MICROFLUIDIC POINT-OF-CARE ECARIN BASED ASSAYS FOR MONITORING DIRECT THROMBIN INHIBITOR THERAPY

by

Benjamin J. Alouidor

A Thesis Submitted to the Faculty of the

COLLEGE OF MEDICINE

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN MEDICAL PHARMACOLOGY
PERFUSION

In the Graduate College

THE UNIVERSITY OF ARIZONA

2018

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SIGNED: *Benjamin Alouidor*

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Jeong-Yeol Yoon, Ph.D.
Professor, Biomedical Engineering

04/24/2018
Date

Raymond Wong, Ph.D., CCP
Director, Perfusion Science Graduate Program

04/24/2018
Date

Zain Khalpey, Ph.D., MD
Director, Mechanical Circulatory Support

04/24/2018
Date

Table of Contents

List of Figures

Abstract

Direct thrombin inhibitors (DTIs), such as dabigatran, have maintained steady outpatient use due to their high oral bioavailability and relatively safe "on-therapy" range. The creation of new intravenous antibody reversal agents, like Idarucizumab, have renewed DTI monitoring interests. Current clinical methods within the United States lack the capacity to quantify DTI concentrations across wide ranges. At present, the international gold standard in quantifying DTI therapeutic thresholds is the ecarin clotting time (ECT). The linear prolongation of the ECT is directly proportional to specific DTI concentrations and inversely proportional to absorbance. This work focused on the development of a microfluidic paper analytic device (μPAD) that can quantify 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 sample migration are then automatically captured by our self-coded Raspberry Pi and/or the Samsung Galaxy S8 smartphone camera. Commercial wax printers were used to create these disposable low-cost μPAD chips. Both the flow length and the blue absorbance from the plasma front on the μPAD were measured, allowing simultaneous, dual assay of ecarin clotting test (ECT) and ecarin chromogenic assay (ECA). Statistically significant ($p < 0.05$) changes in flow and absorbance were observed within our translational research study. Currently there are no quantitative commercially available point of care (POC) tests for the ECT within the U.S. Implementation of the ECT will differentiate between true supratherapeutic incidents and limit the unwarranted use of reversal agents. Additionally, DTIs have proved useful in maintaining anticoagulation during mechanical circulatory support in heparin induced cytopenia (HIT) patients. Our hopes are that our device will provide

caregivers and patients the tools to monitor DTI therapy within both in and/or outpatient settings.

Introduction

Direct Thrombin Inhibitors (DTIs) as Anti-Coagulants

The coagulation pathway has intrinsic and extrinsic origins that converge on the Stuart-Prower Factor (Factor X). Factor X complexes with Factor V to form the prothrombinase complex, which is responsible for cleaving Factor II (prothrombin) into its active form thrombin (17). Thrombin's hallmark feature encompasses platelet stimulation, the enzymatic conversion of fibrinogen to fibrin, and its ability to activate the clot stabilizer Factor XIII. Several key ancillary functions involve a positive feedback mechanism which stimulates Factor V, Factor VIII, and Factor XI (5).

Direct thrombin inhibitors (DTIs) are novel anticoagulants which do not require cofactors to achieve blood thinning. Hirudin, the most potent DTI, was first isolated from the salivary glands of medicinal leeches. These drugs produce a state of reduced hemostasis which decreases the occurrence of thrombotic emboli. DTIs' mechanism of action is made through the allosteric inhibition of thrombin, thus preventing fibrin generation (16). Traditional therapeutics, such as heparin, requires the cofactor antithrombin and can only achieve 20-40% thrombin inhibition whereas DTIs attain 70% inhibition. DTIs' enhanced ability to inhibit thrombin, in comparison to heparin, is due to thrombin's enzymatic structure. Thrombin has one active (or enzymatic) site and two exosites; anion-binding exosite II is known as the heparin binding domain whereas

exosite I binds fibrinogen (7). Heparin and other low-molecular-weight heparins form a heparin-antithrombin-thrombin complex. The heparin-antithrombin complex has reduced affinity to pre-existing fibrin-bound thrombin. This is problematic because thrombin upregulates its own release due to positive feedback loops. DTIs however can inhibit free, fibrin-bound, or even heparin-bound thrombin. Given thrombin's role as a potent platelet activator, DTIs also increase anticoagulation by indirectly inhibiting platelet aggregation (23).

Univalent DTIs include dabigatran, ximelagatran, inogatran, and argatroban. Univalent DTIs inhibit thrombin by blocking its active site. Bivalent DTIs, such as bivalirudin, hirudin, and lepirudin, block both thrombin's active and fibrinogen binding site. These bivalent anticoagulants form irreversible 1:1 complexes with thrombin (18). However, bivalirudin's anticoagulant effects are transient due to thrombin's ability to cleave bivalirudin.

Pharmacodynamics & Pharmacokinetics of DTIs

Major adverse events of DTI therapy, such as intracerebral bleeding, can occur through interactions with other drugs. Recombinant hirudins, desirudin and lepirudin, have 10x reduced affinity to thrombin but are the strongest clinically available DTIs (28). Their primary route of elimination is renal clearance with the remainder removed with bile. Bivalirudin and univalent DTIs undergo hepatic proteolysis with 20% renal clearance. Prolonged exposure to ximelagatran has been linked to small incidents of hepatoxicity, which lead to FDA failure of regulatory approval. However, most DTIs' hepatotoxicity is rather minimal, since *in vivo* studies have confirmed little to no

reactivity of DTIs with cytochrome P450 proteins (31), which activate or breakdown pharmacological compounds during hepatic metabolism. DTIs' little reactivity with cytochrome P450 proteins reduces the incidence of drug-drug interactions that occurs during CYP receptor modulation; i.e. CYP stimulation or inhibition influences drug clearance. Nonetheless, decreased elimination of DTIs can occur by inhibiting the permeable glycoprotein (P-gp) transport system (2). Dabigatran for instance, can experience decreased elimination when paired with antiarrhythmic ion channel blockers, such as verapamil or quinidine, given the P-gp systems similarity to ion channels (15).

Clinical Considerations for DTIs

DTIs have gained increased use in heparin induced thrombocytopenia (HIT) and within extracorporeal membrane oxygenation (ECMO) device patients. HIT occurs due to cross-reactive platelet autoantibodies responding to heparin binding with platelet factor 4 (12). Lepirudin is an alternative anticoagulant therapy following the diagnosis of heparin induced thrombocytopenia with thrombosis (HITT). Thrombosis can occur once activated platelets release aggregation mediators. Note that 40% of HIT patients can develop hirudin antibodies post lepirudin treatment (10). Lepirudin immune complex accumulation will lead to decreased renal excretion and subsequently increased toxicity.

Bivalirudin (administered intravenously) has been shown to decrease percutaneous coronary intervention (PCI) morbidity when compared to heparin (6). Thrombin's ability to cleave bivalirudin paired with its short half-life can reduce adverse events of bleeding. Prospective studies involving bivalirudin's use with mechanical circulatory support (MCS) devices remains limited. Retrospective reports indicate

bivalirudin as a viable alternative to heparin, but large variability exist between centers in terms of target activated clotting time (ACT) and bivalirudin loading and infusion dose (1, 25).

Dabigatran's (administered orally) wide therapeutic range combined with little food and drug interactions has improved its outpatient therapeutic indications. Dabigatran has been used to prevent deep vein thrombosis, pulmonary embolism, and strokes in non-valvular atrial fibrillation (3). Though considered relatively safe, supratherapeutic incidents can occur. Quantification of the patients' response to dabigatran therapy can prophylactically detect and/or enable precise 1:1 reversal of these incidents. Point-of-care (POC) DTI testing could be instrumental before surgery, during bridging of anticoagulant therapies, or when gauging both sub or supratherapeutic DTI levels.

Conventional Testing for DTI Based Therapy

Hospitals within the United States have traditionally used the plasma-based activated partial thromboplastin time (aPTT) and the whole blood-based activated clotting time (ACT) to monitor the extent of blood coagulation during DTI based therapy. Though these assays are effective in monitoring heparin levels, they lack sensitivity to intravenous (bivalirudin) and oral (dabigatran) DTI anticoagulants (29). The aPTT remains curvilinear throughout a wide range of dabigatran concentrations; whereas the ACT is only linear up to 250 ng/mL dabigatran (Figure 1).

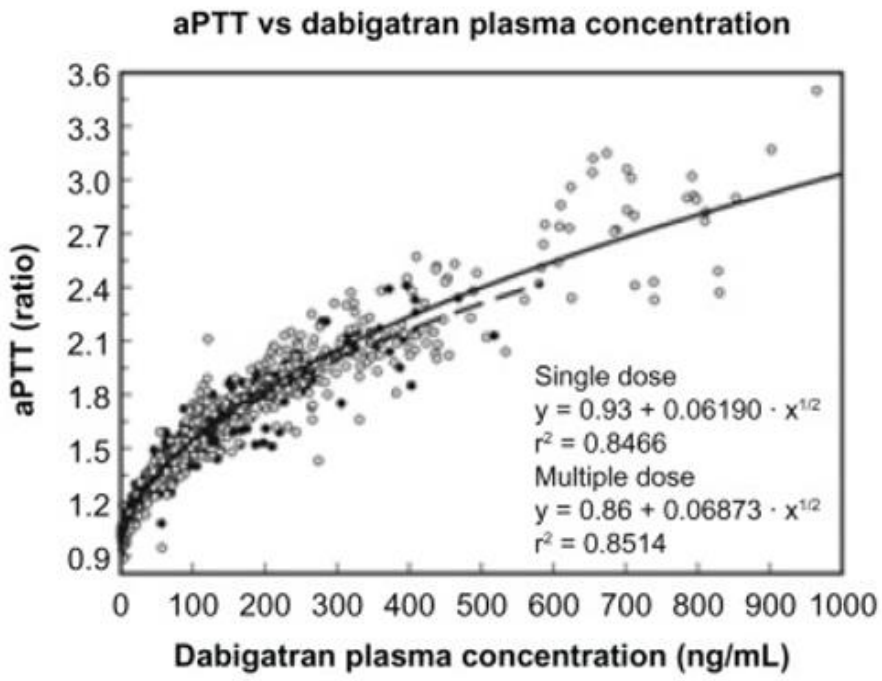


Figure 1. Dabigatran plasma concentration vs. aPTT. Reproduced from (11) with permission, © 2013 Dove Medical Press Ltd.

The prothrombin time (PT), an alternative measure for quantifying blood coagulation, primarily measures plasma Factor VII activation in the presence of Tissue Factor. These extrinsic factors are traditionally used to monitor warfarin therapy. An international normalized ratio (INR) is used to standardize the PT given Tissue Factor reagent variation in international sensitivity indices (ISI); $INR = (PT_{patient} / PT_{control})^{ISI}$. Again, dabigatran concentrations were found to correlate poorly to the PT/INR assay following orthopedic surgery (27).

Haemonetics (Braintree, MA) recently released the TEG 6s® POC analyzer and utilizes resonance technology to measure platelet activity and the extrinsic & intrinsic factors capacity to form clots. Clot strength is determined by exposing the specimen to a fixed vibrational frequency (14). Blood meniscus motion is then captured using a light-emitting diode (LED); weak clots will exhibit lower resonant frequencies and lower TEG

readouts. Given the lack of domestic POC DTI testing platforms, Haemonetics added a prototypical ecarin based cartridge for qualitative detection (8). The commercially unreleased cartridge can only distinguish between the presence of DTIs or direct Factor Xa inhibitors; it cannot provide quantitative assessments. Similarly, the thrombin time (a measure of thrombin activity) cannot reliably differentiate between DTI concentrations and should only be used to indicate the presence of drug (4).

Ecarin Clotting Time (ECT) and Ecarin Chromogenic Assay (ECA)

In contrast to the above mentioned 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 to 10% without ecarin), converts fibrinogen to fibrin, and is inhibited by DTIs; thus, conventional coagulation analyzers can quantify its capacity to form clots (22). Note that low levels of prothrombin or hypofibrinogenemia will cause falsely elevated clotting times unproportionate to DTI concentrations (Figure 2).

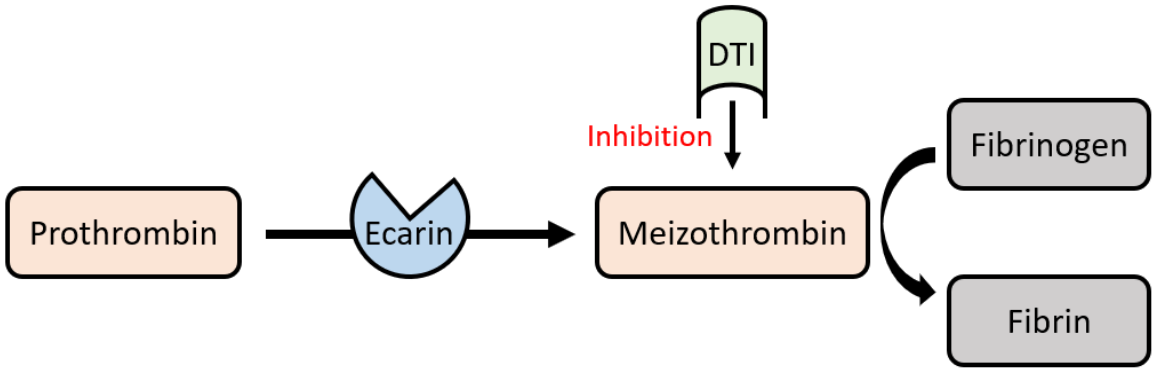


Figure 2. Ecarin clotting time (ECT).

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 DTI concentration increases (26) (Figure 3). Given the oxyhemoglobin's and methemoglobin's peak absorption at 415 nm and 405 nm, respectively, the ECA is performed on plasma to avoid red cell interference (9). ECA assays are mainly affected by hypothrombinemia.

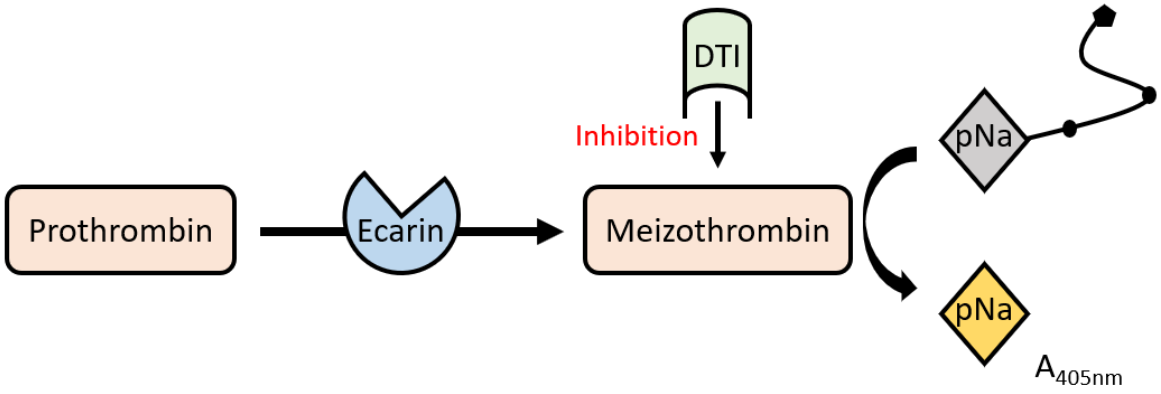


Figure 3. Ecarin chromogenic assay (ECA).

Paper Based POC Tests

Though conventional hemostasis analyzers could be used to monitor coagulation status, novel paper-based methods have proven effective. Previous efforts used a paper-based lateral flow assay (LFA) device to visualize blood coagulation in a POC setting. Sodium citrate anticoagulated animal blood was reversed with fixed concentrations of $CaCl_2$; decreased distance traveled correlated with restored hemostasis (19). Nitrocellulose paper (negatively charged) has emerged as the preferred coagulative LFA substrate given its unique characteristics in resisting cell adhesion and protein fouling. The electronegative environment of nitrocellulose fibers mimics the exposed phospholipid surface, which prevents platelet aggregation and subsequent coagulation (24).

Microfluidic paper analytical devices (μPADs), more sophisticated and multi-channel 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 (30). Smartphone based imaging has advanced LOC devices as well as paper based POC tests (LFA and μPAD) due to its wide availability, as well as its ability to compute, display, and collect data (13). Smartphones also contain a white LED flash, which could be used for various optical sensing (21). Pairing smartphones' robust features with microfluidics can create inexpensive POC testing devices which could rapidly detect endpoints of interest.

Hypothesis and Specific Aims

Hypothesis

Our aim is to develop an ecarin based microfluidic paper analytic device (µPAD) that can quantify the DTI concentration within a patient's whole blood sample. We hypothesize that increased DTI concentrations will I) increase the distance of sample flow and II) decrease the rate of chromogenic substrate cleavage in the fixed presence of ecarin and chromophore. Effective application will provide a quantitative paper-based method to monitor DTI therapy within inpatient and/or outpatient settings.

Specific Aims:

I. Design, construct, and test a prototypical microfluidic paper analytic device (µPAD) for DTI quantification in whole blood.
    a. Clinically relevant concentrations of DTI agents (dabigatran and bivalirudin) will be added to sodium citrated whole blood, then used to monitor hemostasis after ecarin activation.
    b. Whole blood will be separated through paper fibers, generating plasma front and red blood cell (RBC) front.

II. Design, construct and test a user-friendly touchscreen device to capture and interpret µPAD data.
    a. Flow characteristics (length of plasma front at given time) will be recorded via Raspberry Pi and/or Smartphone technology and correlated to ecarin clotting time (ECT).

b. Chromogenic substrate cleavage will be quantified within the plasma front using smartphone (Android and iOS) imaging and correlated to ecarin chromogenic assay (ECA).

III. Optimization of assay by varying incubation time and ecarin concentration.

IV. Verification of data collected using analytical statistical tests.

Materials and Methods

Specimen Collection

Sodium citrated human whole blood specimens were initially purchased from commercial vendors. Fresh whole blood was then collected from human volunteers per University of Arizona's IRB amended protocol 1612094853. Specimens were collected by a licensed Medical Laboratory Scientist and stored in 1:10 volume 0.129 M sodium citrated vacutainers prior to testing. Unfiltered human sodium citrated plasma was purchased in bulk. 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 8 channel μPAD chips. Sample loading

zones are 4 x 4 mm with perpendicular 1 mm wide x 30 mm long travel channels. Channels were partitioned with hydrophobic wax ink and printed using a Xerox ColorQube 8580 solid wax color printer (Norwalk, CT). Wax ink RGB values are 0, 255, and 255 respectively; zero red intensity is used to distinguish between the presence and absence of blood. The hydrophobic wax layer was imbedded into the µPAD chip via hotplate (150 seconds at ~130°C). G041 glass fiber conjugate pad sheets (Millipore Sigma) were cut into 4 x 4 mm segments and used as sample incubation pads.

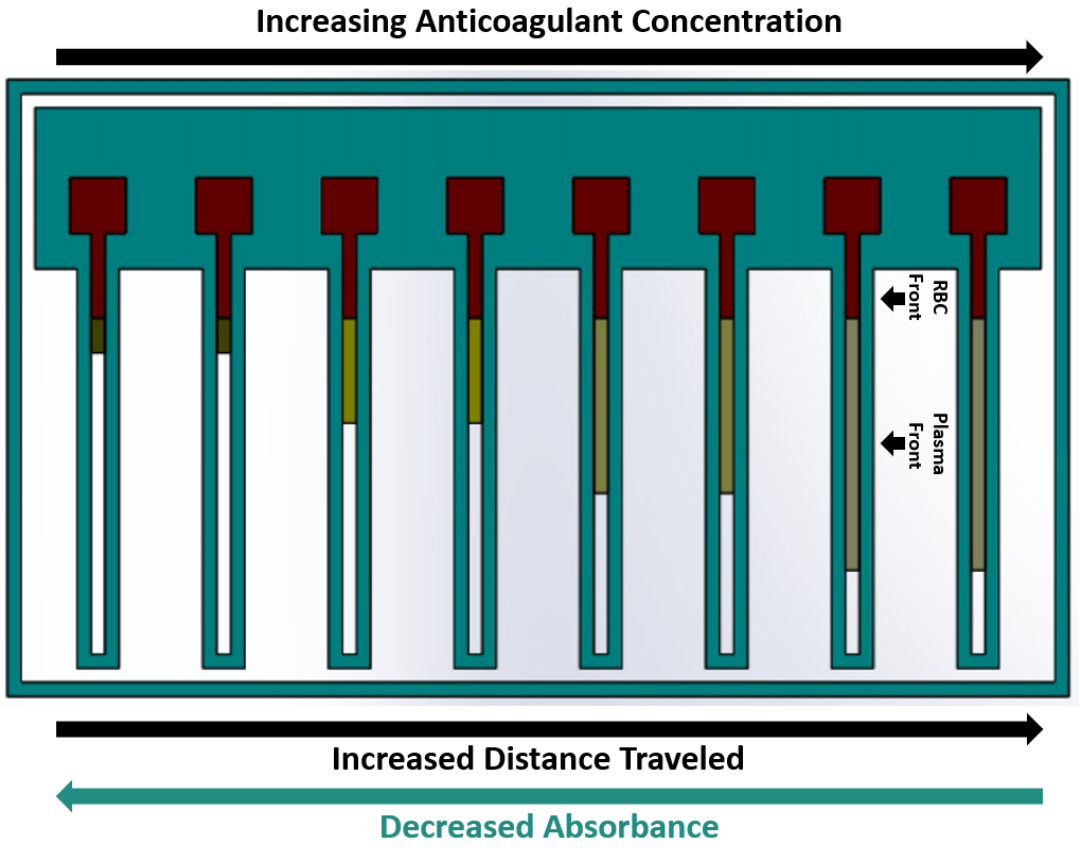


Figure 4. µPAD ECT/ECA chip.

Solution Preparation

Given its physiological similarity to human plasma, Plasma-Lyte A (Baxter Healthcare Corporation) was used to dilute DTI drugs: bivalirudin (Sigma Aldrich) from

0, 7, 14, to 21 µg/mL and dabigatran (TargetMol, Boston, MA) from 0, 200, 400, to 600 ng/mL. Ecarin (Sigma Aldrich) was reconstituted in 1x tris-buffered saline to a final concentration of 5 EU/mL; aliquots were stored at –20^{o}C. Pefachrome Thrombin (H-D-Cyclohexylglycine-Ala-Arg-pNA · 2AcOH) (5-Diagnostics, Independence, MO) was diluted with Plasma-Lyte to a working solution of 0.5 mM.

Ecarin Clotting Test (ECT) Procedure

Fiber glass sample pads were housed on a 3D printed stamper platform as shown in Figure 5. After thawing at room temperature, 5 µL of 5 EU/mL ecarin is preloaded onto the sample pads. After blood collection, whole blood is aliquoted into an 8 well plate and spiked with fixed amounts of DTIs. A multichannel pipette (ThermoFisher Scientific, Waltham, MA) transfers 5 µL of the anticoagulated blood onto the sample pads. Subsequently after a 2-minute room temperature incubation, the stamper platform is flipped onto the µPAD's specimen loading zone. Figure 5 illustrates the ECT microfluidic assay procedure.

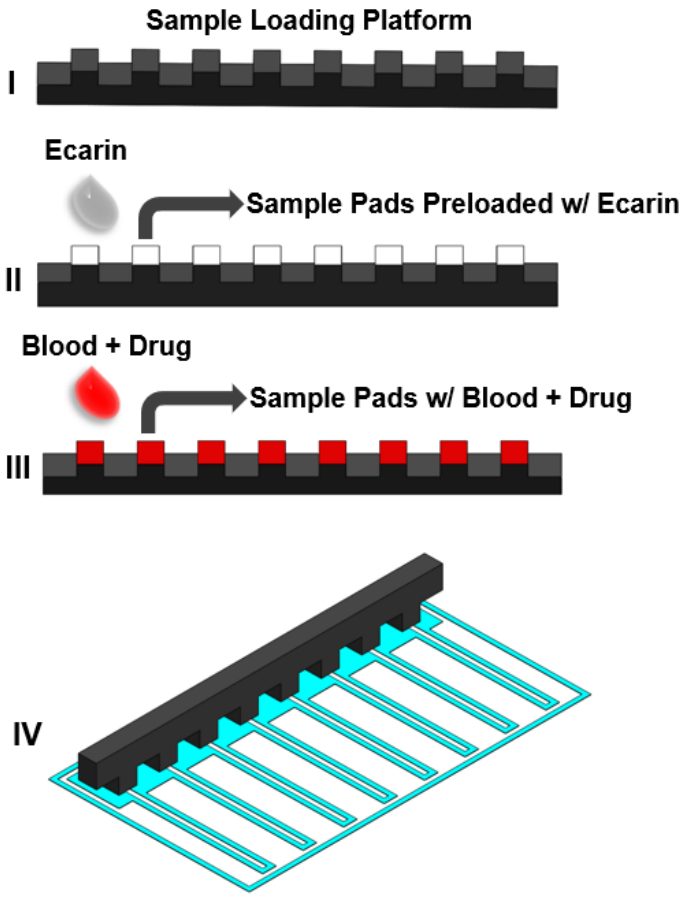


Figure 5. Ecarin clotting test on µPAD.

A custom coded Raspberry Pi 3 was retrofitted with an 8-megapixel Raspberry Pi Camera Module V2 and a touchscreen 7" display. The Pi automatically takes pictures at set time intervals (10, 20, 30, 40, 50, 60, 120, and 180 s). Images were also taken manually with the Samsung Galaxy S8 12-megapixel rear camera.

Ecarin Chromogenic Assay (ECA) Procedure

Conditions are similar to Figure 5 except:

I) 1.75 µL of 4 mM pNa is preloaded together with 5 EU/mL ecarin.

II) A 3-minute room temperature incubation on a stamper after loading blood samples.

In preliminary studies to establish a positive control, unfiltered human plasma was analyzed using the ecarin chromogenic assay 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 V.1.6.7 software (Ocean Optics) was used to obtain and analyze the spectra from the miniature spectrophotometer. Reflectance was also measured using the Samsung Galaxy S8 (Suwon, South Korea) 12-megapixel rear camera in lieu of a miniature spectrophotometer.

Following these positive control experiments, the images were collected from the above mentioned μPADs with LED excitation and imaging by Raspberry Pi or smartphone (Galaxy S8). The plasma front areas were analyzed as on the ECA.

Data Analysis

Images taken from the Raspberry Pi or Samsung Galaxy S8 were analyzed using the National Institutes of Health (NIH)'s ImageJ software. ECT images were divided into red, green, and blue (RGB) components with a focus on blue pixels. The average length from the loading zone to the end of plasma front was used as distance traveled at given time point. The reliable length of the hydrophobic wax channel was used as a correction factor to correct for chip tilt or rotation. Similarly, ECA images were separated 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 a fixed area. Absorbance (A) was obtained by comparing measured intensity (I) to our reference sample where DTI concentration is zero (I_0), using the definition $A = \log (I_0 / I)$.

Results

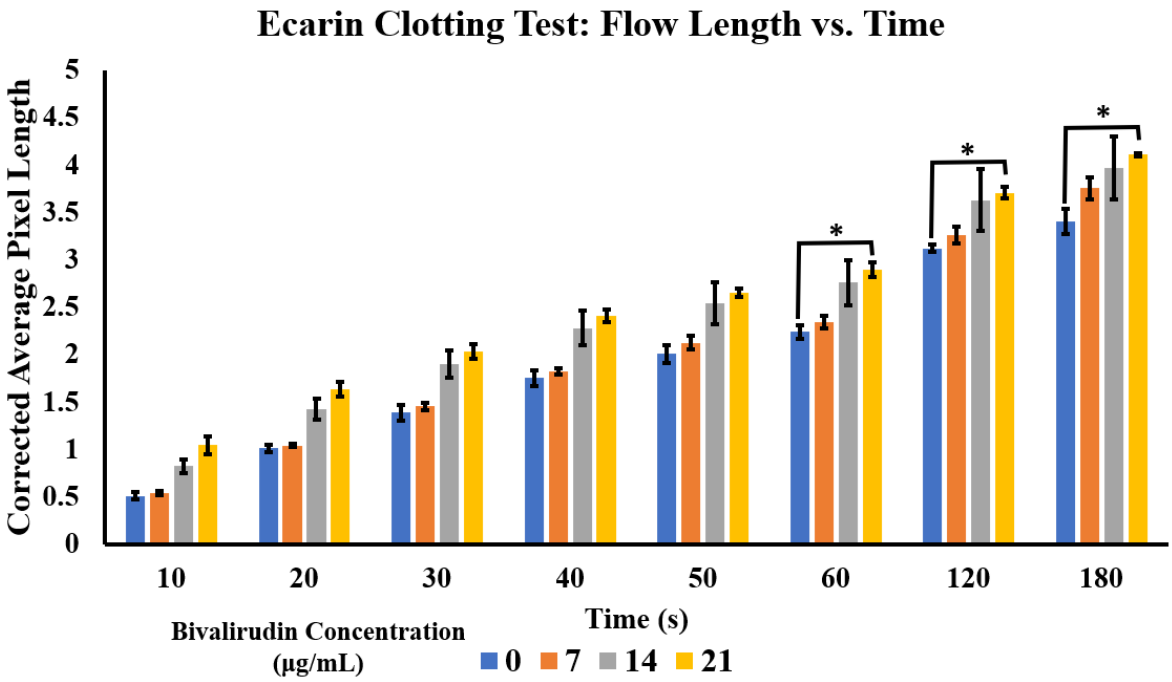


Figure 6. Ecarin clotting test (ECT) on µPAD: plot of flow length vs. time with varied bivalirudin concentration. Assay performed in duplicate. Error bars represent standard mean error.

The flow lengths from the inlet to the end of plasma front on each µPAD channel were analyzed automatically using Raspberry Pi system and software. The corrected flow lengths were plotted against time with varying bivalirudin concentrations (Figure 6; 0, 7, 14, and 21 µg/mL). All data show the expected linear increase in distance traveled over time and bivalirudin concentrations. The multiple time data points display that the

increasing trend begins at 10 s (although this time point is not reliable between subjects) and becomes more pronounced over time. Regression analysis (ANOVA) confirms the significance of the increasing trend ($p < 0.05$) at 60, 120, and 180 s (indicated with *), indicating that the minimum assay time was 60 s (= 1 min).

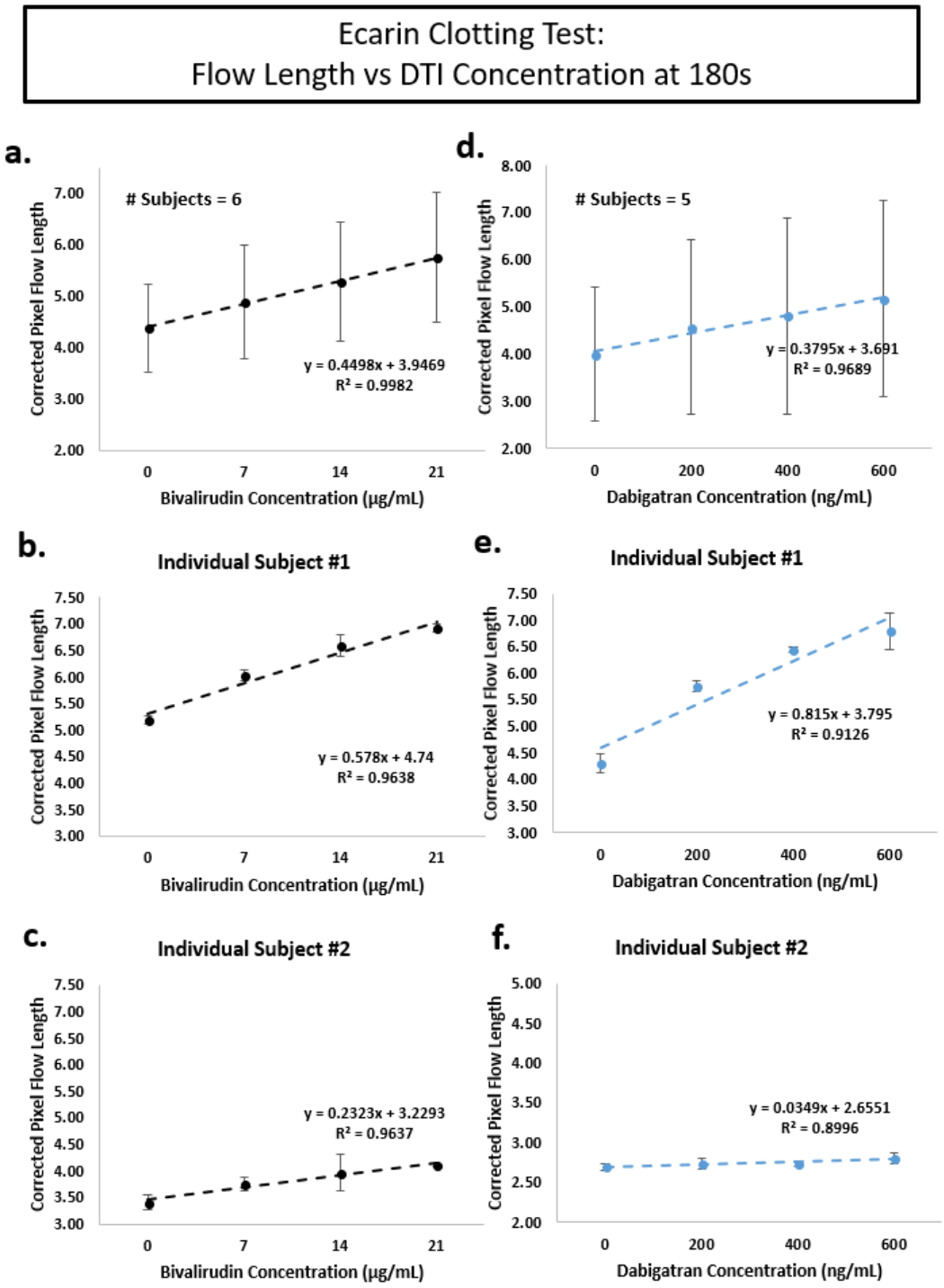
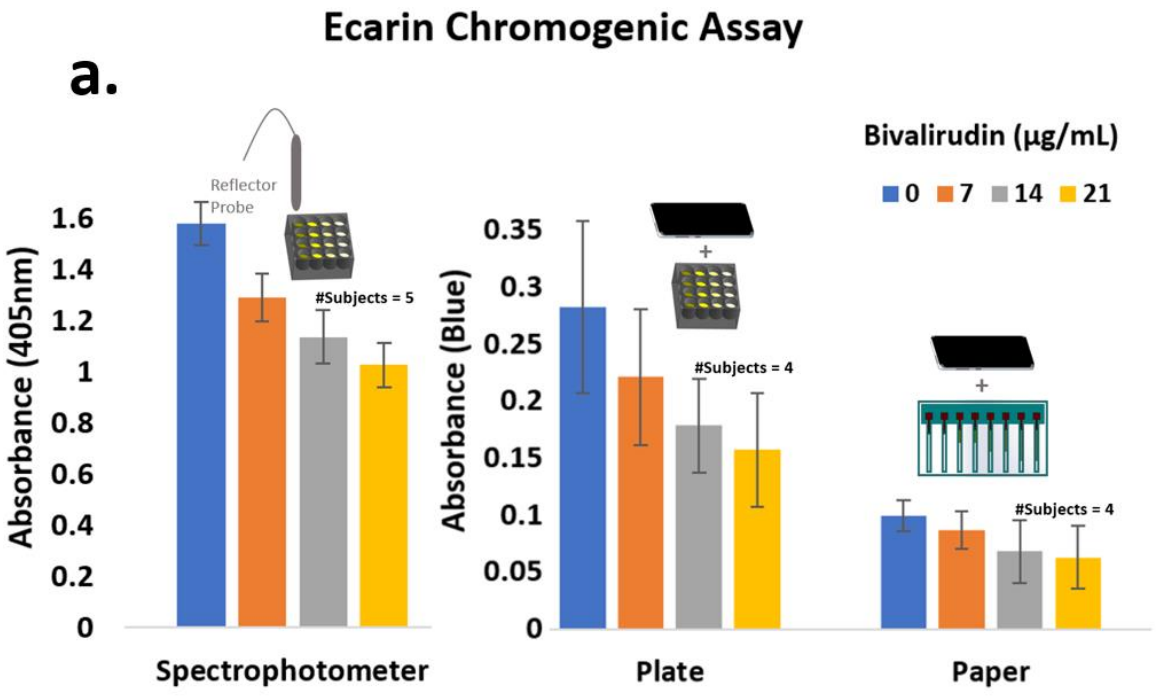


Figure 7. Cumulative ECT data as evaluated by the flow length on µPAD at 180 s, with varied concentrations of bivalirudin and dabigatran. Assay performed in duplicate for each subject.

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 images collected using a Samsung Galaxy S8. Figure 7 shows the flow length data at 180 s, plotted against bivalirudin (A) or dabigatran (B) concentration, again showing the expected increasing trend. Grouping all the patients together increased standard error because each subject responded differently to DTIs. Flow lengths from two specific subjects (#1 and #2) are separated plotted in Figure 7B-F, which confirms this variation with subject #2's almost flat response to DTI presence. Analyzing each subject individually decreased standard error and ANOVA analysis confirmed the statistical significance (B and E: $p < 0.001$; C: $p < 0.05$). All subjects' linearity is further confirmed with R^2 values; B – 0.9638, C – 0.9637, D – 0.9126, and F – 0.8996.



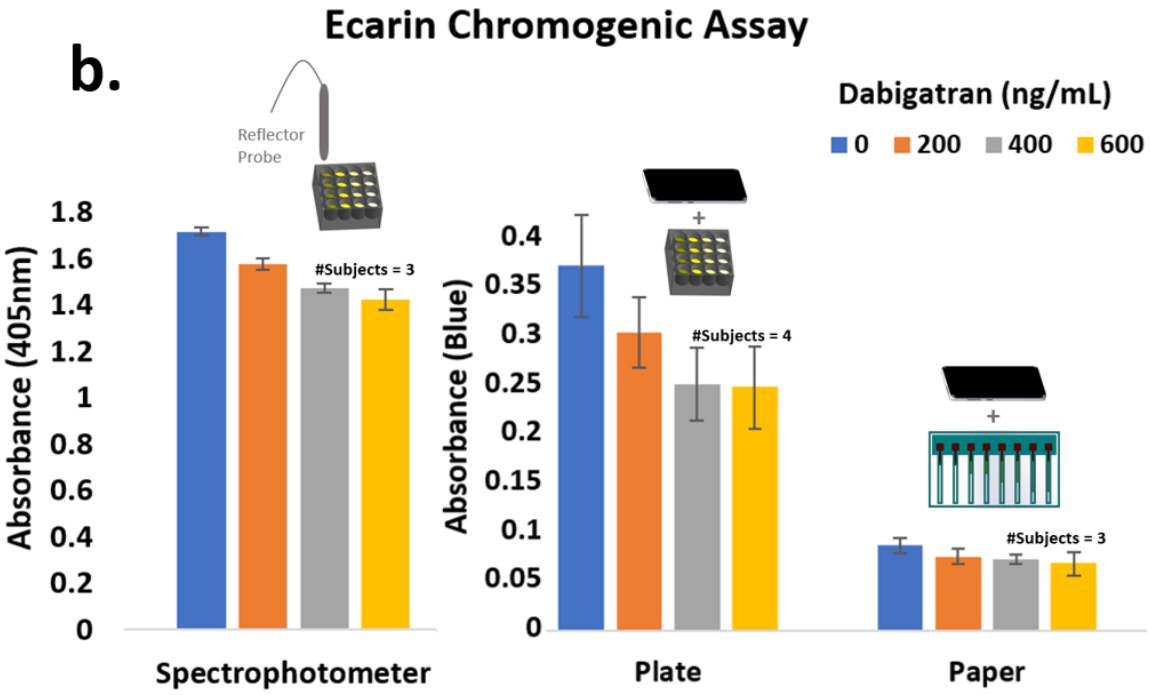


Figure 8. Ecarin chromogenic assay (ECA) with varied concentrations of bivalirudin and dabigatran on 96 well plate (using plasma) or μPAD (using whole blood), using either a reflection probe or a smartphone. Well-based assays performed in triplicate for each subject; paper analysis performed in duplicate for each subject.

Ecarin chromogenic assays (ECAs) were performed for I) the plasma solutions in a 96 well plate using a reflection probe and a miniature spectrophotometer, II) the same using a smartphone, and III) whole blood on a μPAD using a smartphone (Figure 8). The highest absorbance values were obtained from the plasma solutions in a 96 well plate using a reflection probe and a miniature spectrophotometer. Linear regression analysis highlights the expected decrease of bivalirudin and dabigatran concentrations ($p < 0.001$). T-test analyses (Appendix A-b) show that we can differentiate between the presence and absence of drug and between higher drug concentrations ($p < 0.001$). Next, we substituted

the expensive reflection probe and the miniature spectrophotometer with a smartphone and took pictures of the plasma reaction solutions in a 96 well plate. Similarly, ANOVA analyses were consistent ($p < 0.001$) and we could distinguish from the negative control ($p < 0.05$). Bivalirudin assays had trouble discriminating between 7 vs. 14 µg/mL whereas dabigatran assays could not distinguish between 400 vs. 600 ng/mL (Appendix A-c). Lastly, 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). Bivalirudin and dabigatran data displayed a decrease in linear regression that is statistically significant ($p < 0.05$). Though we could detect the absence of drug (Appendix A-b), there was a further decrease in sensitivity with higher drug dosages (Appendix A-c).

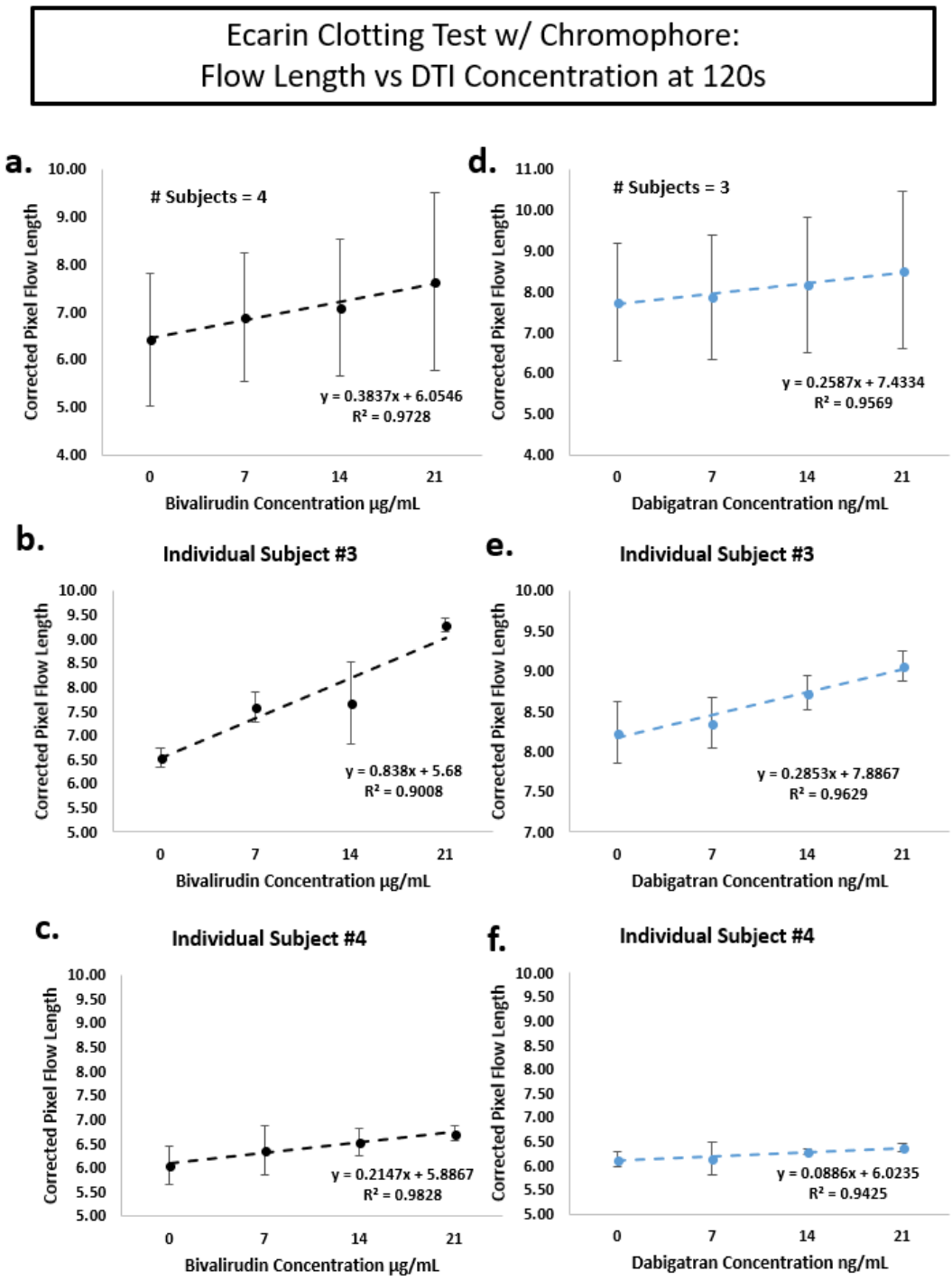


Figure 9. Cumulative ECT data with chromophore (ecarin) as evaluated by the flow length on µPAD at 120 s, with varied concentrations of bivalirudin and dabigatran. Assay performed in duplicate for each subject.

The flow lengths from the inlet to the end of plasma front on each μPAD channel were analyzed manually from images collected using a Samsung Galaxy S8. ECTs analyses were repeated using the whole blood added with ecarin, and the results are shown in Figure 9, showing almost identical trends as those shown in Figure 7. Subject #3's response to bivalirudin and dabigatran was significant and confirmed via ANOVA regression analysis ($p < 0.05$). Likewise, standard error decreased when analyzing individual subjects.

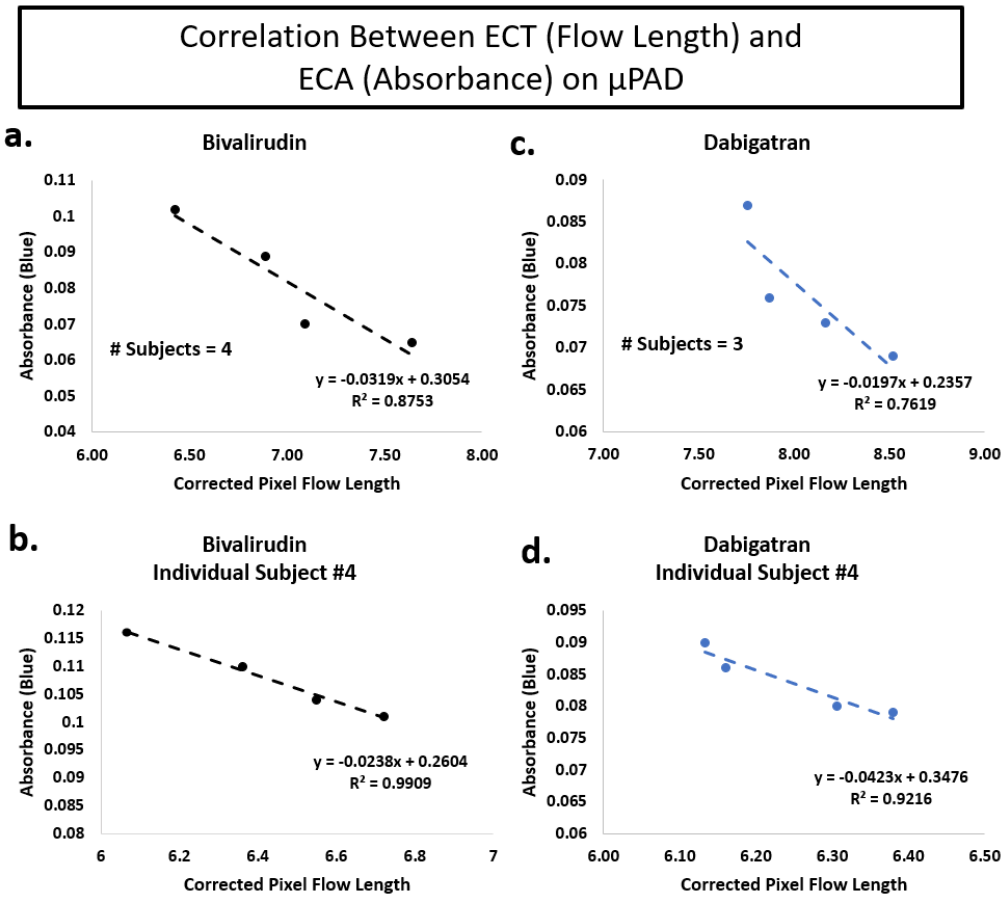


Figure 10. Correlation between ECT (as measure by the flow length, derived from Figure 9 data) and ECA (as measured by blue absorbance, derived from Figure 8 data) on the same μPAD.

Correlation between ECT and ECA from the same μPAD assay of whole blood was attempted in this analysis. Next, the blue absorbance readings from μPAD, taken from Figure 8 data representing ECA, were plotted against the flow lengths from μPAD, taken from Figure 9 data representing ECT (Figure 10). As expected, increasing drug concentration increased flow length and decreased absorbance. The expected trend is confirmed with R^2 values; A – 0.8753, B – 0.9909, C – 0.7619, and D – 0.9216.

Discussion

Thrombosis prevention is a fine balance between inhibiting hypercoagulability, stasis, and vessel wall injury. Direct thrombin inhibitors promote a hypocoagulopathic state by inhibiting the common pathway via Factor IIa. DTIs are more efficacious than other anti-coagulants (heparin and warfarin) given its higher capacity for inhibiting both free and bound thrombin, relatively safe pharmacological profile, and no 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 point-of-care (POC) prototypical devices for monitoring changes in blood and plasma when exposed to DTIs. We hypothesized that increased DTI concentration will lead to decreased coagulation; thus, increased distance traveled in ECT. We also theorized that lower DTI concentrations will provide a detectable increase in chromophore (pNa) cleavage in ECA. With these goals, we designed a novel paper microfluidic analytical device (μPAD) with the ability to quantify bivalirudin and dabigatran concentrations from various human subjects and their effects on the extent of coagulation.

As expected, the ECT length-based analyses correlated with increased DTI concentration. The use of paper microfluidics allowed us to perform the ECA without centrifuging the blood samples. Paired with a Raspberry Pi device or a smartphone, these innovations decrease assay time, reduces specimen volume requirements, and improves the POC practicality of ecarin based testing. Our POC ECA assay cannot differentiate between higher drug concentrations. The decrease in sensitivity could be due 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. Additionally, red blood cell (RBC) or free hemoglobin contamination could explain the decreased sensitivity with whole blood µPAD testing. The fluorophore rhodamine 110 could be utilized to improve the µPAD's sensitivity. Rhodamine 110 emission and excitation spectra are 520 nm and 496 nm, respectively (20). The longer wavelengths will reduce the effects of hemoglobin interference. It will also allow the use of Samsung Galaxy S8's flash instead using of ambient light. Unfortunately, fluorometric analyses would require a band pass filter and increase assay complexity and cost.

A higher-grade nitrocellulose would reduce flow rate which could provide better plasma front separation and decrease RBC contamination. Prefixing ecarin onto our microfluidic chip would decrease blood handling and eliminate the need for sample pads or a sample loading platform. Lastly, introducing fixed concentrations of fibrinogen and/or prothrombin will remove patient specific problems with both ECT and ECA assays; i.e. assays will not be affected by rate-limiting reactants.

Combining both chromogenic and clotting assay can overcome the intrinsic limitations of each assay 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 were hemolyzed, lipemic, or had poor ambient light conditions, ECA assays would be unreliable. Figure 10 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 Ecarin Clotting and Chromogenic Test (ECCT) has never been implemented before and is thus novel in overcoming the limitations of either assay performed individually.

Clinical Translation

Further optimization is required to improve the sensitivity of our paper-based assays. With increased sensitivity, our methods could identify specific drug concentrations and help physicians titrate drug dosages overtime. Exact quantification could also lead to improved perfusion and ECMO management of HIT patients due to decreased clotting events. Current plasma-based tests (aPTT, PT, etc.) require centrifugation whereas the whole blood ACT becomes curvilinear. Our μPAD assays are linear throughout DTI concentration ranges and provides results without centrifugation. In addition to increasing total sample size and testing other direct thrombin inhibitors, one could code and install a dedicated heater into the Raspberry Pi. ECA and ECT assay validation could confirm superiority versus current DTI testing platforms (ACT, aPTT, etc.). Furthermore, reversing dabigatran's anticoagulation with Idarucizumab will expand our assay's clinical utility. Evaluating efficacy in various disease states, such as

mechanical circulatory support induced hemolysis will be necessary to fully define the limitations of our chromogenic methods.

Conclusion

We have described a novel microfluidic paper-based assay for DTI quantification in patients' whole blood samples. These ecarin based μPAD assays have the potential to quantify patient specific drug levels in a variety of clinical situations. Using only 5 μL of whole blood per channel, several assays show statistical significance with a total assay time of 5 minutes. The obtained data suggest we could further decrease assay time and improve sensitivity with further device optimization. Direct thrombin inhibitors are gaining increase use within a myriad of clinical situations. Our efforts lay the groundwork for a viable paper-based method for DTI therapy monitoring.

Appendices

a.

		ANOVA	R Squared
Bivalirudin (µg/ml)	Spec.	0.0000	0.950
	Plate	0.0001	0.957
	Paper	0.0409	0.955

		ANOVA	R Squared
Dabigatran (ng/ml)	Spec.	0.0000	0.958
	Plate	0.0000	0.891
	Paper	0.0100	0.904

b.

		ttest		
		0v7	0v14	0v21
Bivalirudin (µg/ml)	Spec.	0.0000	0.0000	0.0000
	Plate	0.0238	0.0014	0.0001
	Paper	0.0331	0.0065	0.0137

		ttest		
		0v200	0v400	0v600
Dabigatran (ng/ml)	Spec.	0.0000	0.0000	0.0000
	Plate	0.0011	0.0000	0.0000
	Paper	0.015	0.003	0.0060

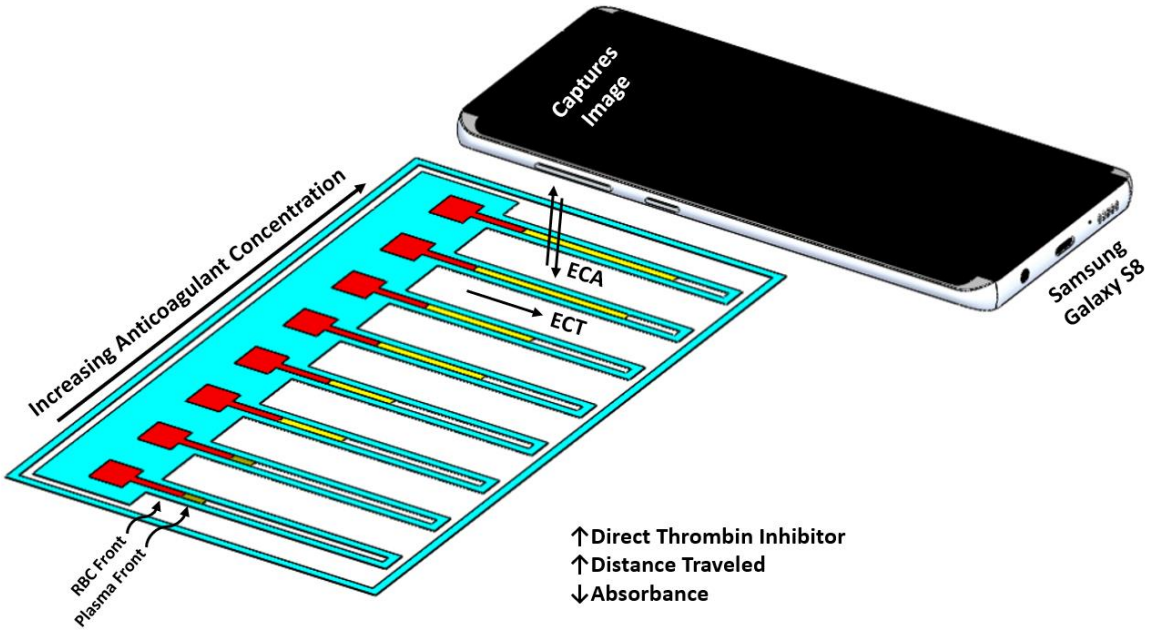
c.

ttest		
0v7	7v14	14v21
0.0000	0.0001	0.0020
0.0238	0.1178	0.0339
0.0331	0.0826	0.5065

ttest		
0v200	200v400	400v600
0.0000	0.0000	0.0030
0.0011	0.0415	0.0736
0.015	0.2396	0.2265

Legend		
$p < 0.001$	$p < 0.05$	$p > 0.05$

Appendix A. Ecarin Chromogenic Assay Statistical Analyses



Appendix B. µPAD Graphical Representation

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