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From laboratory to clinical practice: Dabigatran effects on thrombin generation and coagulation in patient samples

Tuukka A. Helin [a], Marja Lemponen [a], Paul Hjemdahl [b], Yuko Rönquist-Nii [c], Riitta Lassila [d,1], Lotta Joutsi-Korhonen [a,*]

[a] Coagulation Disorders Unit, Clinical Chemistry, HUSLAB Laboratory Services, Helsinki University Central Hospital, POB 340, 00029, Helsinki, Finland
[b] Clinical Pharmacology Unit, Department of Medicine, Solna, Karolinska Institutet, and Karolinska University Hospital, 17176, Stockholm, Sweden
[c] Clinical Pharmacology Unit, Karolinska University Hospital, 17176, Stockholm, Sweden
[d] Coagulation Disorders Unit, Hematology and Cancer Center and Clinical Chemistry (HUSLAB Laboratory Services), University of Helsinki, Helsinki University Central Hospital, POB 340, 00029, Helsinki, Finland

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ABSTRACT

Introduction: Dabigatran (Dabi) is not routinely monitored. However, in emergency cases quantitative assessment is required and laboratories must provide suitable tests at all hours. Little is known about Dabi effects on thrombin generation.
Materials and methods: Patient samples (n = 241) were analyzed for functional Dabi concentrations (Dabi-TT) using a combination of the Hemoclot Thrombin Inhibitors assay (HTI®) and, for samples with low levels, undiluted thrombin time (TT). Results were compared to prothrombin time (PT) and activated partial thromboplastin time (APTT). In 49 samples Dabi effects were further investigated with Calibrated Automated Thrombogram (CAT®) for thrombin generation and with Russell's viper venom time (RVVT), prothrombinase-induced clotting time (PiCT®), chromogenic Anti-IIa® and ecarin clotting assay (ECA®). Fibrinogen and D dimer were assessed to reflect the coagulation status of the patient. A subset of these samples (n = 21) were also analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).
Results: Dabi-TT correlated with RVVT ($R^2 = 0.49$), PiCT® ($R^2 = 0.73$), ECA® ($R^2 = 0.89$), Anti-IIa® ($R^2 = 0.90$) and LC-MS/MS ($R^2 = 0.81$). APTT correlated curvi-linearly with Dabi-TT ($R^2 = 0.71$), but was normal in many cases (18/70) despite Dabi-TT > 40 ng/mL. There was no association between Dabi-TT and fibrinogen or D dimer levels. Increasing Dabi concentrations prolonged lag time ($R^2 = 0.54$) and, surprisingly, elevated the ETP and Peak of CAT® ($p < 0.001$).
Conclusions: Thrombin-specific tests measure Dabi accurately, whereas coagulation time based assays depend more on other factors. The enhanced thrombin generation in Dabi-treated patients may predict clinically relevant hypercoagulability and warrants further investigation.

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Introduction

Dabigatran etexilate (Pradaxa®, Boehringer Ingelheim; Dabi) is a direct thrombin inhibitor (DTI) anticoagulant. Due to what is considered predictable pharmacokinetics, routine laboratory monitoring is claimed to be unnecessary [1, 2]. Nevertheless, under special circumstances, e.g. renal or hepatic dysfunction, acute bleeding complications or thrombosis, and emergency surgery, assessment of anticoagulation becomes necessary [3, 4]. Coagulation laboratories must provide readily available (all hours) and practical tests for measurements of Dabi anticoagulation. In the absence of routine methodologies for assessments under clinically stable situations, it becomes challenging to evaluate anticoagulation during medical emergencies.

The screening tests prothrombin time (PT) and activated partial thromboplastin time (APTT) are of limited value [5–9]. To better assess Dabi effects, more sensitive methods must be used. Thrombin time (TT)

Abbreviations: Dabi, dabigatran; DTI, direct thrombin inhibitor; PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; ECA, ecarin clotting assay; HTI, Hemoclot Thrombin Inhibitors; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PiCT, prothombinase-induced clotting time; FXa, activated coagulation factor X; RVV-V, Russel's viper venom V; RVVT, Russel's viper venom time; IIa, thrombin, Anti-IIa, anti-thrombin activity; PPP, platelet-poor plasma; Dabi-TT, functional dabigatran concentration; CAT, Calibrated Automated Thrombogram; ETP, endogenous thrombin potential.

* Corresponding author at: Clinical Chemistry and Hematology, HUSLAB Laboratory Services POB 340, 00029 Helsinki, Finland. Tel.: +358 50 427 2402, fax: +358 9 471 74016.
E-mail addresses: tuukka.helin@helsinki.fi (T.A. Helin), marja.lemponen@hus.fi (M. Lemponen), paul.hjemdahl@ki.se (P. Hjemdahl), yuko.ronquist@karolinska.se (Y. Rönquist-Nii), riitta.lassila@hus.fi (R. Lassila), lotta.joutsi-korhonen@hus.fi (L. Joutsi-Korhonen).
[1] CSO of Aplagon Ltd.

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is linear, highly sensitive, and can be calibrated with Dabi to depict its effects [10]. Chromogenic ecarin clotting assay (ECA®) uses the snake venom ecarin to generate meizothrombin, [11–13] with less interferences with, e.g., lupus anticoagulant and warfarin than clot-based assays [14]. TT calibrated with Dabi, Hemoclot® Thrombin Inhibitors (HTI®), and ECA® correlate well with actual Dabi concentrations in plasma as measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), whereas correlations with APTT are modest and with PT non-existent [8, 9].

Prothrombinase-induced clotting time (PiCT®) uses activated coagulation factor X (FXa), phospholipids and Russel's viper venom V (RVV-V) for activation, leading to prothrombinase complex activation and thrombin formation. Modified PiCT® has been used to measure both DTIs and FXa inhibitors [15]. Russell's viper venom time (RVVT) with a FX-specific activator is sensitive to FXa inhibitors, but might also be useful to assess DTIs [16]. The chromogenic anti-thrombin (IIa) activity (Anti-IIa®) assay detects DTIs. In this assay, excess thrombin is added to the sample and the amount of residual thrombin is measured. Again, interference is less pronounced than in clot-based assays [17].

Since most coagulation assays measure the time to fibrin formation (i.e., the initiation phase of coagulation), Dabi's many biological effects remain underestimated. Thrombin generation assays measuring the full spectrum of thrombin formation seem justified and potentially more informative [18]. DTIs have been reported to decrease thrombin formation, but the effects vary with different DTIs and are somewhat controversial [19].

Previously, we have studied the suitability and variability of coagulation assays using *in vitro* spiked plasma samples shipped to several European laboratories [7], and compared methods in anonymized patient samples [8, 9]. The relationships between Dabi plasma concentrations by LC-MS/MS and the risks of suffering thromboembolic or major bleeding events in the RE-LY study were recently published, yielding an excellent basis for the interpretation of Dabi concentration data [20]. As patients referred for laboratory testing likely vary in their coagulation status, it is important to evaluate Dabi also in real life patient samples (i.e., beyond the selected trial patients with standardized sampling). Here, we aimed to assess how well different clotting assays detect Dabi in actual patient samples using indirect measurements by Dabi-calibrated TT (Dabi-TT) as a reference (where Dabi-TT is undiluted thrombin time for dabigatran < 40 ng/mL, and HTI for dabigatran ≥ 40 ng/dL). The performance of the functional analysis was confirmed by gold standard measurements by LC-MS/MS in a subset of the samples. Our additional aim was to gain more insight into the impact of Dabi on thrombin generation.

Materials and Methods

Study samples

Patient samples sent to the Meilahti hospital coagulation laboratory (HUSLAB Laboratory Services, Helsinki University Central Hospital, Finland) for Dabi concentration analysis were collected during a 5 year period (between 2008 and 2013). A total of 241 random plasma samples (from 85 patients) were accumulated. The specific clinical situation was not recorded, but at that time, Dabi was indicated for postoperative thromboprophylaxis after elective orthopedic surgery (150 or 220 mg once daily) and in patients with atrial fibrillation (either 150 or 110 mg twice daily). The Dabi concentration analysis was freely available in our hospital district with the recommendation to order the test only under special circumstances, such as major bleeding complications, thrombosis or emergency surgery and to order simultaneously PT and APTT. Blood samples were collected into sodium citrate anticoagulant (3.2%; 109 mM) tubes according to the local sampling protocol as part of hospital routine, centrifuged (at 2500 g for 15 min) and the platelet-poor plasma (PPP) was separated within 2 hours and stored at -80 °C before analysis.

Original Dabi Concentration Analysis Supplemented with Screening Tests

Dabi concentrations were assessed using diluted, Dabi-calibrated TT with HTI® (Aniara) in the 241 stored samples, the analytical range being 40-1000 ng/mL (85-2120 nmol/L) with intra-assay and inter-assay CVs of 7 % and 10 %, respectively. The screening tests TT (BC Thrombin Reagent®, Siemens Healthcare Diagnostics), APTT (Actin FSL®, Siemens Healthcare Diagnostics) and PT (Nycotest PT®, Axis-Shield; Owren-type assay) were performed in parallel according to our routine hospital protocol. TT had a local reference range of 17-25 s and an analytical range of 12-140 s and APTT 23-33 s and 18-180 s, respectively. PT (standard human plasma, Siemens Healthcare Diagnostics) had a reference range of 19-24 s and an analytical range of 16-180 s. The analyses were performed using the BCS® XP automatic analyzer (Siemens Healthcare Diagnostics).

Since very low Dabi concentrations could not be measured using HTI® (with the the lower detection limit of 40 ng/mL), we needed to estimate undiluted TT values as Dabi concentrations: TT < 60 s was set to correspond to 0 ng/mL of Dabi; TT 60-100 s to 10 ng/mL; TT 100-120 s to 20 ng/mL and TT 120-140 s to 30 ng/mL. The decision of these arbitrary categories was based on the literature and our data; TT has been shown to be linear with Dabi [10] and here, all the samples with measurable quantities of Dabi using HTI®, had an undiluted TT > 60 s. TT values < 60 s were chosen to represent 0 ng/mL instead of the upper local reference range for TT (25 s), since TT is not specific for Dabi effects and the prolongation at TT values ≥ 60 s most likely reflect Dabi effects. We then combined the TT data (<40 ng/mL) with HTI® data (≥40 ng/mL) to obtain functional estimates of Dabi concentrations (Dabi-TT) covering the entire concentration range.

Further Analysis of Dabi with Thrombin-specific Assays

We further analyzed the stored patient samples and assessed them with a large panel of clotting assays and thrombin generation as described below. We included only samples with comprehensive results from all methods, i.e., a total of 49 samples (35 patients).

The chromogenic Anti-IIa® (Direct Thombin Inhibitor assay®, Siemens Healthcare Diagnostics), the chromogenic ECA® (Haemosys® ECA-T, JenAffin), the RVVT (DVVTest 10®, Sekisui Diagnostics) and the PiCT® (Pefakit® PiCT, Pentapharm) were evaluated regarding their potential to quantify Dabi. All analyses were performed using BCS® XP.

In the Anti-IIa® assay, 25 μL of sample was mixed with 50 μL of substrate and the reaction initiated with 250 μL of thrombin reagent. In the ECA®, 25 μL plasma sample was diluted with 100 μL ECA® prothrombin buffer and mixed with 25 μL ECA® substrate, incubated (37 °C, 1 min), and the reaction was started with 50 μL of the ECA® ecarin reagent. Both the Anti-IIa® and the ECA® assays were calibrated for Dabi using calibrators from Aniara to achieve plasma Dabi concentrations of 0, 30, 250 and 510 ng/mL.

The RVVT, which is part of a screening panel in lupus anticoagulant testing, had the local upper normal reference value of 41 s. The PiCT® assay was performed using the 2-step protocol: 50 μL of sample was mixed with 50 μL of activator (containing RVV-V, FXa and phospholipids). At 180 s incubation 50 μL of 25 mM $CaCl_2$ was added and the clotting time measured. The PiCT® reagents induce antithrombin inhibition of FXa. The manufacturer reports a reference range of 19-31 s for PiCT®.

Thrombin generation was measured using Calibrated Automated Thrombogram® (CAT®, Diagnostica Stago) with the Stago PPP reagent (tissue factor 5 pM and phospholipids 4 μM) without corn-trypsin inhibitor. The lag time of the initiation of thrombin generation, and the endogenous thrombin potential (ETP), peak, time to peak, and time to the end of thrombin generation (start of tail) were measured according to the manufacturer's instructions. For comparison, ten plasma samples from three healthy volunteers were analyzed in parallel with the patient samples. These healthy controls had averages of 2.1 min (range 1.6-

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