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Therapeutic Drug Monitoring of Apixaban Using Chromogenic Kits

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Abstract

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by

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Apixaban is a novel oral anticoagulant that prevents clotting by directly inhibiting Factor Xa in the coagulation cascade. Due to its different pharmacokinetics, previous standards for testing anticoagulant concentrations are ineffective at measuring apixaban. In this study, Hyphen Biomed Biophen Direct Xa Inhibitor and Biophen Heparin chromogenic kits from Aniara Diagnostica were used with a NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer to see if either of these kits provide acceptable precision and accuracy for the quantification of apixaban in plasma samples, and to evaluate if there is a significant difference in these two kits at varying concentrations of apixaban.

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Introduction

Anticoagulants (blood thinners) are medications prescribed to patients to reduce the risk of blood clotting. Patients are usually prescribed anticoagulants following hip/knee replacement surgery, pulmonary embolisms, strokes, heart attacks, or patients that have atrial fibrillation (1). While they are prescribed frequently, there are risks to taking blood thinners such as increased risks of major bleeding, thrombocytopenia, and osteoporosis, etc. (2).

Anticoagulants work by blocking the formation of a blood clot by affecting different factors in the coagulation cascade (Figure 1) (3). Based on the type of anticoagulant, different steps of the coagulation cascade are affected. For example, the first anticoagulant used was Unfractionated Heparin (UFH), which binds to antithrombin and increases the ability to inactivate thrombin, Factor Xa, and Factor IXa (2). However, Unfractionated Heparin also nonspecifically binds to endothelial cells, monocytes, and plasma proteins which leads to its unpredictability and the need of monthly monitoring (4). The suggested of monitoring unfractionated heparin was the activated partial thromboplastin time (aPTT) (4). The activated partial thromboplastin time is a method of measuring blood coagulation by adding different solutions to the patient's plasma sample, and then measuring the clotting time (4). The aPTT is commonly around 22 to 40 seconds, and if it is less than 22 seconds the prescription would be increased, but if greater than 40 seconds, the prescription would be decreased (4). Following UFH was the creation of new Low Molecular

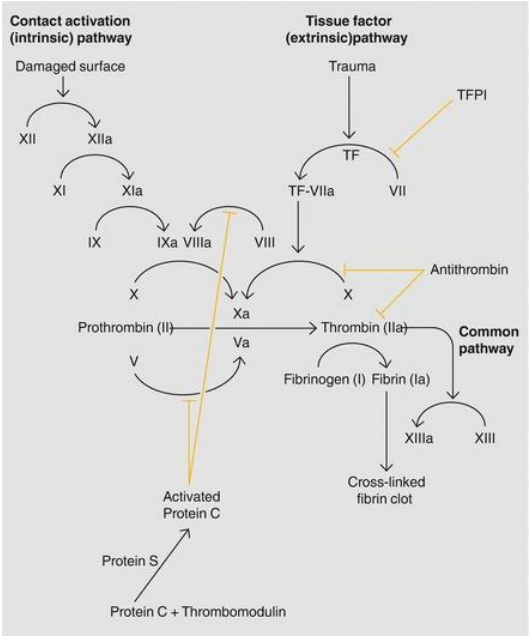

***Figure 1. Coagulation Cascade** (3).*

Weight Heparin (LMWH) which is one-third of the weight of UFH (2). This lower weight made the LMWH more predictable in its binding to antithrombin and lowered its affinity for cells and plasma proteins, which led to it becoming the recommended treatment over the UFHs (2). While LMWH was more predictable, close monitoring was still required, commonly still using aPTT. Warfarin is another common anticoagulant that was produced in the 1950s and is a Vitamin K antagonist. Warfarin affects coagulation by interfering with the conversion of vitamin K to its 2, 3 epoxide, which is required to activate different coagulation factors (1). This can be seen in Figure 2 (5). Warfarin also needs to be monitored to keep levels in the appropriate range; however, warfarin is commonly measured by the Prothrombin time/ International Normalized Ratio (PT/INR) (4). Prothrombin time is measured by adding a thromboplastin reagent containing a tissue factor with calcium to the patient's plasma sample and then the clotting time (4). Due to differences in the thromboplastins that can be used, the PT can vary, so the International Normalized Ratio was created by dividing the patients PT by the mean normal PT (4). The latest anticoagulant type is the thrombin inhibitors or Factor Xa inhibitors, which include apixaban. These type of inhibitors act by reversibly blocking Factor Xa at its active site (6). These new anticoagulants are different in that they generally do not require monitoring unless the patients have impaired renal function, are elderly, or have extreme high or low body weights (6). They do not require monitoring due to the specific affinity of the drugs to Factor Xa and their consistent peak and trough times. The

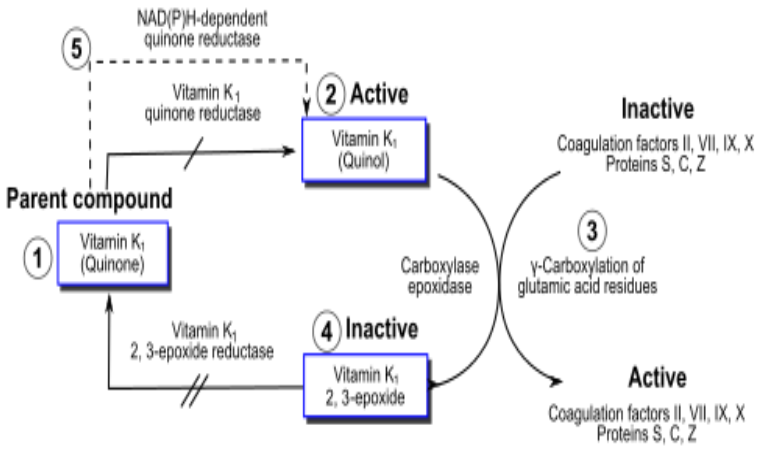

Figure 2. Vitamin K Conversion Cycle *(5).*

major downside to these new anticoagulants is that they cannot be measured using the past standards of aPTT or PT/INR because these methods are not sensitive to Factor Xa inhibitors (7).

Apixaban is considered a direct Factor Xa inhibitors. Apixaban is usually prescribed after major orthopedic surgeries such as hip or knee replacements at a dosage of 2.5mg twice a day, or in the case of preventing a stroke/atrial fibrillation a dosage of 5mg taken twice a day (8). Apixaban has a peak concentration after three hours post dosage and has no interactions with food, and has been found to be just as effective as warfarin in preventing deep vein thrombosis, risk of stroke, and atrial fibrillation in patients (9). While regular monitoring is not needed for apixaban, there are cases in which knowing the exact concentration would be important, such as bleeding recurrence in thrombosis, before surgery in an invasive procedure, identification of patients taking other drugs with possible interactions, suspicion of overdose, or need of reversal of anticoagulant (10). As mentioned earlier, apixaban cannot be effectively measured using aPTT or PT/INR methods,; however, there is some evidence suggests that chromogenic Xa assays can be effective in measuring apixaban concentrations (11). There are several different types of chromogenic kits available to measure Antifactor Xa activity, but this research exclusively investigates the Hyphen Biomed Biophen Direct Xa Inhibitor and Biophen Heparin chromogenic kits from Aniara Diagnostica.

The purpose of this research is to compare the specific Biophen Direct Xa Inhibitor (DiXaI) chromogenic assay to the non-specific Biophen Heparin LRT chromogenic assay and determine if there are any differences between the two in terms of sensitivity, specificity, or reproducibility in measuring the Direct Xa inhibitor Apixaban.

Methods

Heparin LRT Kit

The procedure for the Heparin Kit is the same as stated in the BIOPHEN Heparin LRT Kit's packaged instructions. The BIOPHEN Apixaban Standard Range Calibrator set and BIOPHEN Apixaban Standard Range Control set was used for this experiment. The first step was to reconstitute the BIOPHEN Apixaban Calibrators (Cal 1-3) and Controls (C1, C2) with 1mL of distilled water, mix, then let stabilize to room temperature for thirty minutes. Meanwhile, the Heparin Reagent 1 (R1) and Reagent 2 (R2) were equilibrated to room temperature for thirty minutes. While waiting for them to stabilize, a water bath was set to 37°C and 2 sets of microcentrifuge tubes were labelled the following: R1, R2, Cal 1, Cal 2, Cal 3, C1, and C2. At the end there were 14 total labelled microcentrifuge tubes. One set of empty microcentrifuge tubes was placed into the water bath to get them to 37°C. In the R1 and R2 tubes in the water bath, 250µl of each reagent was pipetted into the corresponding microcentrifuge tube and allowed to reach 37°C. Next the calibrators and controls were diluted. For the Apixaban Standard Range set of Calibrators and Controls, a 1/15 dilution was needed with 50µl of each calibrator/control in 735µl of physiological saline. Next, a 10ml of a 20% acetic acid solution was prepared with 2ml of acetic acid in 8ml of water. After everything had stabilized, 100µl of each calibrator and control were placed into corresponding microcentrifuge tube that was in the water bath. The entire experiment was then conducted in the water bath. The experiment started Calibrator 1 and 250µL of preincubated R1 were put into the Cal 1 preincubated microcentrifuge tube, and sat in the bath for 2 minutes undisturbed. Then after 2 minutes, 250µl of the preincubated R2 was added. Two minutes after the addition of R2, the reaction was stopped with 400 µl of the 20% acetic acid. This process was repeated for each of the calibrators

and controls. Then the samples were run at 405nm using a program on a NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer.

DiXaI Kit

The procedure for the DiXaI Kit is the same as stated in the BIOPHEN DiXaI Kit's packaged Instructions. The BIOPHEN Apixaban Standard Range Calibrator set and BIOPHEN Apixaban Standard Range Control set was used for this experiment. The first step was to reconstitute the BIOPHEN Apixaban Calibrators (Cal 1-3) and Controls (C1, C2) with 1mL of distilled water, mix, and then let stabilize to room temperature for thirty minutes. Meanwhile, the DiXaI Reagent 1 (R1) and Reagent 2 (R2) were reconstituted with 2.5mL of distilled water, mixed, and let stabilize to room temperature for thirty minutes. The pre-constituted DiXaI Reagent 3 (R3) was also set to room temperature for thirty minutes. While waiting for them to stabilize, a water bath was set to 37°C and 2 sets of microcentrifuge tubes were labelled the following: R1, R2, Cal 1, Cal 2, Cal 3, C1, and C2. At the end there were 14 total labelled microcentrifuge tubes. One set of empty microcentrifuge tubes was placed into the water bath to reach 37°C. In the R1 and R2 tubes in the water bath, 1000µl of each reagent was pipetted into the corresponding microcentrifuge tube and let reach 37°C. Next the calibrators and controls were diluted. For the Apixaban Standard Range set of Calibrators and Controls, a 1/40 dilution was needed with 50µl of each calibrator/control in 1985µl of Reagent 3 (R3).Next, 10ml of a 20% acetic acid solution was prepared with 2ml of acetic acid in 8ml of water. After everything had stabilized, 200µl of each diluted calibrator and control was place into its corresponding microcentrifuge tube that was in the water bath. The entire experiment was then conducted in the water bath. Starting with Calibrator 1, 200µL of preincubated R1 was added to the microcentrifuge tube labelled R1 that was in the water bath. After 1 minute, 200µl of the preincubated R2 was added. Exactly 45

seconds later the reaction was stopped by adding 400 µl of the 20% acetic acid. This process was repeated for each of the calibrators and controls. Afterwards, each of the calibrators and controls were placed on a NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer and run at 405nm. Each of the calibrators were run five times to test reproducibility.

Results

Table 1 summarizes the apixaban concentration (± standard deviation) measured at 405nm using the DiXaI and Heparin kits from Aniara Diagnostics and the NanoDrop instrument from Thermo Scientific. Since all samples tested were standardized calibrators and controls prepared in lyophilized plasma, the target values for apixaban in these samples is also shown in the table. The mean and standard deviation for the Heparin and DiXaI kits were computed using Prism GraphPad 8 software. Before calculating results, out of range values and outliers were taken from the data using the ROUT function in GraphPad. Both a Welch's t-test and an independent t-test was done for each of the data sets. An independent t-test was used to see if there was a significant difference in the mean between the Heparin LRT kit and the DiXaI kit. A Welch's t-test was done to compare each the DiXaI and Heparin kit's values to the target values at each of the calibrators and controls, with an a priori level of significance assigned at $p=0.05$. As seen in Table 1, there was a significant difference in the target value and measured value for both the Heparin and DiXaI kits at Calibrator 1 and Calibrator 2. There was also a significant difference in the target value and measured value at Calibrator 3 for the Heparin Kit, and Control 2 for the DiXaI Kit.

	Target Value	Kit	N	Mean	Std Deviation	Sig. (2-tailed)
Calibrator 1	0.000	DiXaI	26	0.0249	0.0153	<0.005
		Heparin	22	0.0250	0.0188	<0.005
Calibrator 2	0.290	DiXaI	30	0.2588	0.0276	<0.005
		Heparin	30	0.2680	0.0394	0.005
Calibrator 3	0.578	DiXaI	10	0.5713	0.0148	0.185
		Heparin	18	0.5456	0.0433	0.006
Control 1	0.178	DiXaI	29	0.1734	0.0266	0.596
		Heparin	29	0.1825	0.0394	0.548
Control 2	0.384	DiXaI	28	0.4211	0.0328	0.000
		Heparin	28	0.3701	0.0555	0.196

Table 1. Welch's T- Test Results for DiXaI and Heparin Kit at p,0.05.

An independent-t-test was also done between the two kits at each of the calibrator and control concentrations. For Calibrator 1, both the DiXaI and Heparin kits had similar mean values and both kits had at least five out of range values; however, the DiXaI kit had a slightly smaller standard deviation than the Heparin kit but was further away from the target value than the Heparin kit, as seen in Figure 1. Calibrator 2 had similar results with both kits not being significantly different from each other, as well as DiXaI having a smaller standard deviation and Heparin being closer to the target value as seen in Figure 2. Calibrator 2 was different from the other calibrators and controls in that there were no out of range values measured. Figure 3 displays the results of the independent t-test for Calibrator 3. Calibrator 3 not only had the most out of range values compared to any of the other concentrations but was the only concentration that had a significant difference between the Heparin and DiXaI kit values. For Calibrator 3, the Heparin kit was significantly different than the DiXaI kit, with the DiXaI kit having a smaller standard deviation and being closer to the target value than the Heparin kit. In Figure 4 is the t-

test results for Control 1. For Control 1, neither kits were significantly different from each other; however, once again DiXaI had a smaller standard deviation and was closer to the target value. Lastly, Control 2 results for the independent t-test is found in Figure 5. For Control 2, there was a significant difference between the two kits' results at p=0.05, and the Heparin had the smaller standard deviation and was closer to the target value than the DiXaI kit.

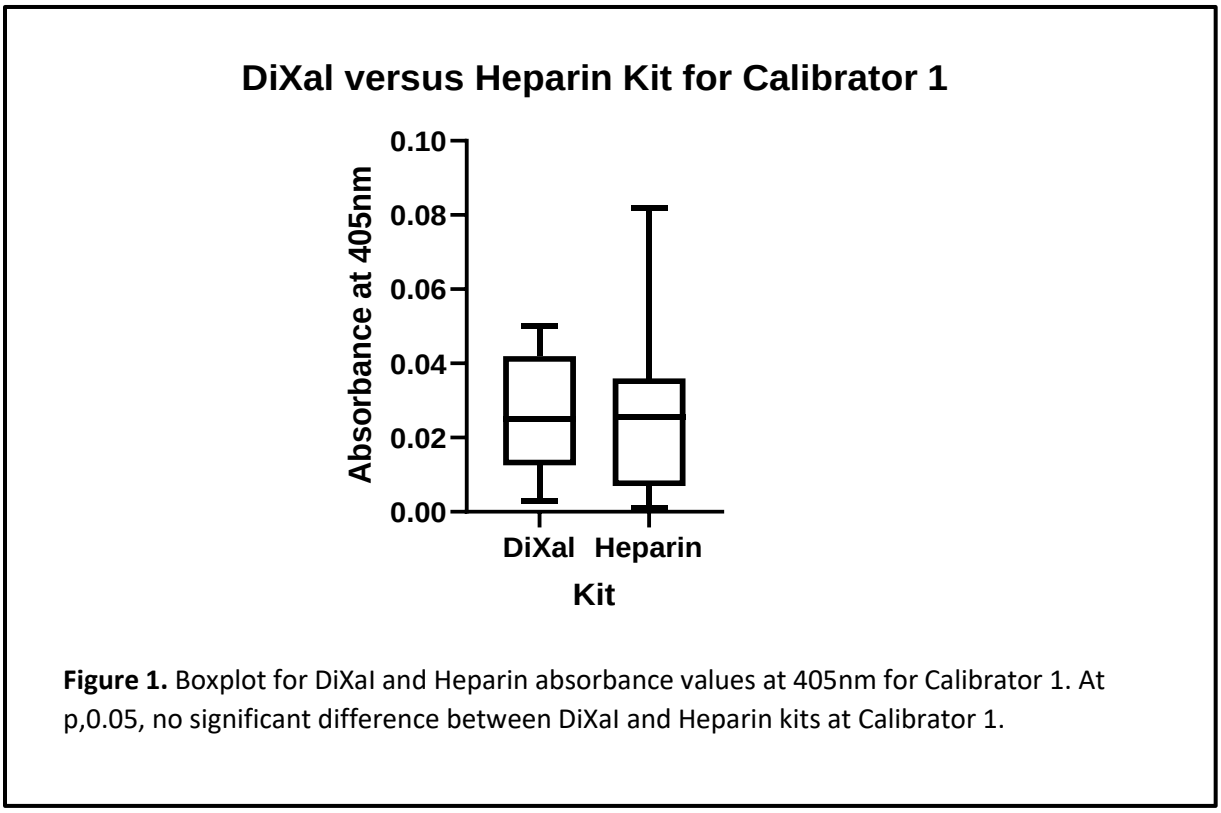

Figure 1. Boxplot for DiXaI and Heparin absorbance values at 405nm for Calibrator 1. At p,0.05, no significant difference between DiXaI and Heparin kits at Calibrator 1.

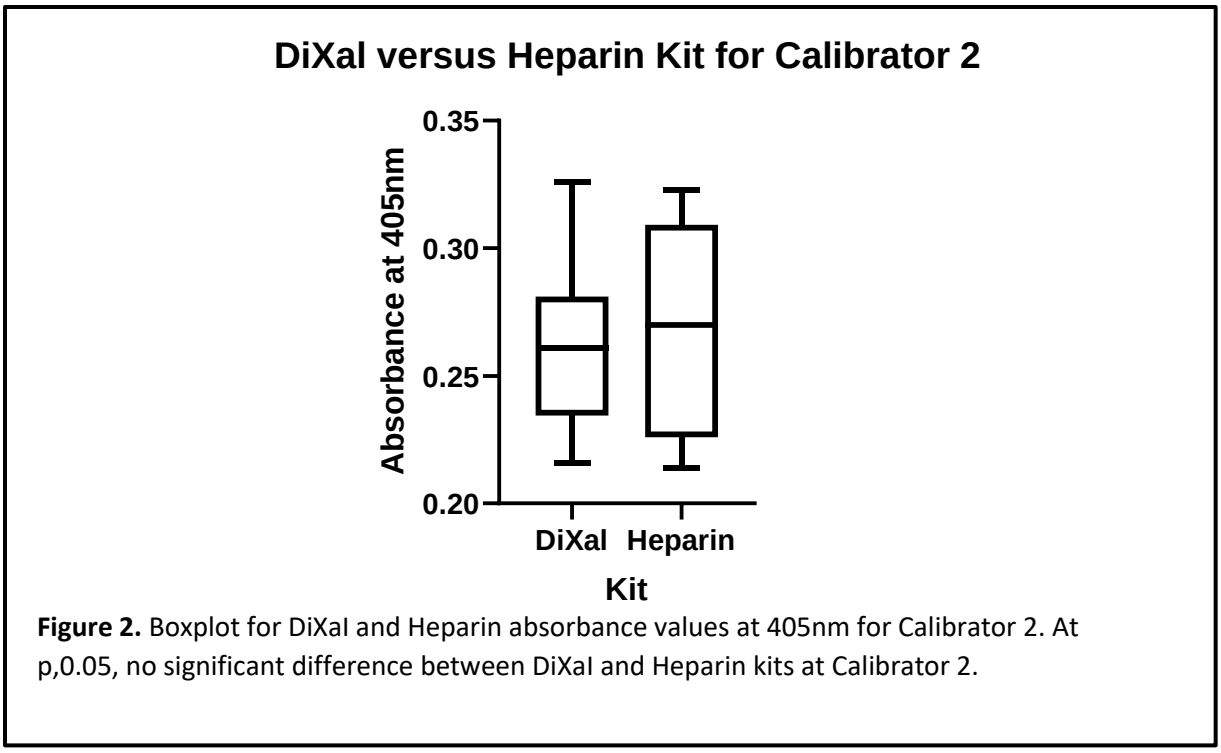

Figure 2. Boxplot for DiXaI and Heparin absorbance values at 405nm for Calibrator 2. At p,0.05, no significant difference between DiXaI and Heparin kits at Calibrator 2.

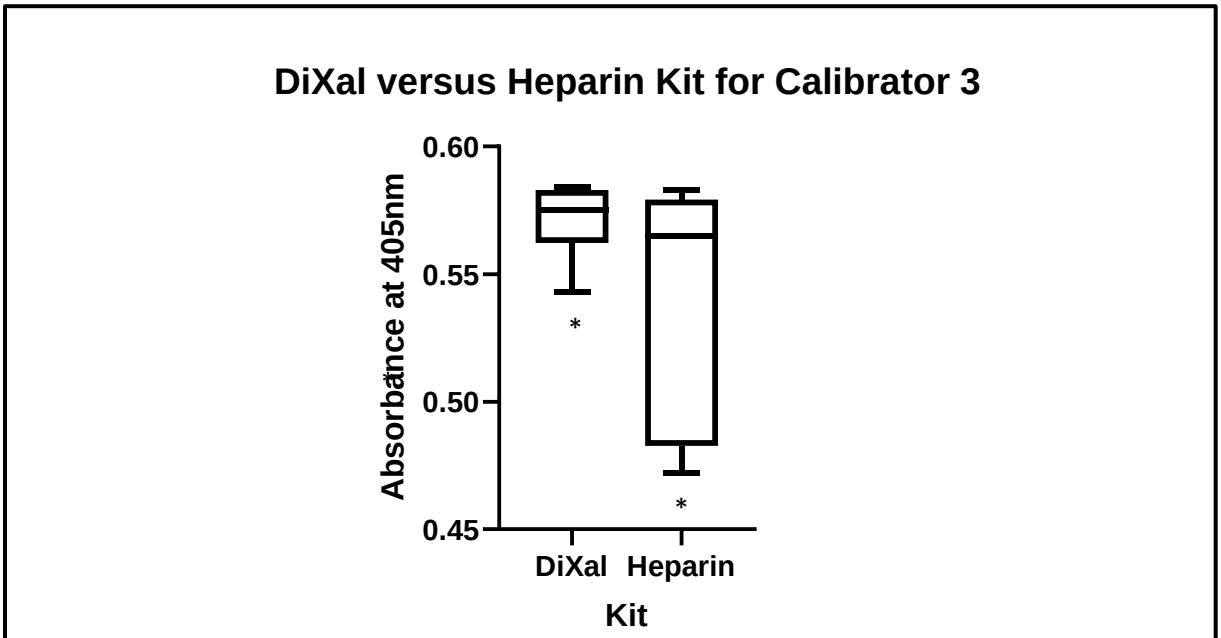

Figure 3. Boxplot for DiXaI and Heparin absorbance values at 405nm for Calibrator 3. At p,0.05, there is a significant difference between DiXaI and Heparin kits at Calibrator 3, indicated by (*).

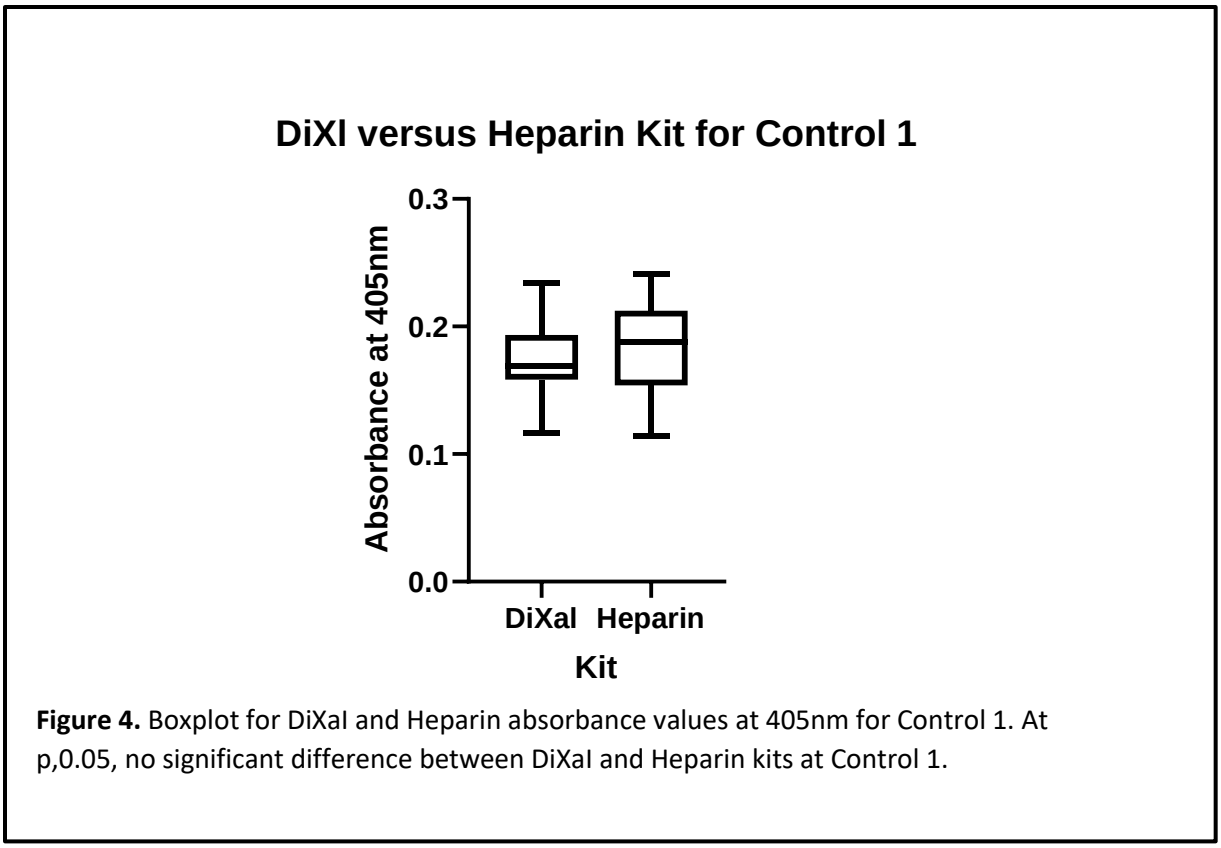

Figure 4. Boxplot for DiXaI and Heparin absorbance values at 405nm for Control 1. At p,0.05, no significant difference between DiXaI and Heparin kits at Control 1.

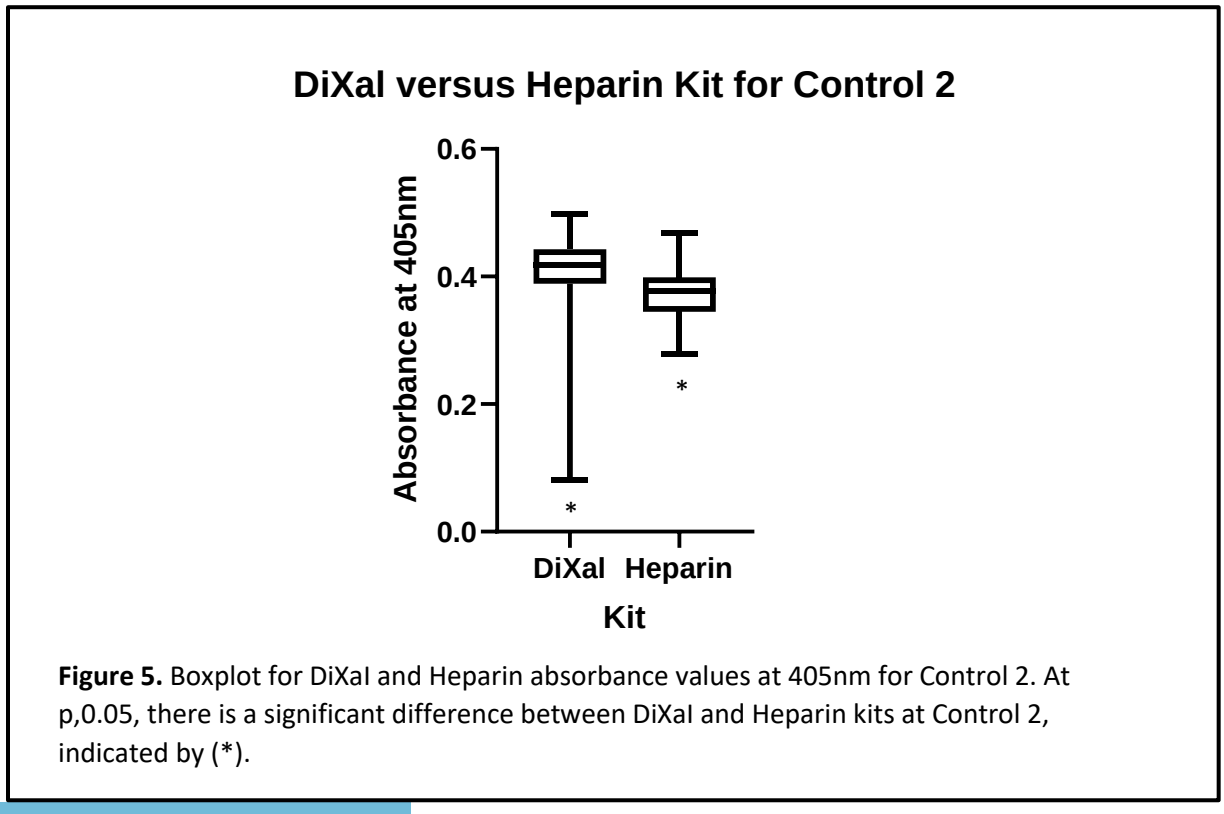

Figure 5. Boxplot for DiXaI and Heparin absorbance values at 405nm for Control 2. At p,0.05, there is a significant difference between DiXaI and Heparin kits at Control 2, indicated by (*).

Discussion

The DiXaI kit was more precise as shown by the smaller standard deviation at every concentration level when compared to the Heparin kit. For example, at Calibrator 1 DiXaI kit's standard deviation was 0.0153, while the Heparin kit's standard deviation was 0.0188. This trend continues for every concentration. However, the Heparin kit tended to be more accurate when comparing the measured concentration to the target concentration value. At Calibrator 2, Control 1, and Control 2, the Heparin kit's mean was closer to the target concentration value than the DiXaI kit. For Calibrator 1 and Calibrator 3 the DiXaI kit was more accurate for example Calibrator's target value was 0.578, and the DiXaI kit′s mean value was 0.5713 while the Heparin kit′s mean value was 0.5456. Still, both kits still had a significant difference between their mean values and the target values for every concentration except for Control 1. There is no clear relationship between which kit performs more accurately at different concentrations, nor is there a significant difference between either kit except for the higher concentrations which were Calibrator 3 and Control 2. This difference in Calibrator 3 could be explained by both kits having disproportionately high amounts of out of range values at this concentration. While measuring the samples for Calibrator 3 the spectrophotometer repeatedly read out of range, which could mean the measurement was too high seeing as this calibrator had the highest concentration of apixaban. This could be fixed with another dilution; however, this protocol is already lacking in accuracy and another dilution could add to it.

Conclusions

Overall, there was not a statistically significant difference between the two kits at almost every concentration; however, the DiXaI kit tended to be more precise while the Heparin kit tended to be more accurate. For the most part, both kits gave results significantly different from

the target values of each calibrator and control, and would not be the best option to quantify apixaban blood levels. These kits could be useful to detect whether or not apixaban is in the blood stream, if the concentration is not too high and does not give an out of range reading such as with Calibrator 3. This could lead to less accurate readings and could alter the applicability for the antidote Andexxa, if an overdose is suspected. These kits also have limited utility for therapeutic drug monitoring, where dose adjustment for individual patients is predicated on accurate drug measurements. Furthermore, the execution of these kits require finesse to achieve accurate results. Not only does it require a UV-Vis spectrophotometer to be precisely tuned, it also requires precise timing in allowing solutions to reach the proper temperatures, reagents to be mixed at exact times, and samples that are only good for two hours after being mixed. As of now, the gold standard to measure plasma drug concentrations is mass spectrometry which is limited and expensive, so other techniques are being looked at such as using Dilute Russell's viper venom time which is commonly used for detection of lupus anticoagulants but has been seen to be sensitive to determine the anticoagulant effects of apixaban (10).

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