Monitoring direct thrombin inhibitors with a plasma diluted thrombin time

Jason E. Love, Chris Ferrell, Wayne L. Chandler
Department of Laboratory Medicine, University of Washington, Seattle, Washington, USA

Summary
Activated partial thromboplastin time (aPTT) monitoring of direct thrombin inhibitors (DTIs) is vulnerable to interference from many sources. If the baseline aPTT is prolonged, as occurs with lupus inhibitors, alternative methods are required to monitor DTI levels. We compared the plasma diluted thrombin time (1:4 dilution of patient plasma with normal plasma) and the aPTT in patient samples spiked with argatroban, bivalirudin, or lepirudin at three concentration levels. Each drug was spiked into five samples with lupus inhibitors, five samples with deficient vitamin K-dependent factors, three samples with elevated D-dimers, and eight samples with normal baseline aPTT values. The aPTT overestimated the spiked DTI concentration in all samples with lupus inhibitors, low levels of vitamin K-dependent factors, and elevated D-dimers. In samples with normal baseline aPTTs, the aPTT failed to correctly estimate the spiked drug concentration in four of 24 samples spiked with argatroban, seven of 24 spiked with lepirudin, and three of 24 spiked with bivalirudin. The plasma diluted thrombin time was not affected by lupus inhibitors, low vitamin K-dependent factor levels or elevated D-dimer levels and correctly estimated the spiked drug level in 63 of 63 samples spiked with argatroban, 63 of 63 samples spiked with bivalirudin, and 62 of 63 samples spiked with lepirudin. In conclusion, the plasma diluted thrombin time appears to be a viable alternative to the aPTT for monitoring DTI levels, especially in patients with lupus inhibitors or low levels of vitamin K-dependent factors.

Keywords
Direct thrombin inhibitors, therapeutic drug monitoring, lupus inhibitors

Introduction
Direct thrombin inhibitors (DTIs) have emerged as effective alternative anticoagulants in the acute management of heparin-induced thrombocytopenia (HIT) (1). Patients with HIT are at risk for severe thrombosis and require anticoagulation. Heparin must be discontinued, because additional heparin would fuel the underlying autoimmune reaction (1). Warfarin is not an immediate option, because it may precipitate thrombosis in acute HIT (2).

For most DTIs, therapeutic drug monitoring is required to ensure adequate dosing and to prevent overdosing (1). In non-cardiopulmonary bypass settings, DTI therapy is typically monitored with the activated partial thromboplastin time (aPTT) (3). aPTT monitoring of DTIs is problematic for many reasons. Results vary depending on aPTT reagent selection and lot number (4). The aPTT can be prolonged or shortened due to deficiency or excess of coagulation factors (5). In addition, the aPTT contains phospholipids that make the assay vulnerable to interference from lupus inhibitors (3). Overall, the aPTT may be prolonged or shortened due to many factors that are independent of DTI level (6). The aPTT cannot accurately monitor DTI therapy if the assay is prolonged at baseline (7). In addition, the aPTT loses sensitivity to DTIs at high drug concentration, and may not be able to discriminate between toxic and therapeutic levels of some DTIs (5, 8). For these reasons, alternative methods to monitor DTI levels are of value.

We investigated a plasma diluted thrombin time as an alternative to the aPTT for monitoring DTI levels. The unmodified thrombin time is overly sensitive to DTIs, and not suitable for therapeutic drug monitoring (8). We theorized that one volume of patient plasma diluted into three volumes of normal plasma could blunt the sensitivity of the thrombin time and make the test suitable for therapeutic drug monitoring. We found the plasma diluted thrombin time to be an accurate indicator of argatroban, lepirudin, and bivalirudin concentration, with no interference due to lupus inhibitors, deficiency in vitamin K-dependent factor levels, or elevated levels of D-dimers.

Correspondence to:
Wayne L. Chandler, MD
Department of Laboratory Medicine
Box 357110, University of Washington
Seattle, WA 98195, USA
Tel.: +1 206 598 6131, Fax: +1 206 598 6189
E-mail: wlc@u.washington.edu

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Material and methods

Human subjects
Studies on human subjects were carried out according to the principles of the Declaration of Helsinki. The study was approved by the University of Washington Human Subjects Review Committee.

Blood sampling
Venous blood samples were anticoagulated by adding 4.5 ml of blood to 0.5 ml of 0.105 M citrate. All samples were centrifuged immediately at 3,600g for 2 minutes at room temperature, divided into aliquots and frozen at −80°C until analyzed.

Coagulation analysis
All measurements were made on Diagnostica Stago analyzers (STA-R Evolution and STA-Compact). Measurements were made using Diagnostica Stago STAP TT Automate for the aPTT, STA Thrombin for the thrombin time, STA Fibrinogen for the kinetic fibrinogen assays, STA Liatest D-DI for the D-dimer assay, and Neoplastine C1 Plus reagents for the prothrombin time. Diagnostica Stago STA PTT LS aPTT reagents were also used as a second aPTT screen for the purpose of lupus inhibitor testing, and only for this purpose. The ISI and geometric mean normal of the prothrombin time reagent were 1.27 and 13.2 seconds (sec). Pooled normal plasma was purchased from Precision Biologic. Positive lupus inhibitor samples had 1) a prolonged aPTT using either the STA PTT Automate or STA PTT LS reagents, 2) incomplete correction after mixing with pooled normal plasma, 3) complete correction after addition of hexagonal phase phospholipids, and 4) no evidence of other coagulation factor inhibitors (normal aPTT after phospholipid addition and incubation at 37°C, normal prothrombin time, and normal thrombin time). For the plasma diluted thrombin time one volume of citrated plasma was added to three volumes of pooled normal plasma. One hundred µl of this diluted plasma was added to 100 µl of STA thrombin reagent that contains 1.5 NIH units of human thrombin/ml. The final thrombin concentration in the assay was 0.75 NIH units of human thrombin/ml. The manufacturer reports the on-instrument stability of STA thrombin after reconstitution to be seven days. Controls run before and after each run were in control and similar in value.

Figure 1: APTT and plasma diluted thrombin time standard curves for argatroban, bivalirudin, and lepirudin.
Standard curves
Argatroban, bivalirudin, and lepirudin were prepared according to the manufacturers instructions. The drug solutions were serially diluted into normal saline to achieve stock solutions prior to 1:20 dilution into plasma samples. For argatroban the final concentrations ranged from 0.1 – 6 µg/ml, for bivalirudin the final concentrations ranged from 0.1 – 5 µg/ml, and for lepirudin the final concentrations ranged from 0.1 – 5 µg/ml. Standard curves were generated by measuring the aPTT and plasma diluted thrombin time in each spiked sample. Controls run before and after each standard curve showed similar values.

Estimating therapeutic ranges
We extrapolated aPTT ratio ranges from our standard curves to achieve estimated therapeutic ranges for argatroban, bivalirudin, and lepirudin. For argatroban, 1.5–3.0 x the baseline aPTT not to exceed 100 sec is considered therapeutic for anticoagulation in HIT (4). Extrapolating this range to our standard curve gave argatroban concentrations of 0.2 – 2.2 µg/ml. Published therapeutic argatroban concentrations vary, with reported ranges of 0.5 – 1.5 µg/ml, 1.0 – 2.0 µg/ml, and 0.4 – 1.1 µg/ml (5, 12, 13). We elected to truncate our extrapolated 0.2 – 2.2 µg/ml range down to 0.6–1.8 µg/ml for use in this study, the approximate mean of prior published ranges.

For lepirudin 1.5–2.5 x the baseline aPTT has been considered therapeutic for anticoagulation in HIT (9). Extrapolating this range to our standard curve gave lepirudin concentrations of 0.2 – 1.5 µg/ml. Published therapeutic lepirudin concentrations vary, with reported ranges of 0.1 – 2.0 µg/ml, 0.75 – 1.5 µg/ml, and 0.5 – 1.5 µg/ml (8, 12, 15). We elected to truncate our extrapolated 0.2 – 1.5 µg/ml range to 0.5–1.5 µg/ml for use in this study, approximately the mean of prior ranges.

For bivalirudin 1.5–2.5 x the baseline aPTT has been considered therapeutic in studies of anticoagulation for HIT (9). Extrapolating this range to our standard curve gave bivalirudin concentrations of 0.25–1.5 µg/ml. Well defined therapeutic concentration ranges have not been defined for the use of bivalirudin in HIT therapy. Our range of 0.25 – 1.5 µg/ml is roughly in accord with what other authors have found when comparing aPTT ratios to bivalirudin concentration (14).

Figure 2: APTT and plasma diluted thrombin time log scale standard curves for argatroban, bivalirudin, and lepirudin.
Figure 3: APTT and plasma diluted thrombin time results for patient specimens spiked with argatroban at 0.4, 1.0, and 3.0 µg/ml. Solid lines represent the standard curves for argatroban in pooled normal plasma for the aPTT and plasma diluted thrombin time. The grey boxes represent the target therapeutic range.
Testing patient plasma
We collected a total of 63 patient samples: 15 with lupus inhibitors that prolonged the aPTT, 15 with low levels of vitamin K-dependent factors (INR > 1.5), nine with D-dimer levels greater than 5 µg/ml, and 24 with varying fibrinogen levels and normal baseline aPTTs. We spiked 1/3 of the samples with argatroban, 1/3 with bivalirudin, and 1/3 with lepirudin. We set our spiked DTI concentration levels to subtherapeutic, therapeutic, and supratherapeutic levels based on the ranges stated above. For argatroban we spiked the samples to 0.4, 1.0, and 3.0 µg/ml. For bivalirudin we spiked the samples to 0.1, 1.0, and 3.0 µg/ml. For lepirudin we spiked the samples to 0.1, 1.0, and 2.0 µg/ml. aPTT and diluted thrombin time measurements were made on all samples.

Results
Increasing concentrations of argatroban, bivalirudin, and lepirudin resulted in a dose-dependent increase in both the aPTT and the plasma diluted thrombin time (Fig. 1). Log scale plots demonstrate the linearity of the standard curves (Fig. 2). The plasma diluted thrombin time was more sensitive to lepirudin than it was to bivalirudin or argatroban. At lepirudin concentrations above 2.0 µg/ml, the plasma diluted thrombin time returned values of greater than 700 sec which was set as the maximum for our assay. The aPTT showed similar sensitivities to all three drugs, but the assay had a tendency to plateau at high drug concentrations.

Results for argatroban
The aPTT failed to accurately estimate the spiked argatroban level in 29 of 63 samples (Fig. 3). Failures mainly occurred in samples with lupus inhibitors, low levels of vitamin K-dependent factors, or elevated D-dimer levels, all of which had prolonged baseline aPTTs. The aPTT overestimated the spiked argatroban concentration in these samples. Failures also occurred in four of 24 samples with a normal baseline aPTT, underestimating the spiked argatroban level in all cases. In contrast, the plasma diluted thrombin time accurately estimated subtherapeutic, therapeutic, and supratherapeutic levels in 63 of 63 samples (Fig. 3).

In samples spiked with argatroban at 0.4 µg/ml the plasma diluted thrombin time predicted a mean argatroban concentration of 0.42 µg/ml with a CV of 17%. For samples spiked at 1.0 µg/ml the plasma diluted thrombin time predicted a mean argatroban concentration of 1.1 µg/ml with a CV of 15%. For samples spiked at 3.0 µg/ml the plasma diluted thrombin time predicted a mean argatroban concentration of 3.0 µg/ml with a CV of 15%.

Results for bivalirudin
The aPTT failed to accurately estimate the spiked bivalirudin level in 28 of 63 samples (Fig. 4). Failures mainly occurred in samples with lupus inhibitors, low levels of vitamin K-dependent factors, or elevated D-dimer levels, all of which had prolonged baseline aPTTs. The aPTT overestimated the spiked bivalirudin concentration in these samples. Failures also occurred in three of 24 samples with a normal baseline aPTT, overestimating the spiked bivalirudin concentration in all cases. In contrast, the plasma diluted thrombin time returned accurate estimations of subtherapeutic, therapeutic, and supratherapeutic concentration in 63 of 63 samples (Fig. 4).

In samples spiked with bivalirudin at 0.1 µg/ml the plasma diluted thrombin time predicted a mean bivalirudin concentration of 0.09 µg/ml with a CV of 15%. For samples spiked at 1.0 µg/ml the plasma diluted thrombin time predicted a mean concentration of 1.0 µg/ml with a CV of 15%. For samples spiked at 3.0 µg/ml the plasma diluted thrombin time predicted a mean bivalirudin concentration of 3.4 µg/ml with a CV of 21%.

Results for lepirudin
The aPTT failed to accurately estimate the spiked lepirudin level in 32 of 63 samples (Fig. 5). Failures mainly occurred in samples with lupus inhibitors, low vitamin K-dependent factor levels, or elevated D-dimer levels, all of which had prolonged baseline aPTTs. The aPTT overestimated the spiked lepirudin concentration in these samples. Failures also occurred in seven of 24 samples with a normal baseline aPTT levels, overestimation of the spiked lepirudin level occurred six times and underestimation once. In contrast, the plasma diluted thrombin time returned accurate estimations of subtherapeutic, therapeutic, and supratherapeutic concentration in 62 of 63 samples (Fig. 5). The single failure occurred in a sample with elevated D-dimer levels and a fibrinogen level of 1,043 mg/dl. The supratherapeutic, 3.0 µg/ml aliquot from this sample returned a plasma diluted thrombin time that was within the therapeutic range.

In samples spiked with lepirudin at 0.1 µg/ml the plasma diluted thrombin time predicted a mean lepirudin concentration of 0.1 µg/ml with a CV of 23%. For samples spiked at 1.0 µg/ml the plasma diluted thrombin time predicted a mean concentration of 1.0 µg/ml with a CV of 13%. For samples spiked at 2.0 µg/ml the plasma diluted thrombin time predicted a mean lepirudin concentration of 1.8 µg/ml with a CV of 12%.

Dependence on fibrinogen
The plasma diluted thrombin time was slightly dependent on fibrinogen level (Fig. 6). For samples with fibrinogen values >600 mg/dl, the plasma diluted thrombin times were 10% less than expected in argatroban spiked samples, 20% less than expected in lepirudin spiked samples, and a 5% less than expected in bivalirudin spiked samples.

Discussion
The ideal assay for monitoring DTI therapy has not been clearly established. The aPTT is widely available, but the utility of the assay is limited due to interference from many sources (5). In particular, if the baseline aPTT is prolonged, accurate aPTT based monitoring of DTIs is not possible (7).

The ecarin clotting time (ECT) and quantitative thrombin time (QTT) have been used to accurately monitor DTI therapy in such settings (8, 10). Both assays activate the clotting cascade at the level of thrombin generation, so neither is greatly affected by variation in most coagulation factors (8, 10). Phospholipid is not present in either assay so neither suffers inhibition from lupus inhibitors (7). Unfortunately these assays are not widely available (7, 11).
Figure 4: APTT and plasma diluted thrombin time results for patient specimens spiked with bivalirudin at 0.1, 1.0, and 3.0 μg/ml. Solid lines represent the standard curves for bivalirudin in pooled normal plasma for the aPTT and plasma diluted thrombin time. The grey boxes represent the target therapeutic range.
Figure 5: APTT and plasma diluted thrombin time results for patient specimens spiked with lepirudin at 0.1, 1.0, and 2.0 µg/ml. Solid lines represent the standard curves for lepirudin in pooled normal plasma for the aPTT and plasma diluted thrombin time. The grey boxes represent the target therapeutic range.
We based our assay on the QTT, a modified thrombin time designed to monitor bivalirudin and lepirudin levels. Without modification the thrombin time is overly sensitive to DTI concentration and not suited for therapeutic monitoring (8). The QTT overcomes the thrombin time’s sensitivity by adding varying concentrations of thrombin and diluting the sample into various buffers. The technique is accurate but not widely used, possibly because multiple reagents are required, and different reagents are used for different DTIs. We noted that the QTT required dilution into normal plasma in order to measure bivalirudin levels greater than 1.75 µg/ml. We hypothesized that dilution into normal plasma alone could overcome the thrombin time’s sensitivity to DTIs, and turn the thrombin time into a simple method to monitor DTI therapy.

We found that a 1:4 dilution into normal plasma made the thrombin time an effective indicator of lepirudin, argatroban, and bivalirudin concentration. The plasma diluted thrombin time, like the thrombin time, contains no phospholipids and is not affected by lupus inhibitors (17). Dilution into normal plasma likely blunts the assay’s dependence on factor levels. We found no interference due to D-dimer levels or vitamin K-dependent factor levels, and little patient to patient variation (CV of 12–22%). The method is simple, amendable to automation, and can be performed at any institution that has normal plasma and can perform a thrombin time. The plasma diluted thrombin time successfully measured argatroban, bivalirudin, and lepirudin levels, and we hypothesize that the method could be adapted for use with any DTI.

There are limitations to our study. Because this assay is based on the thrombin time, entities that interfere with the thrombin time could interfere with the plasma diluted thrombin time. If a patient has a prolonged baseline thrombin time due to disease, drug effect, or other processes, the plasma dilution in this assay may not be adequate to overcome the interference.

We were able to investigate the effect of fibrinogen level on the plasma diluted thrombin time, and we found the assay to be somewhat dependent on fibrinogen level. Such interference has also been noted in the ECT, especially in monitoring lepirudin levels (8). The effect of low fibrinogen on the assay is limited by the 1:4 dilution into normal plasma. Our normal plasma pool had fibrinogen levels of 274 mg/dl making 205.5 mg/dl the lowest possible fibrinogen concentration after dilution. An upper limit for high fibrinogen samples does not exist, and interference due to high fibrinogen levels is a possibility. For bivalirudin and argatroban the dependence on fibrinogen level was 10% or less and would not likely be clinically significant. However, in lepirudin-spiked samples, the plasma diluted thrombin time was decreased by 20% when fibrinogen levels exceeded 600 mg/dl. This level of interference could be clinically significant. In such situations, further dilution into normal plasma could be a reasonable way to reduce the assay fibrinogen level, but we did not test this in our study. Overall, some degree of fibrinogen level dependence is to be expected in any assay based on the conversion of fibrinogen to fibrin.

A common limitation in testing any DTI monitoring method is the lack of well-defined therapeutic concentration ranges. The therapeutic ranges for DTIs are generally based on prolongation of the aPTT, and not on drug concentration levels. Without a defined concentration range, alternative monitoring by any assay is not possible. We tried to overcome this problem by extrapolating therapeutic concentration ranges from our aPTT standard curves, and adjusting our therapeutic range based on published values. Overall, the optimal therapeutic concentration ranges for DTIs have yet to be determined. Each laboratory should establish its own therapeutic ranges for monitoring DTI therapy. This should be done for aPTT-based monitoring or for monitoring using an alternative assay.

It has been our experience that the standard curves for this assay do not change significantly over time, provided that the same lot unit of reagents is used for each standard curve. If the...
source of pooled plasma is changed, and the fibrinogen level of the pool changes significantly, the standard curve would likely also change. Since laboratories may set this assay up using different reagents and instruments, each lab will need to determine how often calibration curves are needed for their particular version of the assay.

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References