ORIGINAL ARTICLE

Influence of storage time on stability of routine coagulation parameters (international normalised ratio, activated partial thromboplastin time and fibrinogen) at room temperature

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ABSTRACT

Objectives: Extending the maximum acceptable specimen age for testing/retesting some routine coagulation parameters has many benefits (e.g., reagent evaluation, fibrinogen add-on for disseminated intravascular coagulation, or addressing sample delay during snow in Otago/Southland). This study assessed such possibility, where stability of international normalised ratio, activated partial thromboplastin time and Fibrinogen results were examined in relation to storage time.

Methods: From each participating individual (50 total), four citrate tubes were collected. A baseline tube was centrifuged and tested for international normalised ratio, activated partial thromboplastin time and fibrinogen at time of arrival. The other three tubes were kept as whole blood. After 24, 48, and 72 hours respectively from time of collection, one tube was taken for centrifuging and tested for international normalised ratio, activated partial thromboplastin time, and fibrinogen. In addition, centrifuged tubes were retested for international normalised ratio and fibrinogen after 24, 48, and 72 hours respectively (centrifuged fibrinogen after 96 hours) from time of collection. All specimens were kept at room temperature.

Results: The mean bias of international normalised ratio, activated partial thromboplastin time, and fibrinogen at after 24h, 48h, and 72h (centrifuged fibrinogen after 96h) were calculated and compared with specific allowable limits of performance. For both centrifuged and uncentrifuged specimens, international normalised ratio variations up to 72h all passed Royal College of Pathologists of Australasia allowable limits of performance (±0.3); fibrinogen variations up to 72h (centrifuged fibrinogen up to 96h) all passed European Federation of Clinical Chemistry and Laboratory Medicine allowable limits of performance (±10.7%). Activated partial thromboplastin time had a clear increasing trend, and all of its variations failed the European Federation of Clinical Chemistry and Laboratory Medicine allowable limits of performance (±2.7%).

Conclusion: Evidence supports extension of maximum acceptable age of uncentrifuged specimens for international normalised ratio and fibrinogen tests to 72h. Changing the maximum allowable age of activated partial thromboplastin time is not recommended. For centrifuged specimens, we can extend maximum acceptable age for international normalised ratio to 72h and fibrinogen to 96h.

Key words: International normalised ratio (INR), activated partial thromboplastin time (APTT), Fibrinogen, specimen age, result stability.

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INTRODUCTION

Routine coagulation tests that are done the most in the haematology department of a medical laboratory include PT (prothrombin time)/INR (International normalised ratio), APTT (activated partial thromboplastin time), and fibrinogen. PT/INR measures the ability to clot by extrinsic pathway, which depends on activities of Factor VII and II, V, X, and fibrinogen. INR is the normalised ratio of patients' PT in relation to a mean normal PT, which is standardised depends on the types of tissue factor used by the reagents. Many community patients who are on warfarin, (a vitamin K inhibitory anticoagulant), need to have their INR level measured and monitored regularly for therapeutic reasons. APTT measures the ability to clot by intrinsic pathway and it mainly depends on activities of Factor VIII, IX, XI, XII, as well as Factor II, V, X, and fibrinogen. Fibrinogen (also known as Factor I) is essential in the process of haemostasis for its role in converting to fibrin by enzymatic reaction with thrombin to form the stable fibrin-based blood clot.

In order to maintain high laboratory diagnostic quality, routine coagulation tests require pre-analytical elements of sodium citrated specimens to be strictly controlled before testing. These include storage time, temperature, and the status of the specimens (kept as whole blood or centrifuged to obtain separated platelet poor plasma).

The Clinical and Laboratory Standard Institute (CLSI) guideline requires testing of INR, APTT, and fibrinogen in 0.109M sodium citrate samples (3.2% buffered) (1). The current acceptable age also set by the guideline allows specimens to be kept as whole blood for INR at maximum 24 hours; for APTT at maximum 4 hours (if it is for unfractionated heparin analysis, maximum age is one hour); for fibrinogen at maximum 4 hours (1). Based on this guideline, the maximum acceptable ages for INR, APTT, and fibrinogen tests as either whole blood or centrifuged samples at room temperature in Southern Community Laboratories (SCL) Dunedin Haematology Department are set and listed in Table 1.

The short time windows for these parameters pose some challenges on transportation and storage of the specimens. SCL Dunedin regularly receives out-of-town citrate tubes for INR monitoring. Located in Otago/Southland, delay of sample arrival is common in the event of snow or icy road conditions. In this situation, patients may need to re-bleed, which causes extra time and financial cost. Hence, the original aim of this study was to examine whether we can extend the maximal acceptable time frame of INR testing. Testing for fibrinogen and APTT were included later into the study, adding up more context on examining the extension of routine coagulation sample age.

Halfway during the study, we added two more test groups. Being the largest clinical medical laboratories in Otago/ Southland, SCL Dunedin often receives requests from rural laboratories of sending 'warfarinised' citrate tubes to them for their reagent evaluation due to their lack of patients. However, the laboratory can only send specimens away after the requested tests are done, resulting in them all being centrifuged. Therefore, we evaluated the stability of INR from spun citrate tubes over time. In addition, we also examined the stability of fibrinogen from centrifuged citrate tubes.

For both studies on INR stability from specimens stored as whole blood and centrifuged tubes, we also checked the differences of performance from 'warfarinised' and non-'warfarinised' patients. This is to determine whether presence of warfarin would affect INR stability.

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METHODS

A total of 50 subjects from Dunedin community participated in this study at SCL Filleul Street Collection Centre in the period from 15/3/21 to 4/5/21. We obtained the consent to take blood from all participating subjects by letting them sign consent stickers, which were attached on their laboratory request forms.

In this study, each participating subject was invited to provide four 0.109M sodium citrate samples (3.2% buffered). A baseline tube was centrifuged and tested for INR, APTT, and fibrinogen at time of arrival at the laboratory. The other three tubes were kept as whole blood. After 24, 48, and 72 hours respectively from time of collection, one tube not centrifuged was taken for centrifuging and tested for INR, APTT, and fibrinogen. By doing so, we obtained the variations of INR, APTT, and fibrinogen from unspun tubes of each subject over 72 hours.

Stability of whole blood INR was measured for all participants (50 total). Among those, 24 subjects were on warfarin, one on dabigatran, and 25 subjects not on any anticoagulation. 34 subjects had their whole blood APTT variation measured, and 39 subjects had their whole blood fibrinogen variation measured. Since APTT and fibrinogen tests were not included in this study initially, the amounts of subjects tested were less than total amount of participants.

Test tubes for stability of centrifuged INR were obtained from 30 accessioned patient specimens requested for real routine coagulation tested at the laboratory, after the tests were completed. This was approved by Yii Sen Wee, the Head of Haematology Department and Rhonda Lucas, the Coagulation Specialist. Among these specimens, 20 patients were on warfarin and 10 were not. All tubes were reused and tested for INR after 24, 48, and 72 hours respectively from their collection time.

Fibrinogen stability from centrifuged tubes was evaluated by re-using the baseline tubes from those 50 test subjects. In total, 29 tubes were selected based on their haematocrit (to provide an assessment of how much plasma would be available). After 24, 48, and 96 hours respectively from collection time, fibrinogen from these tubes was measured.

Storage temperature was maintained at the room temperature (circa 21°C) of SCL Dunedin for all samples throughout the period of the study. All tests were performed on either one of the two Sysmex CS2500 automated coagulation analysers in SCL Dunedin Haematology, depending on which one was rostered for routine coagulation testing. Reagents used include Dade Innovin™ for INR testing; for APTT there are Actin FS™ and 0.025mol/L calcium chloride solution; for fibrinogen there are Siemens Thrombin and Owren's Veronal Buffer. Both analysers performed QC daily and maintained good diagnostic capability.

RESULTS

The individual value variation from each test groups was illustrated as line charts. For all five test groups, with the help of software Analyse-it in Excel, the mean and median, and Bland-Altman of INR, APTT, or fibrinogen at baseline, after 24, 48, and 72 hours respectively were plotted as graphs. The mean bias for each time points were calculated and listed in Table 2.

All the raw data are presented in the Supplementary Table available online from the NZIMLS website.

Whole blood INR

Figure 1 showed the INR variations of each individual over 72 hours. Figure 2 provides the mean and median of INR at different time points. Both figures separated the subjects into 'warfarinised' and non-'warfarinised'. The changes in INR over time for both groups showed similar stable patterns. Therefore, presence of warfarin was not observed to cause significant INR variation in whole blood. We then combined warfarin and non-warfarin groups together for whole blood INR interpretation. The combined mean and median plot for whole blood INR was also shown in Figure 2.

Figure 3 provides three Bland-Altman plots. From left to right, each plot showed the whole blood INR differences of all individuals between baseline and after 24, 48, and 72 hours respectively. The mean bias was calculated for each storage time: 0.02 (24 hours), 0.05 (48 hours), and 0.12 (72 hours). They were assessed against the RCPA Analytical performance specification of INR, which is set at ±0.3 (2). All mean differences passed the RCPA guideline, indicating that INR is stable up to 72 hours when stored as whole blood at room temperature.

Whole blood APTT

Figure 4 included a line chart of the APTT variation of each individual over 72 hours and a mean and median APTT plot at different time points. An increasing trend was observed from the line chart. The mean and median APTT also increased significantly (mean over 12 seconds; median over 9 seconds). The Bland-Altman plots (Figure 5) for APTT between baseline and after 24, 48, and 72 hours respectively also showed significant increase in APTT results over time. The mean bias for each time point was shown in percentage for comparison with EFLM (European Federation of Clinical Chemistry and Laboratory Medicine) desirable within-individual biological variation of APTT, which is 2.7% (3, 4). The results were 13.87% (24 hours), 26.62% (48 hours), and 34.77% (72 hours). Clearly, none of the mean bias result passed the EFLM guideline, which means APTT is not stable from 24 hours as whole blood at room temperature.

Whole blood fibrinogen

Figure 6 included a line chart of fibrinogen variation of each individual over 72 hours and a mean and median fibrinogen plot at different time points. Subjects showed generally flat lines from the line chart. The mean and median fibrinogen showed little variation (maximum increase of 0.1 from median baseline result). Figure 7 demonstrates the Bland-Altman plots for

fibrinogen within-individual variations between baseline and after 24, 48, and 72 hours respectively. for each time point, the mean bias was shown in percentage for comparison with EFLM desirable within-individual biological variation of fibrinogen, which is 10.7% (3, 4). The mean bias values were -0.50% (24 hours), -0.03% (48 hours), and 0.80% (72 hours). All the mean bias results passed the EFLM guideline, which means fibrinogen is stable up to 72 hours as whole blood at room temperature.

Centrifuged INR

Figure 8 showed the INR variations of each individual over 72 hours. Figure 9 provides the mean and median of INR at different time points. Both figures separated the subjects into 'warfarinised' and non-'warfarinised'. The changes in INR over time for both groups showed similar stable patterns. Therefore, presence of warfarin was not observed to cause significant INR variation in reused centrifuged tubes. We then combined warfarin and non-warfarin groups together for spun INR interpretation. The combined mean and median plot for whole blood INR was also shown in Figure 9.

Figure 10 gave three Bland-Altman plots. From left to right, each plot demonstrated the whole blood INR differences of all individuals between baseline and after 24, 48, and 72 hours respectively. The mean bias was calculated for each time point: -0.03 (24 hours), 0.03 (48 hours), and 0.09 (72 hours). These values were assessed against the RCPA analytical performance specification, which is the same ±0.3 as mentioned above. All mean bias results passed the RCPA guideline, indicating that for centrifuged citrate tubes at room temperature, INR is stable up to 72 hours.

Centrifuged fibrinogen

Figure 11 included a line chart of fibrinogen variation of each individual over 96 hours and a mean and median fibrinogen plot at different time points. Subjects showed generally flat lines from the line chart. The mean and median fibrinogen showed little variation (maximum increase of 0.1 from median baseline result). Figure 12 demonstrates the Bland-Altman plots for fibrinogen within-individual variations between baseline and after 24, 48, and 96 hours respectively. The mean bias for each time point was shown as a percentage for comparison with EFLM fibrinogen allowed limit of performance, which is 10.7 as mentioned above. Overall, the mean bias values were 1.85% (24 hours), -0.64% (48 hours), and 0.48% (96 hours) respectively. All the mean differences passed the EFLM guideline, which means fibrinogen is stable up to 96 hours in centrifuged citrate tubes at room temperature.

Table 1. The current standard for acceptability age of coagulation samples in SCL Dunedin Haematology Department.

Test	Whole blood (room temperature)	Centrifuged (room temperature)
PT/INR (On Warfarin)	<24h	<24h
PT/INR (Not on Warfarin)	<8h	<8h
APTT (On Heparin)	<1h	<2h
APTT (For Special Coagulation)	<4h	<4h
APTT (For Routine Coagulation)	<8h	<8h
Fibrinogen	<24h	<24h

Table 2. Calculated mean bias of all five test groups done in the study, along with indications of pass/fail against allowed limits of performance.

	Calculated mean bias	Baseline vs. after 24h	Baseline vs. after 48h	Baseline vs. after 72h	Baseline vs. after 96h	Allowed limit of performance (ALP), guideline used	Test group passed/failed ALP
INR (Whole blood)	In absolute number	0.02	0.05	0.12	/	RCPA (6) ±0.3	PASSED
APTT (Whole blood)	In %	13.87%	26.62%	34.77%	/	EFLM (2,3) ±2.7%	FAILED
Fibrinogen (Whole blood)	In %	-0.50%	-0.03%	0.80%	/	EFLM (2,3) ±10.7%	PASSED
INR (Centrifuged)	In absolute number	-0.03	0.03	0.09	/	RCPA (6) ±0.3	PASSED
Fibrinogen (Centrifuged)	In %	1.85%	-0.64%	/	0.48%	EFLM (2,3) ±10.7%	PASSED

Table 3: Comparison of mean within-subject % variation in PT/INR from previous studies and our study.

Test	Number of test subjects	Mean % change from baseline after 24h	Mean % change from baseline after 48h	Mean % change from baseline after 72h
Our study (whole blood INR)	50	1.13	3.08	6.45
Totzke *et al* (PT whole blood) (11)	14	-5.4	-12.2	-12.1
Zürcher *et al* (PT whole blood) (12)	59	-4.2	-10.8	NA
Our study (centrifuged INR)	30	-1.23	1.88	4.30
Heil *et al* (PT plasma) (13)	40	0 to -10%	0 to -10% (healthy) -10% to -20% (heparin)	-10% to -20%
Linskens *et al* (PT plasma) (14)	20	-3.9	-0.1	NA

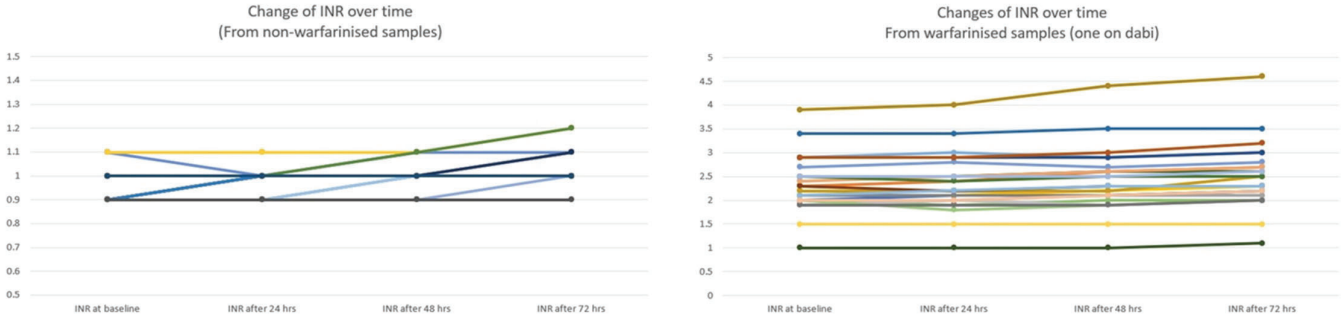

Figure 1. Line Charts of whole blood INR variation over time from non-warfarinised (left) and warfarinised (right) samples. Note one result from a patient on dabigatran is included in the chart on the right (The bottom line). However, its trend showed no significant difference from other lines.

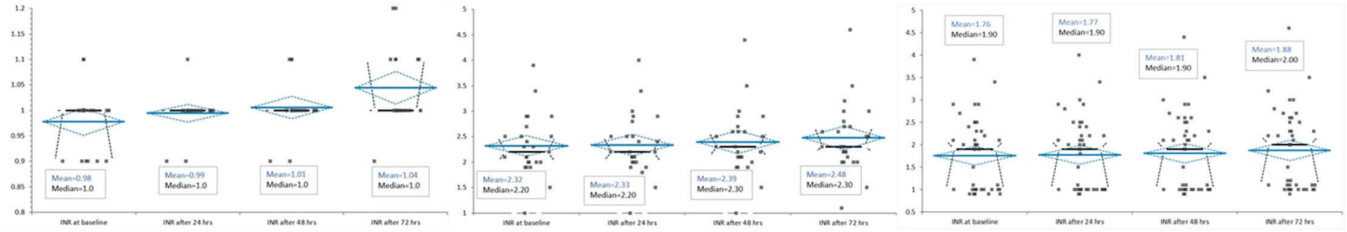

Figure 2. Mean and Median plots of whole blood INR over time from non-warfarinised (left), warfarinised (middle), and combined (right) samples.

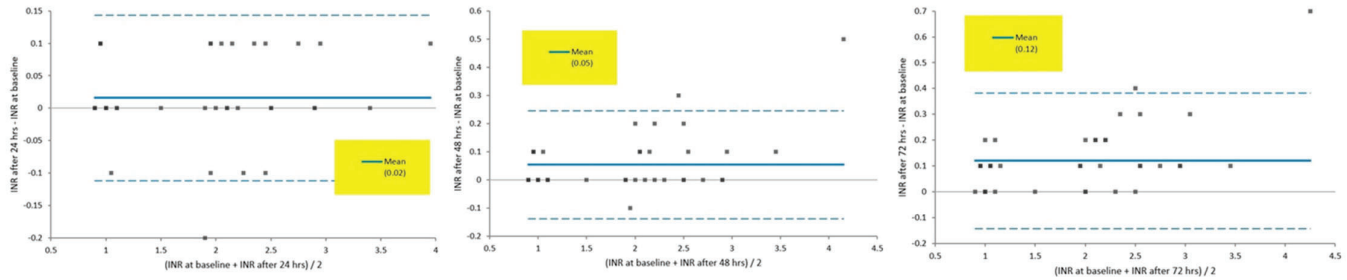

Figure 3. Bland-Altman plots of whole blood INR variation for all individuals between baseline and after 24h (left), 48h (middle), and 72h (right).

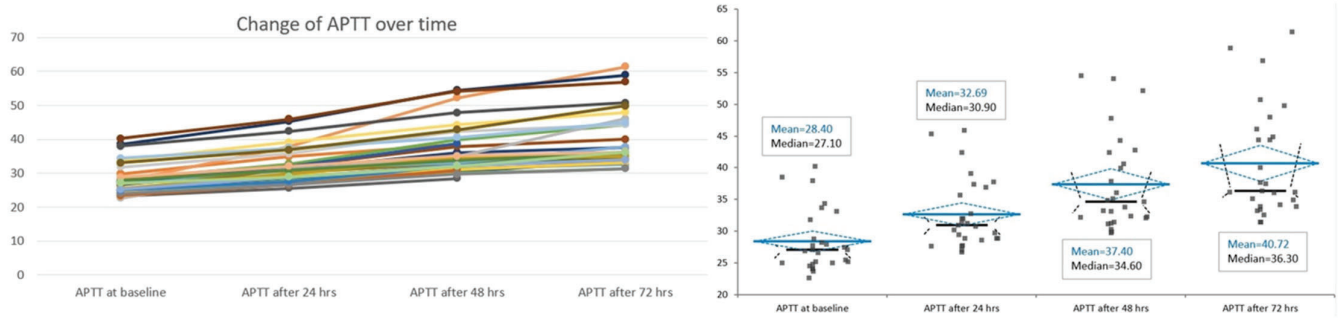

Figure 4. Line chart of whole blood APTT variation over time (left) and Mean and median plot of whole blood APTT variation over time (right).

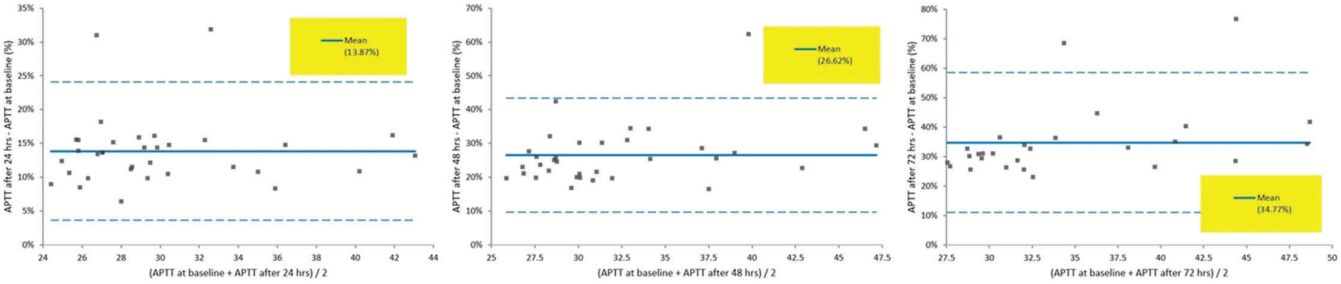

Figure 5. Bland-Altman plots of whole blood APTT variation for all individuals between baseline and after 24h (left), 48h (middle), and 72h (right).

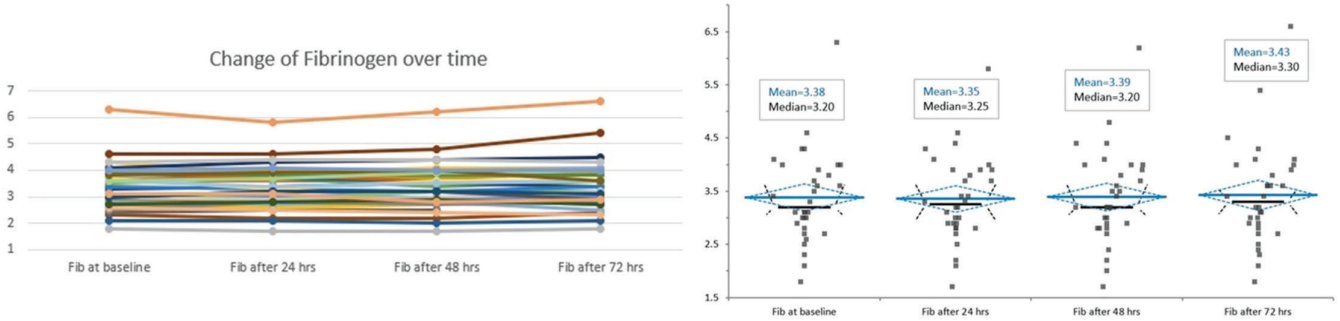

Figure 6. Line chart of whole blood fibrinogen variation over time (left) and Mean and median plot of whole blood fibrinogen variation over time (right).

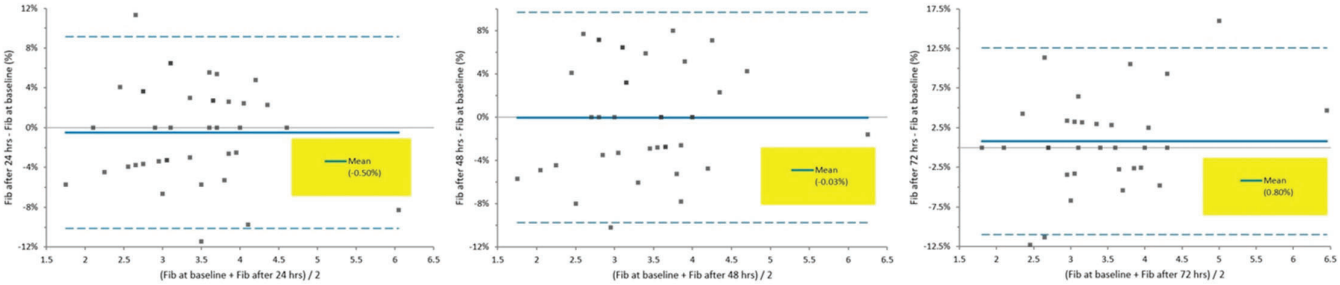

Figure 7. Bland-Altman plots of whole blood fibrinogen variation for all individuals between baseline and after 24h (left), 48h (middle), and 72h (right).

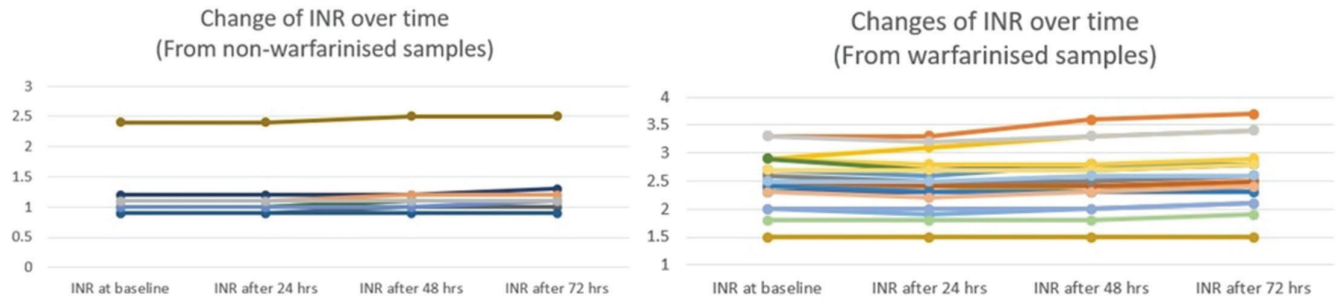

Figure 8. Line Charts of centrifuged INR variation over time from non-warfarinised (left) and warfarinised (right) samples. (The isolated top line on the left belongs to a patient with liver cirrhosis.)

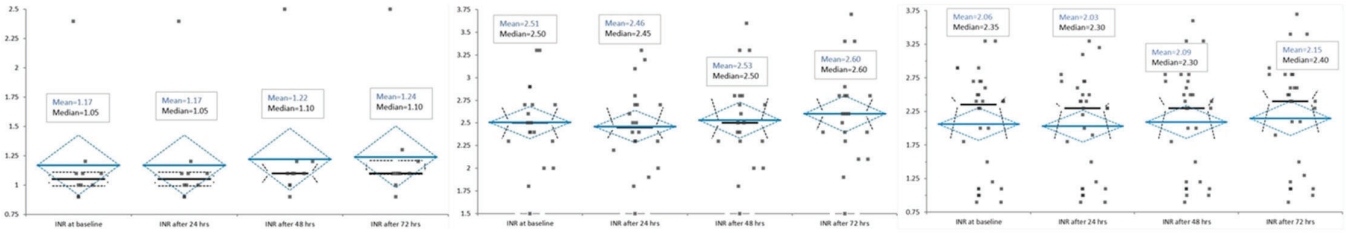

Figure 9. Mean and Median plots of centrifuged INR over time from non-warfarinised (left), warfarinised (middle), and combined (right) samples.

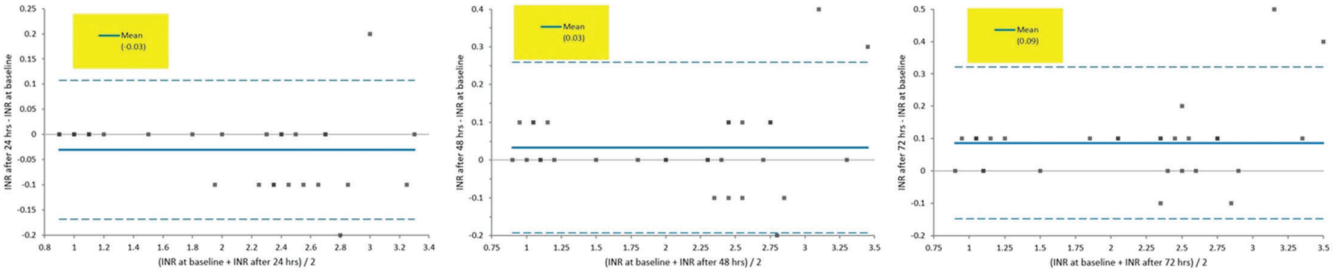

Figure 10. Bland-Altman plots of centrifuged INR variation for all individuals between baseline and after 24h (left), 48h (middle), and 72h (right).

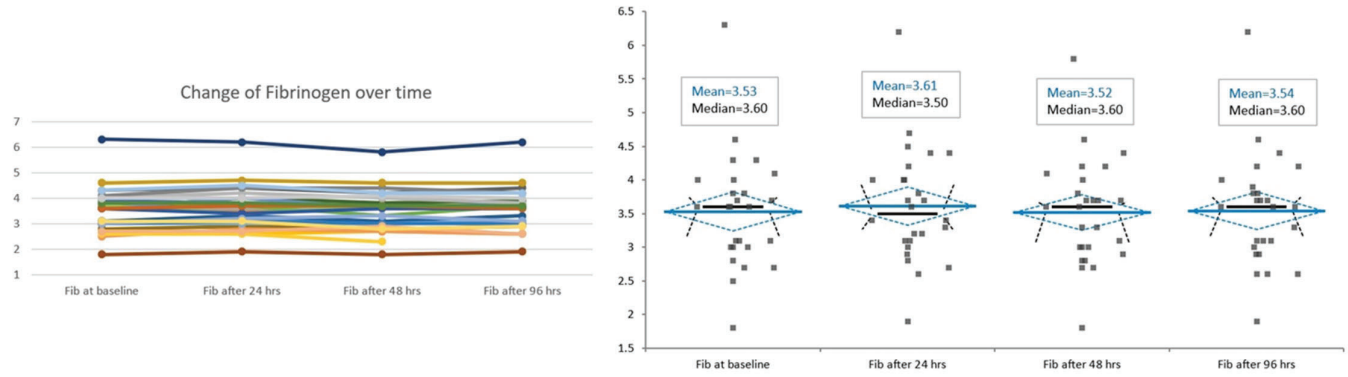

Figure 11. Line chart of centrifuged fibrinogen variation over time (left) and Mean and median plot of spun fibrinogen variation over time (right).

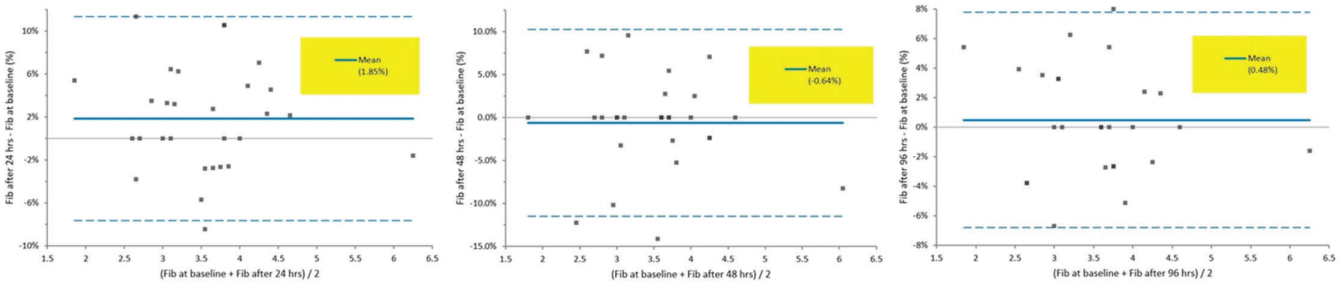

Figure 12. Bland-Altman plots of centrifuged fibrinogen variation for all individuals between baseline and after 24h (left), 48h (middle), and 96h (right).

DISCUSSION

Many previous studies undertaken on INR/PT stability only tested specimens over a storage time of maximum 24 hours, for both samples stored as whole blood (5,6), or separated into plasma (centrifuged) (7-10). Challenges to find supporting studies on INR stability over 72 hours at room temperature did exist. Of the four publications that include INR/PT stability testing over 24h were found, two were based on centrifuged plasma and the other two were based on whole blood. Table 2 summarises the performances of the previous studies and compared them with the current study. Overall, our INR variation over time showed slight increase but was not clinically significant according to the RCPA standard.

From whole blood

Totzke *et al.* (11) showed PT results from whole blood tubes stored at room temperature had significant decrease at 5.4% at 24 hours, 12.2% at 48 hours, and 12.1% at 72 hours following collection. However, this study had smaller number of samples (n=14). Zürcher e*t al.* (12) stated change in PT at 24 hours was less than 10% and less than 15% at 48 hours. Our results showed similar variation patterns as the two studies listed.

From centrifuged tubes

Heil *et al.* (13) stated the stored plasma at room temperature is stable (variation less than 10%) for up to 48 hours among healthy subjects and 24 hours among heparinised patients. Furthermore, Linskens et al (14) stated PT in plasma stored at room temperature is stable up to 48 hours. Our results were more stable than the two studies listed.

The presence of warfarin did not affect performances of INR testing on either on-warfarin or not-on-warfarin patient groups (whole blood: Figure 1,2; Centrifuged: Figure 8,9). A supporting study by Baglin *et al.* demonstrated that INR was stable for 'warfarinised' patients up to three days and Innovin™ (the thromboplastin reagent we used in the current study) gave the most stable INRs (15). It further proved that within 72 hours of storage at room temperature, testing on 'warfarinised' samples would not cause clinically significant change from baseline results. There was indeed one warfarinised individual in our study (Figure 1, the top line on the chart to the right) whose INR result varied from 3.9 to 4.6 over 72 hours. Due to sample size, we might not rule out a different stability pattern of INR for over-anticoagulated patients (INR>3.0), but it would not cause critical concern by our lab protocol (INR≤5.0). A subject on dabigatran was also included in the study (Figure 1, the bottom line on the chart to the right) but the INR variation was not clinically significant (from 1 to 1.1 over 72 hours).

Whole blood APTT were expected to be significantly elevated over 72 hours. Based on previous studies it is known Factor V and VIII activity will decline significantly after 6-8 hours (5, 12). Statistically significant change of APTT measurements were observed when sample age was >24 hours in the study undertaken by Zürcher *et al.* (12). Totzke *et al.* (11) also showed significant APTT increase over 72 hours. We had proven that samples requested for APTT are not stable after 24 hours and onward. We did not perform APTT on samples stored as whole blood at room temperature within sample age of 24 hours (e.g., test APTT at baseline, after 4, 8, 12, 16 hours, etc., respectively) because we would not want to increase workload for our nightshift lab staff. However, such practice is recommended for future studies as an improvement. Continue to follow the current criteria of APTT maximum allowable age (eight hour maximum) is recommended.

In our study, fibrinogen level has been proven to be stable up to 72 hours in whole blood and up to 96 hours in centrifuged tubes. Studies have shown that fibrinogen level is stable up to seven days when stored at room temperature (13,16), which correlated with our findings. This also justified the action to extend centrifuged fibrinogen measurement to 96 hours and omit measurement at 72 hours, allowing the laboratory to perform fibrinogen add-on from centrifuged citrate tubes over a time course of five days. No significant trend of fibrinogen variation over time has been observed in this study. Some occasional negative mean bias (Table 2) might be accounted by a slow declining of fibrinogen activity over time or the Owren's Veronal Buffer we used was close to its maximum acceptable age, but neither of these two factors caused clinically significant change in fibrinogen measurement.

Overall, some potential interfering pre-analytical factors include one slight lipemic subject, two slight haemolysed subjects, and some specimens were not tested at the exact scheduled time point (maximum one-hour delay). Some participating subjects only had three tubes bled, therefore, evaluation of INR/APTT/fibrinogen after 72h could not be undertaken. However, these limitations did not affect the overall results significantly.

CONCLUSION

Based on our study, we are confident to extend the maximum allowable age of uncentrifuged specimens for INR and fibrinogen tests to 72 hours. Age requirement for uncentrifuged APTT should not be changed. For centrifuged specimens, maximum acceptable age for INR up to 72 hours and fibrinogen up to 96 hours can be extended.

AUTHOR INFORMATION

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