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Shanshan Du, Christel Weiss, Giese Christina, Sandra Krämer, Martin Wehling, Roland Krämer and Job Harenberg*

Determination of dabigatran in plasma, serum, and urine samples: comparison of six methods

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Abstract

Background: Assessing the anticoagulant effect of dabigatran may be useful in certain clinical settings. When plasma sampling is not available, serum or urine samples may provide another option for dabigatran determinations.
Methods: Dabigatran was assessed in patients on treatment under real-life conditions in plasma samples by four clotting time-based assays and in plasma, serum, and urine samples by two chromogenic substrate methods.
Results: The concentrations of dabigatran in patients' plasma samples were not different for the Hemoclot test (106.8±89.4 ng/mL) and the ecarin clotting time (ECT, 109.5±74.5 ng/mL, p=0.58). Activated partial thromboplastin time and prothrombinase-induced clotting time showed low correlations with the other assays. Chromogenic assays measured similar concentrations as Hemoclot and ECT. For both chromogenic assays, the concentrations of dabigatran were about 70% lower in serum than in plasma samples (p<0.0001). The intra-class coefficient (ICC, Bland-Altman analysis) was strong comparing ECT, Hemoclot thrombin inhibitor (HTI) assay, and the two chromogenic assays (r=0.889–0.737). The ICC was low for comparisons of the chromogenic assays of serum vs. plasma values (ICC, 0.15 and 0.66). The ICC for the determination of dabigatran in urine samples by the two chromogenic assays (5641.6±4319.7 and 4730.0±3770.2 ng/mL) was 0.737.
Conclusions: ECT, HTI, and chromogenic assays can be used to determine dabigatran in plasma samples from patients under real-life conditions. Chromogenic assays require further improvement to reliably measure dabigatran in serum samples. Dabigatran concentrations in urine samples can also be determined quantitatively.

Keywords: chromogenic substrate methods; clotting time assays; dabigatran; plasma; serum; urine.

Introduction

The direct thrombin inhibitor (DTI) dabigatran is a synthetic oral anticoagulant with a reversible binding to free and fibrin-bound enzyme [1–3]. Non-vitamin K antagonist oral anticoagulants (NOACs) such as dabigatran were developed to overcome the major drawbacks of vitamin K antagonists (VKAs), in particular their need for continuous effect monitoring [4]. Dabigatran etexilate is rapidly hydrolyzed to its active form dabigatran after oral administration and intestinal absorption by non-specific ubiquitous esterases in gut, blood, and liver [5, 6]. Peak plasma concentration are attained 2 h after ingestion [7], and the terminal half-life time was determined to be 12–17 h in patients with normal creatinine clearance [5], allowing for a fixed daily dosing regimen without the need to routinely monitor coagulation for dose adjustment [7]. Dabigatran etexilate has been approved by the European Medicines Agency and the US Food and Drug Administration for prevention and treatment of venous thromboembolism after elective hip or knee replacement surgery and for the prevention of embolic stroke and non-systemic embolism in patient with non-valvular atrial fibrillation (NVAF) [8].

Despite the claim that dabigatran treatment does not to require laboratory-guided dose adjustment, due to the low bioavailability (<6.5%), predominant renal elimination (>80%) [7], and genetic variants' influence on the responses

***Corresponding author: Job Harenberg,** Clinical Pharmacology Mannheim, Medical Faculty Mannheim, Ruprecht-Karls University Heidelberg, Maybachstrasse 14, 68169 Mannheim, Germany, E-mail: job.harenberg@medma.uni-heidelberg.de
Shanshan Du, Giese Christina, Sandra Krämer and Martin Wehling: Clinical Pharmacology Mannheim, Medical Faculty Mannheim, Ruprecht-Karls University Heidelberg, Mannheim, Germany
Christel Weiss: Biometry and Statistics Medical Faculty Mannheim, Ruprecht-Karls University Heidelberg, Mannheim, Germany
Roland Krämer: Institute Inorganic Chemistry, University of Heidelberg, Heidelberg, Germany

to dabigatran [9], assessing the intensity of anticoagulation may be useful in patients at risk of overdose and in high-risk (both for thromboembolism and for bleeding) patients in general [10]. The standard for measuring NOAC concentrations in biological fluids is high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). However, its application is limited to a small number of specialized laboratories [11, 12]. Several methods have been published to assess dabigatran activities, which mainly involved normal plasma samples spiked in vitro with increasing amounts of the NOAC [3, 13–17] or under real-life conditions [10–12, 18, 19]. The investigation aims to add to the literature the measurement of dabigatran in other biological samples than plasma such as serum and urine and to give additional data of blood samples in real-life situations, which are needed to improve medical care of acutely diseased patients. Recently, the preliminary results of the quantification of dabigatran were also reported in serum [20] and urine [21] samples of patients on treatment. Serum and urine samples provided another option when plasma samples are not available. Therefore, we compared six assays mostly used for plasma samples and two chromogenic assays to analyze dabigatran in serum and urine samples from patients on treatment under real-life conditions. To analyze the performance of these assays, we adopted various statistical methods including the Bland-Altman analysis as used previously for the determination of rivaroxaban and apixaban [22].

Materials and methods

Patients

Plasma, serum, and urine samples were obtained from patients with NVAF on treatment with dabigatran 110 mg bid or 150 mg bid for prevention of cerebral or non-cerebral systemic embolism. Patients were recruited in the outpatient care unit of the 4th Department of Medicine at the University Hospital Mannheim, collecting a total of 320 plasma, 177 serum, and 156 urine samples from 2011 to 2013. Serum and urine samples were collected during the last 1.5 years. The Local Ethics Committee approved the study, and participants gave written informed consent prior to the first examination. Blood was taken by venipuncture of an antecubital vein. For plasma sampling, blood was collected into plastic tubes containing 0.109 M sodium citrate (9:1 v/v blood/citrate) and was mixed immediately. Plasma was obtained by centrifugation for 15 min/1800 *g* at room temperature within 30 min. To generate serum, blood was collected into kaolin-containing plastic tubes and incubated for 30 min at room temperature to allow for clotting, followed by centrifugation for 15 min at 1800 *g*. Urine samples were collected at the same time point and centrifuged at 1800 *g* for 15 min at room temperature. Supernatants of plasma, serum, and urine samples were stored at aliquots in cryovials at –80 °C until analyzed.

Origin of dabigatran

Active dabigatran was extracted from commercially available Pradaxa® [23], and its purity was determined by 1H nuclear magnetic resonance spectra and elementary analysis, yielding about 97% purity and high agreement with the literature for carbon, hydrogen, and nitrogen [1]. A stock solution was prepared containing 0.32 mg/mL of dabigatran in dimethylsulfoxide (DMSO). The dabigatran stock solution was spiked to human pooled plasma, serum, and urine with eight concentrations, ranging from 0 to 800 ng/mL, to establish calibration curves. Those calibrators were aliquoted in cryovials and stored at –25 °C until analyzed. The pooled plasma from healthy volunteers was not exactly from the same lot. The final concentration of DMSO in dabigatran solutions was 0.05%, which is assumed to have no impact on coagulation [23]. When using the Hemoclot method, dabigatran reference calibrators and standard plasma samples were taken from the Hemoclot thrombin inhibitor (HTI) assay kit but they were not necessarily from the same lot.

S2238 chromogenic method

In general, chromogenic substrates [24] adopt synthetic tripeptides or tetrapeptides specific for the recognition by factor Xa or thrombin, which are linked to a chromophore, in most instances paranitroaniline. The enzyme splits the chromophore from the specific substrate, and the amount of chromophore released is negatively related to the inhibitor concentration. For this assay, 10 µL of the testing samples (serum, plasma, or urine) or calibrators and 100 µL of Tris buffer (0.05 M Tris, 0.075 M EDTA, 0.175 M NaCl, pH 8.4) were mixed in duplicates and incubated for 5 min in a 96-well microtiter plate at 37 °C. Then, 100 µL of 0.74 NIH/mL human thrombin (Sigma Aldrich, Deisenhofen, Germany) were added followed by incubation for 60 s. Fifty microliters of the chromogenic substrate S2238 (1.59 mM; Chromogenix, Essen, Germany) dissolved in distilled water were added and incubated for 5 min at 37 °C. The reaction was stopped by adding 25 µL of 20% acetic acid. The optical density (OD) was measured at 405 nm using a MultiskanTM FC Microplate Photometer with the software SkanIt 3.1 (Thermo Fisher Scientific, Langenselbold, Germany). The calibration curves were used for every microtiter plate with known concentrations of dabigatran plasma, serum, or urine standard samples vs. the corresponding OD, by the four-parameter logistic model. The concentrations of dabigatran in test samples were calculated from the calibration curves [20, 21, 23].

DTI assays

The DTI assays contained tosylglycyl-L-proly-L-arginyl-5-amino-2-nitrobenzoic acid isopropylamide (tos-gly-pro-arg-ABNA-IPA)] as chromogenic substrate to assess the concentration of dabigatran. According to manufacturer instructions (Siemens Healthcare Diagnostics, Marburg, Germany), 5 µL of the test or calibration samples were mixed as duplicates with 25 µL DTI substrate reagent and then 50 µL of the thrombin reagent were added in 96-well microtiter plate to start the reaction. The reaction was stopped by 10 µL of 20% acetic acid and measured as described in S2238 chromogenic method.

Clotting time assays

Activated partial thromboplastin time (APTT) assays are sensitive to indirect thrombin inhibitors and less sensitive to DTIs [25]. APTT were measured using the reagent PathromtinSL® (mean±SD, 34.5±1.0 s; Siemens Healthcare Diagnostic) [26].

In the prothrombinase-induced clotting time (PiCT) assay [27], Russell's viper venom activates factor V, and the prothrombinase formation only relies on the activity of factors X and II being sensitive toward all inhibitors of these clotting enzymes [28]. The PiCT assay was performed with the two-step method (reagents from Pentapharm, Basle, Switzerland), with a mean±SD of 29.9±4.6 s [29].

The thrombin clotting time test (TT) sensitively determines thrombin inhibitors after addition of bovine or human thrombin to platelet-poor plasma [19]. This assay was found to be too sensitive for determination of dabigatran and was therefore modified for the HTI assay method by pre-diluting patients' samples with normal human poled plasma provided by the commercially available test system [12, 13, 17–19]. The Hemoclot reagent was from commercially prepared diluted TT (HYPHEN BioMed, Neuville-sur-Oise, France); the normal range was 29.3–29.9 s [13]. Linear regression was employed to generate calibration curves.

The reagent for the ecarin clotting time (ECT) assay was from Pentapharm, with a mean±SD of 45.7±0.8 s [30, 31]. In this assay, the activator ecarin converts prothrombin to meizothrombin, which is sensitive to DTIs [30–32]. For the ECT assay, the same calibrators and linear regression model were used as for the HTI method. The experimental procedures of all clotting time assays were performed according to the manufacturer's instructions. All clotting time measurements were all carried out in duplicates on the KC10A micro device (Amelung, Germany)].

Analytical parameters of dabigatran standard samples in plasma, serum, and urine by chromogenic methods

Mean, standard deviation (SD), precision, accuracy, and limit of detection (LOD) were assessed by running calibrator samples in duplicate for 20 times within 1 day. Precision was estimated as coefficient of variation (CV). Accuracy was assessed as mean deviation (MD):

$$\mathrm{MD}\% = \frac{(\text{measured concentration}) - (\text{nominal concentration})}{(\text{nominal concentration})} \times 100.$$

The LOD was calculated by 3.3×SD/S, where SD represents the standard deviation of the blank (n=20) given in absorption units at 405 nm and S represents the slope of the linear curve. The linear curve is established from calibrator concentrations ranging from 0 to 100 ng/mL and their corresponding absorptions.

Analytical parameters of clotting time assays for dabigatran plasma standard samples

The following experiments were performed during four consecutive days: calibrators were run once per day and standard samples were assessed three times every day. Concentrations were read from the calibration curve of the same day. Mean, SD, within-day precision, day-to-day precision, accuracy (MD%), LOD, and limit of quantification (LOQ) were calculated. The LOD was expressed as 3.3×SD/S, and LOQ was expressed as 10×SD/S.

Statistical analysis

Statistical analyses were performed on SAS 9.3 TS for Windows (Cary, NC, USA) and Microsoft Excel 2003. Quantitative variables are given as mean and SD. Furthermore, Pearson's correlation coefficients (r) were assessed to estimate the strength of a correlation. The t-test for two paired samples and the test of Maloney-Rastogi (MR) were performed to compare differences of mean values and error variances. The MR test indicates if measurements differ regarding their precision. Bland-Altman plot and the intra-class correlation coefficient (ICC) were performed to assess the strength of agreement between two measurements. The level of significant differences was set as $p<0.05$.

Of note, the Pearson's correlation coefficient values (r) are bigger than the corresponding ICCs; as for the Pearson's correlation, the data are ranked, thus rendering them insensitive to systematic bias. In that sense, ICC is a more natural measure of association than Pearson's correlation [33].

Results

In vitro results of chromogenic methods

The results of dabigatran standard samples in plasma, serum, and urine are given as mean, SD, sensitivity (CV), accuracy (MD) in Table 1. Dabigatran concentrations in standard plasma samples determined by S2238 and DTI showed significant deviations (p=0.0045). In plasma, S2238 precision between 50 and 100 ng/mL ranged from 13% to 20%, and for the DTI method, from 21% to 30%. The accuracy expressed as MD% was between 1% and 21%. For the plasma, serum, and urine matrix effects, no statistical difference was found for the standard dabigatran samples. The mixed model was employed to analyze two measurements.

The results of the LOD for S2238 are 98, 22, and 21 ng/mL, and for DTI are 117, 117, and 226 ng/mL in plasma, serum, and urine samples, respectively.

In vitro results of clotting time assays

HTI

The concentrations of standard samples were read from the linear regression curves generated at the same-day calibration curve. The mean and SD of the coagulation times determined in 4 days vs. the concentration of in-house

Table 1 Analytical results of human plasma, serum, and urine samples spiked with different concentrations of dabigatran (0–800 ng/mL) determined by S2238 and DTI methods (n=20 for each determination, mean values).

	Added, ng/mL						
	800	600	200	100	50	25	0
	Determined, ng/mL						
Plasma							
S2238							
Mean	891.8	625.1	213.1	111.2	60.5	26.3	2.2
SD	97.75	83.18	30.49	14.5	12.16	6.94	2.35
CV	0.11	0.13	0.14	0.13	0.2	0.26	1.05
MD	0.11	0.04	0.07	0.11	0.21	0.05	nd
DTI							
Mean	807.5	643.7	204.9	98.6	58.4	33.2	22.4
SD	64.08	81.17	24.47	20.42	17.72	12.65	18.02
CV	0.08	0.13	0.12	0.21	0.30	0.38	0.81
MD	0.01	0.07	0.02	–0.01	0.17	0.33	nd
Serum							
S2238							
Mean	903.7	657	208	117.1	53.2	24.6	4.6
SD	88.55	78.05	15.63	9.69	5.84	5.24	4.49
CV	0.10	0.12	0.08	0.08	0.11	0.21	0.97
MD	0.13	0.09	0.04	0.17	0.06	–0.02	nd
DTI							
Mean	777.4	654.6	207.7	104.6	69.6	48.0	26.7
SD	46.12	42.53	40.47	27	15.79	13.23	41.57
CV	0.06	0.06	0.19	0.26	0.23	0.28	1.56
MD	–0.03	0.09	0.04	0.05	0.39	0.92	nd
Urine							
S2238							
Mean	897.8	663.2	211.4	117	68.5	27.7	1.2
SD	63.12	63.73	18.17	12.24	7.22	4.28	1.18
CV	0.07	0.10	0.09	0.10	0.11	0.15	1.01
MD	0.12	0.11	0.06	0.17	0.37	0.11	nd
DTI							
Mean	879.8	663.9	217.5	113.2	63.2	44.2	25.3
SD	53.37	46.05	28.47	14.94	16.81	14.62	13.57
CV	0.06	0.07	0.13	0.13	0.27	0.33	0.54
MD	0.10	0.11	0.09	0.13	0.26	0.77	nd

SD, standard deviation; CV, SD/mean; DTI, direct thrombin inhibitors chromogenic assay; S2238, S2238 chromogenic assay; nd, no definition; $MD\% = \frac{(\text{measured concentration}) - (\text{nominal concentration})}{(\text{nominal concentration})} \times 100$.

and Hemoclot-derived dabigatran calibrators are shown in Figure 1. In-house and Hemoclot calibrator curves almost overlapped, and in-house calibrators showed no statistical difference with Hemoclot dabigatran calibrators (p=0.2097). The LOD and LOQ are shown in Table 2.

ECT

The analytical parameters such as mean, SD, CV, LOD, and LOQ are given in Table 2. The baseline clotting time for the ECT method was 51.1±1.9 s. Linear curve equation determined by in-house calibrators was y=0.8583x+84.885, R^2=0.9864. At a concentration of 50 ng/mL, the within-day precision (CV%) and accuracy (MD%) were 17.57 and –39.42 ng/mL for ECT and for HTI, respectively, and the corresponding CV% and MD% were 17.7 and –15.3 ng/mL, respectively.

APTT

A linear curve was established by plotting the natural logarithm of the concentration as the abscissa and the logarithm of the clotting time as the ordinate. The concentrations from 0 to 100 ng/mL and the corresponding

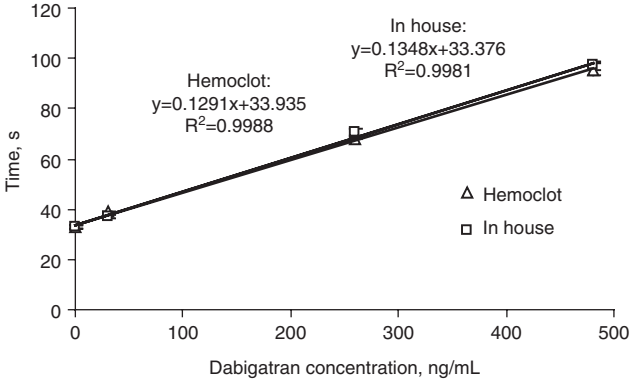

Figure 1 Calibration linear curves were generated by nominal concentrations of dabigatran plasma calibrators and the corresponding mean Hemoclot clotting time of 4 days (n=8, p=0.2097). Triangles represent dabigatran calibrators provided by commercial available HTI kit; diamonds stand for in-house dabigatran calibrators.

clotting times were chosen to fit a linear curve, and from this linear curve, the LOD and LOQ were calculated. The slope for this linear curve was 0.318 and R^2 was 0.9908. Analytical results (n=24) are shown in Table 2.

PiCT

A linear curve was established by plotting the natural logarithm of the concentrations vs. the natural logarithm of the clotting time. Concentrations from 0 to 100 ng/mL and the corresponding clotting time were chosen to form a linear curve to calculate LOD and LOQ. The slope for this linear curve was 0.4757 and R^2 was 0.9663. Analytical assessments for dabigatran effects (n=24) are given in Table 2.

Creatinine clearance

Creatinine was determined from serum samples of patients, and the creatinine clearance was calculated by the Cockcroft-Gault formula [34]. The mean and SD was 71.2±21.5 mL/min. All patients had creatinine clearances >30 mL/min. The coefficient of correlations between creatinine clearance and dabigatran concentration determined by S2238 and DTI in plasma samples were 0.0068 and 0.0014, in serum samples 0.0679 and 0.026,

Table 2 Dabigatran standards plasma samples were measured by Hemoclot, ECT, APTT, and PiCT assays (n=24).

Standard samples	Added, ng/mL	Determined, ng/mL	SD, ng/mL	Accuracy, no. MD, %	Precision, CV%		LOD, ng/mL	LOQ, ng/mL
					Within day	Day to day		
Hemoclot								
Hemoclot	120	129.6	20.0	8.0	15.0	7.3	24.0	74.0
Standard	300	313.4	19.9	4.5	6.0	3.4		
Hemoclot								
In-house	20	7.7	6.2	−61.6	52.1	73.6	26.0	77.0
Standard	50	42.4	8.3	−15.3	17.7	12.2		
	200	230.5	21.2	15.3	5.0	9.0		
	600	575.3	53.0	−4.1	4.6	9.2		
ECT								
In-house	20	−11.7	5.6	−158.5	−32.1	−42.1	7.4	22.3
Standard	50	30.3	6.2	−39.4	17.6	0.1		
	200	255.6	27.0	27.8	9.8	0.1		
	600	697.8	60.7	16.3	8.6	0.0		
aPTT								
In-house	25	28.8	3.1	15.1	1.1	5.0	27.4	83.1
Standard	50	44.7	4.5	−10.7	0.7	6.0		
	200	156.1	15.2	−22.0	0.2	7.0		
	600	587.3	46.2	−2.1	0.1	8.1		
PiCT								
In-house	25	21.1	5.2	−15.6	22.7	14.6	29.7	90.1
Standard	50	43.0	11.1	−14.0	26.5	8.8		
	200	211.0	38.5	5.5	17.7	8.8		
	600	553.4	115.5	−7.8	17.3	14.9		

LOD, 3.3×SD/S; LOQ, 10×SD/S; CV, SD/mean; Hemoclot, Hemoclot thrombin inhibitor; ECT, ecarin clotting time; APTT, activated partial thromboplastin time; PiCT, prothrombinase-induced clotting time; $MD\% = \frac{(\text{measured concentration}) - (\text{nominal concentration})}{(\text{nominal concentration})} \times 100$.

and in urine samples 0.0919 and 0.107, respectively. This indicated no relationship between dabigatran concentrations in plasma, serum, and urine samples with the creatinine clearance in our patients.

Determination of dabigatran in plasma, serum, and urine samples from treated patients by chromogenic assays

The mean concentrations of dabigatran in plasma and serum of patients determined by the S2238 method were 113.5±84.3 and 71.1±67.7 ng/mL, respectively. The concentrations determined by DTI in plasma and serum were 143.1±102.0 and 23.6±21.0 ng/mL, respectively (Table 3). Concentrations in urine were 5641.6±4319.7 and 4730.0±3770.2 ng/mL using the S2238 and DTI chromogenic substrate assays, respectively (Table 3). The MR test proved significantly different of values of the precision of the concentrations of dabigatran measured in plasma and serum by S2238 and the DTI methods. In the Bland-Altman plot (Figure 2), one may see a trend for higher magnitude of differences in precision at higher concentration of dabigatran using the S2238 assay (Figure 2) in contrast to the DTI assay (Figure 3). However, this trend does not influence the conclusion that serum samples are not suitable to determine dabigatran with the presently used methods.

The mean concentrations of dabigatran ($p<0.0001$) and the variances of the results (MR, $p=0.0133$) differed between the S2238 and DTI methods for plasma, serum, or urine samples. As can be seen from Table 4, no method measures always higher or lower values in all the three matrices. The Pearson's correlation and the ICC among the four methods is higher for plasma and urine samples compared with serum samples (Table 4). The values of the Pearson's correlation for dabigatran in plasma and serum samples were r=0.43 and r=0.30 using the S2238 and the DTI assays. As expected, the ICC values were lower than the r-values (ICC=0.36, S2238 assay; ICC=−0.03, DTI assay) (Figures 2 and 3).

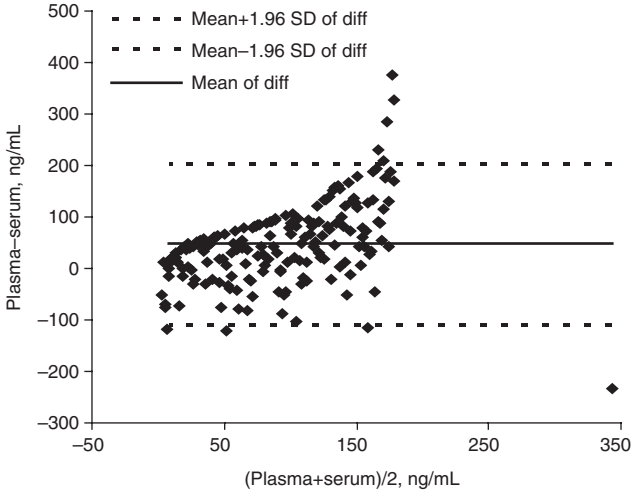

Figure 2 Bland-Altman plot is shown for dabigatran levels in plasma and serum samples from treated patients determined by S2238 method (diff, difference; n=179).

Figures 2 and 3 depict the Bland-Altman plots comparing plasma and serum values of patients' samples determined by S2238 (Figure 2) or DTI (Figure 3) methods, respectively. The differences between dabigatran concentrations in plasma and serum were plotted as means of two values determined in plasma and serum. The mean values of plasma minus serum concentrations were above zero, indicating that they were higher in plasma samples compared with serum samples.

Determination of dabigatran in patient plasma samples by clotting time assays

The results for mean, SD, min, max, 2.5th, 25th, 75th, and 97.5th percentiles of the clotting time coagulation assays HTI, ECT, PiCT, and APTT are given in Table 5. Two of 322 dabigatran-treated patient samples were measured below the normal APTT range (34.5±1.0 s). The 2.5th to 97.5th percentile range of dabigatran patient samples corresponded

Table 3 Concentrations of dabigatran determined by S2238 and DTI chromogenic assays in plasma, serum, and urine samples.

Biological fluid	n	S2238			DTI		
		Mean, ng/mL	CV, %	SD, ng/mL	Mean, ng/mL	CV, %	SD, ng/mL
Plasma	295	113.5	74.3	84.3	143.1	71.3	102.0
Serum	164	71.1	95.2	67.7	23.6	89.0	21.0
Urine	144	5641.6	76.6	4319.7	4730.0	79.7	3770.2

DTI, direct thrombin inhibitors chromogenic assay; S2238, S2238 chromogenic assay.

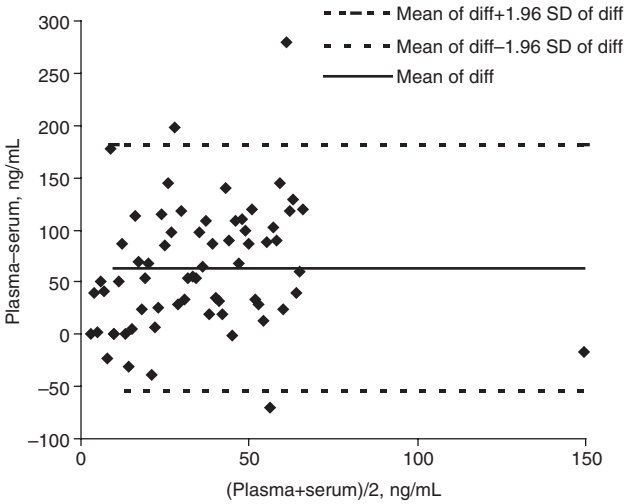

Figure 3 Bland-Altman plot is depicted for dabigatran levels in plasma and serum samples from treated patients determined by DTI method (diff, difference; n=66).

to APTT clotting times from 43.2 to 142.8 s. For the PiCT method, 5.7% of treated patient' samples had coagulation times within the mean±SD (29.9±4.6 s). The 2.5th to 97.5th percentile range of dabigatran patient samples corresponded to PiCT clotting times from 31.1 to 115.6 s.

HTI and ECT clotting times (in seconds) showed a correlation of r=0.91. The other Pearson's correlations between the clotting assays ranged between 0.63 and 0.68.

The concentrations determined by the chromogenic methods S2238 and DTI correlated well with the HTI and ECT assays (r>0.7, Table 6). The ICC values indicated excellent agreement between HTI and ECT assays (ICC=0.89, Table 6). HTI and ECT also revealed the strong correlation with chromogenic assays S2238 or DTI (ICC ≥0.7, Table 6). The variance of the assays indicated no significant differences between HTI and S2238 and a better precision of both assays compared with the DTI. The ECT showed the smallest variance of all assays (MR test, p<0.0001, Table 6).

Discussion

The present investigation demonstrates that dabigatran may be accurately determined under real-life conditions using ECT, HTI, and thrombin-specific chromogenic assays from plasma samples of patients on treatment. This is in agreement with other studies reporting on the lower suitability of the APTT for the determination of dabigatran [17, 19, 35]. APTT and PiCT are global coagulation assays that may be influenced by many biological factors and assay-specific components [36]. As the HTI and ECT assays are both specific modified thrombin clotting time assays, they are less likely to be influenced by these factors. In addition, they can be adapted to all coagulation platforms, and therefore our data support that these two assays are preferable for the analysis of dabigatran in plasma samples from patients; their use depends mainly on the availability of the reagents. In the RE-ALIGN study, mean dabigatran levels were determined using the thrombin-based Hemoclot coagulation assay and were similar [37] to those reported by others [11] and in our patients. Results for the two chromogenic substrates with different selectivity and specificity toward thrombin support this conclusion because a high congruency of data was found [38, 39]. In contrast, another direct synthetic inhibitor, argatroban, could not be detected by chromogenic substrate assays with a sensitivity that was high enough for clinical samples [40]. Therefore, we used two different chromogenic substrates in the present investigation to determine the inhibition by dabigatran in different biological fluids in man.

The clinical interest of measuring dabigatran in serum samples is the non-availability of plasma samples from patients. Serum samples are frequently stored or frozen over a longer time period in contrast to plasma samples from which analyses have to be performed on the same day. The clinical interest of measuring dabigatran in urine samples may refer also to the lack of availability of plasma samples and for forensic purposes in case of suspicion of overdose. A point-of-care (POC) test clearly separated

Table 4 Comparison of S2238 and DTI methods assessing dabigatran in plasma, serum, and urine samples.

Assays	n	Biological fluid	Paired t-test, p-value	r-Value	MR, p-value	ICC
S2238 vs. DTI	295	Plasma	<0.0001	0.70	<0.0001	0.66
	164	Serum	<0.0001	0.38	<0.0001	0.15
	144	Urine	0.0002	0.76	0.0133	0.74

r-value, Pearson's correlation coefficient; MR, Maloney-Rastogi test; ICC, intra-class coefficient; DTI, director thrombin inhibitors chromogenic assay; S2238, S2238 chromogenic assay.

Table 5 Concentrations and coagulation time of dabigatran patients' plasma samples were determined by each test.

Method	Mean	SD	CV	Max	Min	n	2.5th percentile	25th percentile	75th percentile	97.5th percentile
DTI, ng/mL	143.1	102.2	0.72	575.0	7.2	303	10.0	66.1	192.0	365.0
S2238, ng/mL	113.5	84.3	0.74	506.0	1.0	323	10.0	55.0	152.0	321.0
Hemoclot, ng/mL	106.8	89.4	0.84	561.3	0.8	291	1.0	37.8	156.1	303.5
ECT, ng/mL	109.5	74.5	0.68	410.9	1.0	322	1.0	51.4	148.9	274.1
Hemoclot, s	54.8	14.9	0.27	129.9	31.6	291	34.5	43.6	62.9	87.4
ECT, s	199.4	84.5	0.42	535.3	45.5	322	58.2	135.8	244.2	383.3
APTT, s	75.6	25.4	0.34	185.0	34.7	322	43.2	59.0	85.9	142.8
PiCT, s	64.0	20.7	0.32	151.8	25.9	317	31.1	49.6	74.6	115.6

DTI, direct thrombin inhibitors chromogenic assay; S2238, S2238 chromogenic assay; Hemoclot, hemoclot thrombin inhibitor; ECT, ecarin clotting time; APTT, activated partial thromboplastin time; PiCT, prothrombinase-induced clotting time; SD, standard deviation; CV, coefficient of variation.

positive from negative results for dabigatran in urine of patients of treatment [41]. A POC test from whole blood using a PTT reagent also reported a good correlation to plasma concentrations [42]. The development of POC tests for dabigatran is of interest for patients with any acute disease as well as for control of adherence to therapy.

In vitro data indicated that the LOD of the two chromogenic assays showed some differences toward dabigatran, with the DTI assay being less sensitive. This may be caused by different sensitivities of the chromogenic substrates toward thrombin and their competition with dabigatran at the thrombin binding site. Interestingly, in vitro studies using plasma, serum, and urine samples spiked with dabigatran resulted in non-different concentration activity curves for each of the two chromogenic assays. In contrast, serum samples from patients on treatments with dabigatran resulted in about two-third lower values compared with plasma in both chromogenic assays. These differences may be explained by the reversible binding of dabigatran to thrombin, which results in the consumption of dabigatran during coagulation to obtain serum after withdrawal of blood. This trapping in serum samples seems not to occur for the direct factor Xa inhibitors rivaroxaban and apixaban [20, 44].

Table 6 Statistical parameters for comparison of dabigatran concentrations determined by different methods in plasma.

Method	Hemoclot	ECT	DTI
S2238			
t-Test, p-Value	0.15	0.56	<0.0001
MR, p-Value	0.22	0.01	<0.0001
r-Value	0.74	0.77	0.70
ICC	0.74	0.77	0.66
DTI			
t-Test, p-Value	<0.0001	<0.0001	–
MR, p-Value	0.0005	<0.0001	–
r-Value	0.79	0.80	–
ICC	0.72	0.70	–
ECT			
t-Test, p-Value	0.58	–	–
MR, p-Value	<0.0001	–	–
r-Value	0.91	–	–
ICC	0.89	–	–

r-value, Pearson's correlation coefficient; MR, Maloney-Rastogi test; ICC, intra-class coefficient; DTI, direct thrombin inhibitors chromogenic assay; S2238, S2238 chromogenic assay; Hemoclot, Hemoclot thrombin inhibitor; ECT, ecarin clotting time.

Preliminary data showed the feasibility of determination of dabigatran in urine quantitatively [45] and qualitatively [41]. Thus, the biological matrices plasma, serum, and urine themselves do not have relevant effects on the interaction of thrombin with dabigatran. Here we reported the data of several hundred of samples obtained from the patients treated under real-life conditions with dabigatran. Despite the fact that samples of plasma, serum, and urine were taken at the same time point from the patients, no relevant comparisons between urine with plasma or serum levels in patients can be found. This may be due to a variable delay between excretion from blood (50- to 60-fold higher concentrations than in urine) into urine and the collection of urine in the urinary bladder before voiding. Additional information can only be generated from precise phase I studies in volunteers for each NOAC.

The main limitation of the present investigation is that the clinical relevance of measuring dabigatran in serum samples remains to be validated by clinical outcomes in clinical studies. The time of blood sampling after the intake of the last dose of the anticoagulant plays an important role and needs to be standardized in general. Renal impairment may decrease the excretion of dabigatran, thus reduced renal function may limit the validity of the determination of dabigatran in urine [41].

A limitation of these methods refers to a lack of a relation of concentrations of dabigatran in serum and urine with patients' creatinine clearance. However, a week correlation was reported also for plasma levels of dabigatran with creatinine clearance [10]. Limitations include methodological factors that influence the variability of the data: control samples were not used, repeated freezing and thawing experiments were not performed, and not the same lot of reagents were used over the period of analysis of the samples. To reduce variability of assays, we used calibration curves for every assay. The strength of the investigation is to demonstrate the feasibility of using serum samples for the determination of this NOAC by simple and reliable methods, which may be implemented in a coagulation platform connected to a photometer. For patients with normal renal function, urine offers the possibility to analyze the concentration of dabigatran in clinical situations when urine but not plasma samples are available such as in suspicion of overdose or lack of adherence to therapy. However, determination of dabigatran from serum samples requires further improvement before inclusion into clinical routine assessment.

In conclusion, HTI and ECT determine dabigatran sensitively and specifically in plasma samples under real-life conditions of therapy and results are rapidly available and may be easily implemented in all coagulation platforms. APTT and PiCT have a concentration prolongation relationship with dabigatran. Unfortunately, these assays are easily affected by external causes. Chromogenic substrate tests using S2238 and DTI also performed well in this study but require a specific application of the methods to the coagulation platform and lack proper sensitivity at low concentrations. The determinations from serum still require improvement before a general applicability in patients may be achieved. The assignability of the results to other coagulation platforms and other chromogenic substrate methods remains to be proven. The measurement of dabigatran in urine is feasible and may be used to check compliance as has been already demonstrated with a qualitative test [21]. The clinical relevance of the assay results remains to be determined in specific clinical settings under real-life conditions of therapy.

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