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Original article

Study on the stability of PT, aPTT, and fibrinogen in lyophilized plasma for external quality control

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Abstract. *Background and aim:* The stability of Prothrombin Time (PT), Activated Partial Thromboplastin Time (aPTT), and fibrinogen in lyophilized fresh plasma is an important topic in medical research and applications, especially in quality control programs and coagulation testing. To study the stability of PT, aPTT, and fibrinogen indices in lyophilized plasma according to the International Organization for Standardization (ISO) 13528 and ISO 33405 standards, applied to the external quality assessment program for coagulation. *Methods:* An experimental study on external quality control samples of lyophilized plasma with PT, aPTT, and fibrinogen indices. Fresh frozen plasma from the Blood Hospital was negative for Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV), malaria, and syphilis. The t-test assessed sample stability at different time points and temperatures. 200 samples were produce for each lot. Picking randomly total 35 samples for checking stability. The automatic coagulation analyzer Coagulation System 2000i (CS-2000i) from Sysmex was used. *Results:* Lyophilized plasma containing PT, aPTT, and fibrinogen indices maintained stability when stored at temperatures of 2-8°C and -20°C for up to 3 months, with stability during transport for up to 7 days. *Conclusions:* Lyophilized plasma containing PT, aPTT, and fibrinogen indices-maintained stability when stored at two temperatures, 2-8°C and -20°C, and remained stable during transportation throughout the study period. (www.actabiomedica.it)

Key words: aPTT test, coagulation, external quality assessment, fibrinogen test, lyophilized plasma, PT test

Introduction

Blood clotting studies provide valuable information about an individual's coagulation status, helping clinicians assess the risk of bleeding or thrombosis. Understanding the normal and abnormal values of prothrombin time (PT), activated partial thromboplastin time (APTT), International Normalized Ratio (INR), and D-dimer is crucial for accurate interpretation and effective management of coagulation disorders. The PT index is commonly used in a variety of clinical situations, from monitoring patients on anticoagulants to preoperative preparation. However, the variation of this index can be affected by many factors such as sample collection time, storage conditions, and testing methods. Therefore, studying the stability of the PT index has become an urgent issue. APTT is one of the important indicators in evaluating blood clotting ability. It is used to determine the time required for plasma to form a blood clot when certain clotting factors are added. Fibrinogen is a protein produced mainly in the liver and plays an important role in blood clotting. When stimulated by clotting factors, fibrinogen is converted to fibrin, forming strong networks that help form blood clots (1). The stability of PT, APTT, and fibrinogen indicators in freeze-dried

plasma for external blood coagulation test programs is crucial for accurate and reliable results. The Thrombin Generation Assay is a valuable tool for evaluating the coagulation potential of plasma samples. Citrate or acid citrate dextrose-A (ACD-A) are commonly used anticoagulants for preparing platelet-rich plasma, which is essential for regenerative purposes. The Master Limited Service Laboratory Checklist provides guidelines for ensuring quality and accuracy in laboratory testing procedures. Specimen collection protocols, such as those outlined in the Gainesville Test Catalog, are essential for maintaining the integrity of PT, aPTT, and fibrinogen tests. Hemocompatibility evaluations, as per ISO 10993-4 standards, are necessary for assessing the performance of hemostatic agents in blood coagulation tests (2).3. Further research on the stability of these indicators in freeze-dried plasma is needed to enhance the accuracy and reliability of external blood coagulation test programs. Based on the above issues, we evaluated the stability of PT, aPTT, and fibrinogen in lyophilized plasma for external quality control.

Patients and Methods

Criteria for selecting frozen plasma samples

Fresh frozen plasma samples, within their expiry date and meeting safety standards outlined in Circular No. 26/2013/TT-BYT, which covers blood transfusion activities such as donor selection, blood collection, testing, processing, storage, transportation, and reporting. PT analysis results in the sample were from 10 to 12 seconds, aPTT analysis results in the sample were from 20 to 27 seconds, fibrinogen analysis results in the sample were less than 4g/L. The exclusion criteria for frozen plasma samples included: unstable temperature during plasma storage and failing to meet any of the inclusion criteria.

Procedure steps

Preparation of fresh frozen plasma (FFP)

Fresh frozen plasma was defrosted completely within one hour at room temperature. After adding the preservative mixture, the samples were mixed thoroughly using a magnetic stirrer in a thermostatically controlled bath for 30 minutes.

Plasma filtration and PT, aPTT, fibrinogen measurement

The plasma solutions were filtered through a 0.22 mm filter paper into a wide-mouth flask. Plasma values of PT, aPTT, fibrinogen was determined using the Sysmex CS-2000i automatic coagulation system. The system, from Sysmex, Japan, utilizes a multi-wave length optical measurement method with the following reagents: Actin FSL, LOT: 562674; Calcium Chloride, LOT: 563883; Dade Innovation, LOT: 549783A; Dade Thrombin Reagent, LOT: 565131; Owren's Veronal Buffer, LOT: 569915; CA Clean I, LOT: A1127; CA Clean II, LOT: A1221. Quality control materials include Dade Ci-Trol 1, LOT: 548517 and Dade Ci-Trol 2, LOT: 564845.

Sample set creation

Adjust to create a sample set with typical PT, aPTT, fibrinogen values according to the reagent lot used. Dispense 1 ml of plasma into a 3 ml brown vial and refrigerate at -80°C for at least 24 hours, then freeze-dry according to the lyophilization procedure at the control center and per the manufacturer's instructions.

Homogeneity assessment

Assess homogeneity by randomly selecting ten vials of lyophilized samples at each analysis level, recording, and analyzing using a one-way ANOVA test.

Stability evaluation

After freezing the batch of samples, evaluate their stability at different time and temperature milestones. Assess shipping stability over 1, 3, 5, and 7 days and storage stability over 1, 3, and 5 months. At each evaluation time, three samples from each level were randomly selected to test the PT, aPTT, fibrinogen values compared with the initial value using a t-test.

Homogeneity reassessment after distribution

After distributing and packaging the lyophilized plasma sets, we assess homogeneity by randomly selecting ten vials from each batch, reconstituting with distilled water, and analyzing them twice using the Sysmex CS-2000i.

Stability during transportation and storage

Once the sample set is confirmed homogeneous, we assess its stability during transportation and compared the PT, aPTT, and fibrinogen levels at evaluation with the initial homogenization data, using t-tests in Stata 14.1.

Storage conditions of raw materials

We studied the effects of different storage conditions on raw materials from fresh frozen plasma, stored at -20°C or lower for up to six months to ensure sample properties closely resembled plasma. Fresh frozen plasma was selected as the sample source to minimize interference and meet external research requirements.

Proficiency testing compliance

According to ISO 13528:2022, the proficiency testing provider must ensure that the proficiency testing lots are sufficiently homogeneous and stable to meet the objectives of the proficiency testing program.

External control standards

For the standards of external control samples: It is mandatory that samples in the same batch not only be uniform but also maintain the stability of external control parameters over time and across storage temperatures.

Statistical analysis

Data were collected using a standardized form and entered into Excel. Statistical analyses were performed using a standard software package (Stata, version 14.1; StataCorp). For inferential statistics, normally distributed data were analyzed by analysis of variance (ANOVA), and for non-normally distributed data by..... test. Bonferroni or Holm-Bonferroni methods were used to adjust the significance level and to minimize errors arising from conducting multiple comparisons (3). All statistical tests were two-tailed, with a significance level set at <0.05.

Results

The results of the evaluation of PT, aPTT, and fibrinogen stability stored at two different temperature levels at various time points are shown in the Tables reported below.

According to Table 1, in the PT runs, the lowest value is 10.14, and the highest value is 10.55; in the aPTT runs, the lowest value is 27.10, and the highest value is 28.54; in the fibrinogen runs, the lowest value is 2.56, and the highest value is 2.66.

According to Table 2, in the PT runs, the lowest value is 10.27, and the highest value is 10.49; in the aPTT runs, the lowest value is 27.48, and the highest value is 28.54; in the fibrinogen runs, the lowest value is 2.58, and the highest value is 2.66.

From Table 3, using the lyophilization method, samples stored at 2-8°C and at -20°C showed that the PT, aPTT, fibrinogen indices in the normal sample set remained stable throughout the monitoring period of up to 3 months.

According to Table 4, the normal sample achieved PT test, aPTT test, and fibrinogen test stability during transport over a 7-day period. The PT results after 1, 3, 5, and 7 days were compared with the initial concentration results (homogeneity evaluation data).

Discussion

The stability of the PT index in normal samples stored via freeze-drying at 2-8°C remains consistent for up to 5 months, indicating that this method, with proper storage, effectively preserves PT stability over time (4). Bux et al. (5) studied the quality of lyophilized plasma samples and found that the lyophilization process only affected Factor VIII (FVIII) and von Willebrand Factor (vWF), with a reduction of

Table 1. Results of normal PT level, normal aPTT level, and normal fibrinogen level stability evaluation of the sample during storage at 2-8°C.

Temperature	Time (month)	PT (seconds)			aPTT (seconds)			Fibrinogen (g/L)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
	Initial									
	Sample 1	10.37	10.39	10.38	28.32	28.21	28.27	2.64	2.66	2.65
	Sample 2	10.28	10.32	10.30	28.43	28.54	28.49	2.65	2.62	2.64
	Sample 3	10.45	10.49	10.47	28.24	28.48	28.36	2.60	2.59	2.60
	Sample 4	10.48	10.44	10.46	28.38	28.30	28.34	2.62	2.60	2.61
2 - 8^{0}C	**1 month**									
	Sample 1	10.32	10.34	10.33	27.09	27.10	27.10	2.60	2.59	2.60
	Sample 2	10.40	10.40	10.40	28.14	28.11	28.13	2.59	2.60	2.60
	Sample 3	10.50	10.55	10.53	28.16	28.18	28.17	2.61	2.61	2.61
	Sample 4	10.15	10.14	10.15	28.04	27.93	27.99	2.58	2.56	2.57
	3 months									
	Sample 1	10.43	10.42	10.43	27.55	27.51	27.53	2.62	2.60	2.61
	Sample 2	10.45	10.49	10.47	28.48	28.39	28.44	2.58	2.60	2.59
	Sample 3	10.36	10.31	10.34	27.22	27.40	27.31	2.63	2.61	2.62
	Sample 4	10.45	10.44	10.45	27.50	27.50	27.50	2.60	2.57	2.59
	5 months									
	Sample 1	10.43	10.33	10.38	27.90	27.96	27.93	2.64	2.60	2.62
	Sample 2	10.41	10.43	10.42	27.86	27.81	27.84	2.59	2.56	2.58
	Sample 3	10.42	10.31	10.37	27.40	27.48	27.44	2.60	2.63	2.62
	Sample 4	10.37	10.44	10.41	28.01	27.97	27.99	2.61	2.60	2.61

Note: Run 1 and Run 2: respectively, the results of two runs of samples 1, 2, 3, and 4 were randomly selected at the evaluation time. Mean: the average result of two runs of samples 1, 2, 3, and 4 randomly selected at the evaluation time. Initial: results of the homogeneity evaluation.

Table 2. Results of normal PT level, normal aPTT level, and normal fibrinogen level stability evaluation of the sample during storage at -20°C.

Temperature	Time (month)	PT level (seconds)			aPTT level (seconds)			Fibrinogen (g/L)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
	Initial									
	Sample 1	10.37	10.39	10.38	28.32	28.21	28.27	2.64	2.66	2.65
	Sample 2	10.28	10.32	10.30	28.43	28.54	28.49	2.65	2.62	2.64
	Sample 3	10.45	10.49	10.47	28.24	28.48	28.36	2.60	2.59	2.60
	Sample 4	10.48	10.44	10.46	28.38	28.30	28.34	2.62	2.60	2.61
-20^{0}C	**1 months**									
	Sample 1	10.42	10.40	10.41	28.12	28.09	28.21	2.65	2.62	2.63
	Sample 2	10.29	10.33	10.31	28.07	28.07	28.07	2.64	2.61	2.61
	Sample 3	10.47	10.46	10.47	28.20	28.19	28.20	2.60	2.59	2.61
	Sample 4	10.39	10.43	10.41	28.11	28.10	28.11	2.63	2.60	2.62
	3 months									
	Sample 1	10.45	10.44	10.45	27.48	27.50	27.49	2.64	2.63	2.64
	Sample 2	10.27	10.27	10.27	27.52	27.51	27.52	2.63	2.66	2.65
	Sample 3	10.48	10.47	10.48	28.43	28.41	28.42	2.60	2.58	2.59
	Sample 4	10.41	10.40	10.41	27.49	27.52	27.51	2.62	2.64	2.63
	5 months									
	Sample 1	10.39	10.38	10.39	28.05	28.02	28.04	2.66	2.62	2.64
	Sample 2	10.29	10.29	10.29	27.93	27.90	27.92	2.65	2.62	2.64
	Sample 3	10.43	10.41	10.42	27.67	27.64	27.66	2.64	2.60	2.62
	Sample 4	10.44	10.45	10.45	28.10	28.09	28.10	2.64	2.63	2.64

more than 10% compared to pre-lyophilization levels. However, fibrinogen and other coagulation factors remained relatively stable. This is consistent with our study on the stability of PT, aPTT, and fibrinogen when stored at two temperatures (2-8°C and -20°C) for up to 3 month. In a study by Aida Mehmedagić et al (6) on the effect of FVIII activity and the prolongation of APTT caused by heparin, they reported that adding FVIII concentrate to heparinized plasma significantly affected APTT results. Specifically, the activity of FVIII increased, shortening APTT, and this effect was more pronounced when a large amount of heparin was present in the plasma. This is consistent with our study, where aPTT started to become unstable when stored at two temperatures (2-8°C and -20°C) from 5 months. In a similar study on the stability of coagulation factors in plasma stored under different conditions, Woodhams et al (7) collected plasma from a large number of healthy individuals, unaffected by clinical conditions or medications they were using. The samples were unclotted with citrate and processed under optimal conditions, making the data fully applicable to the case of preparing raw materials for the production of standard samples. At -24°C, APTT values increased over storage time and were one of the least stable parameters. This is consistent with our study, where aPTT started to become unstable when stored at temperatures (-20°C) from 5 months. Jennings et al (8) studied the stability of lyophilized plasma samples exposed to temperatures of 31.9°C to 39.7°C during transportation for 1 to 8 weeks. After 6 weeks, PT, APTT, and fibrinogen changes were under 0.5% at 22°C, under 2.5% at 30°C, and up to 9% at 37°C. One-way ANOVA showed no significant differences in these parameters across 2, 4, and 6 weeks at 22°C, 30°C, and 37°C (*P:* > 0.05). The study also confirmed stability across different sample types, including those from healthy volunteers, warfarin users, and hemophilia patients This study is consistent with our research on the stability of plasma samples (PT, aPTT, fibrinogen) when exposed to temperatures ranging from 28°C to 30°C during 7 days of transportation. Fibrinogen is an important factor in the coagulation process, and the fibrinogen level in plasma can reflect the patient's coagulation status. When the fibrinogen results are inaccurate during testing, it can significantly affect clinical decisions and the diagnostic and treatment process. In Zur's et al (9) study,

Table 3. Stability of PT, aPTT, fibrinogen storage for the sample set under different temperature conditions.

Temperature	Time	*P* - value		
		PT	aPTT	Fibrinogen
2 - 8 °C	**1 month**	0.61	0.10	0.11
	3 months	0.81	0.05	0.24
	5 months	0.85	0.03	0.39
– 20 °C	**1 month**	0.84	0.07	0.52
	3 months	0.89	0.07	0.76
	5 months	0.23	0.04	0.35

Table 4. Stability of PT, aPTT, and fibrinogen during transport for the sample lots.

Time (day)	PT				aPTT				Fibrinogen			
	Mean 1	Mean 2	Mean 3	Mean 1	Mean 2	Mean 3	Mean 1	Mean 2	Mean 3	Mean 1	Mean 2	Mean 3
Initial	10.41	10.39	10.40		28.51	28.56	28.56		2.64	2.60	2.60	
1	10.40	10.39	10.39	0.18	28.13	28.46	28.87	0.80	2.58	2.57	2.58	0.09
3	10.40	10.40	10.41	0.67	28.11	28.04	28.14	0.40	2.60	2.60	2.62	0.74
5	10.39	10.40	10.39	0.53	28.26	28.02	28.42	0.12	2.60	2.58	2.58	0.06
7	10.35	10.40	10.38	0.37	28.45	28.13	27.97	0.66	2.61	2.59	2.60	0.27
***P*-value**	*P:>0.05*				*P:>0.05*				*P:>0.05*			

Note: Average 1, Average 2, Average 3: respectively, the average results of two runs of samples 1, 2, and 3 were randomly selected at the evaluation time. Initial: results of the homogeneity evaluation.

lyophilized plasma stability at 25°C showed a significant decrease in fibrinogen levels after 6 and 12 months (*P:* < 0.05), consistent with the manufacturer's data, which reported a 46% reduction after 24 months at 23°C–28°C. At -40°C, fibrinogen decreased significantly after 6 months, particularly after 12 months (*P:* ≤ 0.001), indicating that long-term transport at high temperatures should be avoided. Moreover, the same authors (9) studied lyophilized plasma stability at 4°C, 25°C, and 40°C for up to 12 months. Fibrinogen concentrations significantly decreased after 6 months at 4°C (*P*: 0.04) and 25°C (*P*: 0.02), with a more significant decrease at 40°C (*P:* < 0.001). This aligns with Bux et al (5) study, which found coagulation factors in lyophilized plasma stored at 4°C remained stable for 24 months. Lyophilized plasma samples stored at 2–8°C and -20°C showed stability within 10 weeks, suggesting 4°C is optimal for storage, with stability declining at higher temperatures. Both studies are consistent with our findings, where fibrinogen remained stable during the 3-month storage period at 2–8°C and -20°C. Potential limitations of our study include batch-to-batch variations that may lead to inconsistencies in sample composition or properties, as well as the accuracy of the equipment used in the analysis. Variations in the calibration and performance of instruments, as well as human errors in handling or processing the samples, may also affect the reliability and reproducibility of the results in experimental studies on external quality control samples of lyophilized plasma with PT, aPTT, and fibrinogen indices.

Conclusion

Lyophilized plasma containing PT, aPTT, and fibrinogen indices maintained stability when stored at two temperatures, 2-8°C and -20°C, and remained stable during transportation throughout the study period.

Ethic Approval: This study followed ethical standards set by national committees, the 1964 Declaration of Helsinki and its amendments. It was approved by the Biomedical Research Ethics (approval number 150/HDDD, January 17, 2022). Consent was obtained from all blood donors, and their information remained confidential, with their health and mental well-being safeguarded.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Authors Contribution: Hong Nguyen, Tung Tran and Huy Vu designed the study, Hong Nguyen and Phuc Huynh analysed data and wrote the first original draft of manuscript. All authors revised critically the manuscript content, edited it for intellectual content and contributed to discussion. Hong Nguyen and Tung Tran agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved All authors revised the final version and approved the submitted version.

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