Received: 16 March 2022 | Revised: 31 May 2022 | Accepted: 29 August 2022

DOI: 10.1002/jcla.24695

RESEARCH ARTICLE

WILEY

Validation of high concentrated thrombin time assay for unfractionated heparin monitoring

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Abstract

Background: The high concentrated thrombin time (hcTT), a thrombin time modified by increasing the thrombin concentration, is a possible alternative assay to activated partial thromboplastin time (aPTT) in unfractionated heparin (UFH) monitoring. This study aimed to determine the optimal thrombin concentration used in the hcTT assay for UFH monitoring.

Methods: A total of 30 blood samples obtained from healthy volunteers were included in this study. Thrombin concentrations of 10.0, 15.0, 20.0, and 25.0 IU/ml were used in the hcTT assay. The consistency between the hcTT and anti-FXa assays was evaluated. To validate the hcTT assay, linearity, repeatability, reproducibility, and diagnostic performance of the assay were assessed.

Results: The hcTT assay using thrombin concentration of 15.0 IU/ml showed a strong correlation to the anti-FXa assay with R^2 of 0.72 and the Spearman's correlation coefficient (r_s) of 0.97 (95% CI, 0.96–0.98). Within-run and day-to-day run variabilities of the assay were satisfactory (all coefficients of variation <10%). We found an excellent correlation between the results which were measured using different reagents with intra- or inter-laboratory instruments. Notably, as compared to the aPTT assay, the hcTT assay showed a significantly better performance in identifying the samples which contain UFH at the supratherapeutic level, with an AUC of 0.97 vs. 0.91, $p = 0.049$.

Conclusion: The hcTT assay can be used as an alternative assay for UFH therapy monitoring. A further study using clinical samples is recommended to confirm the appropriateness of the hcTT assay for clinical application.

KEYWORDS
activated partial thromboplastin time, anti-FXa assay, assay validation, high concentrated thrombin time, unfractionated heparin monitoring

1 | INTRODUCTION

Unfractionated heparin (UFH) has been widely used for over the last 50 years as an anticoagulant to treat and prevent thromboembolic events. The anticoagulant effect of UFH is generated by the formation of a complex with antithrombin (AT), which catalyzes the AT to inhibit several activated coagulation factors, including thrombin (factor IIa) and activated factor X (factor Xa).[1] Although it has been largely replaced by newer low molecular weight heparin (LMWH) derivatives and direct oral anticoagulants (DOACs), UFH is still used for many indications, including treating venous thromboembolism, acute coronary syndrome, and other thrombotic diseases, and is widely used in hospitalized settings.[1,2] However, challenges to the use of UFH exist, including its complex pharmacokinetics and pharmacodynamics profile, interpatient variability, complicated administration process, drug-related problems, and its narrow therapeutic range.[3] Therefore, close monitoring of its anticoagulant effect is necessary.

The main laboratory assays for monitoring UFH therapy are activated partial thromboplastin time (aPTT) and the chromogenic anti-factor Xa (anti-FXa) assays.[4–7] The aPTT test is the most widely used method to monitor UFH treatment because the test is simple, relatively cheap, and widely available. However, inter-laboratory variation caused by a different aPTT reagent and analyzer has been noted for the aPTT assay.[8,9] It has been reported that the aPTT from different laboratories shows a different sensitivity to heparin and, as compared to the reference value, a 1.5-to 2.5-fold increase in aPTT does not correlate with the concentration of heparin in the therapeutic range.[10] Moreover, the aPTT assay is affected by various pre-analytical and analytical factors[11] as well as various patients' underlying conditions, including coagulation factor deficiency, lupus anticoagulant (LA), and high coagulation factor level.[11–13] The anti-FXa assay is specific to evaluate the interaction between UFH and AT with a published unique therapeutic range for UFH between 0.3 and 0.7 IU/ml.[14–16] In contrast to the aPTT assay, the anti-FXa assay is more robust and less influenced by the interferences. It is not affected by an increase in factor VIII and fibrinogen levels or coagulation factor deficiency. However, in some circumstances, if AT is not supplemented in the anti-FXa reagents, the assay would be affected in patients with AT deficiency. Limitations of the anti-FXa assay include a high cost of the test, the limited accessibility, and the lack of a standardized protocol.[14–16]

The thrombin time (TT) assay is an alternative assay used in UFH monitoring.[17,18] The TT assay is used to evaluate the conversion of fibrin to fibrinogen in the final common pathway of the coagulation cascade.[18] By adding exogenous thrombin, the phospholipid-dependent extrinsic, intrinsic, and common coagulation pathways are bypassed.[17,18] It is well-known that thrombin is sensitive to heparin, dysfibrinogenemia, and other abnormalities.[18] Various thrombin concentrations are used in the TT assay which results in varied heparin sensitivity. At lower concentrations, thrombin is more sensitive to heparin than in the test which uses a high concentration of thrombin.[18] A concentration of 5.0–10.0 IU/ml of thrombin is used for the TT assay in most laboratories.[19] However, massively prolonged TT is observed in a case of high-dose heparin use.[20] The high concentrated TT (hcTT) assay is a modified TT assay using an increased thrombin concentration, which increases the linearity of the test to cover a full concentration range of heparin use. Previous studies showed that the hcTT assay had a high level of competency for evaluating the anticoagulant effect of UFH.[21–23] However, the thrombin concentration used in the hcTT assay needs to be optimized.

In this study, we used heparin-spiked plasma samples to determine the optimal thrombin concentration and validated the hcTT assay for UFH monitoring by evaluating its consistency, repeatability, reproducibility, and diagnostic performance comparing with the aPTT assay. To the best of our knowledge, this is the first validation study of the hcTT assay used for UFH monitoring.

2 | MATERIALS AND METHODS

2.1 | Study participants and sample collection

The sample size was calculated using a percent coefficient of variation (%CV) of 14.7[15]; a level of significance of 5%; and a level of estimation error of 5%. We estimated that at least 30 blood samples obtained from healthy volunteers were required. The sample size calculated was sufficient for the construction of reference ranges for hemostasis tests used in the investigation of bleeding disorders according to the World Federation of Hemophilia laboratory recommendations.[19] According to the Clinical and Laboratory Standards Institute (CLSI) guidelines and recommendations,[11,24] the samples were collected using 3.2% sodium citrate evacuated polymer tubes (Vacuatte Greiner bio-one, UK). The tubes were centrifuged at 2500× *g* for 15 min within a maximum of 1 h of sample collection to collect platelet-poor plasma (PPP). All PPP samples were evaluated for baseline laboratory parameters, including coagulogram, fibrinogen level, D-dimer, and AT activity. The study protocol was approved by the Committee of Institutional Review Board, Royal Thai Army Medical Department, Bangkok, Thailand (approval No. IRBRTA1338/2564). This study was conducted according to the principles of Declaration of Helsinki. Informed consent was obtained from all participants included in this study.

2.2 | Heparin-spiked plasma sample preparation

Various doses of UFH (Heparin LEO, Ballerup, Denmark) were spiked into each PPP sample to obtain final concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 IU/ml. The spiked samples were aliquoted and stored at −80°C until required.

Anti-FXa activity of all spiked samples was determined using Biophen Heparin LRT reagent (Hyphen Biomed, Neuville-sur-Oise, France). This assay was performed using the Sysmex CS-2500 coagulation analyzer (Sysmex Corporation, Kobe, Japan), according to the industrial protocol and calibrated with Biophen Heparin

calibrators. These spiked samples were prepared in the same laboratory throughout the study.

2.3 | Determination of the optimal thrombin concentration

To optimize the hcTT assay, the concentrations of 10.0, 15.0, 20.0, and 25.0 IU/ml of thrombin (Dade Thrombin reagent, Siemens Healthcare, Marburg, Germany) were freshly prepared and were used to determine the clotting time of all PPP samples in which a concentration range of 0.2–1.0 IU/ml of UFH were spiked. The clotting time was measured using the Sysmex CS-2500 coagulation analyzer. The clotting time obtained from the hcTT and aPTT assays was logarithmically transformed and linear regression analysis was performed to evaluate a correlation between the clotting time and the anti-FXa activity. The thrombin concentration providing the highest R^2 was considered as the optimal concentration and was selected for further study.

2.4 | Determination of consistency and range

Linear regression analysis was used to determine the consistency of the results obtained from the hcTT, aPTT, and anti-FXa assays with the correlation coefficient calculated. The reference range was established according to CLSI guideline H47.[25] The lower and upper limits of the hcTT assay were determined by averaging the clotting time which corresponded to the anti-FXa activity of UFH therapeutic doses of 0.3 and 0.7 IU/ml,[14–16] respectively.

2.5 | Determination of repeatability and reproducibility

To assess the repeatability of the hcTT (using thrombin 15.0 IU/ml), aPTT, and anti-FXa assays, the assays were performed by repeating the within run and day-to-day run (one duplication per day) measurements using all 180 spiked PPP samples. HemosIL Fibrinogen C reagent (Instrumentation Laboratory, Bedford, Massachusetts, USA), was used as a thrombin reagent, to investigate the reproducibility of the optimized hcTT protocol. Additionally, the samples were sent out to a second laboratory, where the medical technologists were blinded to the results, for parallel study using the Sysmex CS-2100i coagulation analyzer (Sysmex Corporation, Kobe, Japan), which shares the same principle as the Sysmex CS-2500. All of the above-mentioned protocols were performed by duplicate measurements.

2.6 | Comparison of diagnostic performance between the optimized hcTT and aPTT

The spiked PPP samples were divided into three groups based on their anti-FXa activity, including subtherapeutic (anti-FXa activity <0.3 IU/ml), therapeutic (anti-FXa activity 0.3–0.7 IU/ml), and supratherapeutic (anti-FXa activity >0.7 IU/ml) groups. The area under the receiver operating characteristic (ROC) curve (AUC) was used to compare the performance of the hcTT and aPTT assays.

2.7 | Statistical analysis

Continuous variables are presented as the mean ± standard deviation (SD), or median and inter-quartile ranges (IQR), based on their distribution. The linear regression analysis was used to demonstrate the linear relationship between test parameters and reported as R^2. The correlation between test results was evaluated using Spearmans coefficient (r_s) with a 95% confidence interval (CI). Within-run and day-to-day run variabilities were determined using the SD and the %CV. The average of the differences in the results obtained from different reagents and instruments and the 95% CI of the limits of agreement (LOA) (95% LOA) were calculated according to the Bland–Altman analysis.[26] Sensitivity and specificity were calculated. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism, Version 9 (GraphPad Software, CA, USA).

3 | RESULTS

3.1 | Baseline characteristics and laboratory parameters of 30 healthy volunteers

A total of 30 healthy volunteers, including 13 (43.3%) men and 17 (56.7%) women, were enrolled. Their average age was 29.3 ± 5.0 years. All participants demonstrated baseline laboratory testing results within the reference range. The mean ± SD for prothrombin time (PT), aPTT, TT, fibrinogen, D-dimer, and AT activity were 12.40 ± 1.30 s, 24.70 ± 2.10 s, 11.00 ± 0.47 s, 287.29 ± 62.74 mg/dl, 0.30 ± 0.02 μg/ml, and 102.30 ± 8.94%, respectively. When the individual plasmas were used to prepare the heparin-spiked samples, the overall correlation between the UFH concentration and anti-FXa activity was determined. We found an excellent correlation with R^2 of 0.98 and slope of 1.07 (95% CI: 1.05–1.09).

3.2 | The optimal thrombin concentration, linearity, and range of the tests

The correlations between the aPTT assay and anti-FXa activity; and the hcTT assays and anti-FXa activity are shown in Figure 1. When linear regression analysis was performed, the clotting time of TT using a thrombin concentration of 15.0 IU/ml showed a superior correlation to anti-FXa activity with R^2 of 0.72, followed by aPTT, TT (thrombin 20.0 IU/mL), and TT (thrombin 25.0 IU/ml) with R^2 of 0.67, 0.56, and 0.49, respectively (Figure 1A). Because the clotting time for conventional TT (thrombin 10 IU/ml) was unmeasurable, that is

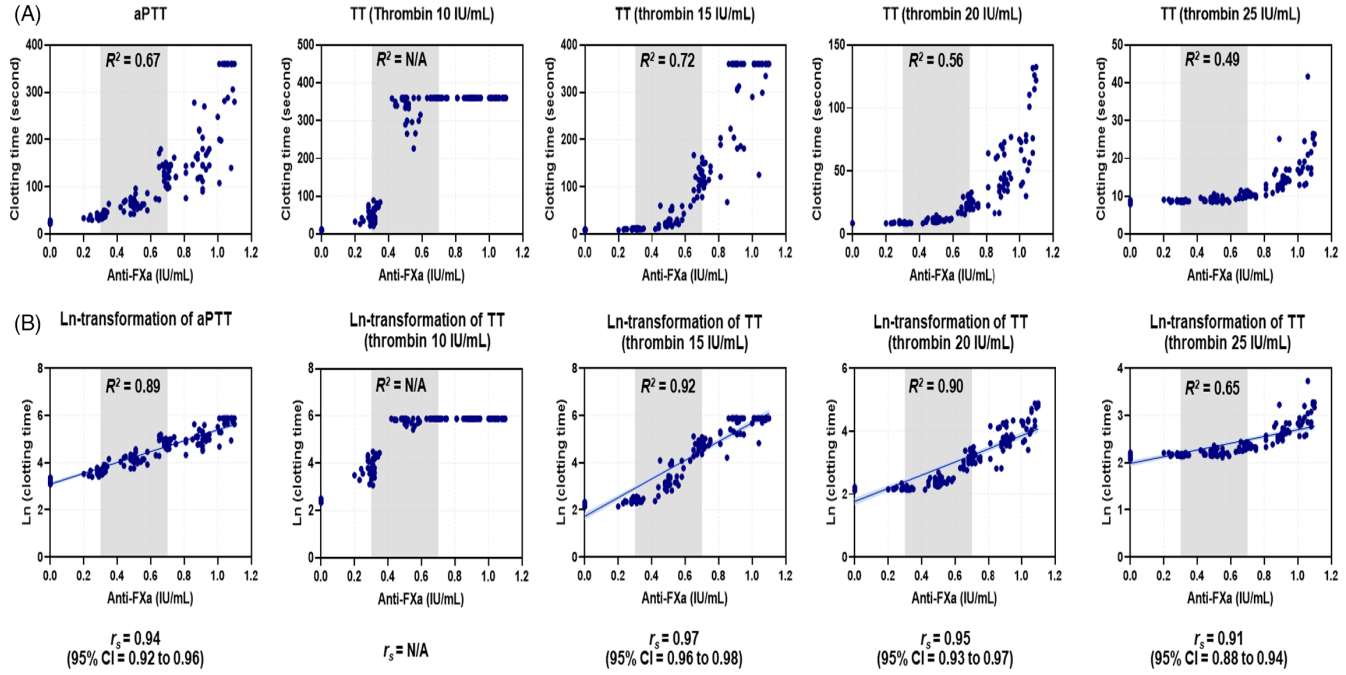

FIGURE 1 Scatterplots demonstrating the correlation between clotting times of different assays for heparin monitoring and anti-FXa activity. (A) The correlation between the clotting times and anti-FXa activity, and (B) correlation between the natural logarithmic (Ln) transformed clotting times and anti-FXa activity. R^2 with regression curves and Spearman's correlation coefficients (r_s) with 95% CI are shown. The shaded gray area represents the therapeutic range of UFH (0.3–0.7 IU/ml). N/A, not applicable

more than 360.0 s, in most of the samples with anti-FXa greater than 0.4 IU/ml; therefore, the correlation of the conventional TT to the anti-FXa activity was not evaluated. To improve linearity of the data, we performed data transformation by taking the natural logarithm (Ln) and re-analyzed the data using linear regression (Figure 1B). It was revealed that the Ln-transformed clotting times have stronger correlations with the R^2 of 0.89 for aPTT, and 0.92 for TT (thrombin 15.0 IU/ml), 0.90 for TT (thrombin 20.0 IU/ml), and 0.65 for TT (thrombin 25.0 IU/ml). The overall Spearman's correlation coefficient (r_s) was 0.94 (95% CI: 0.92–0.96) for aPTT, 0.97 (95% CI: 0.96–0.98) for TT (thrombin 15.0 IU/ml), 0.95 (95% CI: 0.93–0.97) for TT (thrombin 20.0 IU/ml), and 0.91 (95% CI: 0.88–0.94) for TT (thrombin 25.0 IU/ml).

The optimal concentration of thrombin, that is 15.0 IU/ml, was chosen for further validation studies. Within the therapeutic range of UFH (anti-FXa of 0.3–0.7 IU/ml), the correlation of the hcTT to the anti-FXa activity was stronger than that of hcTT to the aPTT assay (R^2 of 0.70 vs. 0.45, Figure S1A). This was reiterated by the data showing that the Ln-transformed hcTT clotting time has a stronger correlation with the anti-FXa activity as compared to the aPTT assay (R^2 of 0.87 vs. 0.72, Figure S1B). For the supratherapeutic range of UFH (anti-FXa greater than 0.7 IU/ml), moderate correlations of clotting time and Ln-transformed clotting time to anti-FXa activity were observed in both hcTT and aPTT assays (Figure S1C-D).

The reference ranges of the aPTT and hcTT assays for UFH monitoring are shown in Table 1. In the UFH therapeutic range, the aPTT values ranged from 36.96 to 123.03 s and the aPTT ratio ranged from 1.29 to 4.29, whereas the optimized hcTT ranged from 11.72 to 121.53 s and the hcTT ratio ranged from 1.22 to 12.60. Interestingly, using the Ln-transformed clotting time showed narrower reference intervals compared with using only clotting time or normalized ratio.

TABLE 1 Therapeutic intervals of different laboratory assays corresponding to the UFH therapeutic range (0.30–0.70 IU/ml)

	Therapeutic interval		
Assay	Lower limit	Median (IQR)	Upper limit
aPTT			
aPTT, s	36.96	64.00 (49.35–108.80)	123.03
aPTT ratio	1.29	2.59 (1.99–4.40)	4.29
Ln(aPTT)	3.61	4.16 (3.90–4.69)	4.81
hcTT			
hcTT, s	11.72	27.00 (13.10–85.50)	121.53
hcTT ratio	1.22	3.01 (1.46–9.54)	12.6
Ln(hcTT)	2.46	3.30 (2.57–4.45)	4.80

Abbreviations: aPTT, activated partial thromboplastin times; hcTT, high concentrated thrombin time; IQR, interquartile range; Ln, natural logarithm.

3.3 | Repeatability and reproducibility

Assessment of the repeatability is shown in Table 2. For the variability, the %CV was 1.05 for aPTT, 2.55 for TT (thrombin 15.0 IU/ml), and 2.33 for anti-FXa heparin assay. For the reproducibility, the optimized hcTT assays were performed in different analyzers

using two different commercially available thrombin reagents. The correlation was evaluated as shown in Figure 2 (A-D). In addition, the Bland–Altman analysis was performed to assess the agreement of the assays. The means and SDs of the biases were calculated as shown in Figure 2 (E-H).

3.4 | The diagnostic performance of the optimized hcTT and aPTT

Using the reference ranges of each assay (Table 1), the sensitivity and specificity of the hcTT and aPTT for the detection of sub- and supratherapeutic UFH levels were calculated as shown in Table 3. The ROC curves were plotted to assess the overall performance of the aPTT and hcTT assays for monitoring UFH therapy (Figure 3). The performance of the aPTT (Figure 3A, left) and hcTT assays (Figure 3B, left) was not different in discriminating the samples bearing UFH at the subtherapeutic range, from those bearing UFH at the therapeutic range (AUC = 0.97 vs. 0.97, $p = 0.822$). Notably, as compared to the aPTT assay (Figure 3A, right), the hcTT assay (Figure 3B, right) showed a significantly superior performance in discriminating the samples bearing UFH at the therapeutic range, from those bearing UFH at the supratherapeutic range (AUC = 0.97 vs. 0.91, $p = 0.049$).

4 | DISCUSSION

Despite the growing use of anti-FXa assay, UFH therapy has been widely monitored by the aPTT assay because of its simplicity, lower cost, and widespread availability.[4–7] Nevertheless, the aPTT assay can be affected by various pre-analytical and analytical factors.[8–13] Several studies have reported the discordance of the results obtained from the aPTT and anti-FXa assays for UFH monitoring.[6,7,12,13,15,16] In addition, the UFH-induced prolongation of aPTT is highly dependent on the reagent and analyzer used. In this study, we aimed to determine the optimal thrombin concentration used in the hcTT assay and establish the potential of using the assay for UFH monitoring.

In this study, heparin-spiked PPP samples were used to determine the optimal thrombin concentration and to validate the hcTT assay. We found that the concentration of 15.0 IU/ml of thrombin was the most optimal concentration for the hcTT assay because the results obtained had the greatest concordance with the anti-FXa assay. As compared to the aPTT assay, it has been reported that the hcTT assay showed better correlation with the anti-FXa assay[15,21] which is consistent with our results. The repeatability of the assay was evaluated by repeating the within-run and day-to-day run measurements. Within-run and day-to-day run variabilities of the hcTT assay were satisfactory with less than 10% of overall %CV. To determine the reproducibility, different reagents and instruments were used. Additionally, the samples were sent out to the second laboratory where the coagulation analyzer which shares the same principle as the Sysmex CS-2500 is used. We found an excellent correlation between the results obtained from the measurements using different reagents and instruments. However, the Bland–Altman plots revealed the essential evidence that biases existed. The biases observed may result from the interference in the clotting wave detection caused by a very high concentration of UFH in the samples which results in various prolongation time measured by different reagents and analyzers. Hence, it can be implied that the test should be validated by each laboratory in its own technical condition prior to implementation. The reference interval of the hcTT was established corresponding to the recommended anti-FXa therapeutic range of 0.30 to 0.70 IU/ml.[14–16] It was observed that the hcTT assay showed a broader range than the aPTT assay. Another point of interest is that the hcTT assay showed a significantly greater ability to identify the supratherapeutic group as indicated by the higher AUC value. Nevertheless, when the concentration of UFH was increased, a nonlinear correlation between the hcTT and anti-FXa assays was observed. Regarding this issue, the clotting time obtained from the hcTT and aPTT assays was logarithmically transformed and linear regression analysis was performed. Strong correlations were observed for both the hcTT and aPTT assays. These findings suggested that the use of logarithmic transformation of the clotting time may be more informative than the aPTT and hcTT clotting time to provide guidance for UFH monitoring. However, when focusing on UFH monitoring at the supratherapeutic range, the hcTT and aPTT clotting times may not reflect the actual anti-FXa activity. Therefore, neither assay may be appropriate for monitoring of UFH at the supratherapeutic range, and the anti-FXa assay potentially plays an important role in such cases.

Unlike the aPTT assay, the TT assay is not affected by LA.[27] Therefore, the hcTT assay could be beneficial in UFH monitoring for patients with prolonged aPTT due to the presence of LA. In addition, it has been stated that the TT assay was not influenced by direct anti-FXa inhibitors, while the anti-FXa and aPTT assays were

TABLE 2 Repeatability of different laboratory assays for heparin monitoring in 180 spiked samples

Assay	Number of measurements	Within run		Day-to-day run		Overall	
		SD	%CV	SD	%CV	SD	%CV
aPTT	180	0.74	0.78	0.86	0.87	0.99	1.05
hcTT	180	0.80	1.47	0.89	2.55	1.02	2.55
Anti-FXa	180	0.01	2.20	0.02	2.27	0.01	2.33

Abbreviations: aPTT, activated partial thromboplastin time; CV, coefficient of variation; hcTT, high concentrated thrombin time; SD, standard deviation.

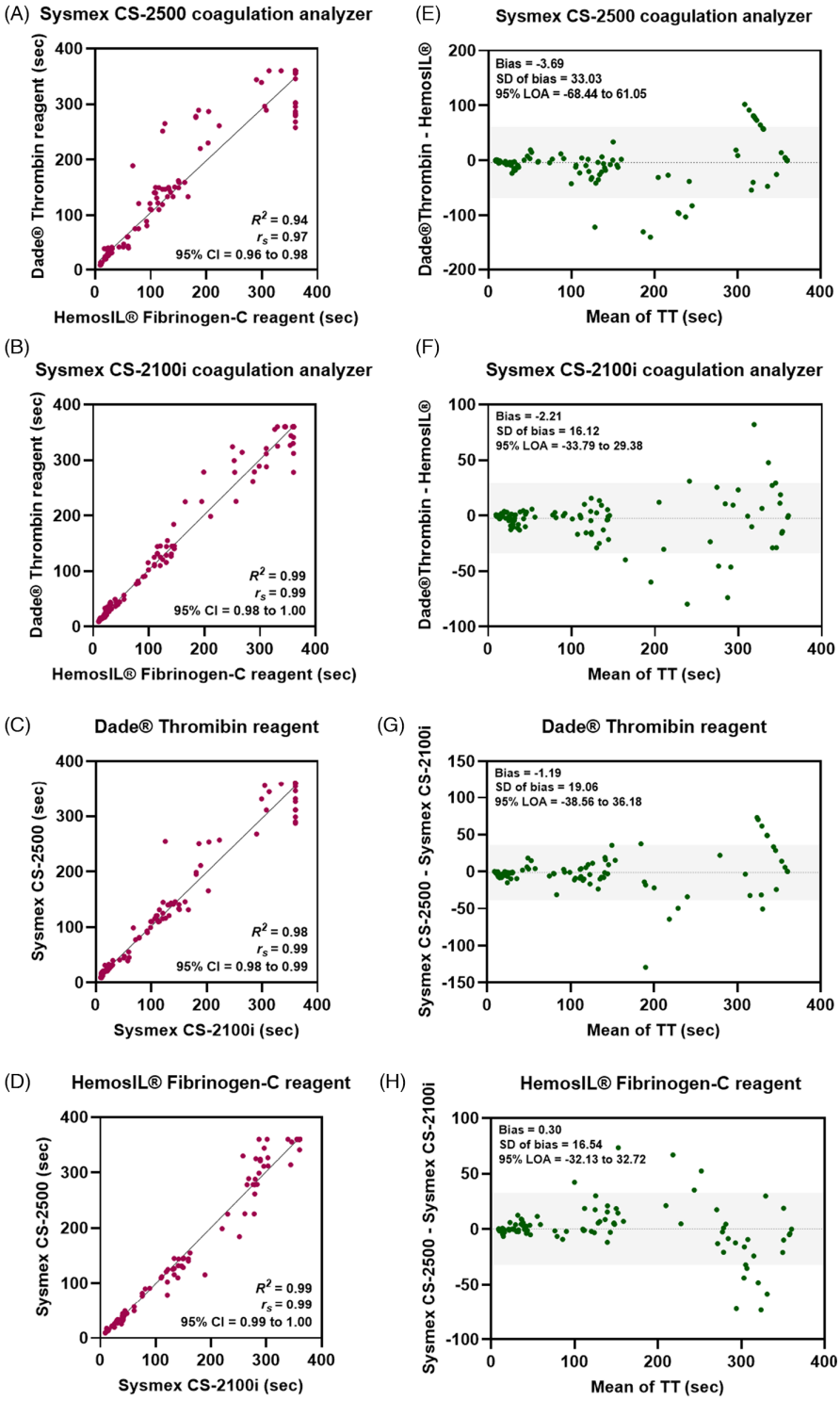

FIGURE 2 Reproducibility assessment of the hcTT measured using different reagents and instruments. (A-D) The scatterplots demonstrating the correlation between two different reagents (Dade Thrombin vs. HemosIL Fibrinogen C reagents) performed by intra- and inter-instruments (Sysmex CS-2500 vs. CS-2100i coagulation analyzers). (E-H) Bland–Altman plots showing assessment of agreement for the hcTT measured using different reagents and instruments. Mean and SD of biases are reported. The shaded gray area represents the 95% LOA range

clearly affected.[28,29] Hence, the advantage of using the hcTT assay for UFH monitoring is that the assay will not be affected in the case of concomitant administration of UFH and direct anti-FXa inhibitors. However, these issues need to be further examined in future clinical studies.

Our study had some limitations. First, the study was performed in vitro using heparin-spiked samples. Therefore, the results may not reflect an in vivo anticoagulant activity of UFH. In addition, the association between our results and the clinical outcomes was not assessed in this study. Evaluation of the use of the hcTT assay in patients treated with UFH is planned. Second, the measurement of clotting time was performed using the same series of analyzer. Hence, the protocol may not be applicable to other analyzers. Third, the effect of potential confounders, including abnormal fibrinogen level, abnormal AT activity, and elevated D-dimer level, which are limitations of the hcTT assay, were not evaluated. These issues need to be further examined in future studies.

TABLE 3 Sensitivity and specificity of aPTT and the hcTT in detecting sub- and supratherapeutic UFH levels

Assay	To detect subtherapeutic levels		To detect supratherapeutic levels	
	% sensitivity (95% CI)	% specificity (95% CI)	% sensitivity (95% CI)	% specificity (95% CI)
aPTT	90.8 (81.3–95.7)	80.0 (66.2–89.1)	77.2 (64.8–86.2)	81.5 (70.5–89.1)
hcTT	84.6 (73.9–91.4)	93.3 (82.1–97.7)	89.4 (78.9–95.1)	87.7 (77.6–93.6)

Abbreviations: aPTT, activated partial thromboplastin time; CI, confidence interval; hcTT, high concentrated thrombin time.

In conclusion, the hcTT assay using a concentration of 15.0 IU/ml of thrombin demonstrated good linearity, repeatability, and reproducibility, and provided a superior diagnostic performance as compared to the aPTT assay. The hcTT could be used as an alternative assay for UFH therapy monitoring. A further study using clinical samples is suggested to evaluate the applicability of the hcTT assay in clinical use.

AUTHOR CONTRIBUTIONS

Dollapak Apipongrat developed the concept, collected the samples, performed the experiment, analyzed the data, and wrote the article. Pornnapa Police performed experiment and performed the statistical analysis. Rattapan Lamool and Punnee Butthep supervised the

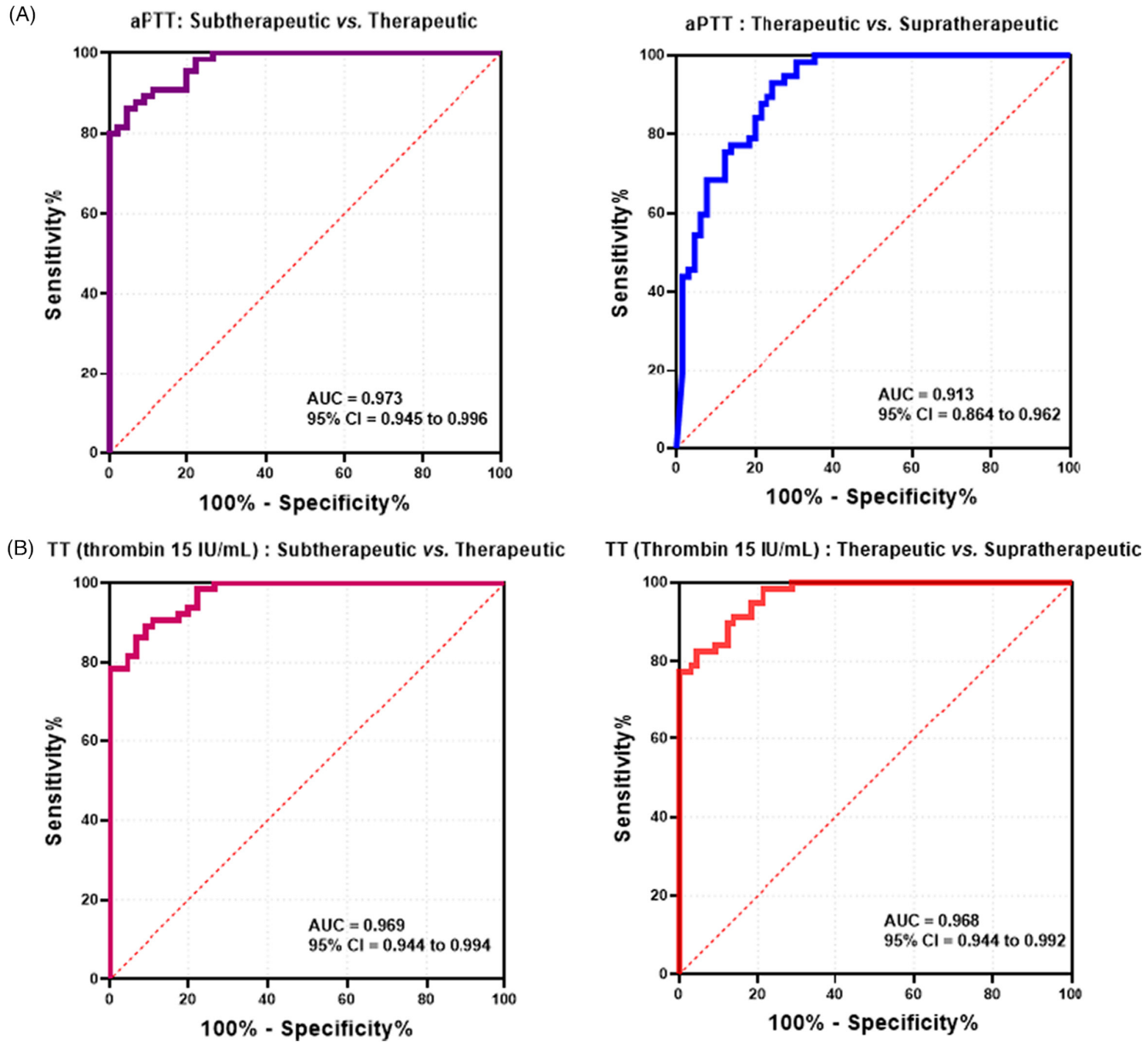

FIGURE 3 Receiver operating characteristic (ROC) curves demonstrating the diagnostic performance of (A) aPTT and (B) the hcTT using thrombin concentration of 15.0 IU/ml

project. Wittawat Chantkran developed the concept, revised the article, and supervised the project. All authors contributed and approved the final version.

ACKNOWLEDGMENT

The authors would like to thank staff members of Special Hematology Laboratory, Phramongkutklao Hospital for their technical support.

FUNDING INFORMATION

The authors received no specific funding for this work.

CONFLICT OF INTEREST

The authors state they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Apipongrat D, Police P, Lamool R, Butthep P, Chantkran W. Validation of high concentrated thrombin time assay for unfractionated heparin monitoring. *J Clin Lab Anal*. 2022;36:e24695. doi: 10.1002/jcla.24695