

Evaluation of coagulation assays versus LC-MS/MS for determinations of dabigatran concentrations in plasma

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Received: 2 May 2013 / Accepted: 4 June 2013
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Abstract

Background Dabigatran is an oral direct thrombin inhibitor for which routine laboratory monitoring is currently not recommended. However, there are situations in which measurements of the drug and its effect are desirable. We therefore compared and validated different coagulation methods for assessments of dabigatran in clinical samples in relation to measurements of plasma dabigatran, without the purpose of establishing effective and safe concentrations of dabigatran in plasma.

Methods Samples were obtained from 70 atrial fibrillation patients treated with dabigatran etexilate. Plasma concentrations were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and were compared with coagulation methods Hemoclot thrombin inhibitors (HTI)

and Ecarin clotting assay (ECA), as well as with prothrombin time-international normalized ratio (PT-INR) and activated partial thromboplastin time (aPTT).

Results A wide range of dabigatran concentrations was determined by LC-MS/MS (<0.5–586 ng/mL). Correlations between LC-MS/MS results and estimated concentrations were excellent for both HTI and ECA overall ($r^2=0.97$ and 0.96 respectively, $p<0.0001$), but the precision and variability of these assays were not fully satisfactory in the low range of dabigatran plasma concentrations, in which ECA performed better than HTI. aPTT performed poorly, and was normal (<40 s) even with dabigatran levels of 60 ng/mL. PT-INR was normal even at supratherapeutic dabigatran concentrations.

Conclusion LC-MS/MS is the gold standard for measurements of dabigatran in plasma. Alternatively, either HTI or ECA assays may be used, but neither of these assays is dependable when monitoring low levels or to infer total absence of dabigatran. The aPTT assay is relatively insensitive to dabigatran, and normal aPTT results may be observed even with therapeutic dabigatran concentrations.

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Keywords Dabigatran · Drug concentration · LC-MS/MS ·
Hemoclot · ECA · PT-INR · aPTT

Dabigatran etexilate (Pradaxa®, Boehringer Ingelheim, Germany) is an oral prodrug that is rapidly transformed in the liver into its active compound, dabigatran, a direct thrombin inhibitor that affects both clot-bound and free thrombin. The drug is approved in Europe and North America for the prevention of stroke and peripheral thromboembolism in patients with non-valvular atrial fibrillation (AF). Dabigatran has a very low oral bioavailability (6.5 % on average), and it is eliminated predominantly in unchanged form by the kidneys (80 %) [1, 2]. These pharmacokinetic features contribute to considerable variability in exposure to dabigatran. For example, trough plasma concentrations of dabigatran in patients treated with

dabigatran 150 mg BID was on average 91.0 ng/ml, with a 25th–75th percentile range of 61.0–143 ng/ml (and a large variation above and below that range) [3].

Although routine laboratory monitoring is currently not recommended, the European Medicines Agency (EMA) recently described that exceeding the 90th percentile of dabigatran trough levels is associated with an increased risk of bleeding, and this level was set at 200 ng/mL for the 150 mg BID dose of dabigatran [3]. There are obvious situations in which measurements of drug concentrations and/or the intensity of anticoagulation are desirable, e.g. in preparation for surgery; with suspected interactions with other drugs; to check compliance and/or anticoagulant effect; and in patients with major bleeds [4]. Patients with moderate renal impairment have an increased exposure to dabigatran, and the drug is contraindicated in patients with severe renal impairment ($\text{ClCr} < 30 \text{ mL/min}$) in Europe [5]. In the U.S., a non-investigated dosage of dabigatran (75 mg BID) was recommended for AF patients with severe renal impairment. Thus, it is easily understandable that renal impairment increases the risk of suffering major bleeds [6], and measurements of dabigatran levels and/or anticoagulant effects may be of particular interest in patients with renal impairment. The lack of a specific antidote makes this issue even more important [7, 8].

Direct measurements of plasma concentrations by Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) may be considered the method of choice for the detection and quantification of dabigatran. However, this method will probably be available for routine use only in specialized centers, and in such centers perhaps only during weekdays. Therefore, functional coagulation tests that indirectly estimate plasma dabigatran concentrations are being developed and tested, the most promising of these being diluted thrombin time (Hemoclot Thrombin Inhibitors® [HTI]) and ecarin clotting assay (ECA). The impact of dabigatran on both standard (prothrombin time [PT] and activated partial thromboplastin time [aPTT]) and new (HTI and ECA) coagulation assays has been tested in several studies *in vitro* [9–15], and recommendations on the selective monitoring of dabigatran treatment are also available [2, 16]. However, experience of the use of these assays in routine clinical settings has not yet been published, and it is not known how these indirect estimates of dabigatran concentrations relate to true drug concentrations in patients treated with dabigatran.

In order to establish laboratory assays appropriate for routine use in various clinical settings, we compared several coagulation tests, including HTI and ECA, with direct measurements of dabigatran by LC-MS/MS in plasma samples from AF patients treated with dabigatran in real life clinical settings. The purpose of this study was to evaluate tests that have been proposed to reflect dabigatran concentrations or

effects, and not to establish effective and safe concentrations of dabigatran in plasma.

Materials and methods

Plasma samples from 70 patients treated with dabigatran and 35 with either no or another anticoagulant that may be expected to interfere with the dabigatran assays from different hospitals in the Stockholm County were tested. The study was approved by the Ethical Review Board in Stockholm, Sweden. Written informed consent was obtained from each donor. Clinical details are not presented since this is purely a laboratory validation study and samples were anonymized.

Blood was taken by antecubital venipuncture and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Belgium) using a 21-gauge needle (Terumo, Belgium). Sampling was usually (in 2/3 of cases; $n=46$) performed at trough (within 10–16 h after the last dose). Seven samples were collected before and additional seven after this time frame, while for the rest of the samples, the exact time between last dose and sample collection was unknown. Plasma was obtained from the supernatant fraction after centrifugation for 20 min at 2,000 g at room temperature. Plasma samples were frozen at $-80 \text{ }^\circ\text{C}$ without delay and heated to $37 \text{ }^\circ\text{C}$ for 5 min immediately before coagulation testing. For drug measurements, heating of the sample is not needed.

Direct measurements of dabigatran in plasma by LC-MS/MS

Plasma concentrations of dabigatran were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The bioanalytical method was validated in accordance with Food and Drug Administration (FDA) Guidelines [17]. Dabigatran was purchased from Alsachim, Strasbourg, France and dabigatran-d3 (internal standard) from Toronto Research Chemicals, Ontario Canada. 150 μL acetonitrile containing internal standard was added to 50 μL plasma. After shaking and centrifugation, a further two-fold dilution with mobile phase A (see below) was performed, after which the sample was gently shaken and re-centrifuged. Three μL of the final extract was injected into the LC-MS/MS system. Chromatographic separation of the analytes was achieved on an Acquity UPLC BEH column (Shield RP18, $2.1 \times 50 \text{ mm}$, $1.7 \mu\text{m}$) using a gradient run with mobile phase A (10 mM ammonium formate pH 4.5) and mobile phase B (0.1 % formic acid in acetonitrile). The analytes were detected using a Waters Quattro Premier XE mass spectrometer operating in positive electrospray ionization (ESI) mode, utilizing selected reaction monitoring (SRM) with ion transitions $472 \rightarrow 289 \text{ m/z}$ for dabigatran and $475 \rightarrow 292 \text{ m/z}$ for the internal standard. The total analysis time was 3 min. The

calibration curve for dabigatran in plasma was linear over the range 1.1–412 ng/mL. Samples above this range are diluted with normal pooled plasma for accurate quantitation. The limit of detection (LOD) was estimated to <0.5 ng/mL. No interfering peaks were observed in 18 blank plasma samples at the retention times of dabigatran or the internal standard. Post-column addition of analytes (dabigatran and internal standard) was used to study ion suppression effects from plasma. No significant ion suppression occurred at the retention time of the analyte. Validation experiments with three levels of control samples (8.1, 202 and 393 ng/mL) on three different occasions using six determinations per concentration, showed an inter-assay precision between 6.0 % and 9.3 % and an inter-assay accuracy between -0.9 % and 3.6 %. A typical chromatogram is shown in Fig. 1.

Indirect measurements of dabigatran in plasma by coagulation assays

LC-MS/MS results were compared with results obtained with new coagulation methods for indirect determination of dabigatran concentrations, as well as screening coagulation assays.

Hemoclot thrombin inhibitors[®] (HTI) (HYPHEN BioMed, Neuville-sur-Oise, France) is a quantitative method for the measurement of dabigatran in human citrated plasma with a modified clotting method. Diluted test plasma is mixed with normal pooled human plasma. Clotting is then initiated by adding a constant amount of human α -thrombin. The clotting time is directly related to the concentration of dabigatran in the tested plasma. Calibration was performed with dabigatran plasma calibrators from the manufacturer with the following concentrations: 0, 30, 280 and 510 ng/mL. The lower limit of quantification (LOQ) given by the manufacturer is 50 ng/mL. The assay was performed according to the manufacturer's instructions, and

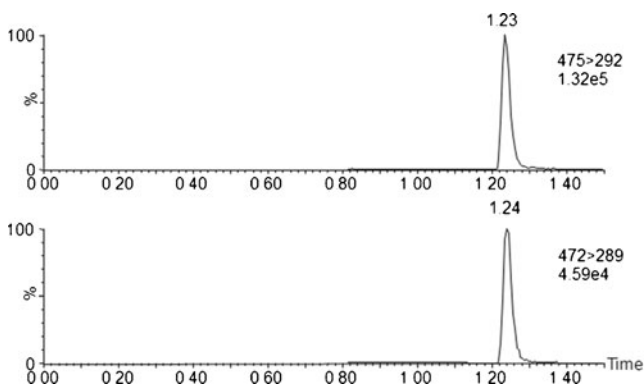


Fig. 1 Chromatogram from the LC-MS/MS analysis of a plasma sample collected from a patient treated with dabigatran etexilate (Pradaxa[®]). The upper panel shows the internal standard with an ion transition from 475 to 292 and a retention time of 1.23 min, and the lower panel dabigatran with an ion transition from 472 to 289 and a retention time of 1.24 min. The calculated concentration of dabigatran was 19 ng/mL in this patient

clotting times were measured on a Sysmex[®] CS2100i (Sysmex, Kobe, Japan). Results of the HTI are given as dabigatran concentrations in ng/mL. Validation experiments with two levels of control samples (110 and 310 ng/mL) on three different occasions using 14 determinations per concentration, showed inter-assay precisions of 7.3 % and 5.7 %, respectively.

Ecarin clotting assay[®] (ECA) (Diagnostica Stago, Asnieres, France) is a quantitative chromogenic method for the measurement of synthetic direct thrombin inhibitors in plasma. The citrated plasma is mixed with a prothrombin buffer and a chromogenic substrate and incubated at 37 °C for 2 min. Addition of ecarin causes the formation of p-nitroaniline and changes the optical density at 405 nm. The ECA reaction time is proportional to the plasma concentration of dabigatran which is expressed in ng/mL. As Diagnostica Stago did not provide any dabigatran calibrator or internal controls; we used the HTI calibrators (HYPHEN BioMed). The calibrator concentrations were 0, 30, 260 and 480 ng/mL. No information on the sensitivity of the ECA test is given by the manufacturer. The assay was performed according to instructions from the manufacturer on a BCS[®] XP System (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Validation experiments with two levels of control samples (150 and 400 ng/mL), on three different occasions using 13 determinations per concentration, showed inter-assay precisions of 4.4 % and 10.3 %, respectively.

aPTT using the Automate[®] reagent (Diagnostica Stago, Asnieres, France) was performed on a Sysmex[®] CS2100i (Sysmex, Kobe, Japan). Results are given in seconds.

PT-international normalized ratio (PT-INR) using the Owren reagent SPA + [®] (Diagnostica Stago Asnieres, France) was performed on a Sysmex[®] CS2100i (Sysmex, Kobe, Japan). Results are presented as INR.

The statistical package of Graph Pad (version 4.0 for Windows) was used for statistical analysis. Data were analyzed by linear regression analysis and slope, intercept and r^2 were determined and presented.

Results

Dabigatran concentrations determined by LC-MS/MS varied considerably in these plasma samples from dabigatran treated AF patients (from <1 to 586 ng/mL).

Correlations between directly measured dabigatran concentrations using LC-MS/MS and the functionally estimated concentrations were excellent for both HTI ($r^2=0.97$) (Fig. 2a) and ECA ($r^2=0.96$) (Fig. 3a) ($p < 0.0001$ for both). An excellent correlation was also observed between HTI and ECA ($r^2=0.94$) (Appendix, Fig. 6). The ECA assay tended to overestimate dabigatran concentrations, with slopes for the regression equations of 1.28 vs. LC-MS/MS results (i.e., true concentrations) and 1.35 vs. HTI. At low levels of dabigatran

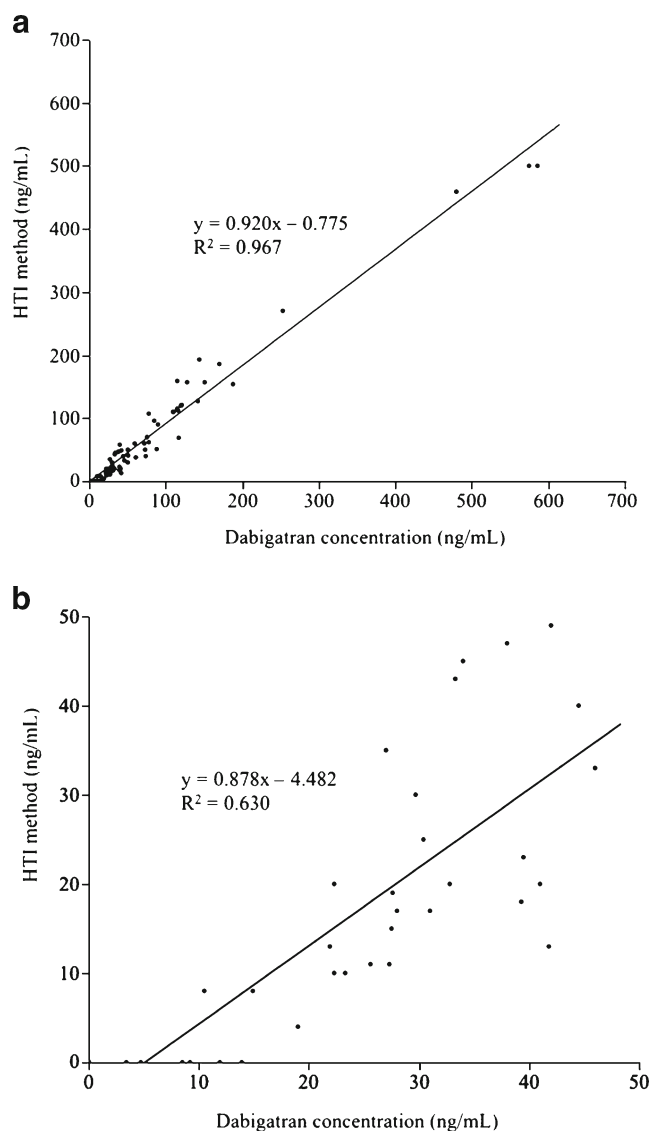


Fig. 2 Correlation between dabigatran plasma concentrations measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and concentrations indirectly estimated by the Hemoclot thrombin inhibitors[®] (HTI) method ($p < 0.0001$). Panel **a** shows the correlation for all samples and panel **b** shows the relationship for low values (<50 ng/mL) with the line of identity ($x=y$) indicated. Six patients with HTI results of 0 ng/mL had measurable dabigatran concentrations by LC-MS/MS

(<50 ng/mL), results with the HTI assay were not well correlated with results obtained by LC-MS/MS. Thus, the HTI assay showed underestimation of dabigatran concentrations and large variability with a CV of approximately 30 % in the range 30–50 ng/mL (Fig. 2b). The ECA test performed better than HTI at low dabigatran concentrations (Fig. 3b).

A significant, but considerably weaker correlation was also observed between dabigatran concentrations and aPTT values in seconds ($r^2=0.58$), and several aPTT values were within the normal range (<40 s), even with dabigatran levels up to 60 ng/mL (Fig. 4).

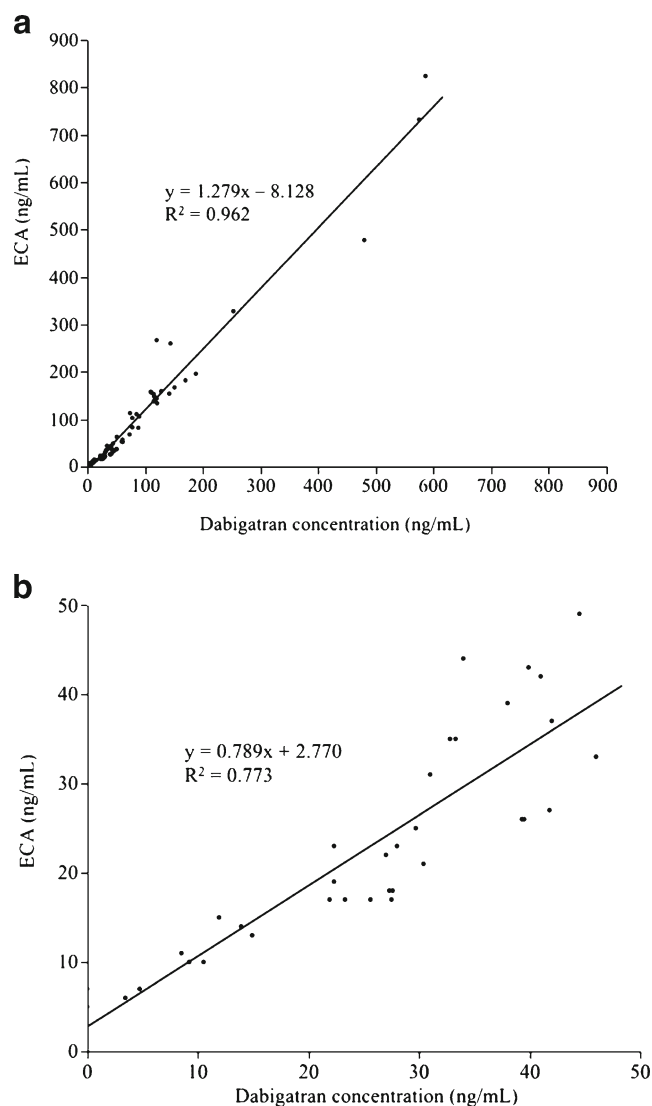


Fig. 3 Correlation between dabigatran plasma concentrations measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and concentrations indirectly estimated by the Ecarin clotting assay[®] (ECA) method ($p < 0.0001$). Panel **a** shows the correlation for all samples and panel **b** shows the relationship for low values (<50 ng/mL) with the line of identity ($x=y$) indicated. Two patients with ECA results above 5 ng/mL had non-measurable dabigatran concentrations by LC-MS/MS

There was a weak correlation between dabigatran concentrations and PT-INR ($r^2=0.48$). Most importantly, however, PT-INR values above 1.2 were only observed with dabigatran concentrations above 400 ng/mL (Fig. 5).

We also tested plasma samples from 35 patients with various bleeding disorders or on standard anticoagulants and no dabigatran treatment. All results for both HTI and ECA were below the detection limit (results not shown), which indicates that neither HTI nor ECA were affected by FVIII deficiency ($n=5$), vWF deficiency ($n=5$) or lupus anticoagulant ($n=5$), by warfarin in the therapeutic—INR 2–3 ($n=5$) or supratherapeutic—INR >3 ($n=5$) range, or by heparin ($n=5$) or LMWH treatment ($n=5$).

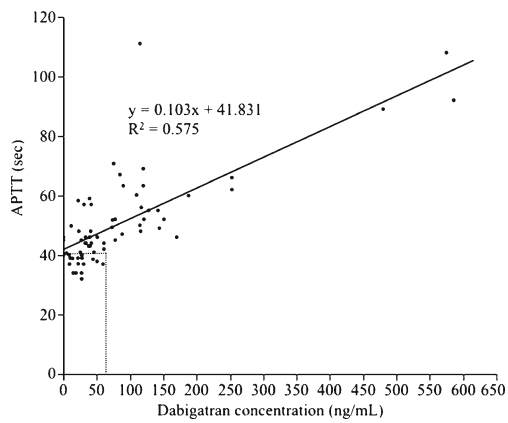


Fig. 4 Correlation between dabigatran plasma concentrations measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and aPTT (Automate®) ($p < 0.0001$). The aPTT reference range is 28–40 s (dot lines represent upper levels of reference range and the respective concentration on the x axis)

Discussion

Routine laboratory monitoring is currently not recommended for treatment with dabigatran. However, there are situations, e.g. preparation for surgery, interactions with other drugs, to check compliance, recurrent thrombosis and major bleeds, when measurements of the drug and its effect are desirable or even necessary. The role of the routine coagulation laboratory that was considered “the mainstay for the management of the old anticoagulants” [18] is under scrutiny in the era of new oral anticoagulants, and novel laboratory measures need to be developed and validated. In contrast to the situation with standard anticoagulants and their well known tests for monitoring, the situation with dabigatran is much more complex. The choice of tests, which should be based on assay availability, linearity, standardization and responsiveness [18], is a challenging task.

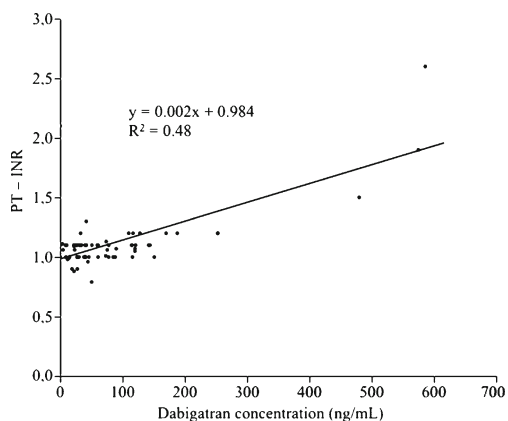


Fig. 5 Correlation between dabigatran plasma concentrations measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and PT-INR (Owren Spa+®) ($p < 0.005$). PT-INR reference range < 1.2

Based on our results, direct measurement of dabigatran plasma concentrations by LC-MS/MS should be considered to be the reference method of choice. The LC-MS/MS method showed both high sensitivity and high accuracy, and every coagulation assay that is proposed for indirect assessments of dabigatran concentrations in clinical samples should be compared to this gold standard. However, LC-MS/MS is currently not widely available, and even if it is available the service is not likely to be 24/7. Therefore, in most places, a well documented coagulation assay is needed for routine use.

Since thrombin is the target of dabigatran, a chromogenic anti-IIa based assay would likely be the first choice for monitoring this drug, and should be relatively easy to run in an ordinary coagulometer. Unfortunately, however, no such test is currently available for dabigatran monitoring [18].

Screening coagulation tests such as PT-INR and aPTT are widely used and may be the only assays available in small laboratories. However, our (Owren reagent based) PT assay was insensitive even to supratherapeutic dabigatran concentrations. The first abnormal PT-INR was observed when the concentration of dabigatran was almost 500 ng/mL. This confirms previous findings from in vitro studies [10–14]. Although the Quick reagent based PT assay may have better sensitivity [11, 13, 14], it seems that PT-INR should not be used for the evaluation of dabigatran treatment.

In vitro studies have shown that aPTT assays are sensitive to dabigatran with curvilinear concentration-response relationships that become linear above 400 ng/ml [10]. In previous studies, aPTT was prolonged independently of reagent with dabigatran concentrations above 100 ng/ml [11] or 120 ng/mL [14] in vitro. In our study with patient samples, the aPTT values were consistently above the upper reference limit (40 s) only at dabigatran concentrations above 60 ng/mL. The results of aPTT may be influenced by coagulometers and reagents [12, 13], but reasonable agreement can be observed between laboratories [14], and it seems that the aPTT could be useful for the assessment of peak effects shortly after taking the drug, or in connection with bleeding associated with high concentrations of dabigatran [19]. Importantly, however, our data show that a normal aPTT cannot exclude the presence of dabigatran in therapeutic concentrations. Hence, aPTT has limited utility for evaluations of the intensity of anticoagulation during dabigatran treatment.

Thrombin time (TT) was too sensitive for use as a dabigatran assay [13, 20]. However, a diluted thrombin time [20] and the commercially available diluted thrombin time reagent, i.e., the HTI assay, showed a concentration dependent prolongation of clotting time with a linear relationship and good reproducibility in vitro [10, 12, 13]. We observed a good correlation between HTI expressed as estimated dabigatran concentration in ng/mL and direct dabigatran plasma concentrations, but assay precision and variability were not satisfactory in the low range. Therefore, the interpretation of low dabigatran concentrations should be cautious, and the HTI cannot be used to infer absence of

dabigatran, e.g. when recurrent thrombosis or compliance are the clinical questions at hand. However, the HTI assay is certainly valuable for urgent assessments of dabigatran treatment, especially in conditions associated with bleeds or a high risk of bleeding.

The ECA assay is specific for thrombin generation. Generally, good linearity and sensitivity was observed with this assay in vitro and, despite potential problems with calibration at the higher end of the response curve [10, 13], the procedure is recommended for dabigatran monitoring [18]. We confirmed those findings in clinical plasma samples, as we found excellent correlations with both true dabigatran concentrations (LC-MS/MS) and the HTI assay. ECA may be more reliable than HTI at low concentrations, but it is still doubtful if ECA could definitely rule out any presence of dabigatran. The ECA assay has, however, been more difficult to establish in our routine 24/7 laboratory with a Sysmex analyser, while the application is available for the less commonly used BCS-XP. Therefore, we believe that HTI will be more commonly used than ECA, despite the superior sensitivity of the latter, for indirect assessments of dabigatran concentrations in clinical routine laboratories.

In spite of the encouraging results observed in our study, which to a great extent confirm data obtained in vitro, estimated dabigatran concentrations measured by ECA and/or HTI should be carefully interpreted. Plasma concentrations of dabigatran vary considerably, with large differences between peak and trough concentrations, even in healthy individuals. Therefore, the results of laboratory tests will be difficult to interpret without knowledge of the exact time when the blood sample was collected relative to intake of the last dose of medication [21]. Both compliance and accumulation may be important issues in real life. When assessing the anticoagulant activity in, e.g., patients with moderate to severe renal impairment or with extreme body weights, samples collected immediately before the next scheduled dose at steady-state will provide the most useful information.

The purpose of this study was to compare and validate different laboratory methods for the assessment of dabigatran in clinical samples, not to establish effective and safe concentrations of dabigatran in plasma. Another limitation is the relatively few samples with dabigatran concentrations considered to be subtherapeutic or supratherapeutic. Thus, the performance of indirect assessments of dabigatran concentrations in these situations is not yet fully characterized. Nevertheless, we consider our results new and informative, and this is our first comparison of plasma concentrations of dabigatran and coagulation assays in samples obtained from patients treated in real non-trial clinical settings. We also believe that the results of our study are sufficient to commence selective monitoring of dabigatran using LC-MS/MS and/or HTI or ECA when this is needed.

In conclusion, LC-MS/MS should be considered the gold standard or reference method to determine plasma concentrations of dabigatran because of its high sensitivity and accuracy. If LC-MS/MS is not available, HTI and ECA are the assays of choice for estimating the intensity of dabigatran anticoagulation and

drug levels in clinical samples. However, neither of these assays can be used to monitor low levels or infer the total absence of dabigatran. The classical coagulation assays, PT-INR and aPTT, have no or very limited utility in this context. aPTT may be used for qualitative assessment if HTI and ECA are not available, with the precaution that a normal aPTT may be observed even with therapeutic levels of dabigatran.

Conflicts of interest JPA has received support for attendance at scientific meetings from Stago. None of the other authors declare any conflict of interest.

Contributions to the manuscript JPA – responsible for the study design, data analysis and interpretation and writing the manuscript.

MS – providing samples, helped in research ethics application, data analysis and reviewing the manuscript.

JE – responsible for the technical installation and evaluation of the HTI assay in the laboratory, data analysis and reviewing the manuscript.

EEB – responsible for the technical installation and evaluation of the ECA assay in the laboratory, data analysis and reviewing the manuscript.

LS – responsible for laboratory analysis of HTI, handling of the study samples and reviewing the manuscript.

EMN – responsible for laboratory analysis of ECA, handling of the study samples and reviewing the manuscript.

LO – performing of some assays, data analysis and reviewing the manuscript.

YR – laboratory analysis of dabigatran concentration, handling of the study samples and reviewing the manuscript.

AP – performing of some assays, data analysis and reviewing the manuscript.

OB – responsible for the LC-MS/MS assay for determination of dabigatran concentration and reviewing of the manuscript.

PH – helped in the study design and interpretation of data and reviewed the manuscript.

REM – responsible for the study design, research ethics application, data analyses and interpretation, writing and reviewing of the manuscript.

Appendix

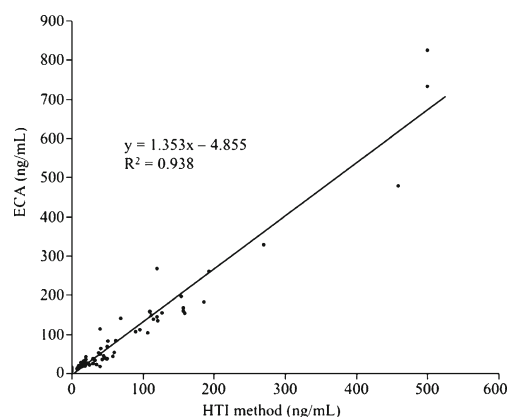


Fig. 6 Correlation between dabigatran concentrations indirectly estimated by the Hemoclot thrombin inhibitors® (HTI) method and the Ecarin clotting assay® (ECA) ($p < 0.0001$)

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