

# Comparison of calibrated dilute thrombin time and aPTT tests with LC-MS/MS for the therapeutic monitoring of patients treated with dabigatran etexilate

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## Summary

Ways to monitor dabigatran etexilate (DE) therapy would be useful in certain situations. Functional assays such as aPTT or Hemoclot<sup>®</sup> Thrombin Inhibitor (HTI) have been proposed to evaluate dabigatran concentrations, but previous findings are based on *in vitro* studies and results must be confirmed in clinical samples. It was the aim of this study to compare aPTT and HTI measurements with liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurements of dabigatran in plasma samples from DE treated patients. Seventy-one plasma samples were included. aPTT was performed using STA-CKPrest<sup>®</sup> and SynthASil<sup>®</sup>. HTI was performed according to instructions from the manufacturer. The LC-MS/MS method utilised dabigatran-d3 as internal standard. The plasma concentration range was 0 to 645 ng/ml as measured by LC-MS/MS. Overall, the HTI and LC-MS/MS analyses correlated well ( $r^2=0.97$ ). The Bland-Altman analysis showed a mean difference of 9 ng/ml (SD: 20 ng/ml). However, the HTI

performed poorly at concentrations <50 ng/ml. LC-MS/MS was sensitive (limit of quantification 1.1 ng/ml) and specific for dabigatran. The aPTT methods did not correlate well with plasma concentrations measured by LC-MS/MS ( $r^2 = 0.59$  with SynthASil<sup>®</sup> and 0.50 with STA-CKPrest<sup>®</sup>). In conclusion, the poor sensitivity, important inter-individual variability, and poor correlation with LC-MS/MS preclude the use of aPTT to estimate dabigatran concentrations. Due to its small inter-individual variability and good agreement with LC-MS/MS measurements, we recommend the use of HTI assays to rather accurately estimate concentrations of dabigatran >50 ng/ml. Quantification of lower dabigatran levels in DE-treated patients requires the "reference" LC-MS/MS method.

## Keywords

Dabigatran, coagulation assay, LC-MS/MS, monitoring, aPTT

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## Introduction

Dabigatran etexilate (DE, Pradaxa<sup>®</sup>, Boehringer Ingelheim, Ingelheim, Germany) has been approved by the European Commission for primary prevention of venous thromboembolic events in adult patients who have undergone elective total hip replacement surgery or total knee replacement surgery as well as for long-term use in the prevention of thromboembolic events in patients with non-valvular atrial fibrillation (AF) (1). The latter indication is also approved by the US Food and Drug Administration (FDA) (2). The net clinical benefit of DE in comparison with warfarin in patients with non-valvular AF (3) and recent recommendations by the European Society of Cardiology (ESC) (4) suggest a wider use of DE in the near future.

The key pharmacokinetic properties of DE include a very low oral bioavailability (mean 6.5%) and a predominantly ( $\geq 80\%$ ) renal elimination (5). Moreover, studies have shown considerable variation in plasma dabigatran concentrations (6). Frequent monitoring of dabigatran levels/effects is not recommended in the majority of patients, but it is anticipated that a non-negligible proportion of patients will achieve either insufficient or supra-therapeutic levels of the drug when given a fixed dose (7, 8). Consequently, possibilities to monitor the intensity of DE treatment may be valuable in some situations such as recurrent thrombosis or bleeding, before urgent surgery, in bridging, and in patients with risk factors for dabigatran accumulation or too low levels. Such factors include some drug-drug interactions, moderate renal impairment, moderate hepatic impairment or extreme body weights. Although some information is available (1, 2), robust data on dabigatran levels that

are associated with good therapeutic or harmful effects are currently lacking.

We have proposed that the plasma levels of dabigatran may be estimated by different coagulation assays such as activated partial thromboplastin time (aPTT) or dilute thrombin time (Hemoclot® Thrombin Inhibitor (HTI, Hyphen BioMed, Neuville-sur-Oise, France), the latter being the most sensitive and dependable one. However, these findings were based on *in vitro* analysis (9) and, to date, the accuracy of these estimates compared to true plasma concentrations from patients treated with DE in real life has not been determined. The aim of this study was to assess correlations between HTI and aPTT with the reference method for measurements of dabigatran in plasma, i.e. liquid chromatography with tandem mass spectrometry (LC-MS/MS), in patients treated with DE.

## Materials and methods

The study was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Centre Hospitalier Universitaire UCL Mont-Godinne –Dinant (Yvoir, Belgium) and the Ethical Review Board in Stockholm, Sweden, respectively. Written informed consent was obtained from each donor. Clinical details are not presented since this is purely a laboratory validation study and samples were anonymised.

### Normal pool plasma and home-made calibrators

Twenty-seven healthy individuals were included in the study. The exclusion criteria were thrombotic and/or haemorrhagic events, antiplatelet and/or anticoagulant medication, hormonal therapy, pregnancy, and use of drugs potentially affecting platelet and/or coagulation factor functions during two weeks prior to sampling. Blood was taken by antecubital venipuncture and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Leuven, Belgium) using a 21-gauge needle (Terumo). Platelet-poor plasma (PPP) was obtained from the supernatant fraction after double centrifugation for 15 minutes (min) at 2,000 g at room temperature. Immediately after centrifugation, PPPs from the 27 donors were mixed to obtain the normal pooled plasma (NPP) which was frozen at  $-80^{\circ}\text{C}$  without delay. Frozen NPP samples were thawed and heated to  $37^{\circ}\text{C}$  for 5 min just before experiments.

Dabigatran for coagulation testing in Belgium was a generous gift from Boehringer-Ingelheim. A stock solution (10 mM) of dabigatran was prepared in DMSO plus HCl 5% according to recommendations from the manufacturer and 25  $\mu\text{l}$  aliquots of this solution were frozen at  $-20^{\circ}\text{C}$ . An intermediate solution (100  $\mu\text{M}$ ) was prepared as follows: 10  $\mu\text{l}$  of stock solution in 990  $\mu\text{l}$  of phosphate-buffered saline (without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) resulting in a concentration of DMSO of 1% (v/v). Thus, the final concentration in DMSO in the analysed plasma was  $\leq 0.2\%$  (v/v) which did not influence the coagulation. Eleven dabigatran concentrations ranging from 0 to 943 ng/ml in the test sample mixture were prepared in NPP.

## Clinical samples

Seventy-one plasma samples from patients treated with DE were included in the study. For aPTT, only 55 plasmas were included in the analysis since we were not able to obtain valid (not expired) batch for further calibrations. Blood was taken by venipuncture and PPP was obtained as described above for the volunteers. Plasma samples were frozen at  $-80^{\circ}\text{C}$  without delay and heated to  $37^{\circ}\text{C}$  for 5 min immediately before coagulation testing. For drug measurements (see below) heating of the sample is not needed.

## Coagulation assays

### Activated partial thromboplastin time (aPTT)

aPTT was measured using two different reagents, STA-CKPrest® (Diagnostica Stago, Asnières, France) and SynthASil® (Instrumentation Laboratory, Lexington, KY, USA). For each reagent the same batch was used in order to reduce possible variability. STA-CKPrest® contains phospholipids (cephalin) and kaolin while SynthASil® contains synthetic phospholipids and micronised silica as activator. The two aPTT tests were performed as previously described on a STA-R Evolution® analyser (9, 10). Results are given in seconds, as ratios (vs NPP), and in ng/ml according to calibration with home-made calibrators (ranging from 0 to 943 ng/ml).

### Hemoclot® Thrombin Inhibitor assay (HTI)

The HTI (Hyphen BioMed) is a specific chronometric assay for the determination of direct thrombin inhibitors (DTIs) in plasma (9, 11). It is based on inhibition by the DTI of a standardised amount of thrombin introduced by the test itself. The test was performed as previously described on a STA-R Evolution® analyser (9). Calibration is performed using a reference, lyophilised preparation of dabigatran (from 40 to 500 ng/ml in the initial sample after reconstitution) (Hyphen BioMed). We, however, used our own calibrators (see above).

### Liquid chromatography coupled with tandem mass spectrometry

Plasma concentrations of dabigatran were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) after sample preparation by protein precipitation of 50  $\mu\text{l}$  citrated plasma with 150  $\mu\text{l}$  acetonitrile containing dabigatran-d3 as an internal standard. Dabigatran for LC-MS/MS analyses in Stockholm was purchased from Alsachim (Strasbourg, France) and dabigatran-d3 from Toronto Research Chemicals (Toronto, ON, Canada). After centrifugation the sample was diluted with an equal amount of mobile phase A (see below), after which the sample was gently shaken and re-centrifuged. An aliquot (3  $\mu\text{l}$ ) of the final extract was injected into the LC-MS/MS system. Separation of the analytes was achieved on an Acquity UPLC BEH column (Shield RP18, 1.7  $\mu\text{m}$ , 2.1 x 50 mm), using a gradient run with mobile phase A (10 mM ammonium formate pH 4.5) and mobile phase B

(acetonitrile). The analytes were detected using a Waters Quattro Premier XE mass spectrometer operating in positive electrospray ionisation (ESI) mode utilising selected reaction monitoring (SRM) for the transitions 472→289 m/z for dabigatran and 475→292 m/z for the internal standard. No interfering peaks were observed in 18 blank plasmas, and no significant ion suppression effect was found. The calibration curve for dabigatran in plasma was linear over the range 1–400 ng/ml and the limit of detection (LOD) was estimated to <0.5 ng/ml. Validation experiments with three levels of control samples (8.1, 202 and 393 ng/ml) on three different occasions (six determinations per concentration), showed an inter-assay precision between 6.00% and 9.25% and an inter-assay accuracy between -0.91% and 3.64%. The method was validated according to European Medicines Agency guidelines. This method represents a simplification of the procedure described by Stangier et al. (12).

### Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Results for the HTI vs the LC-MS/MS methods were compared by Pearson correlation analysis and a Bland-Altman analysis. For the aPTT methods, first order equations were used to fit the relations best. Bland-Altman analyses were also performed. The 95th limits of agreement of the Bland-Altman analyses are calculated as fol-

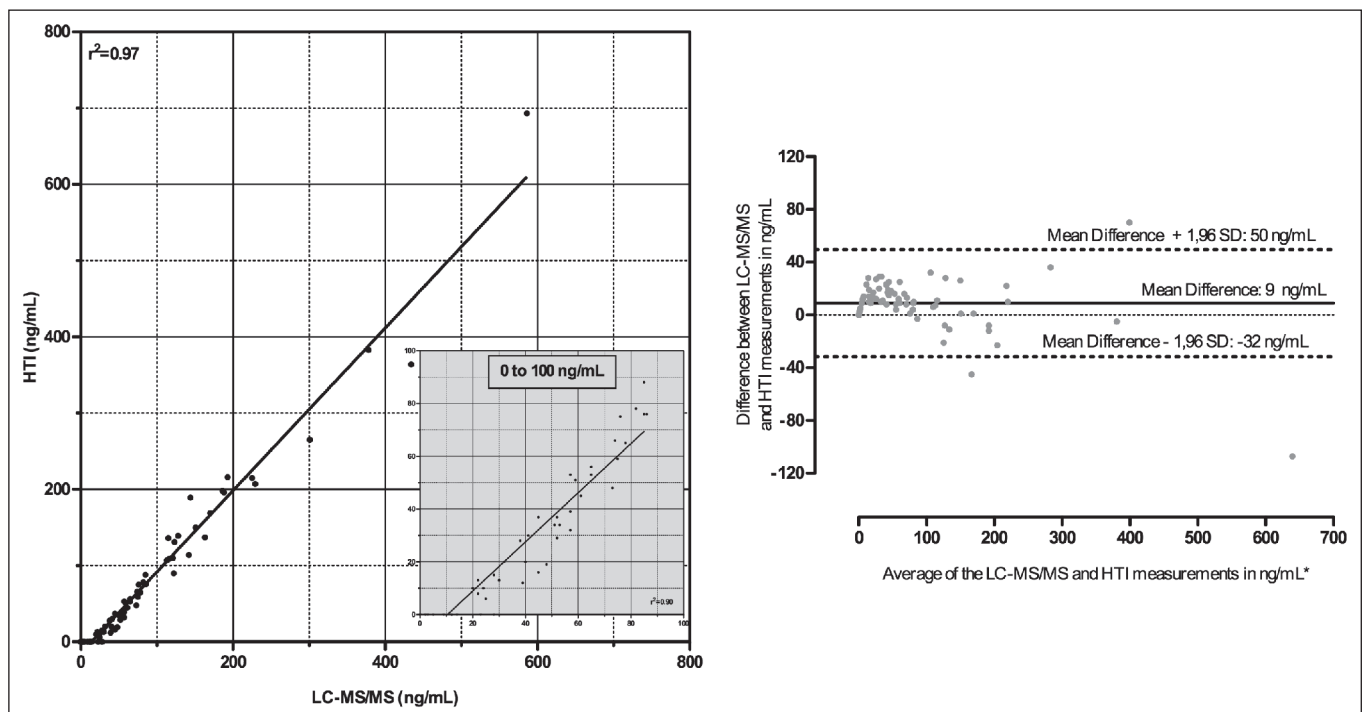
lows: Mean difference – or + 1.96\*standard deviation for the 5th and the 95th limit of agreement, respectively.

## Results

Seventy-one samples were analysed by LC-MS/MS, HTI and aPTT (two reagents). The correlation between the HTI and LC-MS/MS methods is presented in ►Figure 1. ►Figure 2 summarises results obtained with the two aPTT reagents in comparison with LC-MS/MS. Our home-made calibrators gave “estimated concentrations” by the HTI which were within 20% of the expected values when using the Hyphen calibrators.

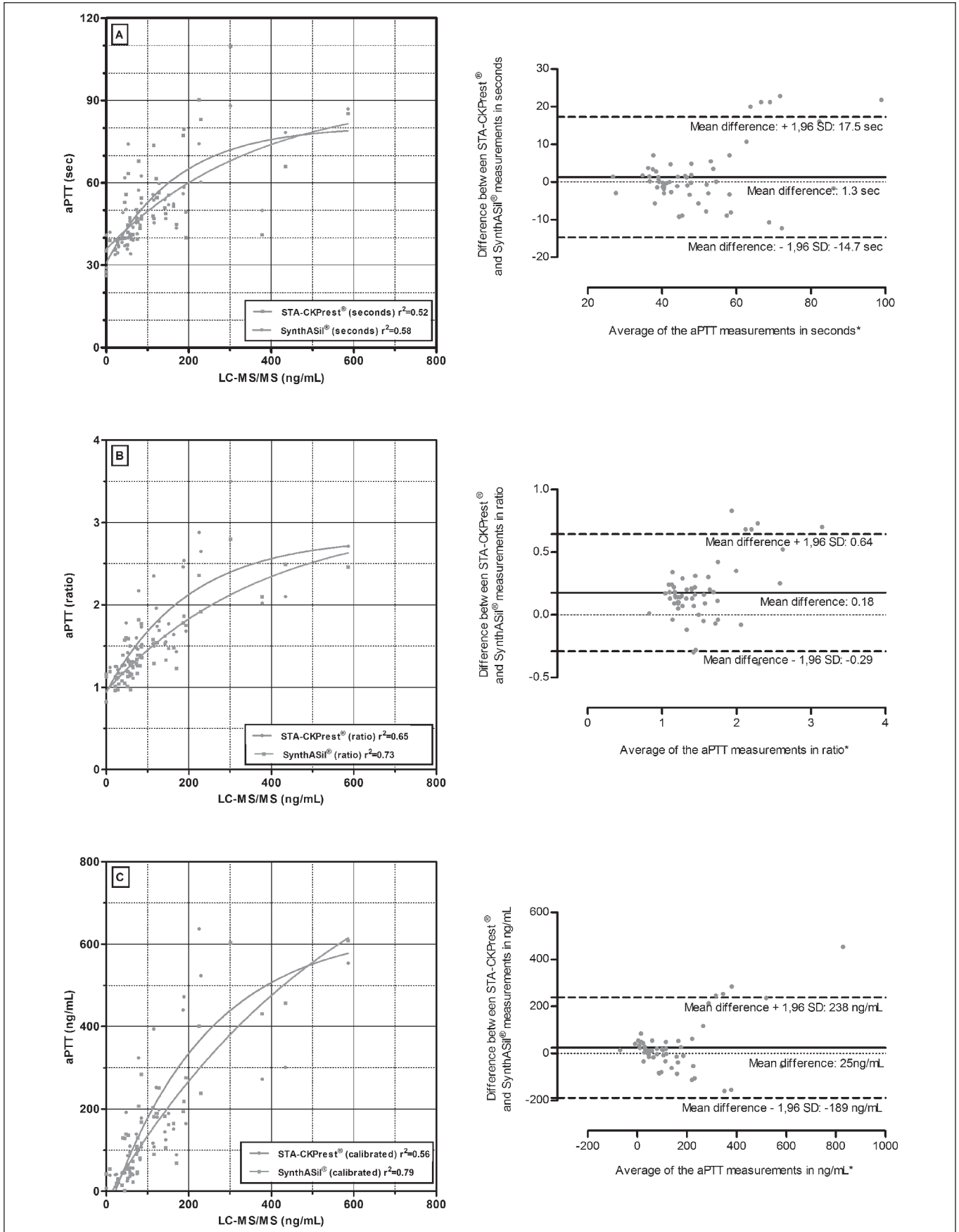
### Correlation between the Hemoclot® Thrombin Inhibitor test and LC-MS/MS

The Pearson correlation between HTI and LC-MS/MS had a coefficient ( $r$ ) of 0.99 (95% confidence interval: 0.98 to 0.99;  $p < 0.0001$ ), and an  $r^2$  of 0.97 (►Figure 1). Results from the Bland-Altman analysis are also provided in the ►Figure 1. For plasma concentrations  $\leq 100$  ng/ml ( $n=50$ ) the Pearson correlation coefficient was 0.95 (95% confidence interval: 0.91 to 0.97;  $p < 0.0001$ ) and the  $r^2$  was 0.90 (►Figure 1; insert). The Bland-Altman analysis for samples with  $\leq 100$  ng/ml dabigatran showed a mean difference of -12 ng/ml (SD: 9 ng/ml; 95% limits of agreement: -29 to 4 ng/ml).



**Figure 1: Correlation and Bland-Altman analysis between liquid chromatography-tandem mass spectrometry (LC-MS/MS) and the Hemoclot® Thrombin Inhibitors (HTI) assay for the measurement of dabigatran concentrations in patient plasma samples. The insert shows the relationship at dabigatran concentrations  $\leq 100$  ng/ml as deter-**

mined by LC-MS/MS. Nine patients with HTI results of 0 ng/ml had measurable dabigatran concentrations by LC-MS/MS. (Highest concentration measured by LC-MS/MS giving 0 ng/ml with HTI is 28 ng/ml.) For the Bland-Altman analysis the difference is calculated as follow: [difference (A-B) vs average] where A is the result of the LC-MS/MS and B the result of HTI.



For HTI, the limit of quantification (LOQ) and the limit of detection (LOD) in plasma given by the manufacturer are 100 and 30 ng/ml, respectively. These values are higher than those obtained with our own fresh calibrator (53 and 22 ng/ml, respectively). For LC-MS/MS, the LOD and lower limit of quantification (LLOQ) were 0.5 ng/ml and 1.1 ng/ml, respectively.

### Correlations between the aPTT assays and LC-MS/MS

The aPTT tests showed concentration-dependent prolongations of clotting time. The first order equations gave  $r^2$  values of 0.50 and 0.59 for STA-CKPrest® and SynthASil®, respectively (► Figure 2A). When expressing results as ratios the  $r^2$  values were 0.65 and 0.73 for STA-CKPrest® and SynthASil®, respectively (► Figure 2B). The dabigatran concentrations estimated with calibrated reagents gave  $r^2$  values of 0.56 and 0.79 for STA-CKPrest® and SynthASil® (► Figure 2C). The corresponding Bland-Altman analyses are also provided in ► Figure 2.

### Discussion

The current EU-SmPC (07/02/2013) mentions that it can be assumed that measures of anti-coagulant activity reflect dabigatran levels and can provide guidance for the assessment of bleeding risk. Thus, exceeding the 90<sup>th</sup> percentile of dabigatran trough levels is considered to be associated with an increased risk of bleeding (1). For patients treated with 150 mg dabigatran twice daily (bid) for stroke prevention in atrial fibrillation, it is stated that the 90<sup>th</sup> percentile of dabigatran plasma concentrations measured at trough (10-16 hours [h] after the previous dose) was about 200 ng/ml, and that an aPTT ratio greater than two-fold upper limit of normal (aPTT prolongation of about 80 seconds (s) in the EU-SmPC) at trough reflects the 90<sup>th</sup> percentile of observations. The FDA database on dabigatran plasma concentrations vs risks of suffering a stroke or a major bleed in the RE-LY study supports this contention, and also shows poor protection against stroke with dabigatran levels below the 10<sup>th</sup> percentile for patients treated with DE 150 mg bid, i.e. approximately 30 ng/ml (2).

Although aPTT has been suggested for the evaluation of pharmacodynamic effects of dabigatran (1, 13), it is not suitable for accurate quantification of its anticoagulant effect for several reasons. First, there is an inter-reagent variability and, as previously stated, an aPTT of about 80 s does not reflect a plasma dabigatran concentration of 200 ng/ml (which was found to be from 48.6 to 62.5 s in our previous *in vitro* study (9) and is confirmed by the present results). The same is observed for the cut-off proposed in VTE prevention for the bleeding

risk (9). Secondly, it was also demonstrated that pre-analytical and inter-individual variables could impact on the results of aPTT testing (9). Finally, the dose-response is not linear, precluding the possibility to differentiate minor versus major overdoses. In the present study, aPTT values with the best reagent (SynthASil®) varied from 50-90 s with dabigatran concentrations  $\geq 200$  ng/ml in patient plasmas, and could be as high as 75 s with only 50 ng/ml dabigatran in plasma. Even with a curvilinear model to fit the data the aPTT tests showed too much variability to reliably quantify dabigatran levels in patient plasma samples. Therefore, a rapid, more sensitive and reproducible assay with a linear dose-response for a wide range of concentrations is required.

Assays used to evaluate thrombin function are more sensitive than those that are not specific for this coagulation factor (9, 11, 14). However, thrombin time (TT) is too sensitive towards dabigatran (9, 11) leading to the necessity to dilute the thrombin reagent to obtain reliable results (15). Thus, the HTI was developed and has been proposed as a rapid, standardised and calibrated assay to determine plasma concentrations of dabigatran (9, 11, 16). Theoretically, the use of diluted plasma allows the HTI assay to measure dabigatran effects at a wide range of concentrations. Moreover, it is also fully automatable and has been adapted to different coagulometers in order to be easily implemented in laboratories. One limitation of this test is that in case of switching therapy (i.e. from heparins/ heparinoids to DE; or from hirudin and derivatives to DE), it will be influenced by the presence of such inhibitors in the plasma. This implies the necessity of an accurate anamnesis of the drugs taken by the patient to avoid overestimation of drug concentrations in plasma. An important issue is whether the dabigatran concentrations estimated with the HTI assay, which hitherto have been based on *in vitro* studies with spiked plasma samples, reliably predict the true plasma drug concentrations in patients treated with DE in real-life conditions.

In this study, we report on the correlations between dabigatran concentrations in patients treated with DE as determined by the calibrated HTI and a newly developed LC-MS/MS method. We also compared the LC-MS/MS method with two aPTT assays in order to evaluate the recommendations provided by the regulators and the literature (1, 13).

### Correlation between Hemoclot® Thrombin Inhibitor and LC-MS/MS

The HTI showed a good correlation with LC-MS/MS and the Bland-Altman analysis revealed an acceptable mean difference (► Figure 1). When focusing on samples  $\leq 100$  ng/ml, the mean difference was approximately the same (► Figure 1). In both cases the 95% limits of agreement suggest reasonable accuracy of HTI to estimate dabigatran plasma concentrations in the normal-high concentration range. However, the LOQ and LOD for HTI (53 and 22 ng/ml, respectively, with home-made calibrators, and an LOQ of 50 ng/ml according to the manufacturer) are much higher than those obtained with the sensitive and accurate LC-MS/MS method (1.1 ng/ml and 0.5 ng/ml, respectively). This limits the use of HTI for accurate quantitative estimates of  $C_{\text{trough}}$  in patients treated for

**Figure 2: Correlations between aPTT (using either of two different reagents) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results are expressed in seconds (A), ratios (B) or ng/ml (C). Graphics of the Bland-Altman analysis comparing the two reagents when results are given in seconds, ratio or ng/ml are also provided. For the Bland Altman analysis the difference is calculated as follow: [difference (A-B) vs average] where A is the result of STA-CKPrest® and B the result of SynthASil®.**



stroke prevention in atrial fibrillation, as  $<43$  ng/ml at  $C_{\text{trough}}$  has been considered to be a sub-therapeutic level (9). In orthopaedic indications, the sub-therapeutic level at  $C_{\text{trough}}$  has been set at 10 ng/ml (9), and the use of HTI obviously cannot be recommended to detect such low concentrations.

Thus, HTI can be used to reliably estimate dabigatran plasma concentrations above 50 ng/ml and thus especially for measurements of  $C_{\text{max}}$ . However, HTI should not be used to rule out relevant dabigatran levels below 50 ng/ml (e.g. before fibrinolytic therapy of acute ischaemic stroke or before acute surgery associated with an increased bleeding risk). Variability of coagulation analysers may increase the imprecision further at these low concentrations. In addition, the commercial calibrators provided with the assay are from 40 to 500 ng/ml which implies that concentrations below 40 ng/ml should not be estimated. However, normal pooled plasma without dabigatran (from the same batch as the dabigatran plasma calibrators) can be purchased separately from the manufacturer and used as blank for the calibration curve. The LC-MS/MS method is more accurate and is useful in the entire range of dabigatran concentrations. LC-MS/MS is thus required for quantification of very low to moderate dabigatran concentrations in clinical samples or for pharmacokinetic studies.

### Correlation between aPTT and LC-MS/MS

The Subcommittee on Control of Anticoagulation of the Scientific and Standardisation Committee of the International Society on

Thrombosis and Haemostasis (ISTH) mentions that the aPTT can be used to determine the relative intensity of anticoagulation due to dabigatran with most available reagents but should not be used to quantify drug plasma concentrations (13). A Swedish *in vitro* study (17) found that almost all patients would be expected to have prolonged aPTT at  $C_{\text{max}}$  (approximately 200 ng/ml) and a large proportion even at 50 ng/ml, irrespective of the reagent used. However, it was previously found that aPTT results for the expected  $C_{\text{trough}}$  concentrations (50 ng/ml on average, but with considerable inter-individual variability [2]) overlapped the range of baseline values obtained among 10 healthy individuals with several reagents (17). In addition, the previously published results are based on *in vitro* analyses of plasma from healthy volunteers spiked with dabigatran and obviously do not include the inter-individual variability one may expect among patients.

In the present study, both aPTT reagents showed very poor correlations with dabigatran concentrations in plasma measured by LC-MS/MS when results were expressed in seconds (► Figure 2A). The use of ratios only partially improved the correlation (► Figure 2B). However, an aPTT ratio below 2.0, whatever the reagent used in this study, could exclude dabigatran concentrations above 200 ng/ml. This needs to be confirmed in a larger cohort. Calibration of each reagent with dabigatran improved the correlation little, and only so for SynthASil® (► Figure 2C). The Bland-Altman analyses comparing the two aPTT reagents demonstrate the high inter-reagent variability (► Figure 2). If aPTT testing is to be used, results should be given as ratios rather than in seconds. However, due to its high variability, the aPTT cannot reliably estimate plasma dabigatran concentrations. It has poor sensitivity and considerable inter-individual variability in actual samples from patients. Thus, a normal aPTT could not exclude therapeutic levels of dabigatran and, conversely, therapeutic levels of dabigatran do not dependably result in an abnormal aPTT.

### Limitations of this study

This study was not intended to correlate the dabigatran plasma concentrations with clinical efficacy or safety outcomes. Although aPTT performed poorly, we cannot exclude that the test may give relevant information in connection with cases of bleeding and perhaps also in cases with recurrence of thrombosis. Although pharmacokinetic studies with these novel agents have identified an expected range of drug concentrations for particular clinical trial patient populations, this range does not necessarily define the limits beyond which the bleeding or thrombosis risk would increase significantly for a particular patient (18). Clinical studies that investigate the relationships between either dabigatran concentrations in plasma (measured by LC-MS/MS and/or estimated via HTI) or aPTT values and clinical outcome are still required.

### Conclusions

The poor sensitivity, the important inter-individual variability, and the poor correlation with LC-MS/MS preclude the use of aPTT to estimate dabigatran concentrations in plasma. Due to its small

#### What is known about this topic?

- Dabigatran etexilate (DE) is an oral anticoagulant used for primary prevention of venous thromboembolic events in adult patients who have undergone elective total hip replacement surgery or total knee replacement surgery as well as for long-term use in the prevention of thromboembolic events in patients with atrial fibrillation.
- Monitoring is generally not required but possibilities to evaluate the intensity of DE treatment may be valuable in some situations.
- Activated partial thromboplastin time (aPTT) can be used for urgent determination of the relative intensity of anticoagulation. The Hemoclot® Thrombin Inhibitor can be used to determine the drug concentration.

#### What does this paper add?

- There is a poor correlation between aPTT and the measured plasma dabigatran concentration. aPTT should not be used to estimate dabigatran concentrations in plasma and poorly reflects the intensity of anticoagulation due to dabigatran.
- The Hemoclot® Thrombin Inhibitor assay correlates well with reference LC-MS/MS measurements for dabigatran concentration above 50 ng/ml.
- Detection and quantification of lower levels of dabigatran in DE treated patients requires the more sensitive LC-MS/MS analysis.

inter-individual variability and good agreement with LC-MS/MS measurements, we recommend the use of HTI assays to rather accurately estimate normal to high plasma concentrations of dabigatran, i.e. plasma levels above 50 ng/ml. Taking into account the lower sensitivity of the HTI method, however, detection and quantification of lower levels in DE treated patients (to check for compliance or in case of recurrence of thrombosis) requires LC-MS/MS analyses. The latter should be considered to be the gold standard but is presently not widely available.

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### Conflicts of interest

None declared.

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