Impact of dabigatran on a large panel of routine or specific coagulation assays

Laboratory recommendations for monitoring of dabigatran etexilate

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Summary

Due to low bioavailability and high inter-individual variability, monitoring of dabigatran may be required in specific situations to prevent the risk of bleedings or thrombosis. The aim of the study was to determine which coagulation assay(s) could be used to assess the impact of dabigatran on secondary haemostasis. Dabigatran was spiked at concentrations ranging from 4.7 ng/ml to 943.0 ng/ml in pooled citrated human platelet-poor plasma. The following clotting assays were performed: prothrombin time (PT); activated partial thromboplastin time (aPTT); thrombin time (TT); ecarin clotting time (ECT); ecarin chromogenic assay (ECA); prothrombinase-induced clotting time (PiCT); activated clotting time (ACT); Hemoclot Thrombin Inhibitor (HTI) and thrombin generation assay (TGA). A concentration-dependent prolongation of PT, dPT, and aPTT was observed with aPTT being the more sensitive test. The results varied mostly due to the clotting reagent. HTI, ECT and TGA were the most sensitive tests but are not available 24 hours a day. In addition, HTI showed a linear correlation with a good reproducibility. Dabigatran induced a concentration-dependent delay and inhibition of tissue factor-induced TGA. Cut-offs related with higher risk of bleedings or thrombosis were defined for each reagent of aPTT and HTI. In conclusion, aPTT could be used for the monitoring of dabigatran and as screening test for the risk of overdose. However, because of its higher sensitivity, good reproducibility, excellent linear correlation at all doses, its simplicity of use, and possibilities of automation, HTI should be considered as the gold-standard.

Keywords

Dabigatran, monitoring, Hemoclot Thrombin Inhibitor, new oral anticoagulant, bleeding, thrombosis

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Introduction

An anticoagulant at least as effective as available anticoagulants, which can be given orally, and is free from the challenges of vitamin K antagonists (VKAs) (1) is currently needed. Dabigatran etexilate (DE, Pradaxa®) which would not theoretically require monitoring (2) and can be given orally, is clearly a good candidate. DE is a potent, synthetic, non-peptide competitive, rapidly acting oral direct thrombin inhibitor (DTI). The first indication granted was primary prevention of venous thromboembolism (VTE) in patients undergoing elective major orthopaedic surgery (MOS) (3). In this indication DE is delivered at 220 mg once daily (qd) giving a maximal concentration (2–4 hours [h]) at steady state ($C_{max,ss}$) of 183 (5^{th}-95^{th} percentile: 67–447) ng/ml and a trough concentration at steady state ($C_{trough,ss}$) after 24 h of 37 (5^{th}-95^{th} percentile: 10–96) ng/ml (4). During the clinical development, the use of DE was associated with a clinical efficacy and a bleeding risk comparable to that seen with enoxaparin (5). In the treatment of atrial fibrillation (AF), for which DE was recently approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) (6, 7), patients are chronically exposed to the drug leading to an increased risk of bleeding and thrombosis due to drug and/or pathologic interactions and the lack of compliance. In this indication, a twice daily (bid) regimen of 150 mg DE is prescribed giving a $C_{max,ss}$ of 254 ± 70.5 ng/ml (mean ± SD) and a $C_{trough,ss}$ after 12 h of 80.3 ± 18.7 ng/ml (mean ± SD) in healthy elderly subjects (8). In the *Summary of Product Characteristics* of the EMA, a close clinical surveillance is recommended throughout the treatment period (3). Moreover, the difficult pharmacokinetic characteristics of dabigatran, the concentration-effect relationship and the bleeding risks strongly suggest that drug monitoring may be recommended. The data on interactions between dabigatran and other drugs are

also a matter of concern and are still limited. Indeed, few clinically significant drug interactions have been noted to date (9). Last but not least, patients with moderate renal impairment have an increased exposure to dabigatran. It is contraindicated in patients with severe renal impairment (10, 11). Recently, five cases of fatal haemorrhage were reported in Japan in patients with severe renal impairment taking DE, leading to regulatory actions and communications by the EMA (12, 13). Moreover, Australian authorities (14) and EMA (15) mentioned cut-off for different coagulation assays suggesting that monitoring is needed in specific clinical settings. This is particularly important due to the absence of an antidote (16–19).

Many coagulation assays are already available and some studies have been performed to evaluate the impact of dabigatran on different coagulation assays (4, 18, 20–22). However, at the present time, no guidelines exist for the monitoring of Pradaxa® (18).

The aim of the present study was to determine which coagulation assay(s) should be used to measure the pharmacodynamic effects of dabigatran among a range of routine or specific tests. Testing solutions of dabigatran used in this paper were chosen to cover the therapeutic range in the orthopaedic and AF indications. Our data will be compared to the existing studies (4, 18, 20–22), and laboratory recommendations for the monitoring of DE will be proposed.

Materials and methods

Dabigatran was spiked at increasing concentrations in pooled citrated normal human platelet-poor plasma (PPP) to measure prothrombin time (PT), dilute PT (dPT), activated partial thromboplastin time (aPTT), prothrombinase-induced clotting time (PiCT), thrombin time (TT), activated clotting time (ACT), ecarin clotting time (ECT), ecarin chromogenic assay (ECA), fibrinogen assay, antithrombin assay, hemoclot thrombin inhibitor (HTI), reptilase time and thrombin generation assay (TGA). All appropriate standards, taken from the literature and from the manufacturer's instructions, were used for calibration of the results. Methods for PT, dPT, PiCT, ACT, fibrinogen assay, antithrombin assay and reptilase time are described in Supplementary materials (available online at www.thrombosis-online.com).

Testing solutions of dabigatran

Eleven dabigatran concentrations ranging from 4.7 ng/ml to 943.0 ng/ml in the test sample mixture were prepared.

The preparation of dabigatran concentrations is presented in Supplementary materials (available online at www.thrombosis-online.com).

Whole blood and normal-pooled plasma

A total of 27 healthy individuals were included in the study. The exclusion criteria were thrombotic and/or hemorrhagic events, antiplatelet and/or anticoagulant medication, hormonal therapy, pregnancy and uptake of drugs potentially affecting the platelet and/or coagulation factor functions during the two weeks prior to the blood drawn. The study protocol was in accordance with the Declaration of Helsinki and was approved by the Medical Ethical Committee of the CHU Mont-Godinne, Université Catholique de Louvain (Yvoir, Belgium). A written informed consent was obtained from each donor. The study population displayed the following characteristics: five females and 22 males aged from 18–61 years (mean age = 26 years) with body mass index (BMI) ranging from 17.2–34.9 $kg.m^{-2}$ (mean BMI = 23.1 $kg.m^{-2}$). Blood was taken by venipuncture in the antecubital vein and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Leuven, Belgium) using a 21-gauge needle (Terumo). PPP was obtained from the supernatant fraction of the blood tubes after a double centrifugation for 15 minutes (min) at 2,000 g at room temperature. Immediately after centrifugation, PPP from the 27 donors were brought together to obtain the normal pooled plasma (NPP) which was frozen at – 80°C without any delay. Frozen NPP samples were thawed and heated to 37°C for a minimum of 5 min just before experiment.

Whole blood was obtained from a healthy member of the laboratory staff, a 24-year-old male with a BMI of 22.0 $kg.m^{-2}$. The exclusion criteria and the blood collection were the same as mentioned above.

Thrombin generation assay (TGA)

The calibrated automated TGA measurement was performed according to previously reported procedures (23, 24). For the thrombin activity measurements, 10 µl of inhibitor in phosphate-buffered saline (PBS, 6.7mM phosphate, pH 7.4), 80 µl of NPP, and 20 µl of PPP-Reagent 5 pM, PPP-Reagent LOW or MP-Reagent were mixed in a 96-well microtitre plate (Thermo Immulon 2HB, Thermo Labsystems, Enschede, The Netherlands) and were incubated for 5 min at 37°C. The plasma clotting was then triggered by the addition of 20 µl of fluorogenic substrate/calcium chloride buffered solution at 37°C. A calibration curve was also performed simultaneously using 80 µl of NPP, 10 µl of PBS, 20 µl of thrombin calibrator and 20 µl of substrate/calcium chloride-buffered solution at 37°C. The substrate hydrolysis was monitored on a microplate fluorometer Fluoroskan Ascent FL (Thermo Labsystems) with a 390/460 nm filter set using the Thrombinoscope software (v 3.0, Thrombinoscope BV, Maastricht, The Netherlands). Dabigatran was tested at nine concentrations ranging from 4.7 ng/ml to 707.3 ng/ml in the test sample mixture.

Activated partial thromboplastin time (aPTT)

Fifty µl of NPP was mixed with 50 µl of PTT-A®, CKPrest®, Cephascreen® (Diagnostica Stago, Asnieres, France); three phospholipid (cephalin) reagents containing silica, kaolin and polyphenols as activator, respectively. Clotting time was measured, after 240 seconds (sec) (300 sec for Cephascreen®) of incubation time (37°C) and then the addition of 50 µl of $CaCl_2$ 25 mM starts the measurement on a STA-R®.

The same experiment was performed with Actin FS® (Siemens Healthcare Diagnostics, Deerfield, IL, USA) which contain soja phospholipids and ellagic acid on a BCS®. A volume of 50 µl of NPP was also mixed with 50 µl of Synthasil® (Instrumentation Laboratory, Lexington, KY, USA) containing synthetic phospholipids and micronised silica. Clotting time was measured, after 300 sec of incubation time (37°C) and then the addition of 50 µl of $CaCl_2$ 20 mM starts the measurement on an ACL-TOP®.

Thrombin time (TT)

STA®-Thrombin (Diagnostica Stago) contains human thrombin at 1.5 NIH U/ml and clotting time was measured on STA-R®. Normal procedure of TT test was experimented on STA-R® by mixing 50 µl of thrombin diluted 10 fold and 50 µl of plasma sample. In addition, different dilutions of TT were assessed using the KC-10® to reduce the sensitivity but results were not conclusive and will be not shown in this paper.

Hemoclot Thrombin Inhibitors® (HTI)

Hemoclot Thrombin Inhibitors® (HYPHEN BioMed, Neuville-sur-Oise, France) is a specific chronometric assay for the determination of DTIs in plasma (4). First, tested plasma was diluted 1:8 for all concentrations of dabigatran in Owren-Koller® buffer. Fifty µl of tested plasma were mixed with 100 µl of NPP (Reagent 1) and were incubated during 240 sec. One hundred µl of highly purified human thrombin, in the α-form (Reagent 2) pre-incubated at 37°C is then added to start the reaction. Clotting times were measured on STA-R®.

Ecarin clotting time (ECT)

Ecarin (Kordia, Leiden, The Nederlands) was tested at two concentrations; 1.67 IU/ml and 5 IU/ml in the final mixture. At the concentration of 1.67 IU/ml, three different lots were tested. Fifty µl of NPP was mixed with 100 µl of ecarin reagent without incubation time. Clotting time was measured on STA-R®.

Ecarin chromogenic assay (ECA)

According to the instructions of the manufacturer (Diagnostica Stago), 100 µl of ECA prothrombin buffer pre-warmed at 37°C, 25 µl of plasma sample and 25 µl of plasma buffer pre-warmed at 37°C were incubated at 37°C for 60 sec. The measurement started after the addition of 50 µl ECA reagent on STA-R®.

Statistical analysis

To assess the advantage of one assay over another for the determination of dabigatran, a statistical comparison of sensitivity, linearity and reproducibility of these assays was performed. The sensitivity is defined as the final concentration in dabigatran needed to double the measured parameter (2 x Clotting Time (CT); 2 x lag time or 2 x OD/min) (see ▶Table 4 in the Suppl. materials, available online at www.thrombosis-online.com). This was achieved by different mathematical models fitting at best all the points. For each assay and reagent, three repeated measures for each concentration of dabigatran were recorded. Results presented are the mean value and the standard deviation. GraphPad Prism 5.01® for Windows® was used to perform statistical analysis. The concentration represented in the figures is the concentration in dabigatran in the initial sample. Ratio expressed the clotting time of a NPP spiked with dabigatran divided by the clotting time of NPP without spiking, both run in the same series.

- Linear regression was used to determine ECT, HTI and ECA (sec).
- The equation one phase association was used for aPTT and dPT.
- The equation exponential growth was used for PT and ACT.
- The equation two phases association was used for PiCT.
- The equation one phase decay was used for ECA (OD/min).
- The mathematical model was accepted if R^2 was higher than 0.99.

Reproducibility was expressed as the coefficient of variation (CV) [(standard deviation/mean)-*100] of the triplicate for each concentration and each test. Minimum, mean and maximum CV were determined for each test and compared between tests (see ▶Table 5 in the Suppl. materials, available online at www.thrombosis-online.com).

The lower limit of quantitation was calculated as follow: [(10*standard deviation of Y0)/ slope]. The upper limit of quantitation reflects the concentration from which results were unreliable (concentration above 941 ng/ml were not tested). For assays that depend on the reagent, the dynamic range presented was the mean of the individual lower and upper limit of quantitation of the different reagents.

For aPTT and HTI, the intra- and inter-assays variability, expressed in mean CV, was also assessed by measuring 10 replicates of 5 different concentrations (500; 200; 67; 43 and 10 ng/ml). The mean CV represented the sum of the CV for the five concentrations divided by 5 (i.e. the number of concentrations). For the inter-

Table 1: Summary of the assays useful for the assessment of the pharmacodynamic effects of dabigatran in plasma. aPTT = activated partial thromboplastin time; HTI = Hemoclot Thrombin Inhibitor®; ECA = ecarin chromogenic assay; ECT = ecarin clotting time; TGA = thrombin generation assay; TT = thrombin time; PT = prothrombin time; PiCT = prothrombinase-induced clotting time; ACT = activated clotting time; dPT = dilute prothrombin time; N.D. = not determined.

	Useful for monitoring		Reliable but requires laboratory experience				Not recommended				
	aPTT	HTI	ECA	ECT		TGA	TT	PT	PiCT	ACT	dPT
				5 IU/ml	1.67 IU/ml						
Sensitivity (ng/ml)†	83–120	8	4	15	21	66 to 80	Too sensitive	175 –248	33	361	35 –93
Dynamic range of quantitation (ng/ml) ‡	58 – 941	53 – 941	6 – 47	24 – 941	21 – 471	N.D.	5 – 25	31 – 941	37 – 200	N.D.	54 – 941
Reproducibility (%) ††	0.4 –8.5	1.0	6.8	0.9	0.7	6.0	N.D.	0.8 – 1.2	3.7	3.9	1.2–7.6
Dependence of reagent	Yes	No	No	No	No	Yes	N.D.	Yes	No	No	Yes
Linearity of the response	No	Yes	Yes	Yes	Yes	No	Yes	No	No	No	No

† Sensitivity expressed the concentration needed to double the evaluated parameter (2 x clotting time (CT); 2 x OD/min; 2 x lag time (LT)). ‡ The lower limit of quantitation was calculated as follow: [(10*standard deviation of Y0)/ slope]. The upper limit of quantitation reflects the concentration from which results were unreliable (concentration above 941ng/mL were not tested). †† Reproducibility was expressed as the coefficient of variation [CV (standard deviation/mean*100)] of the triplicate for each concentration and each test.

assay variability, each of the five concentrations was measured once a day during 10 days with the same lot of reagents.

Results

►Table 1 summarises the sensitivity, the reproducibility, the linearity and the dynamic range of quantitation of the different assays performed in this study. Detailed information on the 2x CT; 2x lag time or 2x OD/min values, linearity and reproducibility results, is presented in ►Tables 4 and 5 in the Suppl. materials (available online at www.thrombosis-online.com).

Thrombin generation assay (TGA)

Dabigatran showed a concentration-dependent prolongation of the lag time of the TF induced pathway (►Fig. 1).

Activated partial thromboplastin time (aPTT)

aPTT showed a concentration-dependent prolongation of clotting time (►Fig. 2). The variability ranged from 0.7% (for CKPrest®) to 1.5% (for Actin FS®) in intra-assay and from 1.2% (for Cephascreen®) to 3.1% (for PTT-A®) in inter-assay.

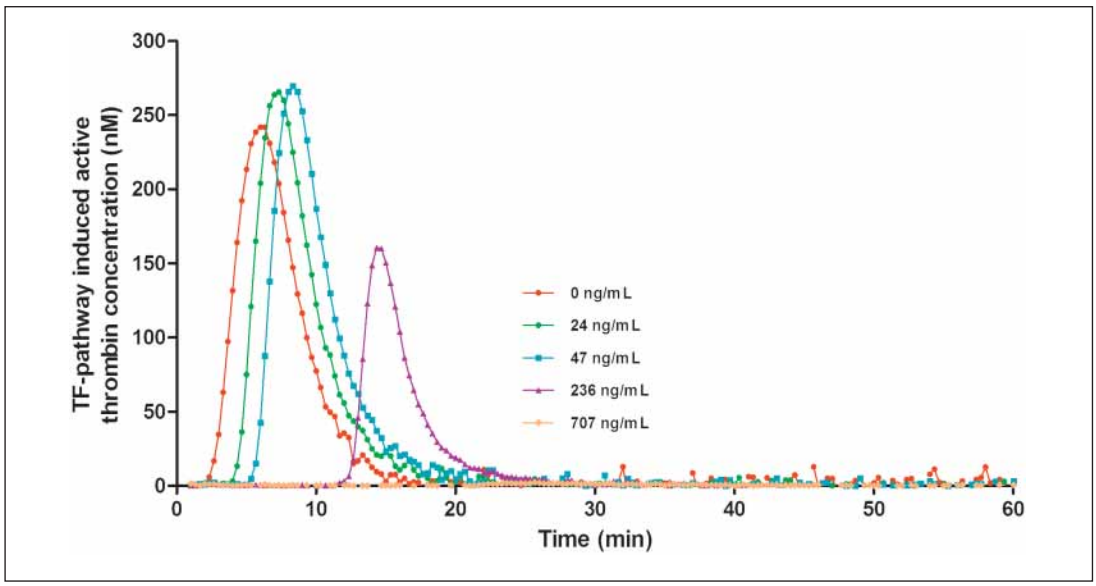

Figure 1: Influence of dabigatran on the thrombin generation assay (TGA). In the TF-induced pathway, dabigatran mainly delayed the initiation phase with a strong dose-dependent increase of lag time and T_{max} and a slight dose-dependent decrease of C_{max} and ETP.

Thrombin time (TT)

TT was too sensitive leading to high variability for concentrations higher than 25 ng/ml in the initial blood sample (data not shown).

Hemoclot Thrombin Inhibitor® (HTI)

HTI showed a concentration dependent prolongation of clotting time (▶Fig. 3). The variability was 0.9% and 2.5% in intra- and inter-assay, respectively.

Ecarin clotting time (ECT)

ECT showed a concentration-dependent prolongation of clotting time. For the two concentrations of ecarin tested, 2 x CT was 15 ng/ml and 21 ng/ml for ECT 5 IU/ml and 1.67 IU/ml in final mixture, respectively (▶Fig. 4). Reproducibility [mean CV (%)] was 0.7% for ECT 1.67 IU/ml and 0.9% for ECT 5 IU/ml, respectively. Inter-lot reproducibility showed a similar response until 470 ng/ml in the initial sample with a CV = 3.1% (2 x CT was 17 ng/ml; 23 ng/ml and 21 ng/ml, respectively) (▶Fig. 4). Nevertheless, with concentration higher than 470 ng/ml, some samples showed a coagulation time exceeding the limit of measurement. Thus, CV increased with a value of 5.1% and sensitivity decreased sharply (38 ng/ml, 41.5 ng/ml and 39 ng/ml, respectively, for the three different lots tested) (data not shown).

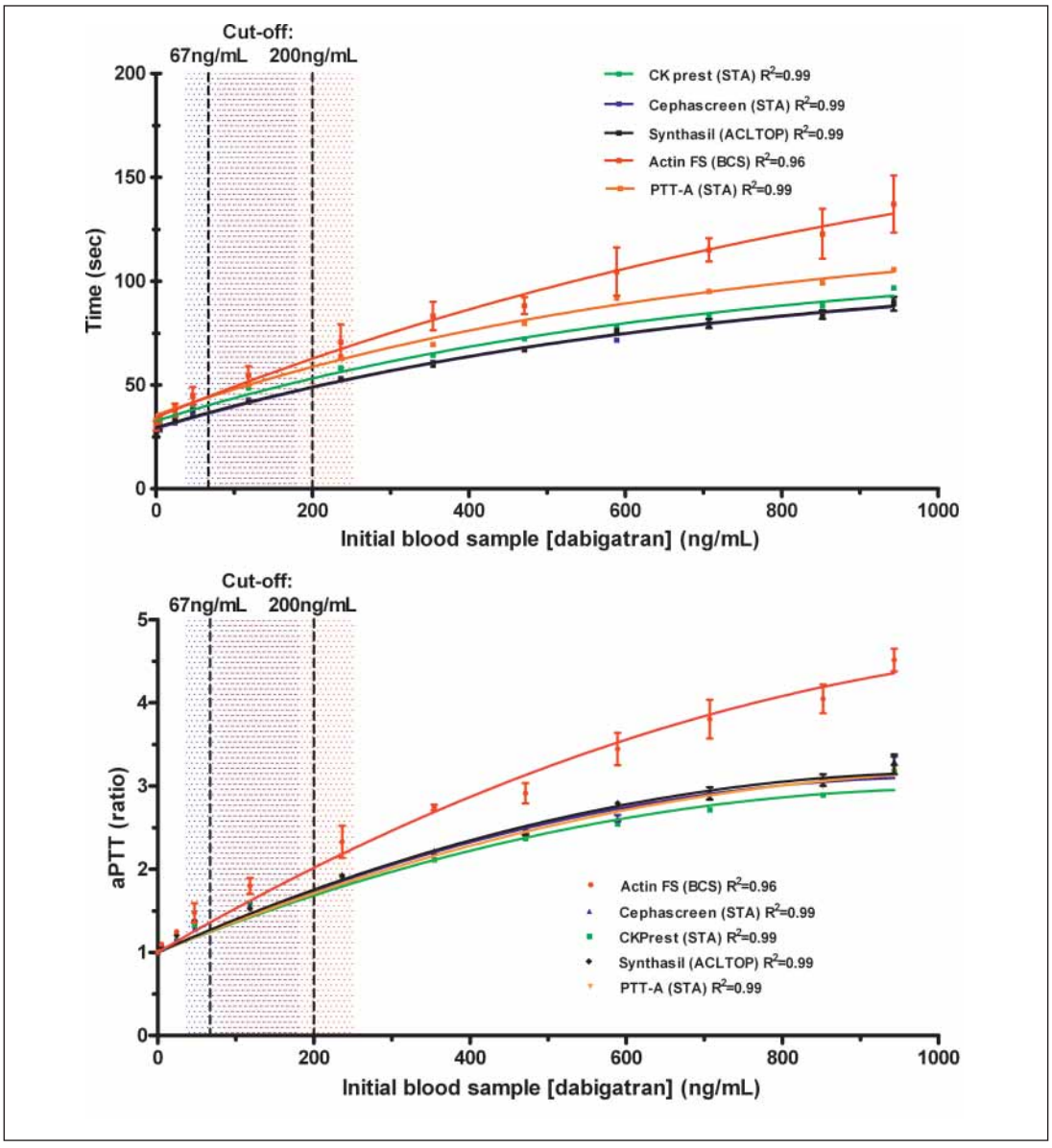

Figure 2: Influence of dabigatran on activated the partial thromboplastin time (aPTT). aPTT showed a concentration-dependent prolongation of clotting time with a loss of sensitivity for higher concentrations in dabigatran. Actin FS® was the most sensitive test with a 2 x CT of 83 ng/ml but suffered from a lower reproducibility. The box in blue and red represents the therapeutic range in the orthopaedic indication and in AF patients, respectively. Cut-offs at C_{trough} of 67 ng/ml and 200 ng/ml are defined for the orthopaedic indication (220 mg qd) and for the AF patients (150 mg bid), respectively (15).

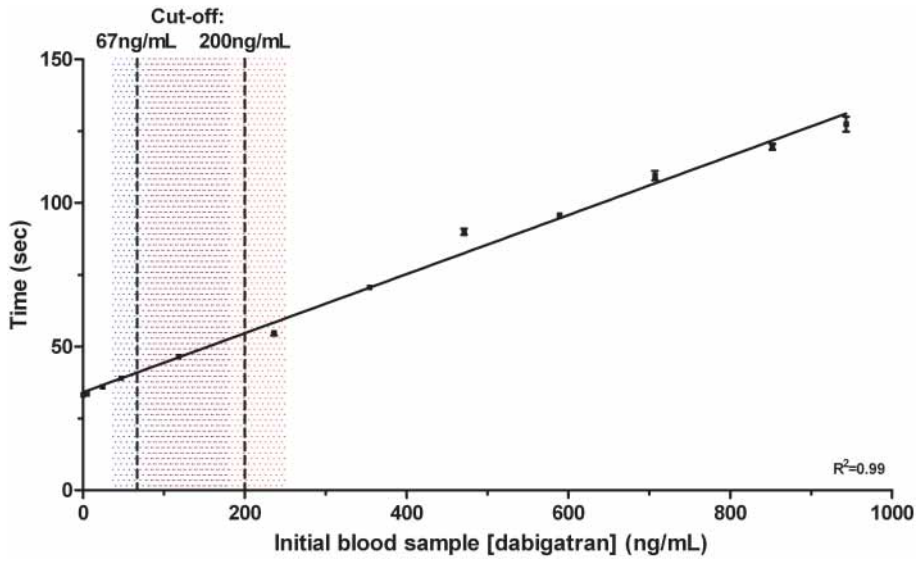

Figure 3: Influence of dabigatran on Hemoclot Thrombin Inhibitor® (HTI). HTI showed a concentration-dependent prolongation of clotting time with a linear relation and a good reproducibility. HTI is the most sensitive assay with a 2 x CT of 8 ng/ml. The box in blue and red represents the therapeutic range in the orthopaedic indication and in AF patients, respectively. Cut-offs at C_{trough} of 67 ng/ml and 200 ng/ml are defined for the orthopaedic indication (220 mg qd) and for AF patients (150 mg bid), respectively (15).

Ecarin chromogenic assay (ECA)

ECA showed a concentration-dependent decrease of OD/min. 2 x OD/min was 4 ng/ml, but the sensitivity decreased with the highest concentrations of dabigatran making the relation non-linear (see ▶ Fig. 9 in the Suppl. materials, available online at www.thrombosis-online.com). By measuring the time necessary to obtain an OD = 1, results showed a concentration needed to double the time necessary to reach the value of 1 in absorbance of 7 ng/ml, but results were not linear for concentrations lower than 50 ng/ml (▶ Fig. 5). For all the concentrations above and until 940 ng/ml, the relation was linear as shown in a previous study performed on argatroban (25).

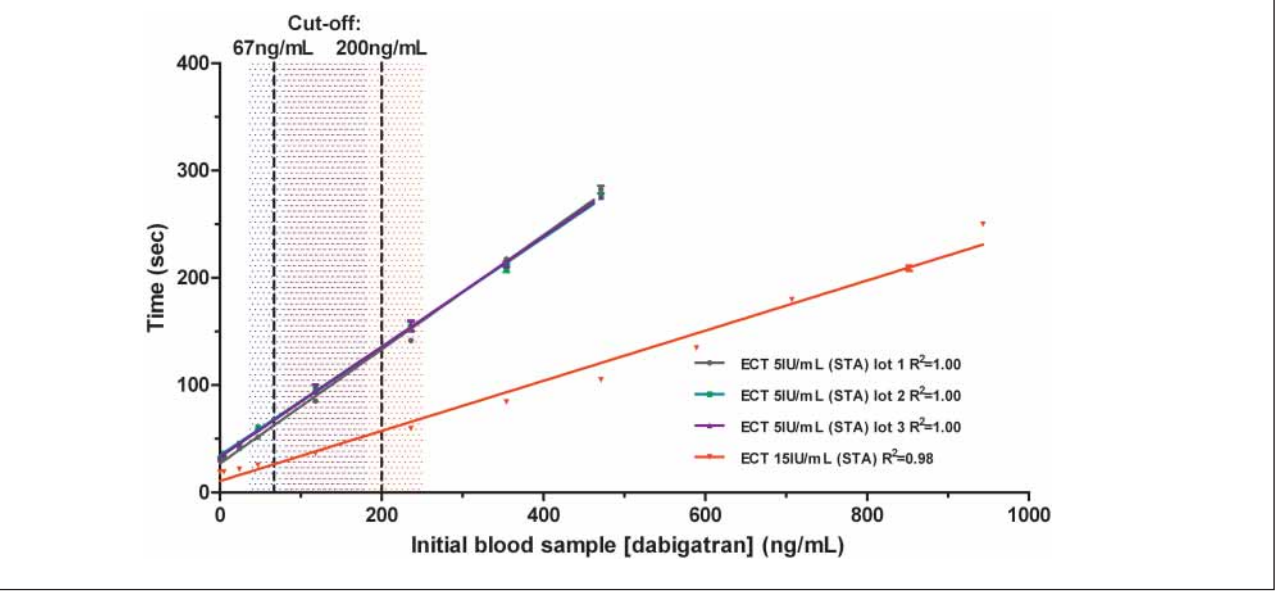

Figure 4: Influence of dabigatran on ecarin clotting time (ECT) 5 IU/ml and 1.66 IU/ml in final concentration. Mean 2 x CT was 21 ng/ml for ECT 1.66 IU/ml versus 15 ng/ml for ECT 5 IU/ml. ECT 1.66 IU/ml showed a better correlation coefficient than ECT 5 IU/ml (R^2>0.99 vs. 0.98). Results obtained inter-lots were very reproducible up to 470 ng/ml. The box in blue and red represents the therapeutic range in the orthopaedic indication and in AF patients, respectively. Cut-offs at C_{trough} of 67 ng/ml and 200 ng/ml are defined for the orthopaedic indication (220 mg qd) and for AF patients (150 mg bid), respectively (15).

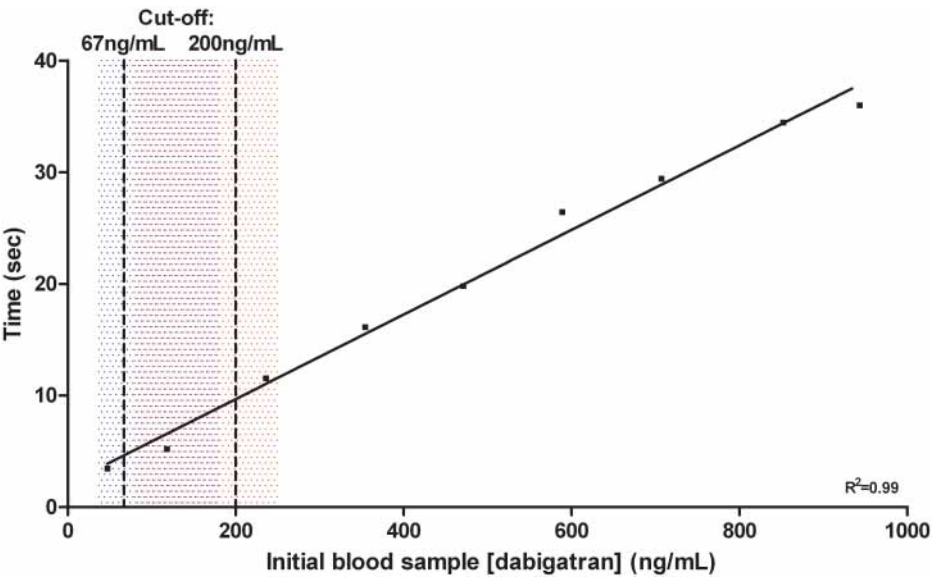

Figure 5: Influence of dabigatran on time needed to obtain an OD=1 with the ecarin chromogenic assay (ECA). By measuring the time needed to obtain an OD=1, sensitivity of the test decreased but a linear relation from 47 ng/ml to 943 ng/ml was observed (results below 6 ng/ml were not reliable). The box in blue and red represents the therapeutic range in the orthopaedic indication and in AF patients, respectively. Cut-offs at C_{trough} of 67 ng/ml and 200 ng/ml are defined for the orthopaedic indication (220 mg qd) and for AF patients (150 mg bid), respectively (15).

Prothrombin time (PT)

Dabigatran showed an exponential concentration-dependent prolongation of PT (see ▶Fig. 6 in the Suppl. materials, available online at www.thrombosis-online.com). The increase in clotting time was dependent on the reagent used.

Dilute prothrombin time (dPT)

dPT showed an exponential concentration-dependent prolongation of clotting time (see ▶Fig. 7 in the Suppl. materials, available online at www.thrombosis-online.com).

Prothrombinase-induced clotting time (PiCT)

PiCT showed a two-phase association relation until a plateau at 360 ng/ml (see ▶Fig. 8 in the Suppl. materials, available online at www.thrombosis-online.com).

Activated clotting time (ACT)

ACT showed a concentration-dependant prolongation of clotting time (data not shown)

Fibrinogen assay (Clauss method) and reptilase time

Dabigatran has no effect on the fibrinogen assay and the reptilase time tested in this study (data not shown).

Antithrombin chromogenic assay

Dabigatran induced a concentration-dependant influence of the rate of functional antithrombin. Basal value of functional antithrombin was 106% and it increased up to 128% with dabigatran concentration in the analytical sample of 943 ng/ml (see ▶Fig. 10 in the Suppl. materials, available online at www.thrombosis-online.com).

Discussion

DE (Pradaxa®) is a direct and reversible thrombin inhibitor. It received a first marketing authorisation by the EMA in March 2008 for the following indication: "*Primary prevention of venous thromboembolic events in adult patients who have undergone elective total hip replacement surgery or total knee replacement surgery*" (3) which was extended to: "*Prevention of stroke and systemic embolism in adult patients with non-valvular atrial fibrillation*" (6) on April 2011. This second marketing authorisation was already approved by the FDA in October 2010 (7). A laboratory control is not con-

sidered to be necessary to adjust the dosage (26). However, drug monitoring remains under passionate debate (26–29) and even if it is not recommended by the marketing authorisation holder, it is strongly suggested by different authors
and the opinion of the regulator has recently changed about this, suggesting that monitoring may be necessary in specific clinical conditions. The following situations might require laboratory control for patient management: patients presenting in emergency with adverse events (thrombosis or haemorrhage), necessity of immediate reversal of anticoagulation, renal failure, liver failure, suspicion or known interaction with other drugs (26) and bridging.

No guidelines are currently available on which assay to use to perform such measurements (18). This is of particular importance for the novel chronic indications of Pradaxa® such as VTE and non-valvular AF (30–33). In this last indication, five cases of fatal haemorrhage were recently reported in Japan in patients taking DE (12). However, these cases should be interpreted with caution since no information about severe renal impairment is provided but they reflect the necessity of monitoring the anticoagulation by a rapid, widely available, specific and sensitive assay. Urgent invasive action may also play a role in the necessity of a gold standard assay to monitor patients under DE despite the fact that there is still no antidote to neutralise the effects of dabigatran (16–18). In addition, monitoring of dabigatran would be necessary for the clinician in different conditions also including misuse, abuse (resulting in adverse events) or lack of compliance (26).

Aim of the study

The aim of the study was to determine the most sensitive and reliable coagulation assay(s) that could be used routinely to assess the pharmacodynamic effects of dabigatran. We tested a large panel of reagents currently available in the majority of biological laboratories with a broad plasma concentration range of dabigatran. Some studies have already been performed with the same goals but were limited by the low panel of reagent used, the lack of information on these reagents, the absence of comparison between reagents, and the weak concentration range used (4, 18, 21). One study performed by Lindahl et al. (22) compared the effect of dabigatran on PT, aPTT, AT and fibrinogen. Nevertheless, these tests are not easily comparable due to the lack of standardisation in the expression of the results. Therefore, it is still difficult for the clinician to be able to make a rational choice for the monitoring of patients treated with DE.

One of the strengths of our study is the large range of dabigatran concentrations used including the therapeutic range in the orthopaedic and AF indications as well as higher concentrations. It also includes an estimation of the raw values obtain for recommended assays to reach the cut-offs above which there is an increasing risk of bleeding (as defined by the Australian authorities regulators [14] and the EMA [15]) or below which the patient is at sub-therapeutic level of anticoagulation (▶Table 3). Comparisons were made in order to select the most sensitive, linear and reproducible type of assay (▶Table 1). More details of these results are presented in ▶Tables 4 and 5 in the Suppl. materials (available online www.thrombosis-online.com).

Routinely used and more specific assays: Their utility in the laboratory assessment of dabigatran

The effects of dabigatran on PT showed a concentration-dependent prolongation of clotting time which can be defined by an exponential function. However, all PT reagents were much less sensitive than aPTT. PT is therefore not recommended for the monitoring of dabigatran. Moreover, the relation was not linear with a lack of sensitivity in lower concentration including the therapeutic range (see ▶Fig. 6 in the Suppl. materials, available online www.thrombosis-online.com). Corollary, the sensitivity for dPT reagent was high ranging from 35 ng/ml for Neoplastin R® diluted 1/256 to 93 ng/ml for Recombiplastin® diluted 1/64. Our results confirm the hypothesis of Samama et al., suggesting that dPT is more sensitive than PT (34) since a prothrombin time using diluted thromboplastin may simulate physiologic events much better than a classical PT (35). However, the relation was not linear with a lack of sensitivity in higher concentration (see ▶Fig. 7 in the Suppl. materials, available online www.thrombosis-online.com). Moreover, the reproducibility was lower than the one of PT probably due to the fact that the assay was performed on KC-10 which is not fully automated. For aPTT, all reagents were equivalent in terms of 2 x CT (83–120 ng/ml). The most sensitive was Actin FS® with reproducibility for the calibration curve about 8.5% which is the worst for aPTT reagents. This value exceeds the inter-laboratory CV of the Belgium National Quality Assessment. Nevertheless, the assessment of the variability ranged from 0.7% for CKPrest® to 1.5% for Actin FS® in intra-assay and from 1.2% for Cephascreen® to 3.1% for PTT-A® in inter-assay showing better reproducibility for Actin FS®. In terms of sensitivity, results are consistent with those from Wienen et al. who claimed a 2 x CT of about 230 nM (108 ng/ml) for the aPTT test. However, the reagents were not specified in their study (21). In the study of Lindahl et al., 2 x CT varied from 227 ng/ml for aPTT-DG® reagent to 286 ng/ml for TriniCLOT® reagent. PTT-A® reagent was also tested by both groups and 2 x CT differed (▶Table 2). The reason for this difference is unclear.

PiCT® is a specific test known to be sensitive to the effect of thrombin inhibitors (36). In our study, we confirmed that this test was one of the more sensitive (2 x CT = 33 ng/ml) but show a plateau at 360 ng/ml (see ▶Fig. 8 in the Suppl. materials, available online www.thrombosis-online.com). Therefore, this test is not useful to adequately assess concentration of dabigatran that may encounter in patients with bleeding risk.

Due to its ease of use, its good sensitivity and its linear relation, ECT could be used to monitor patients on dabigatran. However, some limitations need to be highlighted that may limit its use. Firstly, it has not been standardised as pointed out by other authors (4, 29). Secondly, as illustrated by the present study, there is an inter-lot variability for higher dabigatran concentrations (mean

Table 2: Summary of assays performed in the different studies and expression of the concentration in dabigatran needed to double evaluated coagulation parameter. aPTT = activated partial thromboplastin time; HTI = Hemoclot Thrombin Inhibitor®; ECA = ecarin chromogenic assay; ECT = ecarin clotting time; TGA = thrombin generation assay; TT = thrombin time; PT = prothrombin time; PiCT = prothrombinase-induced clotting time; ACT = activated clotting time; dPT = dilute prothrombin time; N.A. = not applicable.

		Wienen et al. 2007 (21)	van Ryn et al. 2010 (4)	Lindahl et al. 2011 (22)	Present paper
TGA	PPP-Reagent	47 ng/ml	N.A.	N.A.	66 ng/ml
	PPP–Reagent Low	N.A.	N.A.	N.A.	70 ng/ml
	MP-Reagent	N.A.	N.A.	N.A.	80 ng/ml
PT	Innovin[2]	830 nM† (391 ng/ml)	903 nM†‡ (426 ng/ml)	More sensitive than other thromboplastins	175 ng/ml
	Neoplastin CI+[2]			N.A.	230 ng/ml
	Neoplastin R[2]			N.A.	235 ng/ml
	Nycotest PT[1]			Results expressed in INR. Less sensitive than Innovin without any detail.	N.A.
	Owren's PT[1]				N.A
	Simple Simon[1]				N.A.
	SPA +[1]				N.A.
	Recombiplastin[2]			N.A.	248 ng/ml
dPT	Innovin	N.A.	N.A.	N.A.	65 ng/ml
	Neoplastin CI+				62 ng/ml
	Neoplastin R				35 ng/ml
	Recombiplastin				93 ng/ml
aPTT	Actin FS	230 nM† (108 ng/ml)	N.A.	N.A.	83 ng/ml
	Actin FSL			591 nM (279 ng/ml)	N.A.
	APTT-DG			481 nM (227 ng/ml)	N.A.
	APTT-SP			597nM (281 ng/ml)	N.A.
	Cephascreen			N.A.	107 ng/ml
	CKPrest			N.A.	120 ng/ml
	PTT-A			536 nM (253 ng/ml)	112 ng/ml
	Synthasil			N.A.	110 ng/ml
	TriniCLOT			606 nM (286 ng/ml)	N.A.
TT		N.A.	87 nM ‡ (41 ng/ml)	N.A.	Too sensitive
HTI		N.A.	N.A.	N.A.	8 ng/ml
PiCT		N.A.	N.A.	N.A.	33 ng/ml
ECT	5 IU/ml	180 nM (85 ng/ml)	299nM‡ (141 ng/ml)	N.A.	15 ng/ml
	1.67 IU/ml				21 ng/ml
ECA		N.A.	N.A.	N.A.	4 ng/ml
ACT		N.A.	424 nM (200 ng/ml)	N.A.	361 ng/ml
Fibrinogen	Dade thrombin	N.A.	N.A.	Not influenced	N.A.
	Fibri-Prest			Not influenced	N.A.
	Fibrinogen C			Influenced	N.A.
	Multifibren U			Influenced	N.A.
	STA-Fibrinogen			N.A.	Not influenced
Antithrombin	Berichrom ATIII	N.A.	N.A.	Influenced	N.A.
	Coamatic LR			Not influenced	N.A.
	Stachrom AT III			Influenced	Influenced

[1] These PT tests are Owren type PT assays. [2] These PT tests are Quick PT assays. † Reagent not specified. ‡ Calculated from the equation referenced by the authors (4).

CV: 3.1% and 5.1% when excluding and including value above 470 ng/ml, respectively). A specific calibration for each lot is therefore recommended.

ECA was presented early as a method for quantitative determination of direct thrombin inhibitors like hirudin or argatroban by Lange et al. (25). Thus, by measuring time needed to obtain an absorbance of 1, results showed an interesting linear response for concentration from 47 ng/ml to 943 ng/ml (▶Fig. 5). ECA is also the most sensitive assay in our study. This test may be valuable to measure the effect of excess concentrations of dabigatran. Nevertheless, this test is not widely available and the necessity of an adaptation of the results (transformation from results initially in OD/min to times needed to reach an optical density of 1) may limit its use in clinical laboratories.

ACT showed an exponential growth relation with a weak sensitivity which limits its interest.

TT was theoretically useful to assess the effect of dabigatran on the coagulation since it is specifically designed to measure the impact on thrombin. However, this test was found too sensitive for concentration higher than 25 ng/ml, as mentioned previously (4).

We also tested the impact of dabigatran on less routinely used and more specific assays such as TGA and HTI. On TGA, dabigatran induced a concentration-dependent delay and inhibition of the TF-induced thrombin generation with 5 pM TF or 1 pM TF with 4 µM PL and 16.7 mM $CaCl_2$. The drug strongly increased the lag time and T_{max} whereas it slightly decreased the C_{max} and ETP. The lag time was the most informative parameter of the calibrated automated thrombogram (CAT) thanks to a high sensitivity and a low variability (CV ≤6%). These results are in agreement with those published by Wienen et al. (2 x lag time ≈100 nM, ≈47 ng/ml) (21) and by Samama et al. (37). For lower concentrations of dabigatran (from 25 and 50 ng/ml in the TF pathway and most concentration for the two other induced pathways), we noticed an increase in the ETP and C_{max}. These observations were already reported by Wagenvoord et al. (38) who claimed that it represents *in fine* a pure artefact. This artefact was explained by the fact that CAT may overestimate the action of thrombin by confounding a peak produced by the α2-macroglobulin-thrombin complex in presence of DTIs. This peak normally does not appear in the presence of other anticoagulants and the initial algorithm of CAT is not able to interpret correctly this issue.

Another specific test for DTIs, HTI was one of the most sensitive with a 2 x CT of 8 ng/ml. Reproducibility for the calibration curve was quite good (1.0%) and the variability was 0.9% and 2.5% in intra-assay and inter-assay, respectively. Moreover, the linear correlation coefficient (r^2) was 1.00 and the addition of NPP (reagent 1) renders it insensitive to fibrinogen. This test has been already proposed by van Ryn et al. (4), but its sensitivity was not defined.

Interference with diagnostic tests

The interference of dabigatran on fibrinogen assay is of particular interest for the clinician facing excessive blood loss (22). However, differences between fibrinogen test reagents have been reported by Lindahl et al. (▶Table 2). In our study STA® Fibrinogen-5 (Diagnostica Stago) was not influenced by the presence of dabigatran whatever the concentration used. On reptilase time, dabigatran did not show any effect as well.

The influence of dabigatran on the measurement of antithrombin activity is also of importance for physicians because antithrombin deficiency is associated with a risk of venous thrombosis (39). In our study, antithrombin activity measured by a factor IIa-based antithrombin assay (STA®-Stachrom® ATIII) was influenced by dabigatran. The antithrombin activity can also be measured by a factor Xa -based assay (39). In the study of Lindahl et al., the factor Xa-based antithrombin assays were less influenced by dabigatran (22). It is therefore recommended to use factor Xa-based antithrombin assays to measure antithrombin activity in patient on dabigatran.

Proposal of good laboratory practise to assess the pharmacodynamic effects of dabigatran in patients

Thus, as recently recommended by Australian regulatory authorities, aPTT could be used as a screening test to exclude a bleeding risk associated with DE administration (14). Another important point is the standardisation of the time between the last intake of dabigatran and the time of blood collection as these influence dabigatran concentration and thus the results of the coagulation assay. C_{trough} may be more interesting than C_{max} since the absorption phase and C_{max} are more variable than C_{trough}. Nevertheless, if C_{trough} could be used to measure a risk of bleeding with accurate define cut-off, it seems inappropriate to evaluate compliance. Indeed, the range of local normal values for different aPTT (▶Table 3) showed minor differences in comparison with C_{trough} in AF. Moreover, in real life, the baseline clotting time (before drug administration) will often not be determined precluding to detect a relative change in aPTT. Thus, in this case, more specific and sensitive assays should be used.

In a patient taking 150 mg of DE bid, a C_{trough} higher than 200 ng/ml is correlated with an increased risk of bleeding while this value is reduced to 67 ng/ml for patients in primary prevention of VTE (15). In ▶Table 3, cut-offs expressed in seconds and ratios are proposed for HTI and different aPTT reagents for both the risk of bleeding and the sub-therapeutic level of anticoagulation according to the different indications. Above these cut-off values, cautionary measures should be taken together with patient history and renal function to judge whether the patient is at risk or not.

Nevertheless, cut-offs in seconds have already been proposed by the Australian Authorities. Thus, in patient taking 150 mg DE bid regimen, an aPTT above 80 sec at trough (corresponding normally to a C_{trough} about 200 ng/ml) is correlated with an increased risk of bleeding (14). This cut-off is reduced to 45 sec (corresponding normally to a C_{trough} of 67 ng/ml) for patients taking 220 mg DE qd for primary prevention of VTE (15). However, as illustrated in our study, these cut-offs have to be adapted according to the aPTT

Table 3: Baseline, mean C_{trough} and C_{max}, and cut-offs associated with a risk of bleeding or with sub-therapeutic level for aPTT (Actin FS®; Cephascreen®; CKPrest®; PTT-A®; Synthasil®) and Hemoclot Thrombin Inhibitor® in major orthopaedic surgery (MOS) (A) and in atrial fibrillation (AF) (B). A) In MOS: dabigatran etexilate 220 mg qd. B) In AF: dabigatran etexilate 150 mg bid. The results are expressed in seconds and/or ratio of the clotting time of a NPP spiked with dabigatran divided by the clotting time of NPP without spiking. NPP = normal pooled plasma; AF = atrial fibrillation; MOS = major orthopaedic surgery; N.D.: = not determined.

A) In major orthopaedic surgery (MOS): dabigatran etexilate 200 mg qd

Reagent	Local normal values	Baseline time	Clotting time corresponding to a sub-therapeutic level in MOS at C_{trough} (i.e. 10 ng/ml) (4)†		Clotting time corresponding to a sub-therapeutic level in MOS at C_{max} (i.e. 62 ng/ml) (4) †		Clotting time corresponding to mean C_{trough} in MOS (i.e. 37 ng/ml) (4)		Clotting time corresponding to mean C_{max} in MOS (i.e. 183 ng/ml) (4)		Clotting time corresponding to a risk of bleedings in MOS at C_{trough} (i.e. 67 ng/ml) (15)	
	Sec	Sec	Sec	Ratio	Sec	Ratio	Sec	Ratio	Sec	Ratio	Sec	Ratio
Actin FS®	25.8–33.2	30.3	35.9	1.18	43.7	1.44	40.0	1.32	60.5	2.00	44.3	1.48
Cephascreen®	N.D.	27.4	30.1	1.10	35.7	1.30	33.0	1.20	47.2	1.72	36.2	1.32
CKPrest®	26.7– 37.6	30.5	33.9	1.11	39.7	1.30	37.0	1.21	51.6	1.69	40.2	1.32
PTT-A®	28.0–39.0	33.2	36.8	1.11	43.5	1.31	40.3	1.21	57.0	1.72	44.0	1.32
Synthasil®	25.8– 33.2	27.5	30.7	1.12	36.2	1.32	33.6	1.22	47.6	1.73	36.7	1.33
Hemoclot Thrombin Inhibitor®	N.D.	33.3	35.1	1.05	40.5	1.22	37.9	1.14	52.9	1.59	40.9	1.23

† Sub-therapeutic level in MOS is defined as the lower 5th percentile at C_{trough} and C_{max}. (4)

B) In atrial fibrillation (AF): dabigatrain etexilate 150 mg bid

Reagent	Local normal values	Baseline time	Clotting time corresponding to a sub-therapeutic level in AF at C_{trough} (i.e. 43 ng/ml) (8)‡		Clotting time corresponding to a sub-therapeutic level in AF at C_{max} (i.e. 113 ng/ml) (8)‡		Clotting time corresponding to mean C_{trough} in AF '(i.e. 80 ng/ml) (8)		Clotting Time corresponding to mean C_{max} in AF (i.e. 254 ng/ml) (8)		Clotting time corresponding to a risk a bleeding in AF at C_{trough} (i.e. 200 ng/ml) (15)	
	Sec	Sec	Sec	Ratio	Sec	Ratio	Sec	Ratio	Sec	Ratio	Sec	Ratio
Actin FS®	25.8–33.2	30.3	44.4	1.46	51.0	1.68	46.3	1.53	69.5	2.29	62.5	2.06
Cephascreen®	N.D.	27.4	33.7	1.23	40.8	1.49	37.5	1.37	53.1	1.94	48.6	1.77
CKPrest®	26.7– 37.6	30.5	37.6	1.23	45.0	1.48	41.6	1.36	57.7	1.89	53.0	1.74
PTT-A®	28.0–39.0	33.2	41.1	1.24	49.5	1.49	45.2	1.36	64.0	1.93	58.6	1.77
Synthasil®	25.8– 33.2	27.5	34.2	1.24	41.3	1.50	38.0	1.39	53.5	1.94	49.0	1.78
Hemoclot Thrombin Inhibitor®	N.D.	33.3	38.5	1.16	45.7	1.37	42.3	1.27	60.2	1.81	54.7	1.64

‡ Sub-therapeutic level in AF is defined as: mean-2*standard deviation; for C_{trough} and C_{max}. (8).

reagent. Using five different reagents, the aPTT was lower than 80 sec for this concentration in dabigatran. For example, as represented in ▶Table 3, the clotting time related to a concentration of 200 ng/ml varies from 48.6 to 62.5 sec, depending on the reagent. Accordingly, the aPTT ratio for this concentration ranges from 1.74 to 2.06. These differences may lead to a misinterpretation if inappropriate cut-offs are used. In addition, the response of an aPTT reagent varies according to the lot number, and coagulometers showed differences in endpoint detection (40). Therefore, each laboratory should calibrate each lot of its aPTT reagent on one instrument by spiking a NPP with at least six dabigatran concentrations ranging from 0 to 1,000 ng/ml. In one particular laboratory, the aPTT is also affected by pre-analytical (inappropriate collection, handling and/or storage) and biological variables (lupus anticoagulant, hereditary or acquired factor deficiencies, hepatic insufficiency, vitamin K deficiency, disseminated intravascular coagulopathy, increased risk of thromboembolic events, hyperthyroidism, patients with diabetes, women in pregnancy, cancer and myocardial infarction) (40–42). However, on one hand, a prolonged aPTT is not strongly predictive of haemorrhage, and, on the other hand, patients may experience bleeding while displaying a normal aPTT (41). Finally, the kidney plays in minor role in haemostasis (43), but we have to keep in mind that the elimination of dabigatran is affected by renal impairment that may lead to an

increased risk of bleeding due to excessive exposure to the drug. Consequently, if the aPTT is above the cut-off associated with a bleeding risk as mentioned in ▶Table 3, we proposed the use of HTI to measure the concentration of dabigatran in plasma. The ratio for HTI was less pronounced than aPTT, but this test is highly reproducible and linear on a broad range of concentrations, in comparison with aPTT showing a loss of sensitivity for higher concentration and variations depending on different reasons mentioned above. Moreover, dabigatran calibrators and specific methodologies are actually available from Hyphen Biomed® to easily perform these measurements on BCS®, ACL 7000®, ACL-Top®, STA-R® and provisory on Sysmex CA1500®. As aPTT and HTI are global assays, it is also necessary for the clinical biologist to know which anticoagulant is administrated to choose the adequate assay with accurate normal ranges. This is in opposition with the oral anti-Xa rivaroxaban for which there is a specific chromogenic assay insensitive to the presence of LMWH or fondaparinux (44).

Limitation of this study

The limitation of our study is the use of spiked normal pooled plasma samples. Thus inter-individual variation must be investigated with the recommended assays. Moreover, these results should be validated in patients receiving Pradaxa®. Indeed, it is currently unknown how predictive coagulation tests are of the bleeding risks (45). However, we consider that it is not ethically acceptable to expose patients to high-risk overdoses of dabigatran to study the impact on coagulation tests. Moreover, the study of Freyburger et al. explored the impact of DE on patients undergoing MOS (18). The results showed a correlation with those obtained *in vitro*. TGA were also used and showed the same tendency with a concentration dependent delay in the lag time. They also used a dilute thrombin time based on the same principle as HTI to assess the impact of dabigatran on the coagulation. The results showed a good correlation with the results obtained with the ECT which suggest that this kind of test may be useful for the monitoring of patient on Pradaxa®. An inter-individual variability is also mentioned in the study confirming the hypothesis that monitoring may be valuable to minimise the risk of the product.

Conclusions

Our study provides a comparison of the impact of broad plasma concentrations of dabigatran on specific and routinely used coagulation assays with a large panel of reagents. Due to its widespread use, 24 h a day accessibility, low cost and relatively good sensitivity, aPTT could be used for the monitoring of dabigatran and as a screening test for the risk of bleeding. In addition, HTI, ECT and TGA are the most sensitive tests. Besides, HTI showed good reproducibility, excellent linear correlation at all doses, simplicity of use, automation capabilities and should therefore be seen today as the gold-standard assay for the monitoring of dabigatran after a positive screening test. Information about the time between sampling and dabigatran ingestion is mandatory for appropriate interpretation of the tests.

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Conflicts of interest
None declared.

What is known about this topic?

- Regulatory authorities have recently suggested that some monitoring of dabigatran may be necessary in specific clinical cases like misuse, abuse or lack of compliance.
- No guideline/guidance documents are currently available on the assay to use to perform such a monitoring.
- aPTT was recently proposed by Australian authorities to screen patients with an increased risk a bleeding.

What does this paper add?

- Our study provides a comparison of the impact of broad plasma concentrations of dabigatran on specific and routinely used coagulation assays with a large panel of reagents.
- We determined cut-offs associated with a bleeding risk for five different aPTT reagents.
- We recommended HTI as the gold-standard assay for the monitoring of dabigatran after a positive screening test.

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