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Using a Single Calibration Curve With the Anti-Xa Chromogenic Assay for Monitoring Heparin Anticoagulation

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- Each study subject had 6 evacuated blood collection tubes (2 sets of each: 3.8% sodium citrate, 3.2% sodium citrate, CTAD) collected by an atraumatic venipuncture. One tube from each set had a blood to anticoagulant ratio of 9:1. The other tube had an intentional "short-draw" of approximately 6:1 blood to anticoagulant ratio. All specimens had a chromogenic anti-Xa assay performed on each specimen regardless of heparin type measured against the appropriate calibration curve.
- A hybrid curve combining calibrators from the UFH and LMWH assays was run to compare with each specific UFH and LMWH result. None of the mean anti-Xa levels when comparing drug specific curves for the UFH and LMWH heparin subjects were statistically or clinically different (ANOVA P=0.9878 for UFH and LMWH P=0.9100). The hybrid curve compared to specific UFH curves had a P-value of 0.9956. The LMWH subject results ANOVA compared favorably with the specific LMWH curve at a P-value of 0.9512. The hybrid curve LMWH ANOVA results had a P-value of 0.9379.
- The short draw tube did not affect the anti-Xa assay regardless of the anticoagulant. The calibration curves for the UFH, LMWH, and test results compared favorably with the hybrid results.

The purpose of this study was to determine if a single calibration hybrid curve could be used to calibrate the chromogenic anti-Xa assay for monitoring patients on either unfractionated heparin (UFH) or low-molecular weight heparin (LMWH). The APTT is the assay most commonly used to monitor the effects of UFH therapy. Unfractionated heparin potentiates the activity of antithrombin and covalently neutralizes thrombin and factor Xa.[1] Low-molecular weight heparin such as enoxaparin selectively catalyzes the neutralization of factor Xa over thrombin and the drug effect cannot be effectively measured using the APTT assay.[1] The method of choice for monitoring LMWH and other heparin analogues is the anti-Xa chromogenic assay. This procedure can also be used to measure the amount of UFH present.[1,2] Previous publications have cited the interference of different anticoagulant concentrations in evacuated collection tubes, factor deficiencies, interfering substances, specimen collection problems dealing with time of processing and specimen handling, APTT reagent sensitivity, and instrumentation on the APTT results.[3-12] Other studies have shown situations where a therapeutic anti-Xa level of UFH was achieved but dosage changes may have been indicated due to a non-therapeutic APTT level result.[11,13] The antithrombin in these studies were sometimes from different sources. Some were from the assay and other studies used an assay where the patient's antithrombin was the source for the test. This protocol used different concentrations of sodium citrate anticoagulant in the evacuated collection tubes and amount of the blood to anticoagulant ratio to see if these variables affected the chromogenic anti-Xa assay results in samples from patients receiving either a UFH or LMWH dosing regimens.

Materials and Methods

This protocol was approved through the local institutional review board in accord with the tenets of the Helsinki protocol for human subjects experimentation. This study was also monitored and approved by the United States Air Force Surgeon General's Office. Twenty-six subjects (13 male and 13 female, age range of 26 to 91 years of age) receiving LMWH (enoxaparin) in varying concentrations were randomly selected and consented with their approval for participation in this study. Twenty individuals (10 male and 10 female, age range of 20 to 85 years of age) receiving UFH were also randomly selected and consented. The many pre-existing conditions in the protocol subjects necessitated them being prescribed anticoagulant therapy. These included but were not limited to coronary artery disease, deep vein thrombosis, antiphospholipid antibody syndrome, and recurrent spontaneous abortion. Some of the subjects were also receiving oral anticoagulant therapy.

Each study subject had 6 tubes of citrated blood obtained by venipuncture in a 1-time blood draw. Two vacutainer tubes were collected using 3.8% sodium citrate (0.129 M), 2 had 3.2% (0.105 M) sodium citrate, and 2 contained an anticoagulant called CTAD (0.109 M sodium citrate, 15 mM theophylline,

3.7 mM adenosine, and 0.198 mM dipyridamole). All of the tubes were non-wettable, siliconized glass. All of the tubes were purchased from BD Vacutainer Systems (Franklin Lakes, NJ). The CTAD tube is a specially designed evacuated blood collection tube to prevent platelet aggregation in vitro, which minimizes the release of platelet products such as the heparin inhibitor platelet factor 4 (PF4) that may occur between the time of specimen collection, processing, and sample testing.[14,15] One tube for each anticoagulant had a blood to anticoagulant ratio of 9:1 (ND=normal draw) and the second tube had an intentional "short-draw" of approximately 6:1 (SD=short draw) ratio. This ratio was picked at random because there has been no previously published data on the affects of a short draw of blood collection on the anti-Xa assay. Further studies will be necessary to compare other blood to anticoagulant ratios on this assay. All of the tubes were of the 4.5 mL collection size. All of the specimens were centrifuged for 15 minutes at 2,500 *g* to achieve platelet-poor plasma to ensure a residual platelet count of less than 10,000 platelets/µL. All of the specimens were processed within approximately 1 hour after collection. The supernatant plasma was then separated into cryovials and stored at approximately -70°C until ready for testing. The plasma was thawed at 37°C for 5 minutes before testing. None of the specimens had any evidence of clotting, lipemia, or hemolytic interfering substances.

All 6 specimens from each subject had a chromogenic anti-Xa assay performed regardless of the type of heparin they were receiving. The UFH and LMWH levels were measured using a chromogenic anti-Xa assay from Diagnostica-Stago. The STA-Rotachrom Heparin assay is used for measuring a chromogenic anti-Xa activity on the STA-R analyzer. In this assay, the antithrombin is supplied by the individual subject's sample. The detection threshold of the assay stated by the manufacturer is 0.10 IU/mL. Each chromogenic anti-Xa heparin assay was performed against calibration curves using the specific UFH or LMWH calibration curve and the hybrid calibration curve which uses a specific combination of calibrators.

The UFH curve used STA-Hepanorm H calibrators 0, 3, and 6 (UFH concentrations: 0.0, 0.33, 0.49 IU/mL) from Diagnostica-Stago, product #00684. Controls were STA-Heparin controls 2 and 5 from product #00683. The LMWH curve was prepared from STA-calibrator HBPM/LMWH from Diagnostica-Stago, product #00685. The calibrators were HBPM 0, 9, and 18 (LMWH concentrations: 0.0, 0.86, 1.85 IU/mL). The controls were from Diagnostica-Stago, STA-HBPM/LMWH 8 and 14 prepared from product #00686. The hybrid curve combined calibrators of the LMWH and UFH curves using STA calibrators HBPM 0, 9, 18 and STA-Hepanorm H calibrators 0, 3 and 6 (concentrations previously described). This set of calibrators was used in the protocol because of the success of a previous publications data and their ability to run a hybrid curve for measuring anti-FXa results.[16] An ANOVA statistical test and descriptive statistics were used to compare each set of results.

Results

Our institution wanted to find a method that could simultaneously measure both UFH and LMWH subjects with a single calibration curve. Currently, the 3.2% sodium citrate tube with a 9:1 blood to anticoagulant ratio of specimen is considered the standard draw tube and collection ratio for routine coagulation testing. This protocol wanted to compare collection conditions that have in the past affected the APTT assay for monitoring subjects on heparin. These conditions include different citrate conditions, evacuated blood collection tubes, and the blood to anticoagulant ratio.

The UFH anti-Xa mean range result comparing all 6 tubes was 0.32 to 0.37 IU/mL (total range assayed: 0.05 to 2.0 IU/mL). The coefficient of data spread for all subject's results for UFH was 5.7% versus 5.4% for the hybrid curve. The ANOVA for the UFH anti-Xa assay was excellent with a *P*-value of 0.9878 (**Table 1**). The normal draw CTAD tube did yield the highest amount of UFH present. However, it was not statistically or clinically significant. Our definition of clinical significance in this protocol is the following: Would this result make the attending physician alter his treatment of the patient anticoagulation regimen? The LMWH anti-Xa assay had a mean range of 0.37 to 0.46 IU/mL (total range assayed: 0.06 to 1.22 IU/mL) with the CTAD normal draw tube again having the highest recovery of heparin. The ANOVA results had a *P*-value of 0.9100 (**Table 2**). The hybrid curve had a mean range of anti-Xa results of 0.34 to 0.39 anti-Xa IU/mL (total range assayed: 0.11 to 1.24 IU/mL) in comparison to the UFH with an ANOVA *P*-value of 0.9956. The coefficient of data spread for all subject's LMWH results was 7.1% vs 5.7% for the hybrid assay. The ND CTAD tube also gave the highest amount of heparin present with a value of 0.49 IU/mL. The ANOVA results for the

Table 1_Anti-Xa Results On UFH Subjects Compared To The Hybrid Curve

ANOVA single factor, n=20 subjects	UFH	HYBRID
Groups	Average IU/mL	Average anti-Xa IU/mL
3.8 ND	0.36	0.37
3.8 SD	0.32	0.34
3.2 ND	0.37	0.37
3.2 SD	0.33	0.34
CTAD ND	0.37	0.39
CTAD SD	0.36	0.38
Coefficient of data spread in all subjects (%)	5.7	5.4

ND stands for the 9:1 blood to anticoagulant ratio specimen collection.
SD stands for the 6:1 blood to anticoagulant ratio specimen collection.
ANOVA FOR UFH: P-value = 0.9878
ANOVA for HYBRID: P-value = 0.9956
ANOVA for HYBRID vs UFH: P-value = 0.9961

Table 2_Anti-Xa Results On LMWH Subjects Compared To The Hybrid Curve

ANOVA single factor, n=26 subjects	LMWH	HYBRID
Groups	Average IU/mL	Average anti-Xa IU/mL
3.8 ND	0.42	0.46
3.8 SD	0.37	0.43
3.2 ND	0.43	0.47
3.2 SD	0.38	0.42
CTAD ND	0.46	0.49
CTAD SD	0.43	0.46
Coefficient of data spread in all subjects (%)	7.1	5.7

ND stands for the 9:1 blood to anticoagulant ratio specimen collection.
SD stands for the 6:1 blood to anticoagulant ratio specimen collection.

UFH versus the HYBRID curve had a *P*-value of 0.9961. For the LMWH, the HYBRID curve had a mean range of 0.42 to 0.49 anti-Xa IU/mL with an ANOVA *P*-value of 0.9379. The ANOVA data comparing the LMWH versus the HYBRID curve had a *P*-value of 0.9512.

Discussion

Previous investigators have described problems that can occur with various pre-analytical conditions on clottable assays such as the anticoagulant, specimen collection and processing, factor deficiencies, and inhibitors both specific and non-specific. It has been known for many years that the response of the APTT to heparin may vary greatly depending on the coagulation reagents responsiveness, reagent/instrument combinations, patient response to heparin, and to some degree the source of the heparin.[1-12] However, there have been no studies uncovered in our literature search that describe how the same pre-analytical variables may affect the anti-Xa chromogenic assay for monitoring both UFH and LMWH. In this protocol, our laboratory duplicated many of the pre-analytical variables used in some of the other studies that influenced the APTT.

The CTAD tube usually yielded the highest amount of heparin regardless of the blood to anticoagulant ratio. This is probably due to the CTAD tube specifically being designed to negate anti-heparin neutralizing factors for assays such as the platelet-factor 4 and beta-thromboglobulin assays. However, this finding did not appear to be clinically or statistically significant in these data sets. The short draw tube consistently had slightly lower levels on anti-Xa results. This is probably due to the dilution effects of the 6:1 blood to anticoagulant ratio in the specimen collections.

The chromogenic anti-Xa heparin results were not statistically or clinically affected by any of the collection tubes or blood to anticoagulant ratio in any of the data sets on either the UFH or LMWH specimens. The CTAD tube usually yielded the highest amount of heparin regardless of the blood to anticoagulant ratio. However, this finding did not appear to be clinically or statistically significant in these data sets. The hybrid curve did seem to always have a slightly higher bias in anti-Xa results; however, it was not statistically or clinically significantly different. This may be due to the slight difference in the curve optical density readings. The possibility of using a single calibration curve for monitoring subjects on UFH and a variety of low molecular heparinoids such as enoxaparin, ardeparin, and dalteparin had previously been attempted with some success in a limited study.[16] There is also information in the literature of the validity of a single calibrator for LMWH for the anti-Xa assay for assaying most commonly used LMWH preparations.[17] Anecdotal evidence has been communicated between several large research and coagulation centers stating that they are using a form of this hybrid curve with some success. At our institution, the hybrid curve compared to those determined on either the UFH and LMWH were excellent with little or no clinical or statistical differences. The STA-Rotachrom Heparin colorimetric assay for measuring chromogenic anti-Xa activity on the STA-R analyzer was extremely stable. The calibration curves and controls for the UFH, LMWH, and hybrid assays were stable for at least 6 months in this research protocol. In other words the laboratory did not have to recalibrate any of the assays from the initial set-up. No controls were out of range for the entire duration of the study. From the data analyzed at our institution, the coagulation laboratory now has the ability to use a single calibration curve to monitor subjects on standard UFH and enoxaparin LMWH. This saves the laboratory time and expense of maintaining separate calibration curves and controls. It is also apparent that this chromogenic anti-Xa assay for monitoring heparin is not affected clinically or statistically by the citrated collection tube, or the blood to anticoagulant ratio when the specimen is obtained with a normal draw of 9:1 or a short draw of a 6:1 ratio. LM

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