Thrombosis Research 171 (2018) 160–166

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

The anticoagulant effect of dabigatran is reflected in the lag time and time-to-peak, but not in the endogenous thrombin potential or peak, of thrombin generation☆

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ARTICLE INFO

Keywords:
Anticoagulant
Dabigatran
Idarucizumab
Thrombin generation

ABSTRACT

Introduction: Calibrated automated thrombinography (CAT) is a sensitive method to assess coagulation. Dabigatran inhibits both free thrombin and the $\alpha_2$macroglobulin (α_2M)-thrombin complex, which results in an erroneously increased peak and endogenous thrombin potential (ETP) without affecting lag time and time-to-peak. The aim of this study was to elucidate the artefacts in CAT when dabigatran is present.

Materials and methods: Thrombin generation (TG) was measured in vitro by using CAT in the presence or absence of 6 μM idarucizumab in plasma spiked with dabigatran. Additionally, ex vivo measurements were performed in plasmas of 63 patients using dabigatran in the presence and absence of idarucizumab.

Results: The in vitro experiments confirmed that the ETP, peak and velocity index were artificially increased. This was mainly due to the inhibition of the calibrator by dabigatran and partly due to CAT algorithms. The calibration artefact could be resolved by adding idarucizumab to the calibrator well. However, the second, mathematical artefact remains when dabigatran is present in the TG well. These findings were corroborated by ex vivo experiments i.e. the lag time and time-to-peak were significantly reduced in patients upon addition of idarucizumab, but the ETP and peak were not significantly affected. The velocity index did change significantly, since this is a combination of time-dependent factors and the peak.

Conclusions: The peak, ETP and velocity index do not represent the anticoagulant effect of dabigatran on TG measured with CAT. The lag time and time-to-peak, however, do reflect the effect of dabigatran.

1. Introduction

Dabigatran is a direct oral anticoagulant (DOAC) inhibiting thrombin. Dabigatran and DOACs inhibiting factor Xa (rivaroxaban, apixaban, edoxaban) have been shown to be at least non-inferior to vitamin K antagonists (VKA) with respect to the prevention and treatment of venous thrombosis. Additionally, large clinical trials have shown less severe haemorrhage during DOAC treatment compared to treatment with VKA [1–7]. However, bleeding remains the main concern during DOAC treatment and severe bleeding does still occur, which makes reversal therapy necessary. For the reversal of dabigatran, the humanized monoclonal antibody fragment idarucizumab (Praxbind®, Boehringer Ingelheim) was developed [8]. Idarucizumab binds both free and thrombin-bound dabigatran in a 1:1 ratio with an affinity 350 times stronger than the affinity of dabigatran for thrombin [8]. Studies have shown that administration of idarucizumab successfully restores coagulation [8,9]. Measuring the effect of dabigatran around administration of idarucizumab is needed, not only to evaluate reversal, but also to assess the remaining bleeding risk of patients [10–12]. Measuring dabigatran levels before administration helps to select patients who would benefit from reversal thus avoiding unnecessary administration of expensive reversal agents. Measuring after reversal could help detect patients with a rebound effect of plasma dabigatran levels. Moreover, monitoring the effect of dabigatran on coagulation could detect an underlying coagulopathy unrelated to treatment [13–15]. This can only be achieved by reliable assays that provide information

☆ This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.
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https://doi.org/10.1016/j.thromres.2018.10.005
Received 25 June 2018; Received in revised form 21 September 2018; Accepted 3 October 2018
Available online 06 October 2018
0049-3848/

on the global coagulation status.

At the moment, few sensitive methods are available to measure the dabigatran concentration and consequently the neutralizing effect of idarucizumab. Mass spectrometry, the diluted thrombin time (e.g. the Hemoclot thrombin inhibitor® (HTI), Hyphen BioMed), the ecarin clotting assay as well as the ecarin chromogenic assay (e.g. STA-ECA II, Stago) can be used to determine drug levels of dabigatran [16–19]. However, none of these assays are able to provide information on the differential effect of dabigatran on coagulation or on endogenous coagulopathies.

Thrombin generation (TG) is an assay that provides insight in the global coagulation characteristics of patients, and it was shown that TG parameters are associated with both bleeding and thrombotic tendencies in various patient populations [20–23]. Some studies have been carried out towards the effect dabigatran or other reversible, direct thrombin inhibitors on TG. Dabigatran, as expected, prolongs the lag time and time-to-peak, but counterintuitively, increases the peak height and endogenous thrombin potential (ETP) [24–26]. We postulate that these artefacts are due to the calculations used in calibrated automated thrombinography (CAT) [27] and have to be elucidated and solved for CAT to become a clinical tool able to assess patients using direct thrombin inhibitors. Thrombin bound to $\alpha_2$macroglobulin (α_2M) can still cleave small molecules such as the fluorescent substrate used in CAT [20]. Additionally, direct thrombin inhibitors, such as dabigatran, can also inhibit thrombin in complex with α_2M. In order to discern the effects of dabigatran on CAT measurements and calculations, one needs to understand which algorithms are applied in the conversion of the fluorescent signal to the final concentration of free thrombin. By determining the first derivative of the fluorescent data and comparing the slope of the TG curve to that of the calibrator curve, the total thrombin concentration can be determined. This is the first part of the calculations that is affected by dabigatran. The second algorithm is the calculation of α_2M-thrombin present in the measurement (TG) well. After the conversion of the fluorescence the total thrombin concentration is represented. In order to obtain the concentration of free thrombin, the α_2M-thrombin needs to be subtracted from the total amount of thrombin [28]. We assume that in the presence of dabigatran this is the second part of the calculations that could be disturbed. Therefore, unlike what previous reports [25,26] have stated, we postulate that dabigatran not only affects the calibrator, but also the correction for the signal caused by α_2M-thrombin in the measurement well.

The aim of the current study was to situate and clarify the artefacts (increased ETP and peak) found in CAT when measuring samples containing dabigatran, as well as to investigate to what level these artefacts could be corrected by idarucizumab.

2. Materials & methods

2.1. Plasma samples

Sixty-three plasma samples from patients treated with dabigatran etexilate for stroke prevention in non-valvular atrial fibrillation were included in the study. The sample collection was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Centre Hospitalier Universitaire UCL Namur in Yvoir, Belgium (number of approval BU3920096633). Blood was taken by venipuncture from the antecubital vein and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Heverlee, Belgium) using a 21-gauge needle (Terumo). Platelet poor plasma (PPP) was obtained from the supernatant fraction after double centrifugation at 1500*g* for 15 min at room temperature. Afterwards, plasma was aliquoted and frozen at −80 °C without delay. Frozen plasma sample aliquots were thawed and heated to 37 °C for 5–10 min before experiments.

Normal pooled plasma (NPP) was prepared from blood collected from 116 healthy volunteers. Plasma was pooled after an initial centrifugation step (2500*g*, 5 min), followed by ultracentrifugation at 100,000 *g* for 10 min. Aliquots of 500 µl were snap frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Reagents

Synthetic phospholipids (PL) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and used as vesicles consisting of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine (1:1:3, mol:mol:mol). Recombinant tissue factor (TF) was Innovin® (Dade-Behring, Marburg, Germany). The fluorogenic substrate *Z*-Gly-Gly-Arg-aminomethylcoumarin (ZGGR-AMC) was purchased from Bachem (Basel, Switzerland). The calibrator (α_2M-thrombin) was prepared as described by Hemker et al. [29]. Hepes buffers containing 5 mg/ml or 60 mg/ml bovine serum albumin (BSA5 and BSA60) were prepared according to Hemker et al. [30]. Dabigatran was purchased from Alsachim (Illkirch Graffenstaden, France) and idarucizumab (Praxbind®) was from Boehringer Ingelheim (Ingelheim am Rhein, Germany).

2.3. Calibrated automated thrombinography

TG was performed in duplicate in platelet poor plasma using CAT as described previously by Hemker et al. [29]. The calibrator wells contained a final concentration of α_2M-thrombin equivalent to 100 nM thrombin activity. The final concentration of TF for the in vitro experiments was 1, 5 or 20 pM added together with 4 µM PL in the TG well. The patient samples were measured at 5 pM TF and 4 µM PL. These samples were measured in the presence of 6 µM of idarucizumab in the calibrator wells and in presence and absence of 6 µM idarucizumab (final concentration) in the TG well. This concentration was chosen since it can neutralize concentrations of dabigatran up to 6 µM, i.e. 2830 ng/ml (since the idarucizumab binds dabigatran in a 1:1 ratio) and is expected to cover the range of concentrations expected in this population. Additionally, the mean idarucizumab concentration in the REVERSE-AD study after 4 h was around 5–6 µM [9]. Data were analysed with specialized software from Thrombinoscope (Maastricht, The Netherlands). The velocity index was calculated as: peak/(time-to-peak − lag time). α_2M-thrombin concentration curves were deducted from the raw, total thrombin fluoresence data as described in Hemker et al. [28].

2.4. Dabigatran concentration determination

The dabigatran concentration was determined from the inhibition of α_2M-thrombin activity by the dabigatran present in a plasma sample. Samples were prediluted in Hepes buffers containing 5 mg/ml BSA. The diluted samples were incubated (in duplicate) with 30 nM and 70 nM α_2M-thrombin and the remaining α_2M-thrombin activity was measured kinetically as the cleavage of thrombin substrate ZGGR-AMC on a Fluoroskan Ascent fluorimeter (Thermo Labsystems, Helsinki, Finland). The inhibition of α_2M-thrombin was compared to a dose-response series of dabigatran to enable the determination of the plasma dabigatran level. Dabigatran levels between 50 and 500 nM (i.e. 23.6–236 ng/ml) were calculated based on the reference curve containing 30 nM α_2M-thrombin and levels above 500 nM (i.e. 236 ng/ml) were calculated from the curve containing 70 nM α_2M-thrombin. Dilute thrombin time using Hemoclot Thrombin Inhibitors® (Hyphen BioMed®) was also performed for all samples on the STA-R Evolution® coagulometer (Diagnostica Stago®), as previously described [19].

2.5. Statistical analysis

Statistical analyses were performed with Graphpad Prism software (version 5.00). Normality of the data was assessed using the Shapiro-Wilk test. Data are represented as median with interquartile range

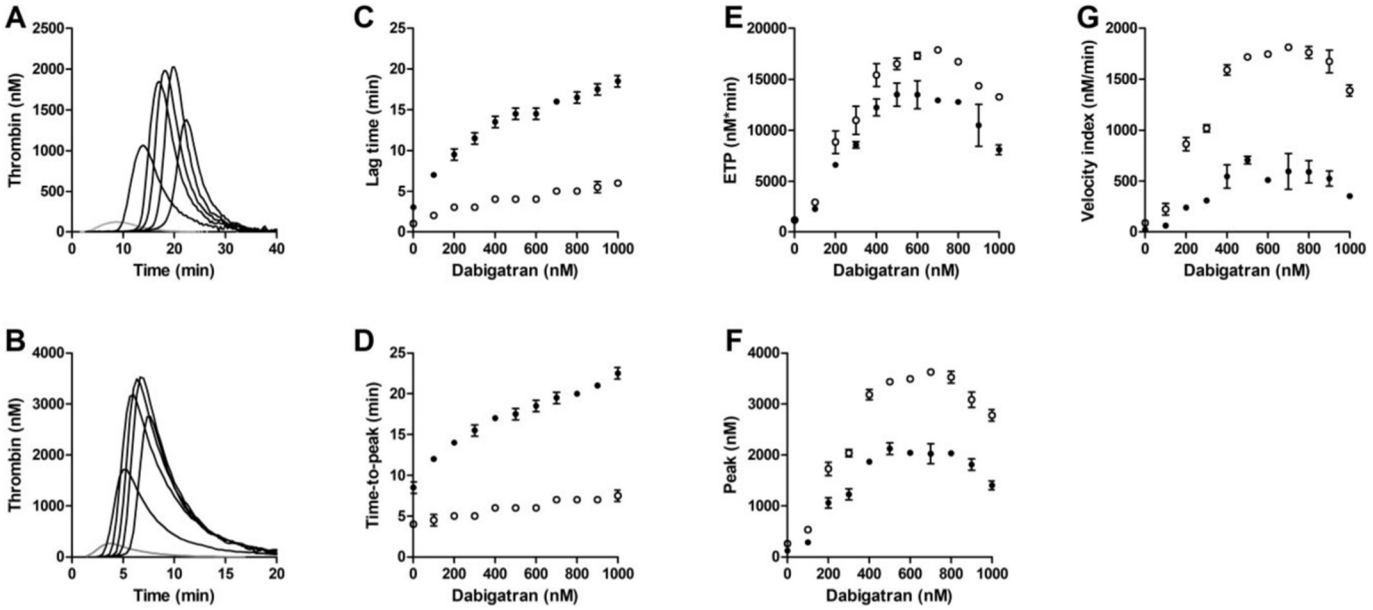

Fig. 1. The effect of dabigatran on a calibrated automated thrombinography measurement. (A–B) Thrombin generation was measured at 1 pM TF (A) and 5 pM TF (B) in normal pooled plasma containing 0 (grey line), 200, 400, 600, 800 and 1000 nM dabigatran (left to right) (n = 2). (C-G) The dose-dependent effect of dabigatran on the lag time (C), time-to-peak (D), endogenous thrombin potential (ETP) (E), peak height (F) and velocity index (G) at 1 pM TF (●) and 5 pM TF (○) using concentrations between 0 and 1000 nM dabigatran (represented as mean ± SD, n = 2).

(IQR). The Wilcoxon signed rank test was used to determine the difference between patient samples before and after addition of idarucizumab. A two-sided p-value of 0.05 was considered statistically significant. Data are represented as mean with standard deviation (SD).

3. Results

3.1. Inaccurate ETP, peak and velocity index in the presence of dabigatran

TG was measured by the CAT method in normal pooled plasma spiked with 0–1000 nM dabigatran, which corresponds to 0–472 ng/ml (Fig. 1). Dabigatran (present in both calibrator and TG wells) caused a counter-intuitive increase of the peak height and the ETP (Fig. 1C–F), since a reduction of these parameters would be expected. The minimal and maximal values of the peak at 1 pM TF were 123 nM (in the absence of dabigatran) versus 2142 nM (500 nM dabigatran) and 263 nM (in the absence of dabigatran) versus 3625 nM (700 nM dabigatran) at 5 pM TF; for the ETP these were 1064 nM·min (in the absence of dabigatran) versus 13,478 nM·min (500 nM dabigatran) at 1 pM TF and 1159 nM·min (in the absence of dabigatran) versus 17,302 nM·min (700 nM dabigatran) at 5 pM TF; and for the velocity index these were 22 nM/min (in the absence of dabigatran) versus 708 nM/min (500 nM dabigatran) at 1 pM TF and 88 nM/min (in the absence of dabigatran) versus 1812 nM/min (700 nM dabigatran) at 5 pM TF. The anticoagulant effect of dabigatran is reflected in the prolonged lag time (6.2 times and 6 times) and time-to-peak (2.6 times and 1.9 times) at both 1 and 5 pM TF, respectively.

Dabigatran reduces the activity of the TG calibrator on the fluorescent substrate dose-dependently (Fig. 2). The addition of 6 μM idarucizumab to the calibrator well is sufficient to neutralize the inhibitory effect of at least 1000 nM (472 ng/ml) of- dabigatran.

3.2. ETP, peak and velocity index remain inaccurate after neutralization of dabigatran in calibrator wells

The addition of 6 μM idarucizumab to the calibrator well enables the correct measurement of the calibrator activity in samples containing dabigatran (Fig. 3). However, at lower dabigatran concentrations (< 300 nM (142 ng/ml)), a stimulatory effect of dabigatran on the TG peak height remains, particularly at the lower tissue factor concentration tested (Fig. 3A). At 1 pM tissue factor the peak without addition was 121 nM, this increased to maximally 156 nM at 200 nM dabigatran (28%). In the presence of 1000 nM of dabigatran the peak was 44 nM. At 5 pM TF the increase was only detectable at 100 nM dabigatran with a peak value of 275 nM as compared to 264 nM in the absence of dabigatran. At 1000 nM dabigatran the peak was 90 nM. The ETP and velocity index follow the same pattern, at 1 pM TF there was an increase from 1099 nM·min (no dabigatran) to 1320 nM·min (200 nM dabigatran) for the ETP and from 19 nM/min (no dabigatran) to 38 nM/min (100 nM) dabigatran for the velocity index. At 5 pM TF the ETP increased from 1276 nM·min (no dabigatran) to 2050 nM·min (100 nM dabigatran), the velocity index, however, gradually decreased from 132 nM/min (no dabigatran) to 79 nM/min (1000 nM dabigatran).

3.3. Neutralizing dabigatran in both calibrator and TG wells allows measurement of 'uninhibited' TG

Idarucizumab can also be used to neutralize dabigatran in the TG well itself. Fig. 4 shows the neutralization of 100 and 400 nM dabigatran in normal pooled plasma by 6 μM idarucizumab during TG. Both doses of dabigatran prolong the lag time and time-to-peak, which is restored upon the addition of idarucizumab. The addition of idarucizumab alone to normal pooled plasma does not affect TG (Fig. 5). When TG is initiated with 1 pM TF, the increase of the peak at the low dabigatran (here 100 nM) concentration is the most visible. With increasing TF concentrations this effect diminishes and is no longer visible at 20 pM TF. However, upon examination of the α_2M-thrombin levels during the TG reaction, it is clear that this complex is inhibited by dabigatran from low to high TF levels (Fig. 4D–F). Therefore, a lower α_2M-thrombin concentration is subtracted from the total thrombin concentration and consequently the ETP and peak are overestimated. Since the velocity index is a function of the peak, this will also be overestimated.

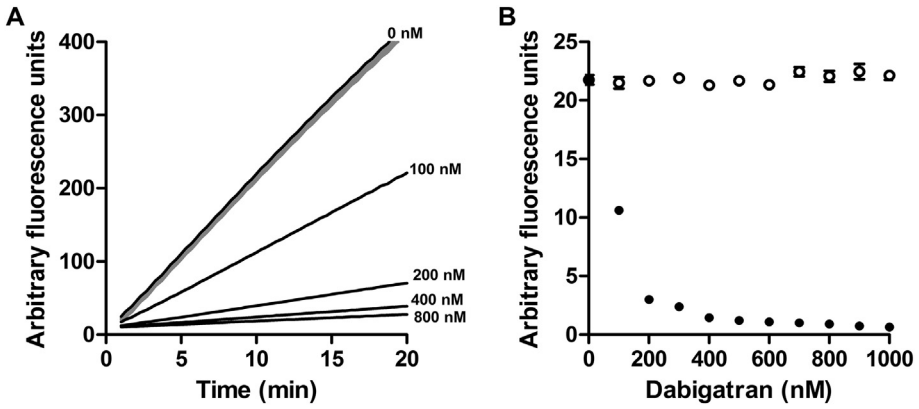

Fig. 2. The effect of dabigatran and idarucizumab on the fluorescent activity of the calibrator. (A) The effect of different dabigatran concentrations on the measurement of the calibrator curves in the absence (black) and presence of 6 μM idarucizumab (grey). The curves containing different dabigatran concentrations in the presence of idarucizumab are all overlapping (n = 2). (B) Quantification of the dose-dependent inhibition of the thrombin generation calibrator by dabigatran (0–1000 nM) in the absence (●) and presence of idarucizumab (○) (represented as mean ± SD, n = 2).

3.4. Proof of principle in patient samples

The use of idarucizumab to reverse the effect of dabigatran was tested in a group of patients treated with dabigatran etexilate (n = 63). The time since last intake was available for 50 of the patients and the median [IQR] time was 17 h [3 h–72 h] with a minimum of 0 h and a maximum of 540 h. The amount of dabigatran in the patient samples ranged from 0 nM (0 ng/ml) to 1362 nM (643 ng/ml), with a median [IQR] concentration of 205 nM [113 nM–440 nM] (97 ng/ml [53 ng/ml-208 ng/ml]). As was demonstrated in NPP, the addition of idarucizumab shortens the lag time and time-to-peak in patients with higher dabigatran levels, whereas TG in the sample with low dabigatran levels is almost unaffected. The same trend was found in all 63 patients treated with dabigatran (Fig. 6). The addition of idarucizumab causes a significant shortening of the lag time (median [IQR] without versus with idarucizumab: 4.7 min [3.3 min–7.0 min] versus 2.7 min [2.3 min–3.3 min], $p < 0.0001$) and the time-to-peak (7.8 min [5.6 min–9.7 min] versus 5.7 min [4.7 min–7.0 min], $p < 0.0001$). One outlier can be seen with a small increase in lag time and time-to-peak after addition of idarucizumab (7.7 min to 8 min and 13.7 min to 14.7 min, respectively). This patient took the last dabigatran tablet over 24 h before blood draw and was found to have 0 ng/ml dabigatran by HTI. The increase is therefore likely attributable to experimental variation. As expected, based on the results obtained in normal pooled plasma, no statistically significant difference was detected in peak height (median [IQR] with versus without idarucizumab: 241 nM [189 nM–310 nM] versus 249 nM [196 nM–280 nM], $p = 0.30$) and ETP (median [IQR] with versus without idarucizumab: 1253 nM·min [1054 nM·min-1445 nM·min] versus 1288 nM·min [1120 nM·min-1511 nM·min], $p = 0.18$). However, a clear restoration of ETP and peak can be seen in the samples that were completely inhibited by dabigatran upon addition of idarucizumab. The velocity index is significantly reduced in the presence of idarucizumab (median [IQR]: 108 nM/min [61 nM/min–150 nM/min]) as compared to in the absence (median [IQR]: 86 nM/min [56 nM/min–125 nM/min]).

4. Discussion

In this paper, we confirm the artefacts in TG that are observed in samples containing dabigatran (an increased peak and ETP), which were reported previously [24,26,27]; and we show that in CAT this increase in ETP and peak is caused by two mechanisms. Firstly, a large part is due to inhibition of the calibrator activity and a subsequent overestimation of thrombin activity in the TG measurement. We demonstrate that this effect can be overcome by the addition of dabigatran reversal agent idarucizumab to the calibrator measurement. Secondly, CAT uses a correction algorithm in which the fluorescence generated by α_2M-thrombin activity is subtracted from the total thrombin activity (i.e. free thrombin plus α_2M-thrombin). These calculations do not take into account that α_2M-thrombin is inhibited by dabigatran to varying extents throughout the TG measurement and therefore TG is overestimated, especially at low dabigatran concentrations [27].

In line with what was previously reported [24,26,27], we found an elevation of TG in plasma containing dabigatran. Peak levels of 3625 nM and ETP's of 17,301 nM·min were found. In normal populations analysed in the same conditions, but in the absence of anticoagulants, these values are on average 330 nM and 1555 nM·min, respectively [31]. This elevation in peak and ETP was only partly restored when dabigatran in the calibrator well was either absent or neutralized.

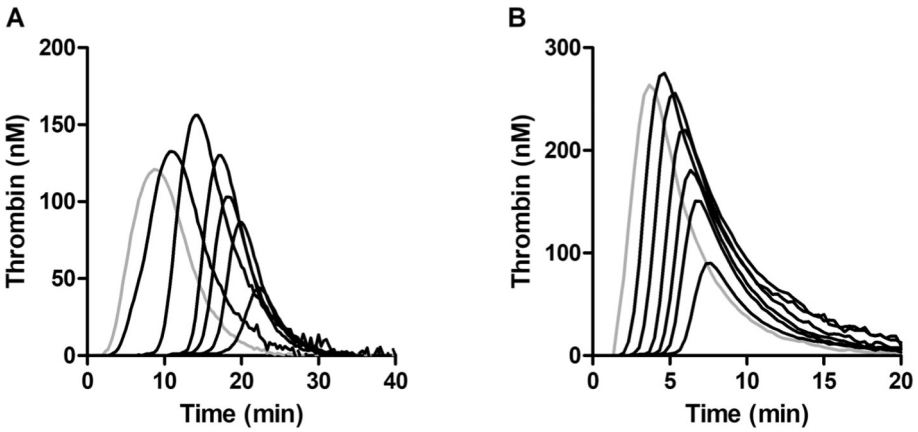

Fig. 3. Thrombin generation curves after a correction of the calibration, achieved by addition of idarucizumab (6 μM) to the calibrator wells. Thrombin generation in normal pooled plasma spiked with 0 (grey line), 100, 200, 400, 600, 800, and 1000 nM dabigatran (left to right) measured at 1 pM TF (A) and 5 pM TF (B) (n = 2).

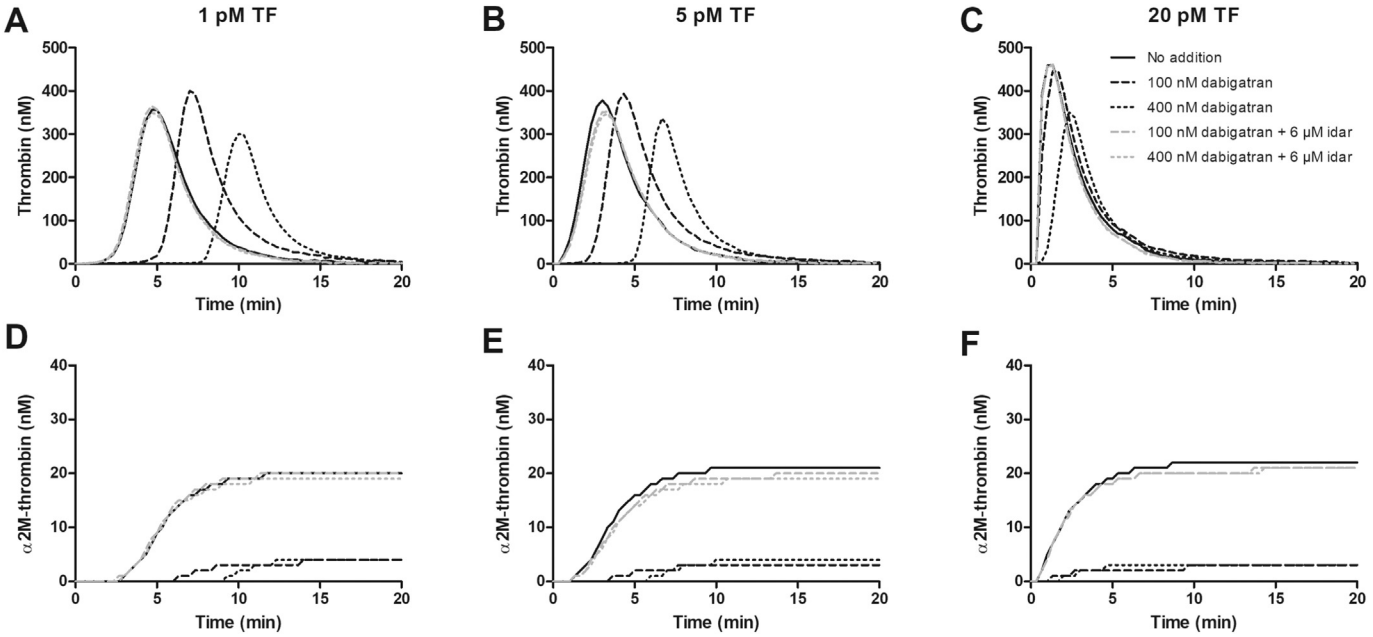

Fig. 4. The effect of idarucizumab on thrombin generation and α_2-macroglubulin (α_2M)-thrombin in normal pooled plasma (NPP) spiked with dabigatran at different tissue factor concentrations. Thrombin generation was measured in the absence (black lines) and presence (grey lines) of 6 μM idarucizumab in normal pooled plasma containing 0 nM (full line), 100 nM (striped line) and 400 nM (dotted line) dabigatran measured at 1 pM (A, D), 5 pM (B, E) and 20 pM (C, F) tissue factor (n = 2). The calculated thrombin generations curves, representing free thrombin, are shown (A–C), as well as the formation of α_2M-thrombin complex over time (D–F).

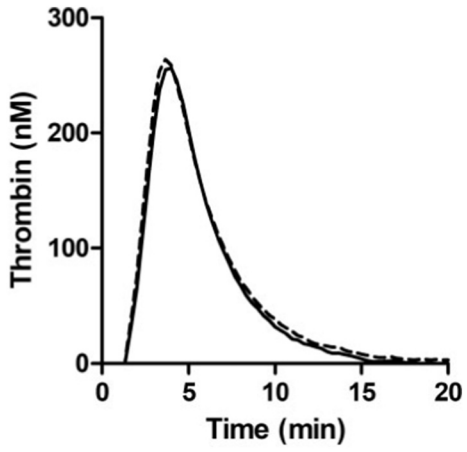

Fig. 5. Thrombin generation in the presence and absence of idarucizumab in the absence of dabigatran. Thrombin generation was measured in the presence (full line) and absence (interrupted line) of idarucizumab at 5 pM tissue factor.

Since the velocity index is calculated by dividing the peak by the time between lag time and peak, this parameter is affected by the increased peak and therefore does not reflect the correct value either. For the neutralization of dabigatran idarucizumab was used, which was previously reported to completely reverse the dabigatran effect on TG [9,32,33]. Dabigatran in the measurement well can also be completely neutralized by idarucizumab, which would allow assessment of TG as if no dabigatran was present ('uninhibited' conditions). However, the effect of dabigatran on the peak and ETP in CAT cannot be assessed this way. Under all circumstances the lag time and time-to-peak in CAT do correctly reflect the effect of dabigatran, since the time-dependent parameters are not altered by the corrections in the CAT algorithms.

Given that an elevation of the peak and/or ETP is still present after correcting the dabigatran effect on the calibrator, a second artefact must be situated in the correction for the α_2M-thrombin in the TG well. A crucial assumption in the CAT algorithm is that the amount of α_2M-thrombin increases over time as more thrombin is formed in pseudo first order reaction and consequently is proportional to the amount of thrombin [28]. When dabigatran is present, the α_2M-thrombin activity at the end of the measurement will be inhibited relatively more than at the beginning, since little thrombin will be left and a lot of α_2M-thrombin is formed. This effect was more pronounced at 1 pM TF as compared to 5 pM TF. At lower TF concentrations, TG is known to be more sensitive and the ratio between free thrombin and α_2M-thrombin will be affected differently compared to at 5 pM TF. We speculate that when TG is initiated with 1 pM TF, the formation of thrombin proceeds slower compared to an initiation with 5 pM TF and therefore relatively more α_2M-thrombin is formed early in the process which is inhibited by dabigatran. At 20 pM TF the artefacts are not visible in the TG curves. However, α_2M-thrombin is inhibited, resulting in an overestimation of the peak and ETP. Additionally, in the presence of higher dabigatran concentrations the peak is reduced compared to the absence of dabigatran. In these conditions the effect of dabigatran on free thrombin is larger than the effect on α_2M-thrombin, therefore the net effect is a decreased, but still overestimated, TG.

The in vitro findings were corroborated in samples from patients containing a wide range of dabigatran concentrations (0–1362 nM). Samples were analysed with idarucizumab present in the calibrator wells, and both in the presence and absence of idarucizumab in the TG wells. We additionally confirmed that idarucizumab does not affect TG in normal plasma [8,33]. The results in the patient samples support the data in NPP spiked with dabigatran: addition of idarucizumab significantly decreased lag time as well as time-to-peak, yet the effect on ETP and peak was not significantly increased. The explanation for this is that about half of the collected samples contained low dabigatran concentrations. In these samples the counterintuitive decrease in peak and ETP after dabigatran neutralization could be seen. The velocity index is significantly reduced upon addition of idarucizumab. This parameter is partly based on time-dependent parameters and partly on the peak. Therefore, although this parameter is significantly altered in the before or after neutralization of dabigatran (due to the effect of the time-dependent factor), this parameter does not correctly reflect the extent of the anticoagulant effect of dabigatran.

Different solutions can be suggested to circumvent the artefacts in

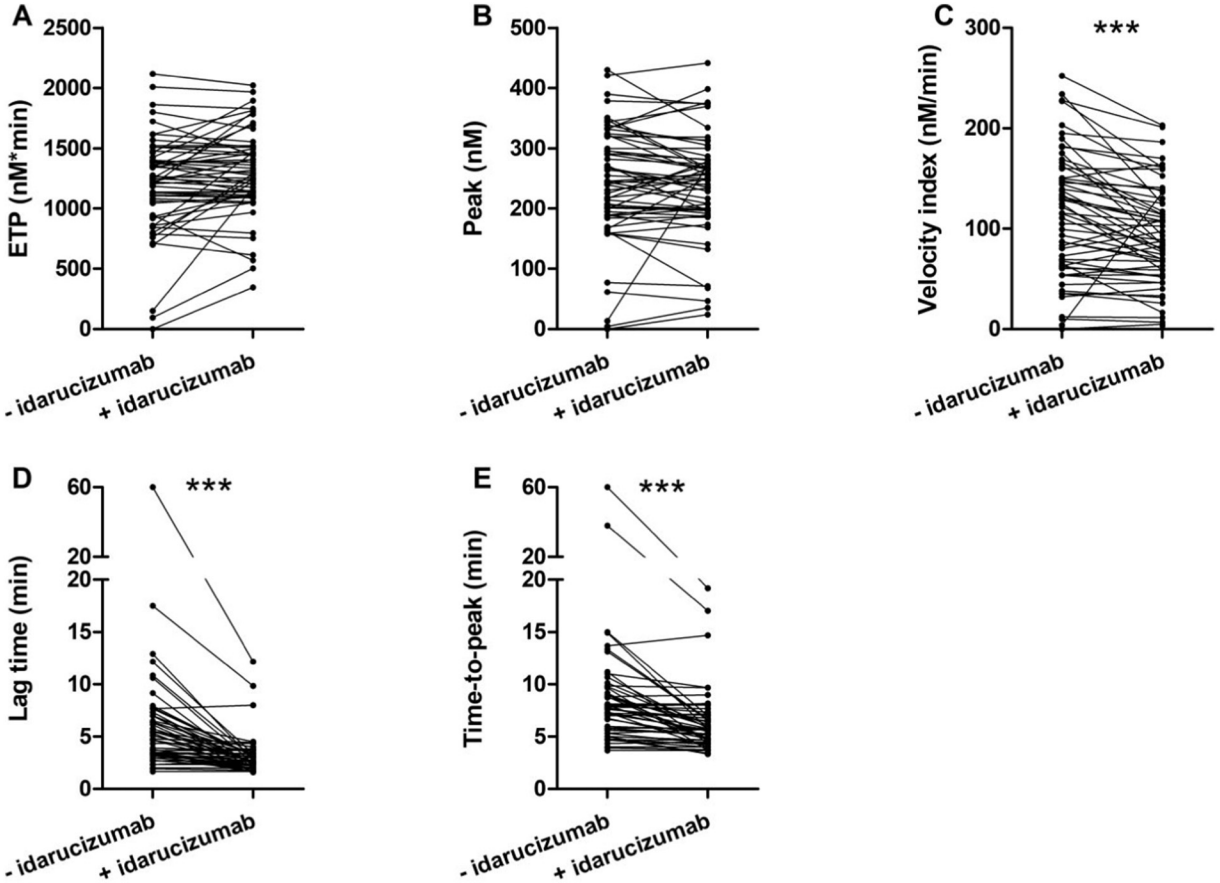

Fig. 6. The effect of idarucizumab on thrombin generation parameters in sixty-three patient samples containing dabigatran. Endogenous thrombin potential (ETP) (A), peak (B), velocity index (C), lag time (D) and time-to-peak (E) before and after the in vitro addition of idarucizumab. Differences between groups were analysed using the Wilcoxon signed rank test. $***p < 0.0001$, $n = 63$.

CAT calculations when dabigatran is present: (1) The measurement method can be altered in such a way that α_2-M-thrombin is not detected in the experiment, or (2) the CAT algorithm can be updated to correctly perform the α_2M-thrombin subtraction step in the TG calculation method. Firstly, to avoid the detection of α_2M-thrombin activity in the TG measurement, a macrosubstrate could be used, which is only cleaved by free thrombin and not by α_2M-thrombin. By using such a substrate the calculation artefacts would not occur. However, substrates with this quality that do not affect TG otherwise are currently not available. Secondly, in order to calculate the correct ETP and peak for samples containing both low and high concentrations of dabigatran, a new computational approach could be developed which allows the correct subtraction of α_2M-thrombin activity. However, this activity varies between different patients since it is dependent on the α_2M and antithrombin concentrations, as well as on the amount and the rate of thrombin generation. The additional complicating factor in patients using dabigatran is the fact that the interactions between these proteins are also influenced by the dabigatran levels. To correct for this an empirical computational approach would have to be developed to predict the amount of α_2M-thrombin formed in individual patient samples with known α_2M and dabigatran levels. However, the development of such an approach would require knowledge of the concentration of dabigatran present in the sample, the kinetic constants of the substrate as well as dabigatran with both thrombin and α_2M-thrombin. Moreover, a complete correction of the artefacts would only be possible when implementing the computational approach together with the addition of idarucizumab to the calibrator. Validation of this algorithm would require devoted experimentation, calculations and moreover an evaluation in large and different patient populations. It would also require the measurement of plasma dabigatran levels in each plasma sample. Currently, we are working on an assay to measure dabigatran levels, which can be analysed in the same experimental set-up as TG accommodating the convenient and simultaneous measurement of dabigatran levels and the effect on coagulation.

The study had some limitations. Idarucizumab was used in a concentration of 6 μM, which can neutralize dabigatran concentrations up to 6 μM (2830 ng/ml). The concentration of idarucizumab that was used, was high in comparison to findings by Jacquemin et al. who used 2.6 μM idarucizumab to neutralize dabigatran in routine coagulation assays [34]. The concentration used, on the other hand, was relatively low compared to patient plasma concentrations at the peak level after a 5 g dose (between 7 μM and 45 μM up to 30 min after infusion) [9]. However, for the purpose of demonstrating artefacts in CAT calculations in the presence of dabigatran, both in vitro and in peri-operative samples, this concentration was sufficient for full neutralization.

Ideally, the TG peak and ETP would be used to evaluate the remaining haemostatic potential of a patient using dabigatran or the level of reversal achieved by the administration of idarucizumab. Using a thrombin generation test for this purpose would provide an insight into the general coagulation status of a patient. In this paper we show that the peak, ETP and velocity index can only be used to assess the 'uninhibited' coagulation potential by adding idarucizumab to the plasma. These parameters might, however, provide an idea of the anticoagulant effect of dabigatran at higher concentrations. Only the lag time and time-to-peak will give an accurate representation of the effect of dabigatran on CAT in all conditions, when idarucizumab is present in the calibrator well. Several studies have shown, though, that the time-dependent parameters tend to be less informative on the bleeding or

thrombotic risk than the functional parameters (peak and ETP) [35,36]. Further research into possible solutions for the dabigatran effect on the CAT algorithms is required.

Acknowledgements

The authors would like to thank Rob Wagenvoord and Bas de Laat for their advice during the preparation of this article.

Disclosures

S. Bloemen, R. Kremers, S. Zwaveling and M. Roest are linked to Synapse Research Institute, a research institute part of the Diagnostica Stago group. F. Mullier reports institutional fees from Stago, Werfen, Nodia, Sysmex and Bayer. He also reports speaker fees from Boehringer Ingelheim, Bayer Healthcare, Bristol-Myers Squibb-Pfizer and Stago all outside the submitted work. J. Douxfils declares not to have any conflict of interest.

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