Effects of pre-analytical variables on the anti-activated factor X chromogenic assay when monitoring unfractionated heparin and low molecular weight heparin anticoagulation

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The purpose of this study was to determine whether the anti-activated factor X (anti-FXa) assay is less affected by pre-analytical variables in monitoring patients on unfractionated heparin (UFH) and low molecular weight heparin (LMWH) than the activated partial thromboplastin time (aPTT). Forty-six subjects receiving either enoxaparin (LMWH) or UFH were randomly selected. Each study subject had six vacutainer tubes (3.8% sodium citrate, 3.2% sodium citrate) drawn 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 an aPTT and a chromogenic anti-FXa assay performed on each specimen regardless of heparin type. The aPTT assay mean with the 3.8% sodium citrate tube short-draw tube was statistically different from the other aPTT assays ($P = 0.06$). However, all six of the mean anti-FXa assays for the UFH and LMWH heparin subjects were not statistically or clinically different (analysis of variance, $P = 0.9878$ for UFH and $P = 0.9060$ for LMWH). The intentional short-draw tube did not affect the anti-FXa assay regardless of the anticoagulant. The anti-FXa assay appears to be a better method for monitoring heparin subjects than the aPTT due to the lack of effect of pre-analytical variables. ***Blood Coagul Fibrinolysis*** **16:173–176**
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Blood Coagulation and Fibrinolysis 2005, **16**:173–176

Keywords: unfractionated heparin, enoxaparin, low molecular weight heparin, anti-activated factor X, activated partial thromboplastin time, anticoagulant, CTAD, sodium citrate

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Received 6 October 2004 **Accepted** 14 January 2005

Introduction

The purpose of this research protocol was to determine whether the anti-activated factor X (anti-FXa) assay is less affected by pre-analytical variables in monitoring patients on unfractionated heparin (UFH) and low molecular weight heparin (LMWH) than the activated partial thromboplastin time (aPTT). The aPTT is the assay most commonly used to monitor the effects of UFH therapy. UFH potentiates the activity of antithrombin and covalently neutralizes thrombin and activated factor X (FXa) [1]. LMWH such as enoxaparin selectively catalyzes the neutralization of FXa over thrombin and usually cannot be measured using the aPTT assay [1]. The method of choice for monitoring LMWH and other heparin analogues is the anti-FXa chromogenic assay. This procedure can also be used to measure the amount of UFH present [1,2]. Previous publications have cited the interference of anticoagulants, factor deficiencies, interfering substances, specimen collection, aPTT reagent sensitivity and instrumentation in affecting the aPTT [3–12]. Other studies have shown situations where a therapeutic anti-FXa level of UFH was achieved but dosage changes were necessary due to a non-therapeutic aPTT level [11,13]. Our study used different concentrations of anticoagulant, collection tubes and amount of the blood to anticoagulant ratio to see whether these variables affected the aPTT and the anti-FXa assay on the UFH and 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 subject 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 26–91 years) 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 20–85 years of age) receiving UFH were also randomly selected and consented. The existing conditions in the protocol subjects that necessitated them being prescribed anticoagulant therapy were many. These included

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coronary artery disease, deep vein thrombosis, antiphospholipid antibody syndrome and recurrent spontaneous abortion, to name a few. Some of the subjects were also receiving oral anticoagulant therapy.

Each study subject had six tubes of citrated blood obtained by venipuncture in a one-time blood draw. Two vacutainer tubes were collected using 3.8% sodium citrate (0.129 mol/l), two used 3.2% (0.105 mol/l) sodium citrate, and two contained an anticoagulant called CTAD (0.109 mol/l sodium citrate, 15 mmol/l theophylline, 3.7 mmol/l adenosine and 0.198 mmol/l dipyridamole). All of the tubes were non-wettable, siliconized glass. All of the tubes were purchased from BD Vacutainer Systems (Franklin Lakes, New Jersey, USA). The CTAD tube is a specially designed vacutainer collection tube to prevent platelet aggregation *in vitro*, which minimizes the release of platelet products such as the heparin inhibitor platelet factor 4 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 (normal draw) and the second tube had an intentional 'short-draw' of approximately 6 : 1 (short draw) ratio. All of the tubes were of the 4.5 ml collection size. All of the specimens were centrifuged for 15 min at $2500 \times g$ to achieve platelet-poor plasma to ensure a residual platelet count of less than 10 000 platelets/μl. The supernatant plasma was then separated into cryovials and stored at approximately −70°C until ready for testing. The plasma was thawed at approximately 37°C for 5 min before testing. No clotted specimens, lipemia, or evidence of hemolysis in any of the specimens were evident.

All six specimens on each subject had an aPTT and an anti-FXa assay performed regardless of the type of heparin they were receiving. The aPTT was performed using PTT-A from Diagnostica-Stago, Inc. (Parsippany, New Jersey, USA) on an STA-R automated coagulation analyzer. The UFH and LMWH levels were measured using an anti-FXa assay from Diagnostica-Stago, Inc., the STA-Rotachrom Heparin colorimetric assay for measuring anti-FXa activity, on the STA-R analyzer. Each anti-FXa heparin assay was performed using either a standard UFH or LMWH calibration curve using a specific combination of calibrators. An analysis of variance (ANOVA) statistical test, a *t* test and descriptive statistics were used to compare each set of results.

Results

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. The 3.8% sodium citrate short draw had the highest mean of all of the aPTT tubes on the UFH subjects and was statistically significantly different (127.2 versus 105.4 s; Student's *t* test, $P = 0.06$). All of the other combinations of collection tubes and anticoagulant and ratio comparisons were not statistically different. The ANOVA assay comparison between the two specimen tubes for each anticoagulant gave an excellent comparison statistically ($P = 0.9481$). The aPTT is not normally used for monitoring the LMWH anticoagulation. However, depending on the molecular weight of the LMWH, and the choice of aPTT reagent/instrument combinations, several LMWHs may be capable of prolonging the aPTT assay. Our protocol was to compare all of the same conditions that were carried out with the UFH subjects. The LMWH aPTT assays had a mean range of 37.1–41.2 s. The highest mean observed was with the CTAD normal collection tube. The ANOVA result *P* value was only 0.4665, which does not give a good comparison. Statistically there is a great difference in the results. Clinically there was very little difference in the data. This is especially true since this assay is not usually relevant to the LMWH levels. See Table 1 for results of all aPTT testing.

The UFH anti-FXa mean range result comparing all six tubes was 0.32–0.37 IU/ml. The ANOVA for the UFH anti-FXa assay was excellent with a *P* value of 0.9878. The normal draw CTAD tube did yield the highest amount of UFH present. However, it was not statistically or apparently clinically significant. The LMWH anti-FXa

Table 1 Activated partial thromboplastin time (aPTT) results on all subjects

	ANOVA: single factor	
Group	UFH average (s)	LMWH average (s)
aPTT 3.8 ND	105.4	37.9
aPTT 3.8 SD	127.2	41.2
aPTT 3.2 ND	107.7	37.1
aPTT 3.2 SD	105.5	39.6
aPTT CTAD ND	104.3	37.7
aPTT CTAD SD	100.1	41.2

Analysis of variance (ANOVA) for unfractionated heparin (UFH), $P = 0.9481$; ANOVA for low molecular weight heparin (LMWH), $P = 0.4665$. ND, normal draw (9 : 1 blood to anticoagulant ratio specimen collection); SD, short draw (6 : 1 blood to anticoagulant ration specimen collection); CTAD, 0.109 mol/l sodium citrate, 15 mmol/l theophylline, 3.7 mmol/l adenosine and 0.198 mmol/l dipyridamole.

Table 2 Anti-activated factor X results on all heparin subjects

	ANOVA: single factor	
Group	UFH average (IU/ml)	LMWH average (IU/ml)
UFH 3.8 ND	0.36	0.42
UFH 3.8 SD	0.32	0.37
UFH 3.2 ND	0.37	0.43
UFH 3.2 SD	0.33	0.38
UFH CTAD ND	0.37	0.46
UFH CTAD SD	0.36	0.43

Analysis of variance (ANOVA) for unfractionated heparin (UFH), $P = 0.9878$; ANOVA for low molecular weight heparin (LMWH), $P = 0.9100$. ND, normal draw (9 : 1 blood to anticoagulant ratio specimen collection); SD, short draw (6 : 1 blood to anticoagulant ration specimen collection); CTAD, 0.109 mol/l sodium citrate, 15 mmol/l theophylline, 3.7 mmol/l adenosine and 0.198 mmol/l dipyridamole.

Table 3 Examples of comparisons of unfractionated heparin (UFH) activated partial thromboplastin time (aPTT) results with anti-activated factor X (anti-FXa) heparin results

Subject	3.8 ND aPTT	3.8 SD aPTT	3.2 ND aPTT	3.2 SD aPTT	CTAD ND aPTT	CTAD SD aPTT	aPTT mean (s)	Anti-FXa mean (IU/ml)
6284	36.9	48.9	38.4	41.4	43.3	47.8	42.8	0.41
0297	114.5	109.9	116.2	107.1	112.8	110.9	111.9	0.35
5793	69.1	64.9	66	66	69.6	67.5	67.2	0.29
1130	68.3	90.8	63.1	74.3	64.4	78.2	73.2	0.45
0807	111.2	133.6	118.6	117	110.6	128.6	119.9	0.45
0447	36.4	63.3	35.8	43.6	34.3	51.1	44.1	0.46
0316	70.7	75.6	78.5	69.5	73.8	66.1	72.4	0.57
6296	114.7	234.2	91.6	114.8	124.3	139.7	136.6	0.05

ND, normal draw (9 : 1 blood to anticoagulant ratio specimen collection); SD, short draw (6 : 1 blood to anticoagulant ration specimen collection); CTAD, 0.109 mol/l sodium citrate, 15 mmol/l theophylline, 3.7 mmol/l adenosine and 0.198 mmol/l dipyridamole.

assay had a mean range of 0.37–0.46 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. See Table 2 for results of all anti-FXa testing.

The aPTT results were skewed for the UFH subjects in comparison with the anti-FXa UFH results in many subjects. For example one subject had a mean of aPTT results of 42.8 s and a heparin level of 0.41 IU/ml. A second subject had an aPTT of 73.2 s with a heparin level of 0.45 IU/ml. Another subject had aPTT results of 111.9 s with a heparin level of 0.35 IU/ml. These few examples corroborate other studies that discussed the disparity in the aPTT and the heparin levels due to other variables besides the effects of the heparin.

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 few studies if any that describe how the same pre-analytical variables may affect the anti-FXa chromogenic assay for monitoring both UFH and LMWH. In this protocol the pre-analytical variables we looked at mirrored some of the other studies results. The 3.8% citrate collection tube aPTT result was affected the most by the short-draw and anticoagulant effect. There were no statistically significant differences between the other collection tubes or anticoagulant ratio on the aPTT results. However, the results may be misleading as several subjects on UFH had aPTT times that were longer than 300 s. These data probably skewed the mean results of the assay. As mentioned in Results, there were several data points that did not correlate with the actual amount of heparin present. 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 datasets.

The anti-FXa heparin results were not statistically or clinically affected by any of the collection tubes or blood to anticoagulant ratio in any of the datasets on either the UFH or LMWH specimens. In theory there should be corresponding results in the therapeutic range of 0.3–0.7 IU/ml heparin units with the anti-FXa assay that the hemostasis laboratory sets up using the *ex vivo* Brill–Edwards method for establishing the heparin therapeutic range of the aPTT [16]. The individual aPTT results and the anti-FXa assay showed a high degree of discordance as seen in previous studies. See selected results in Table 3.

The *in vitro* addition of heparin and aPTT prolongation has shown a high degree of correlation when attempts have been made to establish a therapeutic range of test results. However, when assaying the heparin using *in vivo* samples against the aPTT, the correlations are very poor. In some of the studies less than 50% of the aPTT results correlated with the anti-FXa results for heparinized specimens or explained the differences in the heparin concentrations [11]. In one study the correlation was better for aPTT/anti-FXa testing than for aPTT/protamine sulfate titration comparisons [17].

These findings could lead to inappropriate heparin management and life-threatening complications including thromboses or hemorrhage. It appears the time is right to ‘retire’ the aPTT for heparin monitoring and replace it with an assay that is not affected by any of the variables that may compromise the aPTT result.

Acknowledgements

This research was supported in part by Diagnostica-Stago, Inc. with reagents for performing the anti-activated factor X chromogenic assay. Further funding came from the Surgeon General Office of the United States Air Force.

References

1. Fristsma GA. Evaluation of hemostasis. In: Rodak BF, (editor): *Hematology: clinical principles and applications*, 2nd edition. Philadelphia, PA: W.B. Saunders Co; 2002, p. 738.
2. Adler BK. Unfractionated heparin and other antithrombin mediated anticoagulants. *Clin Lab Sci* 2004; **17**:113–117.
3. Adcock DM, Krestin DC, Marlar RA. Effect of 3.2% vs 3.8% sodium citrate concentration on routine coagulation testing. *Am J Clin Pathol* 1997; **107**:105–110.
4. Adcock DM, Kessin DC, Marlar RA. Minimum volume requirements for routine coagulation testing: dependence on citrate concentration. *Am J Clin Pathol* 1998; **109**:595–599.
5. Storbeck M, Johnson M, Hayward CPM. The effect of low molecular weight heparin on LA testing [Abstract]. *Thromb Haemost* 1995; **73**:1271.
6. Castellone D. Coagulation do's and don'ts. *Advance* 2004; **13**:50–53.
7. Jensen R, Fritsma GA. Preanalytical variables in the coagulation laboratory. *Advance* 2000; **9**:90–94.
8. Castellone D. How to deliver quality results in the coagulation laboratory: commonly asked questions. *Lab Med* 2004; **4**:208–213.
9. Triplett D. Laboratory monitoring of heparin therapy. *Hemoliance Times* 1997; **2**:3.
10. Werner M, Gallagher JV, Ballo MS, Karcher DS. Effect of analytic uncertainty of conventional and point-of-care assays of activated partial thromboplastin time on clinical decisions on heparin therapy. *Am J Clin Pathol* 1994; **102**:237–241.
11. Olson JD, Arkin CF, Brandt JT, Cunningham MT, Giles A, Koepke JA, Witte DL. College of American Pathologists Conference XXXI on Laboratory Monitoring of Anticoagulant Therapy: laboratory monitoring of unfractionated heparin therapy. *Arch Pathol Lab Med* 1998; **122**:782–798.
12. NCCLS Document H21-A3. *Collection, transport and processing of blood specimens for coagulation testing and performance of coagulation assays*, 3rd edition. Villanova, PA: National Committee for Clinical Laboratory Standards; 1998.
13. Evans EW, Turner K. Anti-factor Xa testing for monitoring heparin therapy. *Clin Hemost Rev* 2000; **14**:8–9.
14. Canton MM. Introduction to thrombosis and anticoagulant therapy. In: Harmening DM, (editor): *Clinical hematology and fundamentals of hemostasis*, 3rd edition. Philadelphia, PA: F.A. Davis Co.; 1997, p. 581.
15. Becton-Dickinson. Evaluation of BD Vacutainer™ 2.7 ml glass CTAD (sodium citrate, theophylline, adenosine and dipyridamole) coagulation tube with normal donors, donors on oral anticoagulant therapy and I.V. anticoagulant. *Clinical White Paper VS5816*. Franklin Lakes, NJ: BD Vacutainer Systems; 2001, pp. 1–6.
16. Brill-Edwards P, Ginsberg JS, Johnston M, Hirsh J. Establishing a therapeutic range for heparin therapy. *Ann Intern Med* 1993; **119**:104–109.
17. Kitchen S, Preston FB. The therapeutic range for heparin therapy: relationship between six activated partial thromboplastin time reagents and two heparin assays. *Thromb Haemost* 1996; **75**:734–739.