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Microfluidic Point-of-Care Ecarin-Based Clotting and Chromogenic Assays for Monitoring Direct Thrombin Inhibitors

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Abstract: Direct thrombin inhibitors (DTIs), such as bivalirudin and dabigatran, have maintained steady inpatient and outpatient use as substitutes for heparin and warfarin, respectively, because of their high bioavailability and relatively safe "on-therapy" range. Current clinical methods lack the capacity to directly quantify plasma DTI concentrations across wide ranges. At present, the gold standard is the ecarin clotting time (ECT), where ecarin maximizes thrombin activity and clotting time is evaluated to assess DTIs' anticoagulation capability. This work focused on the development of a microfluidic paper analytic device (μPAD) that can quantify the extent of anticoagulation as well as DTI concentration within a patient's whole blood sample. Capillary action propels a small blood sample to flow through the nitrocellulose paper channels. Digital images of whole blood migration are then captured by our self-coded Raspberry Pi and/or the Samsung Galaxy S8 smartphone camera. Both the flow length and the blue absorbance from the plasma front on the μPAD were measured, allowing simultaneous, dual assays: ecarin clotting test (ECT) and ecarin chromogenic assay (ECA). Statistically significant ($p < .05$) changes in flow and absorbance were observed within our translational research study. Currently, there are no quantitative, commercially available point-of-care tests for the ECT and ECA within the United States. Both the ECT and ECA assays could be instrumental to differentiate between supratherapeutic and subtherapeutic incidents during bridging anticoagulant therapy and limit the unwarranted use of reversal agents. **Keywords:** ecarin clotting time, ecarin chromogenic assay, paper microfluidics, DTI, bivalirudin, dabigatran. *J Extra Corpor Technol. 2019;51:29–37*

Direct thrombin inhibitors (DTIs) are novel anticoagulants which do not require cofactors to achieve blood anticoagulation. DTIs' mechanism of action is through the allosteric inhibition of thrombin, thus preventing fibrin generation (1). Traditional therapeutics, such as heparin, requires the cofactor antithrombin and can only achieve 20–40% of clot-bound thrombin inhibition, whereas DTIs attain 70% of such inhibition (2). The heparin–antithrombin complex has reduced affinity to preexisting fibrin-bound thrombin. This is problematic because thrombin upregulates its own release through positive feedback loops. DTIs, however, can inhibit free, fibrin-bound, and even heparin-bound thrombin (3). Given thrombin's role as a potent platelet activator, DTIs also increase anticoagulation by indirectly inhibiting platelet aggregation (4).

Major adverse events of DTI therapy, such as intracerebral bleeding, can occur through interactions with other drugs and/or altered drug metabolism. Bivalirudin's (administered intravenously) and dabigatran's (administered orally) primary method of clearance is hepatic proteolysis with 20% renal clearance (5). Decreased elimination of DTIs can occur by inhibiting the permeable glycoprotein (P-gp) transport system (6). Dabigatran, for instance, can experience decreased elimination when paired with antiarrhythmic ion channel blockers, such as verapamil or quinidine, given the P-gp system's similarity to ion channels (7).

Hospitals within the United States have traditionally used the plasma-based activated partial thromboplastin

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time (aPTT) and the whole blood–based activated clotting time (ACT) to monitor the extent of blood coagulation during DTI-based therapy. Although these assays are effective in monitoring heparin's effects, they lack sensitivity to intravenous (bivalirudin) and oral (dabigatran) DTI anticoagulants (8). The aPTT remains curvilinear throughout a wide range of dabigatran concentrations, whereas the ACT is only linear up to 250 ng/mL of dabigatran. Prothrombin time (PT), an alternative measure for quantifying blood coagulation, primarily measures factor VII activation in plasma in the presence of tissue factor. These extrinsic factors are traditionally used to monitor warfarin therapy. Again, dabigatran concentrations were found to correlate poorly with the prothrombin time/international normalized ratio (PT/INR) assay following orthopedic surgery (9).

In contrast to the previously mentioned point-of-care (POC) tests, the ecarin clotting time (ECT) can provide a linear response when monitoring DTI therapy. Ecarin is a metalloprotease enzyme derived from Indian saw-scaled viper venom. Ecarin cleaves prothrombin's Arg^{320} peptide bond at the alanine–arginine–aspartic acid (ARG) peptide motif, yielding the thrombin intermediate, meizothrombin. Meizothrombin has up to 97% thrombin activity (compared with 10% without ecarin) and converts fibrinogen to fibrin at a much higher efficiency. Because DTIs can inhibit not only thrombin but also meizothrombin, ecarin-based assays are quite appropriate in assaying DTIs' anticoagulation capability in optimal conditions, compared with conventional coagulation analyses (Figure 1A) (10). However, low levels of prothrombin or hypofibrinogenemia will cause falsely elevated conventional clotting times unproportionate to DTI concentrations (10).

The ecarin chromogenic assay (ECA) can also quantify DTI concentrations. A fixed amount of ecarin and p-nitroaniline (pNA) is added to a patient's plasma sample. The pNA chromophore is commonly linked to a thrombin-specific peptide substrate when determining thrombin activity. Absorbance at 405 nm should decrease as the DTI concentration increases (11) (Figure 1B). Given the oxyhemoglobin's and methemoglobin's peak absorption at 415 and 405 nm, respectively, the ECA is performed on plasma to avoid red blood cell (RBC) interference (12). ECA accuracy is mainly affected by hypothrombinemia.

Although conventional hemostasis analyzers could be used to monitor coagulation status, novel paper-based methods, including simple lateral flow assays (LFAs), have proven effective in terms of portability, cost, and ease of use. Nitrocellulose paper (negatively charged) has emerged as the preferred paper substrate, given its unique characteristics in resisting cell adhesion and protein folding (13). The electronegative environment of nitrocellulose fibers mimics the exposed phospholipid surface of vascular endothelial cells, which slows down platelet aggregation and subsequent coagulation, suitable for clotting assays (14). Microfluidic paper analytical device's (μPAD's) more sophisticated and multichannel versions of LFAs are even more advantageous given small volume requirements, low operative costs, and design versatility. These paper-based lab-on-a-chip (LOC) platforms have gained increased clinical use as diagnostic tools when paired with unique biomarkers (15), although they have not been demonstrated substantially for monitoring the effects of anticoagulant drugs. Smartphone-based imaging (using its digital camera) has advanced LOC devices and paper-based POC tests (LFA and μPAD) because of its

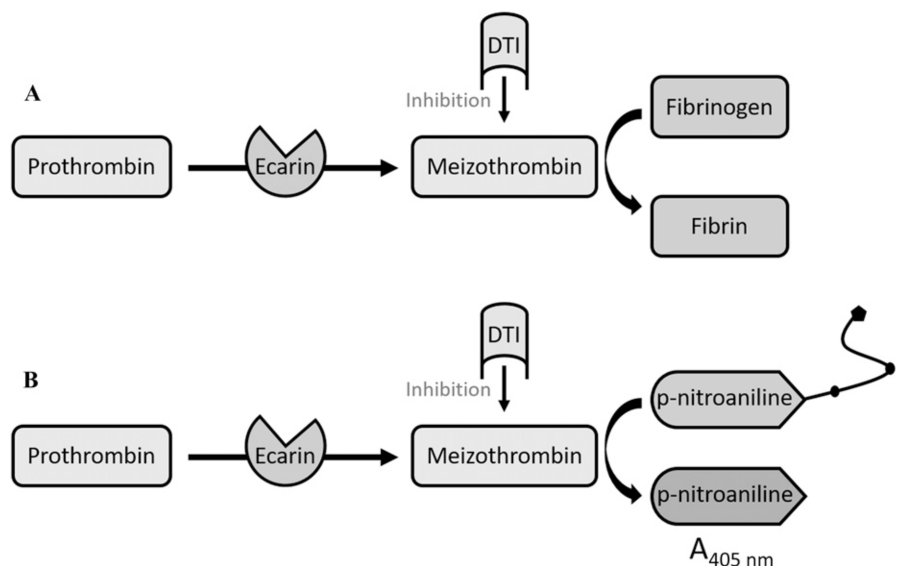

Figure 1. ECT vs. ECA.

wide availability and its ability to compute, display, and collect data (16). Smartphones also contain a white light-emitting diode (LED) flash, which could be used as a light source for various optical sensing applications (17). Pairing smartphones' robust features with paper-based microfluidics can create inexpensive and rapid POC testing devices, especially for assessing the extent of blood anticoagulation upon DTI administration.

The aims of this study were to design and fabricate the microfluidic POC ecarin-based clotting and chromogenic assays and evaluate it for quantifying the DTIs directly from fresh, human whole blood collected from volunteers.

MATERIALS AND METHODS

Specimen Collection

Fresh whole blood samples were collected from healthy human volunteers per the Institutional Review Board (IRB) protocol 1612094853. Specimens were collected by a licensed medical laboratory scientist and stored in 1:10 volume .129 M sodium citrated vacutainers before testing. Written consents from human volunteers were obtained following the previously mentioned IRB protocol. Unfiltered human sodium citrated plasma was purchased in bulk from commercial vendors (VWR International, Radnor, PA). Plasma specimens were placed in 2-mL aliquots and stored frozen at −40°C. Frozen plasma specimens were thawed in a 37°C dry incubator for 15 minutes before analyses.

Paper Microfluidic Chip Fabrication

Millipore HF075 (Millipore Sigma, Billerica, MA) nitrocellulose membranes were used to fabricate the paper-based chips in this study. HF075 has a capillary water flow rate of 75 seconds per 4 cm. SolidWorks (Dassault Systèmes, Vélizy-Villacoublay, France) engineering software was used to design eight-channel μPAD chips, as shown in Figure 2. Sample loading zones are 4 × 4 mm, connected to 1-mm-wide × 30-mm-long travel channels. The channels were partitioned with hydrophobic wax ink and printed using a Xerox ColorQube 8580 solid wax color printer (Xerox, Norwalk, CT). Wax ink red, green, and blue (RGB) values are 0, 255, and 255, respectively; zero red intensity is used to distinguish between the presence and absence of blood. The printed ink was melted throughout the paper substrate using a hot plate (150 seconds at ~130°C) to complete the hydrophobic wax layers. G041 glass fiber conjugate pad sheets (Millipore Sigma) were cut into 4 × 4-mm segments and used as sample incubation pads after placement on top of a 3D printed stamper (refer to the ECT procedure section).

Solution Preparation

Given its physiological similarity to human plasma, Plasma-Lyte A (Baxter Healthcare Corporation, Deerfield, IL) was used to dilute DTI drugs: bivalirudin (Sigma Aldrich, St. Louis, MO) from 0, 7, and 14 to 21 μg/mL and dabigatran (TargetMol, Boston, MA) from 0, 200, and 400 to 600 ng/mL. Ecarin (Sigma Aldrich) was reconstituted in 1× Tris-buffered saline to a final concentration of 5 EU/mL; aliquots were stored at −20°C. Pefachrome thrombin (H-D-

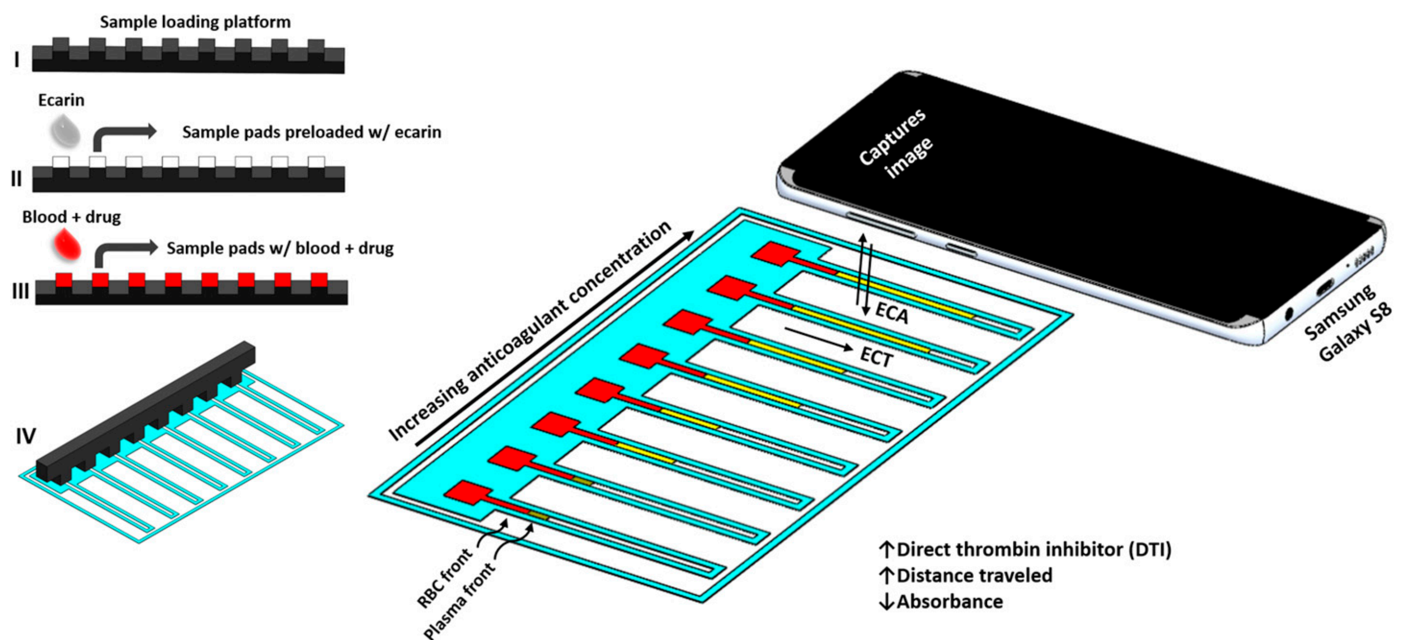

Figure 2. Graphical representation of μPAD ecarin-based assays.

cyclohexylglycine-Ala-Arg-pNA·2AcOH) (5-Diagnostics, Independence, MO) was diluted with Plasma-Lyte to a working solution of .5 mM.

Ecarin Clotting Test (ECT) Procedure

Fiber glass sample pads were housed on a 3D printed stamper platform as shown in Figure 2. After thawing at room temperature, 5 μL of 5 EU/mL ecarin was preloaded onto the sample pads. After blood collection, whole blood was aliquoted into an eight-well plate and spiked with varying amounts of DTIs. A multichannel pipette (ThermoFisher Scientific, Waltham, MA) was used to transfer 5 μL of the anticoagulated blood onto the sample pads. Subsequently after a 2-minute room temperature incubation, the stamper platform was flipped onto the μPAD's specimen loading zone. Figure 2 illustrates the ECT μPAD assay procedure. A custom-coded Raspberry Pi 3 (Raspberry Pi Foundation, Cambridge, UK) was retrofitted with an 8-megapixel Raspberry Pi Camera Module V2 and a 7″ touchscreen display, and automatically took pictures. Images were also taken manually with the Samsung Galaxy S8 (Samsung Electronics, Suwon, South Korea) 12-megapixel rear camera.

ECA Procedure

In preliminary studies to establish a positive control, unfiltered human plasma was analyzed using the ECA on a 96-well plate that contained the plasma reaction mixture (plasma + pNA + ecarin, with varying concentrations of bivalirudin or dabigatran). Reflectance at 405 nm was measured using a premium grade fiber optic reflection probe (Ocean Optics, Dunedin, FL) that was connected to a USB4000 miniature spectrophotometer (Ocean Optics). Ocean View V1.6.7 software (Ocean Optics) was used to obtain and analyze the spectra from the miniature spectrophotometer. Reflectance was also measured using the Samsung Galaxy S8 12-megapixel rear camera in lieu of a miniature spectrophotometer.

Next, ECA was conducted on μPAD using human whole blood spiked with varying amounts of DTIs. Conditions were similar to the ECT procedure except for the following: 1) 1.75 μL of 4 mM pNA was preloaded together with 5 EU/mL ecarin and 2) a 3-minute room temperature incubation on a stamper after loading blood samples. Reflectance was measured from the plasma fronts of each μPAD channel, again using the Samsung Galaxy S8 rear camera.

Data Analysis

Images taken from the Raspberry Pi or Samsung Galaxy S8 were analyzed using the National Institutes of Health's ImageJ software. ECT images were divided into RGB components with a focus on blue pixels. The average length from the loading zone to the end of the plasma front was used as the distance traveled at the given time point. The reliable length of the hydrophobic wax channel was

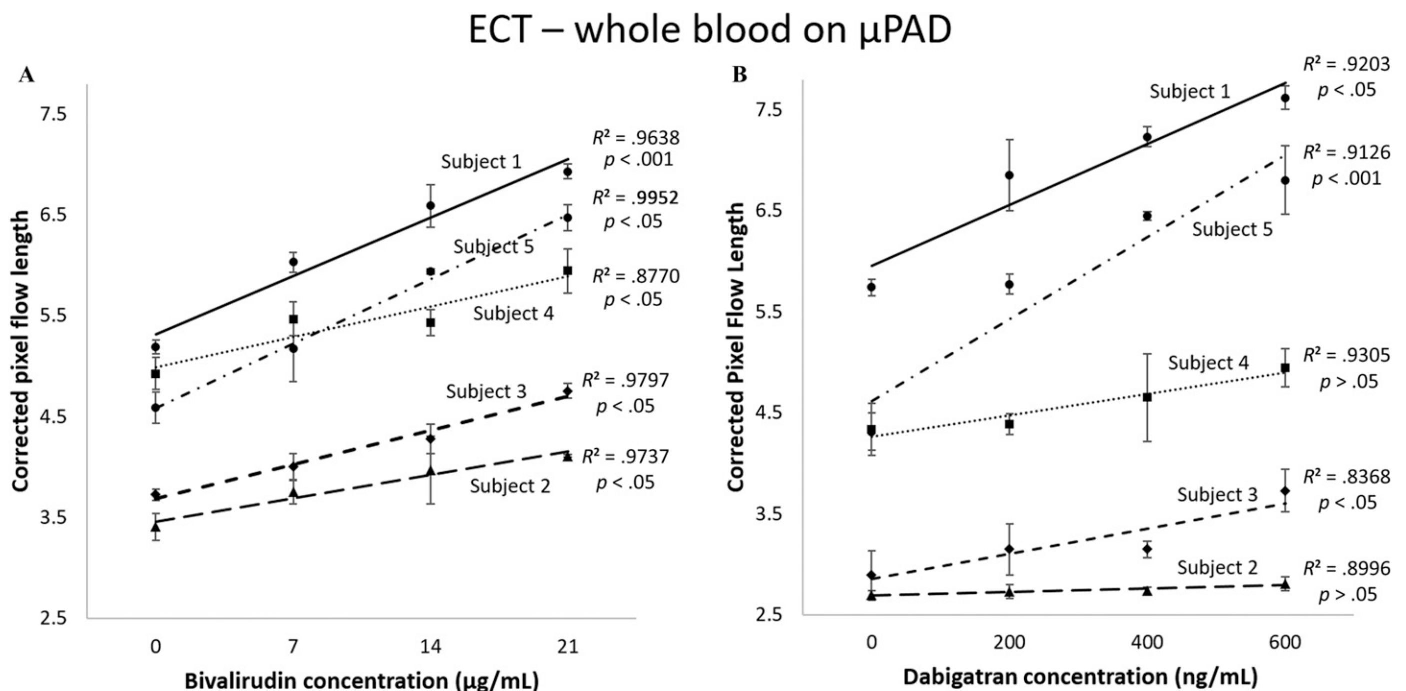

Figure 3. ECT of whole blood on μPAD, without chromophore. Cumulative ECT data as evaluated by the flow length on μPAD at 180 seconds, with varied concentrations of bivalirudin (A) and dabigatran (B); assay performed in duplicate for each subject and each DTI concentration.

used as a correction factor to correct for chip tilt or rotation and the distance between μPAD and the camera. Similarly, ECA images were separated into RGB, and the blue components were used. The average intensity from the RBC front to the end of plasma front, i.e., average intensity from the separated plasma, was determined within such plasma area. Absorbance (A) was obtained by comparing the measured intensity (I) to our reference sample where DTI concentration is zero (I_0), using the definition $A = \log(I_0/I)$.

RESULTS

ECT Results

The flow lengths from the inlet to the end of plasma front on each μPAD channel were analyzed either automatically using Raspberry Pi system and software, or manually from the images collected using a Samsung Galaxy S8. Figure 3 shows the flow length data at 180 seconds, plotted against bivalirudin (A) or dabigatran (B) concentration, again showing the expected increasing trend. Although the overall trend was similar among different subjects, some data show very sensitive response or almost flat response to DTI concentrations. Analysis of variance (ANOVA) confirmed the statistical significance for multiple subjects ($p < .05$ with bivalirudin for all subjects and $p < .05$ with dabigatran for subjects 1, 3, and 5). All subjects' linearity was further confirmed with R^2 values; .8770–.9952 for bivalirudin and .8368–.9305 for dabigatran.

ECA Results

ECAs were initially performed for the purchased plasma solutions in a 96-well plate using a reflection probe and a miniature spectrophotometer or a smartphone (Figure 4). ECAs were repeated for the whole blood on a μPAD using a smartphone (Figure 5). The highest absorbance values were obtained from the plasma solutions in a 96-well plate using a reflection probe and a miniature spectrophotometer (Figure 4A). Linear regression analysis (ANOVA) highlighted the expected decrease of bivalirudin and dabigatran concentrations ($p < .001$) for both bivalirudin and dabigatran. T-test analysis showed that we could differentiate between the presence and absence of drug and between higher drug concentrations ($p < .001$). Next, we substituted the expensive reflection probe and the miniature spectrophotometer with a smartphone and took pictures of the purchased plasma reaction solutions in a 96-well plate

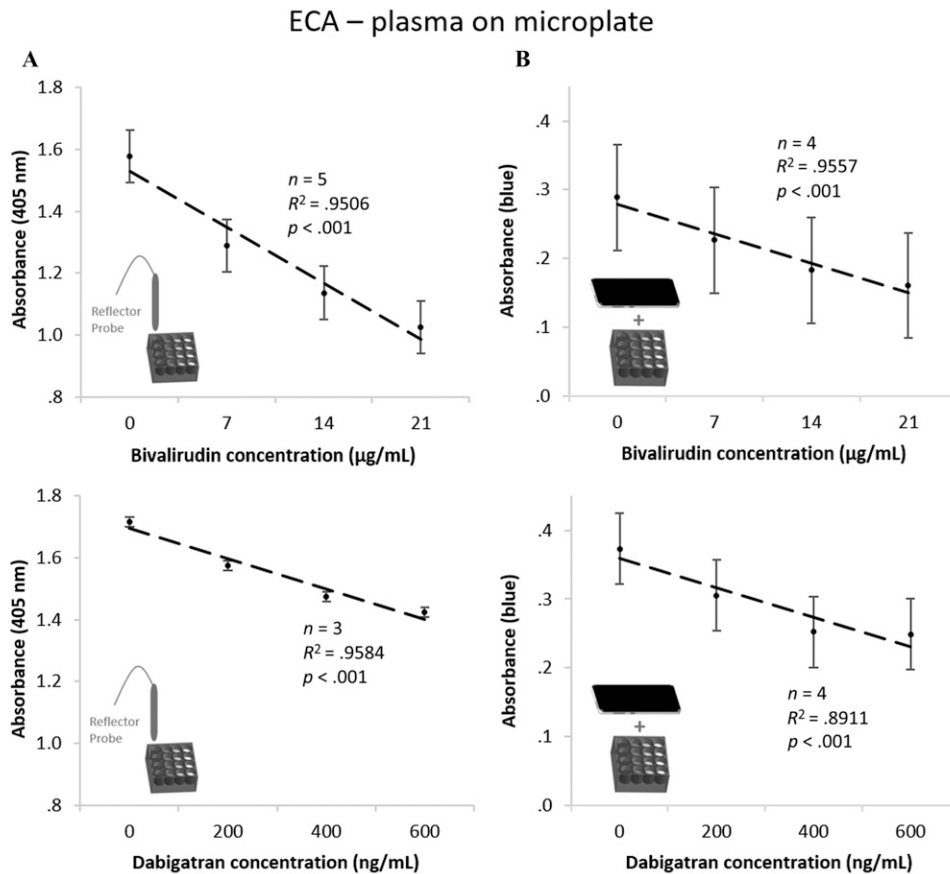

Figure 4. ECA of purchased plasma on a microplate. Ninety-six–well plate results for plasma, using either a reflection probe (connected to a miniature spectrophotometer) (A) or a smartphone camera (B); assay performed in triplicate for each DTI concentration.

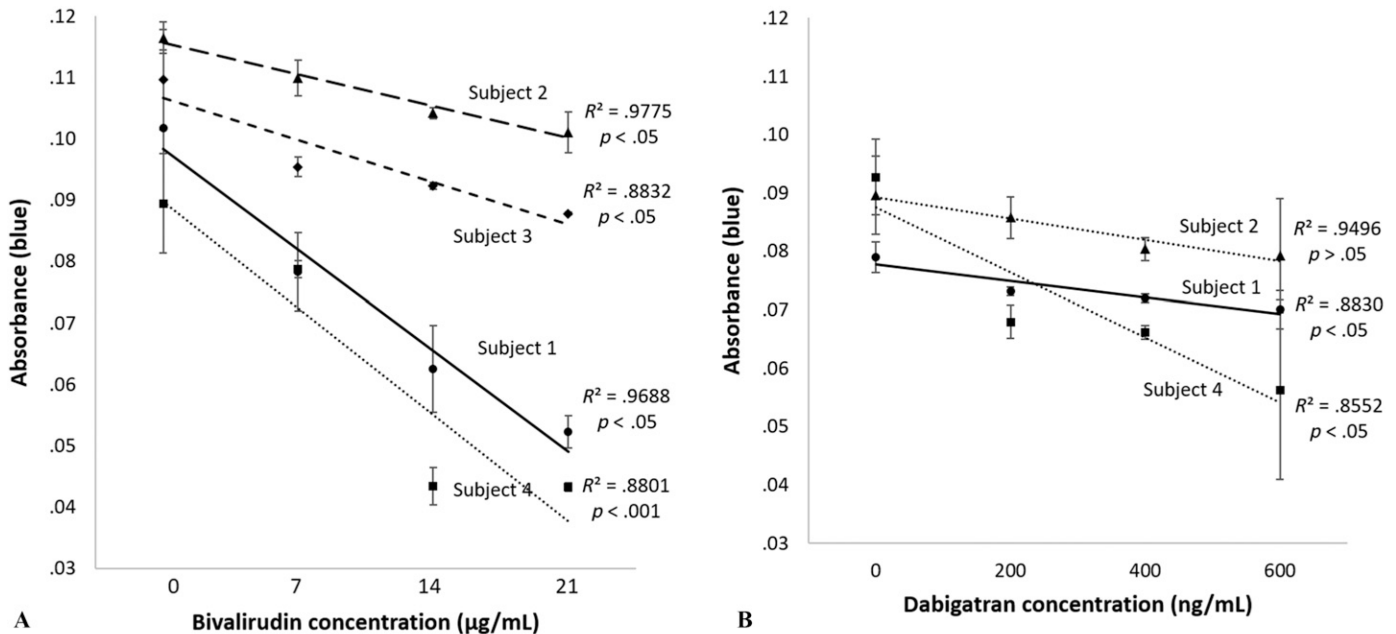

Figure 5. ECA of whole blood on μPAD. Cumulative ECA data as evaluated by the blue absorbance from the plasma front on μPAD at 120 seconds, using a smartphone camera, with varied concentrations of bivalirudin (A) and dabigatran (B); assay performed in duplicate for each subject and each DTI concentration.

(Figure 4B). Similarly, ANOVA analyses were consistent ($p < .001$), and we could distinguish from the negative control ($p < .05$). Bivalirudin assays had trouble discriminating between 7 vs. 14 μg/mL, whereas dabigatran assays could not distinguish between 400 vs. 600 ng/mL. Last, we used whole blood, μPAD, and a smartphone to detect the change in absorbance within the μPAD's plasma front (where there are no RBCs) (Figure 5). Although five subjects were available for the preliminary ECT experiments as shown in Figure 3, the same volunteer subjects were not available for ECA experiments, thus showing the results with four subjects. Most data showed an overall decreasing trend, as expected. Again, some data show very sensitive response or almost flat response to DTI concentrations, similar to the ECT results. ANOVA analysis confirmed the statistical significance for multiple subjects ($p < .05$ with bivalirudin for all subjects and $p < .05$ with dabigatran for subjects 1 and 4). All subjects' linearity was further confirmed with R^2 values; .8801–.9775 for bivalirudin and .8552–.9496 for dabigatran.

Combined Ecarin Clotting and Chromogenic Test (ECCT)

To perform ECT and ECA concurrently from a single μPAD channel, it was necessary to conduct ECT at the same conditions of ECA. Toward this aim, ECT analyses were repeated using whole blood with added ecarin and chromophore, for the same four subjects as those tested in ECA experiments. In fact, these ECT analyses (Figure 6) were concurrently conducted while performing the ECA analyses (Figure 5). Results shown in Figure 6 (measured after 120 seconds) show almost identical trends as those shown in Figure 3 (measured after 180 seconds). ANOVA analysis confirmed the statistical significance for subjects 1 and 4. All subjects' linearity was further confirmed with R^2 values; .8699–.9828 for bivalirudin and .9375–.9629 for dabigatran. Similar to ECT analyses, differences between subjects exist given varied response to DTIs.

Correlation between ECT and ECA from the same μPAD assay of whole blood was attempted in this analysis, and the results are shown in Figure 7. The blue absorbance readings from μPAD, taken from Figure 5 data representing ECA, were plotted against the flow lengths from μPAD, taken from Figure 6 data representing ECT. As expected, increasing drug concentration increased the flow length and decreased the absorbance. The expected trend was confirmed with R^2 values; .6181–.9355 for bivalirudin and .7024–.9216 for dabigatran.

DISCUSSION

Thrombosis prevention is a fine balance between hypercoagulability inhibition, stasis, and vessel wall injury. DTIs promote a hypocoagulopathic state by inhibiting the common pathway via factor IIa. DTIs are more efficacious

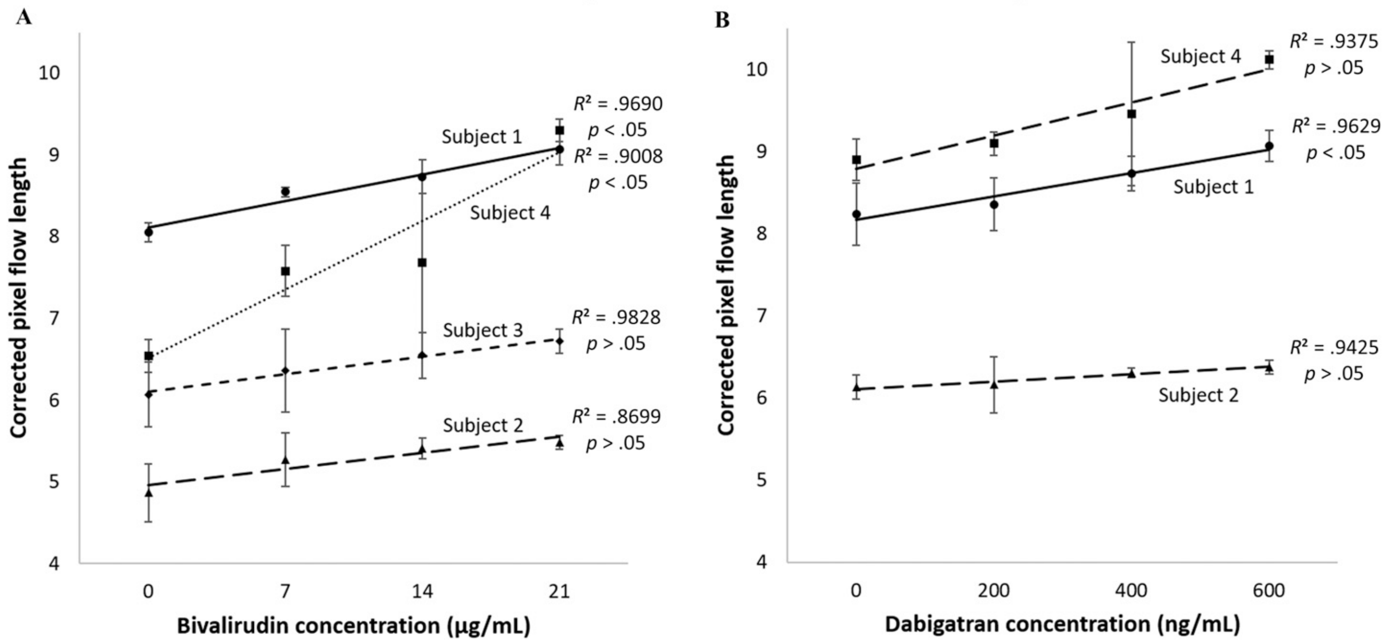

Figure 6. ECT of whole blood on µPAD, with chromophore. Cumulative ECT data with chromophore (ecarin) as evaluated by the flow length on µPAD at 120 seconds, with varied concentrations of bivalirudin (A) and dabigatran (B); assay performed in duplicate for each subject and each DTI concentration.

than other anticoagulants (such as heparin and warfarin) given their higher capacity for inhibiting both free and bound thrombin, relatively safe pharmacological profile, and lack the need for cofactors. Unfortunately, the available and required DTI concentrations in plasma vary greatly by each patient's clinical state. Our study involved the development of POC prototypical devices for monitoring changes in blood and plasma when exposed to DTIs.

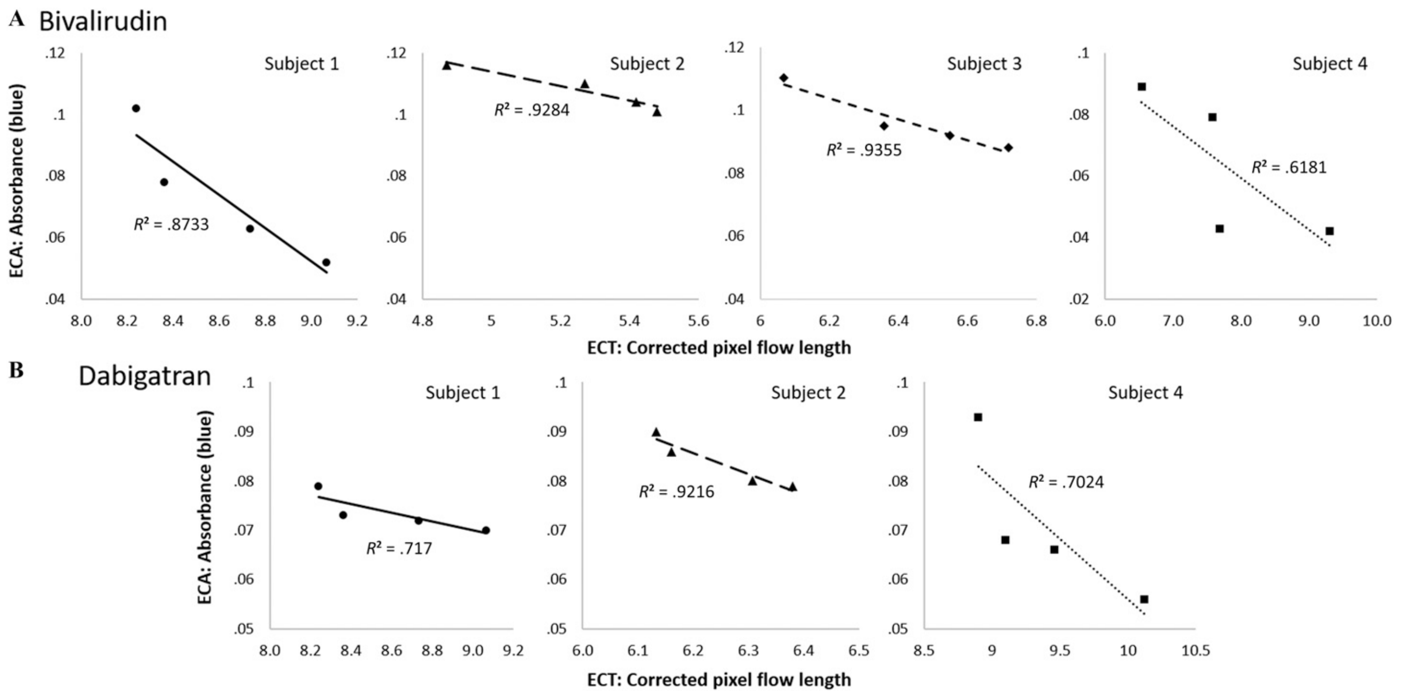

Figure 7. Combined ECCT. Correlation between ECT with chromophore (as measure by the flow length, derived from Figure 6 data) and ECA (as measured by blue absorbance, derived from Figure 5 data) on the same µPAD.

We hypothesized that increased DTI concentration would lead to decreased coagulation, thus increased distance traveled in ECT. We also theorized that lower DTI concentrations would provide a detectable increase in chromophore (pNA) cleavage in ECA. With these goals, we designed a novel μPAD with the ability to quantify the extent of coagulation and thus bivalirudin and dabigatran concentrations from various human subjects.

Current plasma-based tests (aPTT, PT, etc.) require centrifugation, whereas the whole blood ACT is not linear in higher DTI concentrations as discussed in Introduction. Our μPAD assays were linear throughout DTI concentration ranges and provided results without centrifugation. Using only 5 μL of whole blood per channel, several assays showed statistical significance with a total assay time of 5 minutes. ECA and ECT assay validation are being planned to confirm superiority versus current clinical tests (ACT, aPTT, etc.).

In testing our prototype, as expected, the ECT length-based analyses correlated with increased DTI concentrations. The use of paper microfluidics allowed us to perform the ECA without centrifuging the blood samples, as whole blood was separated through paper fibers. Paired with a Raspberry Pi device or a smartphone, these innovations decrease assay time, reduce specimen volume requirements, and improve the POC practicality of ecarin-based testing. Our smartphone and μPAD ECA assays showed smaller absorbance values than those with a spectrophotometer. The decrease in sensitivity could be due to our use of the blue pixels (400–500 nm) of a smartphone or Raspberry Pi camera under ambient lighting conditions, instead of exactly at 405 nm to detect our chromophore. In addition, RBC or free hemoglobin contamination could explain the decreased sensitivity with whole blood μPAD testing. A higher grade nitrocellulose would reduce flow rates which could provide better plasma front separation and decrease RBC contamination. In addition, prefixing ecarin onto our microfluidic chip would decrease blood handling and eliminate the need for sample pads or a sample loading platform.

As demonstrated in this work, there exist significant variations among human subjects which will likely be encountered with cardiac surgery patients deficient in prothrombin. Overcoming such a challenge will involve introducing fixed concentrations of prothrombin to remove patient-specific factors to attain true DTI plasma levels with both ECT and ECA. Also, combining both clotting and chromogenic assays can overcome the intrinsic limitations of each assay when performed individually. For example, hypofibrinogenemia would lead to increased distance traveled irrespective of drug concentration, but the ECA would yield valid results. If a specimen was hemolyzed, lipemic, or had poor ambient lighting conditions, ECA would instead be unreliable. Hyperbilirubinemia patients and pediatric patients on total parenteral nutrition would also lead to unreliable ECA results. With these scenarios, physicians could use the length-based ECT in lieu of the ECA. Figure 7 combines both length (ECT) and absorbance (ECA) assays performed on the same μPAD and reaffirms our hypothesis; increased drug concentration increases distance traveled but decreases absorbance. This combined ECCT has never been implemented before and is thus novel in overcoming the limitations of either assay performed individually.

Further optimization is required to improve the sensitivity of our paper-based assays. With increased sensitivity, our methods could identify specific plasma drug concentrations and help physicians titrate drug dosages over time. Exact quantification would lead to fewer thrombotic events, thus leading to improved perfusion and extracorporeal membrane oxygenation (ECMO) management of heparin-induced thrombocytopenia (HIT) patients. Furthermore, guiding reversal of dabigatran's anticoagulation with idarucizumab will expand our assay's clinical utility.

We have described a novel microfluidic paper-based assay for DTI quantification in patients' whole blood samples. POC DTI testing could be instrumental before surgery, during bridging of anticoagulant therapies, or when gauging sub- or supratherapeutic DTI levels. The obtained data suggest we could further decrease assay time and improve sensitivity with further device optimization. DTIs are gaining increased use within a myriad of clinical situations, and our efforts lay the groundwork for a viable paper-based method for POC monitoring.

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