resistance both assays were strongly influenced by dabigatran already at a concentration of 50 $\mu\text{g/L}$, although Pefakit APC-R which is based on prothrombinase activation more than Coatest APC Resistance V which is based on APTT with and without activated protein C (Figure 5A and B).

Discussion

The effects on coagulation analyses by the new oral anticoagulant dabigatran are concentration dependent which in turn will depend on dose, absorption and clearance through the kidney. Thus the expected trough and maximum concentrations will differ considerably between patients. Young healthy male volunteers reached a C_{\max} of 107 ± 72 $\mu\text{g/L}$, female 76 ± 71 $\mu\text{g/L}$ on a single oral dose of 150 mg. Steady state concentrations measured in the PETRO trial with the dose 150 mg bid were for C_{\max} 184 (64-443) $\mu\text{g/L}$; mean (95% C.I.) and for C_{trough} 90 (31-225) (20). Regarding elderly subjects (mean age 68.4 ± 4.1 years) on 150 mg orally twice daily the male subjects reached C_{\max} of 254 ± 71 $\mu\text{g/L}$ and the female 285 ± 81 $\mu\text{g/L}$ at day 7 (21). The trough concentration is expected to be 50-100 $\mu\text{g/L}$ for 150 mg twice daily. Simulated dabigatran plasma concentrations at moderate renal impairment in orthopedic surgery patients a C_{\max} of 180 $\mu\text{g/L}$ have been calculated (18) based on 150 mg twice daily, which is lower than found for elderly (21). Plasma concentrations are very dependent on renal function in different populations (22). In the RE-LY study on prevention of thrombosis in atrial fibrillation two doses were tested, 110 and 150 mg, twice daily (15). In the RECOVER study on treatment of venous thrombosis one regimen was used, 150 mg twice daily (16). The concentrations used in our tables have been based on assumed trough and maximum concentrations in patients on dabigatran 150 mg twice daily.

In this study we have used plasma samples spiked with dabigatran in a very broad concentration range and common coagulation assays including several reagents in order to generate useable knowledge for the laboratories. A limitation of our study is the use of spiked

plasma samples. However, it is unjustifiable to deliberately expose patients to potentially toxic doses and untenable to collect such samples from patients. Furthermore, dabigatran is the active compound (18, 23) converted *in vivo* from dabigatran etexilate by esterases and is mainly excreted as such through the kidneys, thus for dabigatran spiked samples is a valid methodology. About 20% of dabigatran gets conjugated by glucuronosyltransferases to pharmacologically fully active glucuronide conjugates and is secreted in that form (18, 23). Hepatic impairment had only small effects on bioconversion of dabigatran etexilate to the active compound (20).

Our results for APTT are in accordance with results previously reported by Stangier et al. at Boehringer-Ingelheim (17-18, 22, 24-25). Almost all patients will be expected to have prolonged APTT at C_{max} and a large proportion even at trough concentration irrespective of reagent used at the laboratory.

For PT-assays the dilution of the sample is important since dabigatran is a reversible inhibitor and the effect on PT will decrease with increasing dilution. Our findings are in accordance with previous results on the thrombin inhibitor melagatran (6), i.e. the Quick PT reagent with final plasma dilution 1:3 was affected more than all Owren methods with final dilution 1:21. The reason is not clear why some Owren PT reagents are more sensitive than others despite the fact that all are combined rabbit thromboplastins, even considerably more so than the APTT-reagents. For point of care instruments with no sample dilution an even stronger effect is anticipated, which has been shown previously for melagatran (6). The increase in INR was at high concentrations of dabigatran not linear as the impression is from a figure shown previously (18), but almost exponential. The reason could be many more observations at the low end of the concentration range in contrast to an equal distribution. For Owren methods a

result above the reference interval is not expected for most patients. If a patient on dabigatran has prolonged APTT, >90 sec, and Quick PT INR>2 or Owren PT INR>1.5 over-dosing or accumulation of dabigatran should be considered. These results are indicative of a concentration of dabigatran $\geq 600 \mu\text{g/L}$, based on data from the PETRO trial less than 1% of the patients on 150 mg bid would have $C_{\text{max}} \geq 600 \mu\text{g/L}$ (20).

The result for two of the fibrinogen reagents, i.e. Fibrinogen C from Instrumentation Laboratory and Multifibren U from Siemens Healthcare Diagnostics, with large under-estimation of the concentration in plasma samples from patients receiving dabigatran etexilate is worrying. For example, using these reagents at a concentration of 300 $\mu\text{g/L}$ dabigatran a normal concentration of fibrinogen of 2.3 g/L will be reported by the laboratory as <1 g/L in average. Such a low concentration is associated with an increased risk of bleeding. For the clinician treating a patient with major bleeding it is important to get correct information on the fibrinogen concentration. In our opinion these methods giving falsely low results should be abandoned, physicians should use any of the kits much less sensitive to interference. Underestimation of fibrinogen concentration with some reagents has been reported previously for the irreversible thrombin inhibitor bivalirudin (26).

Antithrombin assays based on thrombin inhibition give falsely high results in contrast to the factor Xa based assay, thus a patient with hereditary deficiency may have the disorder falsely excluded if tested for thrombophilia. For example at a dabigatran concentration of 300 $\mu\text{g/L}$ an antithrombin concentration of 0.68 kIE/L will be falsely reported as 0.80 kIE/L, i.e. in the normal range. However, the new oral anti-Xa inhibitors will likely influence the assays based on Xa inhibition even more in the same direction.

Dabigatran caused interference in the studied methods for APC resistance increasing the ratio, which may could lead to misclassification, making patients heterozygous for factor V Leiden appear normal. However, studies on more APC-resistant donors are needed to confirm this observation. Furthermore, most laboratories nowadays use DNA-analysis to diagnose APC-resistance, i.e. factor V Leiden, and not functional methods. It is not surprising that Pefakit APC-R which is based on prothrombinase activation is affected more by a thrombin inhibitor than Coatest APC Resistance V which is based on APTT. For factor Xa inhibitors the reverse would be expected.

Our conclusion is that dabigatran may have clinically significant, reagent dependent effects on common coagulation analyses. Furthermore thrombophilia investigation should not be done on patients while on dabigatran without knowledge of how the drug affects the locally used methods.

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Table 1

Type of APTT	Baseline(0ug/L) sec., mean (\pm SD) (range)	“Trough”(50ug/L) sec., mean (\pm SD) (range)	“Peak”(200ug/L) sec., mean (\pm SD) (range)	CT ₂ (ug/L)	CV %
TriniCLOT	35,2 (\pm 3,2) (29,4-40,5)	49,1 (\pm 7,0) 36,9-59,4	64,7(\pm 7,8) 54,1-79,7	286 \pm 37	13
Actin FSL	32,2 (\pm 2,5) 27,2-35,6	44,0 (\pm 4,9) 36,6-52,0	59,7 (\pm 6,1) 52,1-72,3	279 \pm 46	16
APTT-DG	31,8 (\pm 3,0) 26,1-36,2	46,3 (\pm 8,3) 32,8-60,5	62,6 (\pm 8,9) 50,3-81,4	227 \pm 50	22
APTT-SP	35,1 (\pm 3,2) 28,9-40,5	49,3 (\pm 6,8) 37,6-59,2	64,1 (\pm 8,1) 52,0-76,6	282 \pm 58	20
PTT-A	33,9 (\pm 2,7) 28,4-38,0	47,1 (\pm 7,0) 37,0-61,2	64,7 (\pm 8,1) 51,5-75,7	253 \pm 49	19

Table 2

PT-assay	Baseline(0ug/L) INR, mean(\pm SD) (range)	“Trough”(50ug/L) INR, mean (\pm SD) (range)	“Peak”(200ug/L) INR, mean (\pm SD) (range)
SPA+	0,98(\pm 0,06) 0,92-1,12	1,00 (\pm 0,08) 0,91-1,16	1,04 (\pm 0,09) 0,94-1,26
Nycotest PT	0,99(\pm 0,06) 0,92-1,12	1,02 (\pm 0,06) 0,96-1,15	1,07 (\pm 0,07) 0,99-1,16
Owrens’s PT	1,03(\pm 0,08) 0,95-1,22	1,06 (\pm 0,08) 0,96-1,25	1,16 (\pm 0,10) 1,05-1,39
Simple Simon	0,93(\pm 0,05) 0,88-1,06	0,95 (\pm 0,05) 0,90-1,06	1,12 (\pm 0,09) 1,01-1,32
Dade Innovin	0,98(\pm 0,04) 0,88-1,06	1,03 (\pm 0,04) 0,97-1,13	1,24 (\pm 0,06) 1,16-1,36

Figure legends

Figure 1. Dabigatran effects on APTT. The obtained APTTs using five different reagents were plotted against the dabigatran concentration in plasma. Triniclot aPTT HS (●), Actin FSL(▼), PTT-Automate (▲), APTT-SP (◆) and DG-APTT (■). Results are mean of 10 different healthy donors \pm SD.

Figure 2. Dabigatran effects on PT assays. Four Owren type PT assays, based on combined rabbit thromboplastin reagents; SPA+ (●), Nycotest PT (·), Owren's PT (▼), and Simple Simon (■), and one Quick PT assay (plain reagent) that utilized recombinant tissue factor, Dade Innovin (▲), were used. Results are shown as the mean INR of 10 different healthy donors \pm SD.

Figure 3. Effects of dabigatran on antithrombin activity. Three different assays based on chromogenic substrates were analyzed using plasma spiked with dabigatran; based on thrombin inhibition were Berichrom ATIII (○) and Stachrom ATIII (●), based on factor Xa inhibition Coamatic LR (▼). Results are shown as the mean activity (IU/ml) of 10 different healthy donors \pm SD.

Figure 4. Effects of dabigatran on fibrinogen concentration results. Four different assays were evaluated; Fibri-Prest (●), Fibrinogen C (▼), Dade Thrombin (○) and Multifibren U(Δ). Results are shown as g/L of 10 different healthy donors \pm SD.

Figure 5. Effect of dabigatran on coagulation-based APC resistance assays. A) APTT-based assay (Coatest APC Resistance V); B, assay based on activation at the prothrombinase level

(Pefakit APC Resistance Factor V Leiden). The results from 8 donors with normal phenotype (●) (mean±SD) and two APC-resistant phenotype (▼), are plotted against the dabigatran concentration in plasma. A ratio below 2.0 and 0.9-1.8 for A and B respectively, indicates APC-resistance.

FIGURES

FIGURE 1

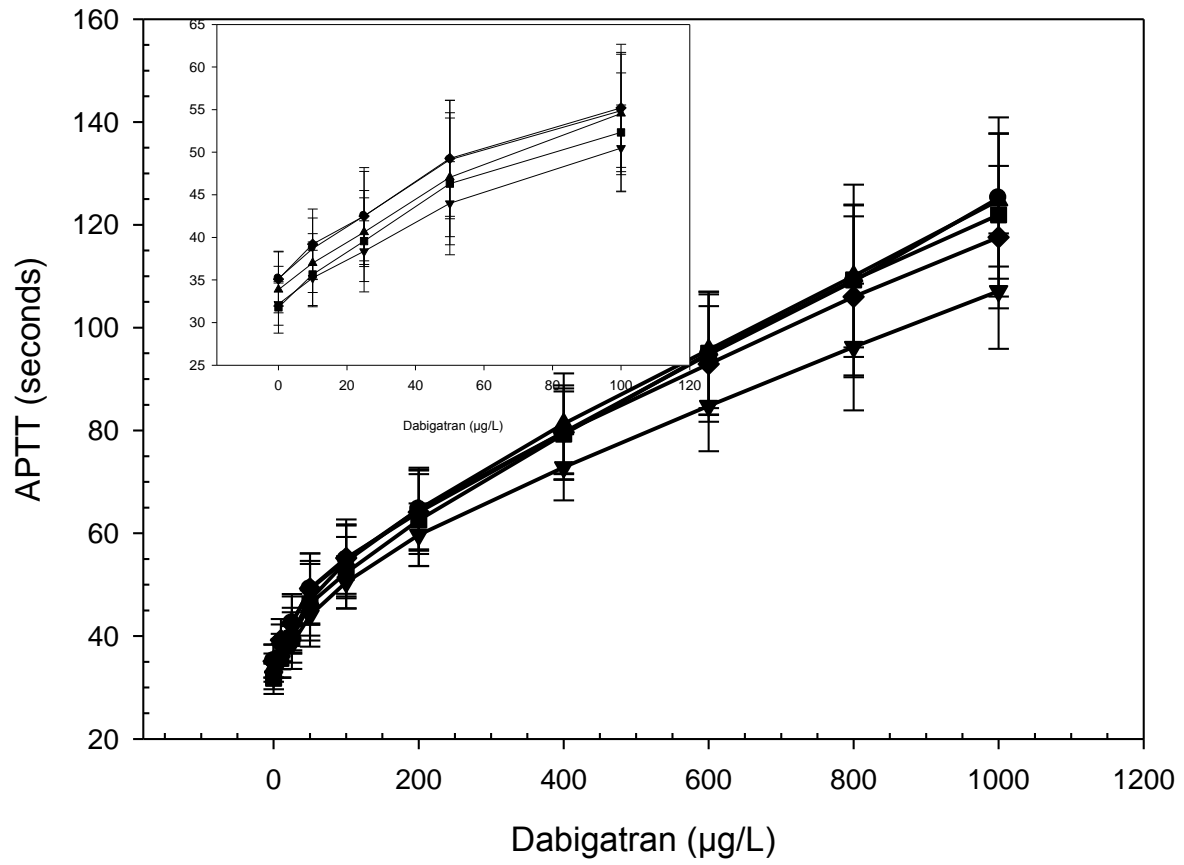


FIGURE 2

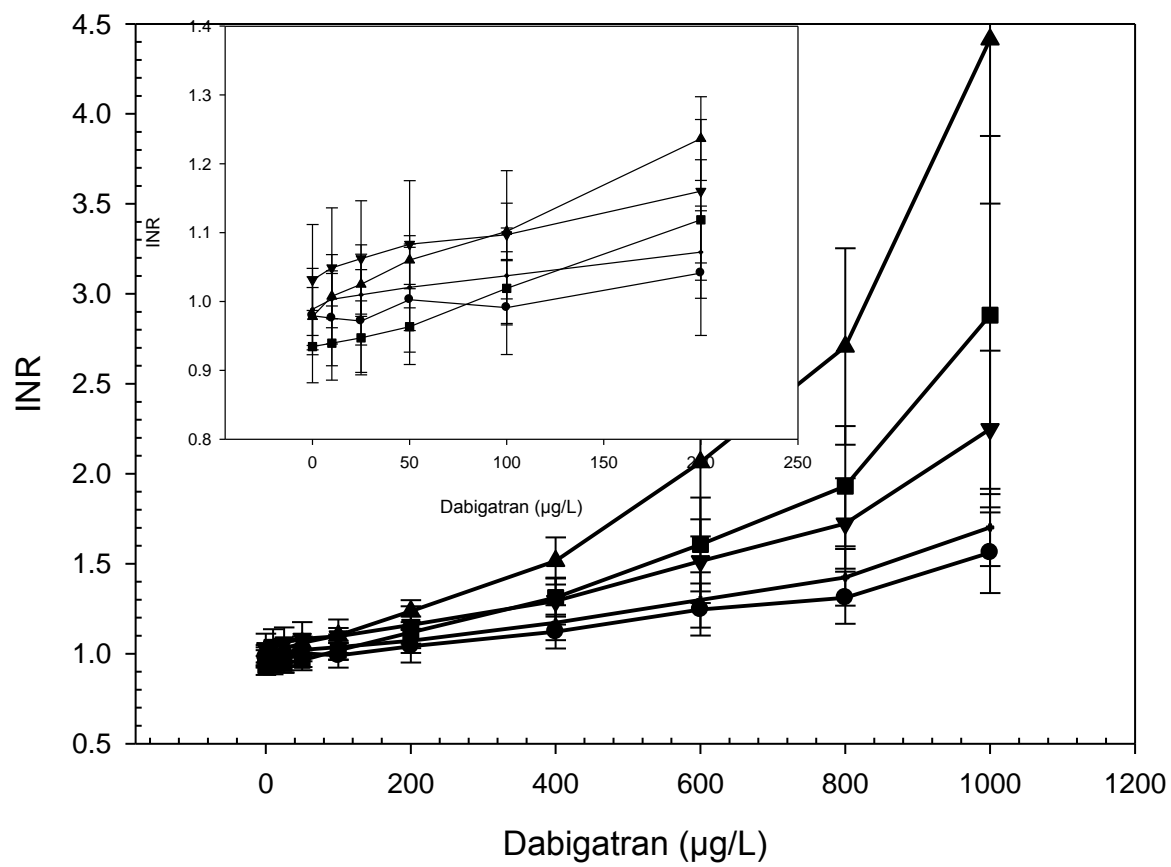


FIGURE 3

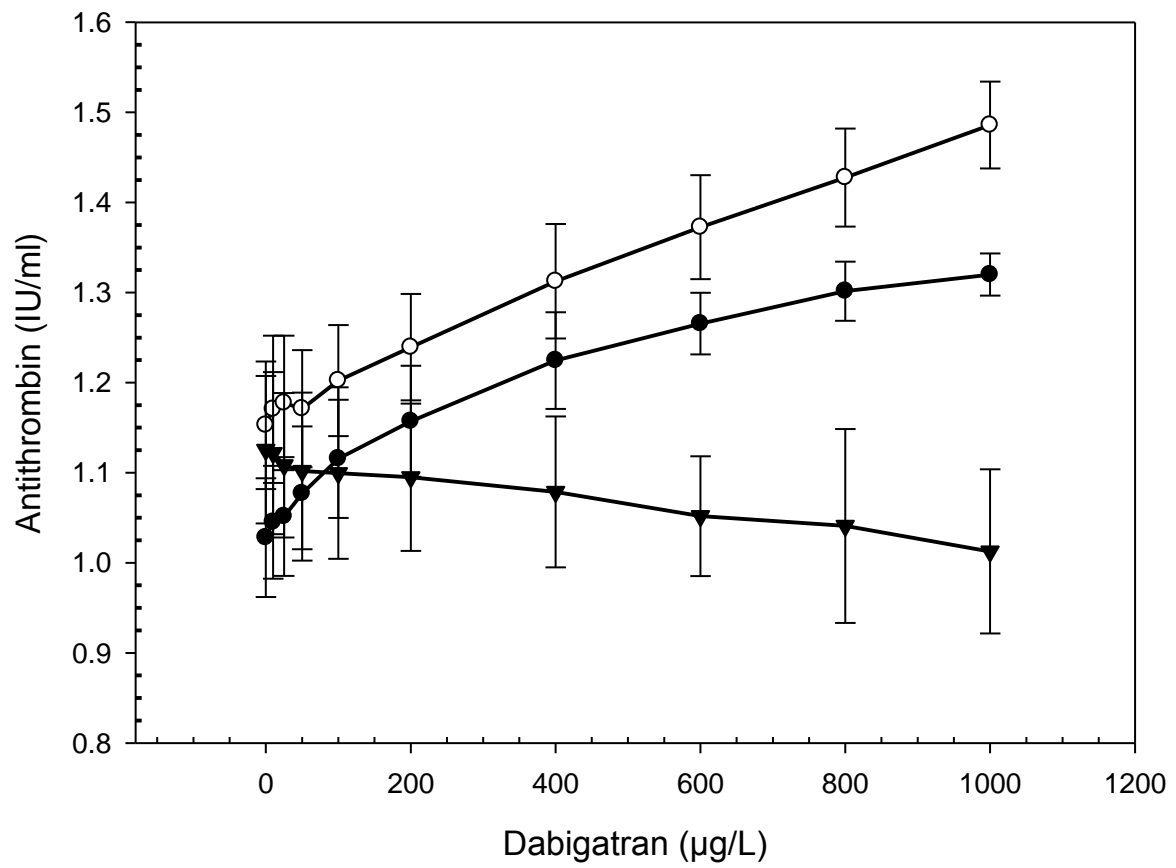


FIGURE 4

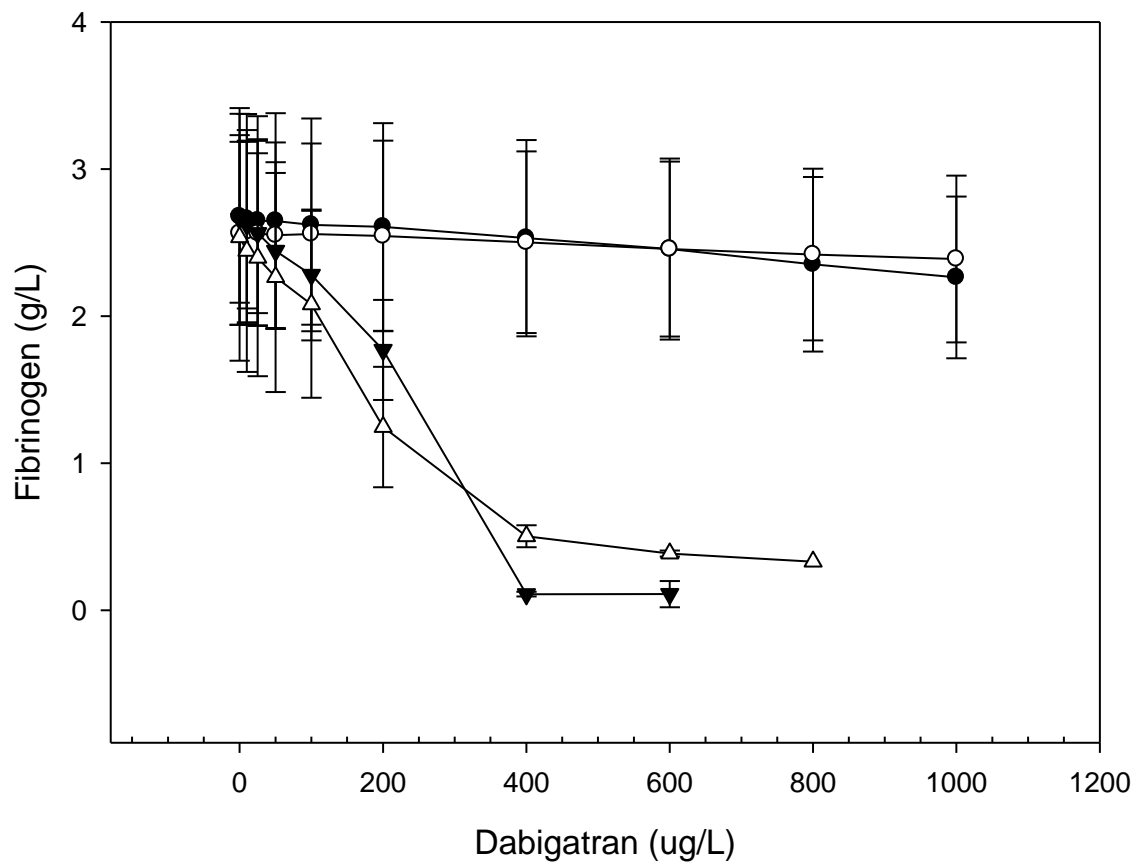


FIGURE 5A

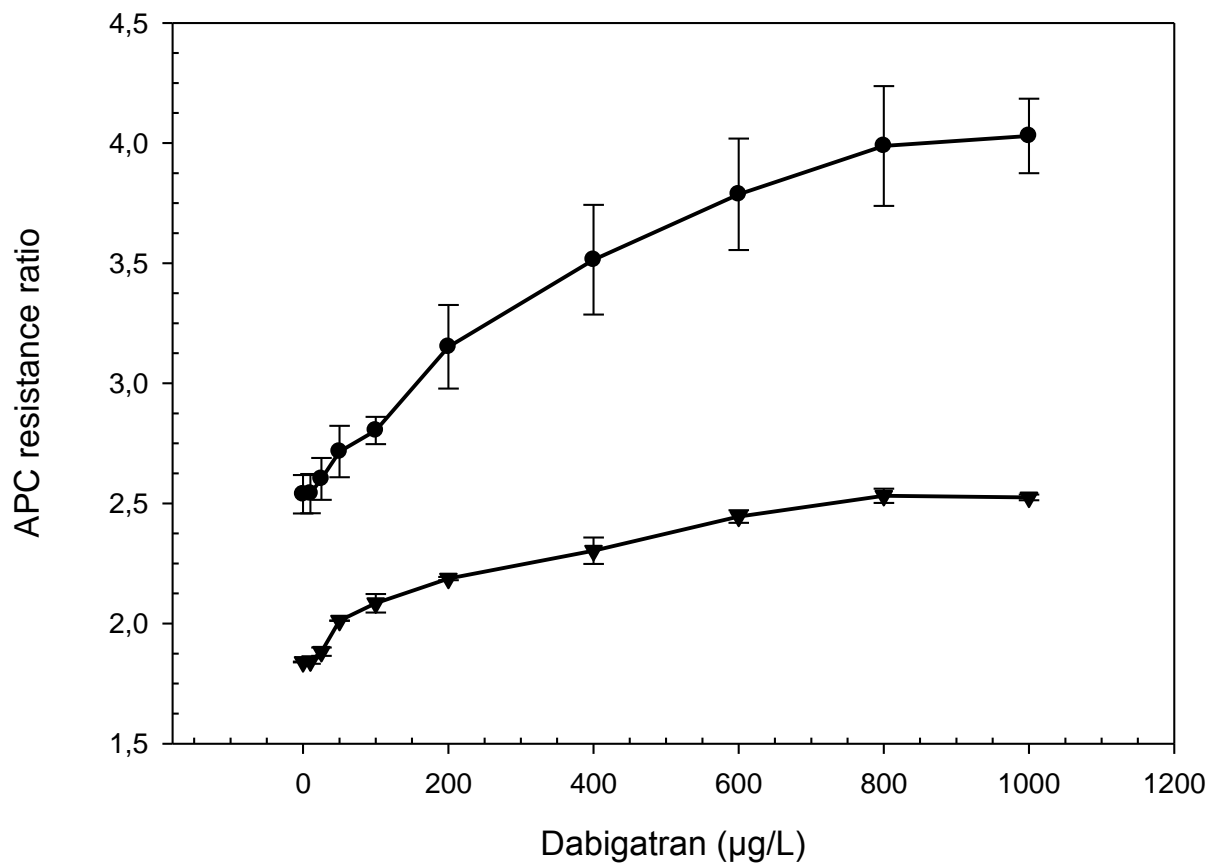


FIGURE5B

