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Original article

Effect of anticoagulants, temperature, and storage time on G6PD activity measured by automated enzymatic UV assay

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Abstract

***Background**:* The effects of anticoagulants, temperature, and storage duration on glucose 6-phosphate dehydrogenase (G6PD) activity measurements using the automated enzymatic UV assay (via clinical chemistry analyzer) are not well established, with no standardized guidelines. This study aimed to clarify these factors to improve the reliability of G6PD testing in stored blood samples.

***Objectives**:* To evaluate the impact of anticoagulants (EDTA and heparin), temperature (room temperature (RT), 4°C, and -20°C), and storage duration on G6PD activity measurement using the automated enzymatic UV assay.

***Methods**:* Leftover EDTA- and heparin-packed red blood cell samples from routine testing were stored at three temperatures. G6PD activity and hemoglobin (Hb) levels were measured daily from Day 1 to Day 7 to assess stability.

***Results**:* G6PD activity in EDTA samples at 0 h was 12.7 ± 0.7 U/g Hb, increasing at RT by Day 3 (15.4 ± 1.3 U/g Hb; P <0.001), but declined by Day 7 (13.5 ± 1.0 U/g Hb; P = 0.152). At 4°C, activity remained stable for four days before rising due to Hb degradation (15.4 ± 0.8 U/g Hb; P <0.001), then declined at -20°C (11.2 ± 0.6 U/g Hb; P = 0.009), suggesting enzyme degradation. In heparin samples, activity started at 11.3 ± 0.6 U/g Hb, showed increased activity at RT (13.0 ± 1.0 U/g Hb; P = 0.019), stability at 4°C (11.7 ± 0.6 U/g Hb; P = 0.133), and decreased at -20°C (9.9 ± 0.6 U/g Hb; P = 0.002). Significant differences appeared only between RT and -20°C for both anticoagulants (P <0.05). Hb levels in EDTA samples decreased significantly at 4°C, from 215.3 ± 5.0 g/L Hb on Day 5 to 196.2 ± 5.5 g/L Hb on Day 7 (P = 0.019), while those in heparin samples remained stable.

***Conclusion**:* Storage at 4°C best preserved G6PD activity for up to four days. EDTA was more effective than heparin in maintaining enzyme stability.

***Keywords**:* Anticoagulants, , G6PD activity, hemoglobin stability, storage duration, storage temperature.

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Glucose 6-phosphate dehydrogenase (G6PD) is the first enzyme in the pentose phosphate pathway (PPP) and plays a crucial role in generating NADPH, an essential coenzyme for glutathione reductase and glutathione peroxidase. These enzymes help eliminate reactive oxygen species (ROS) and maintain cellular redox balance. As a result, G6PD is vital for protecting cells, particularly red blood cells, from oxidative damage.[1] G6PD deficiency, an X-linked recessive genetic disorder, increases the risk of acute hemolytic anemia (AHA) when individuals are exposed to oxidative stressors, such as naphthalene, fava beans, and certain antimalarial drugs. However, under normal conditions, affected individuals are typically asymptomatic.[2] Due to this risk, screening for G6PD deficiency is recommended for malaria patients before administering antimalarial drugs to prevent potential adverse effects.[3] Additionally, newborns with G6PD deficiency are at risk of developing hyperbilirubinemia and neonatal jaundice, which, if left underdiagnosed and untreated, may lead to bilirubin toxicity and intellectual disabilities. Currently, G6PD deficiency affects over 500 million people worldwide.[4] To prevent severe complications caused by delayed diagnosis, the World Health Organization (WHO) recommends newborn screening in regions where the prevalence of G6PD deficiency in males is 3–5% or higher.[5]

G6PD deficiency can be diagnosed using various methods, including the methemoglobin reduction test (MRT), G6PD activity test, and fluorescent spot test (FST). All of these methods require fresh blood samples, which have limited storage stability-24 hours (h) if kept at room temperature (RT) or within three days if stored at 4°C.[6] Although immediate testing with fresh samples is ideal for accurate screening, it is often impractical due to multiple logistical challenges, including:

remote or rural setting malaria patients in remote areas often face limited access to laboratory infrastructure, trained personnel, and electricity-dependent diagnostic tools. Consequently, blood samples must be transported to better-equipped laboratories, where maintaining appropriate temperature and minimizing transport time are critical to preserving sample integrity.

neonatal testing constraints blood collection from newborns is invasive and is usually performed alongside other diagnostic procedures, limiting the opportunity for timely G6PD testing.

infrequent testing schedules some hospitals only conduct G6PD deficiency screening once a week, necessitating extended sample storage and raising concerns about red blood cell degradation and compromise enzyme stability.

Even when blood samples are stored under recommended conditions, variations in storage duration and temperature can impact G6PD activity, potentially leading to inaccurate results. While prior studies have evaluated the effects of time and temperature on sample integrity for G6PD deficiency testing[7, 8], there is still limited research exploring how different anticoagulants in combination with storage conditions, influence G6PD enzyme activity.

This study aims to evaluate G6PD enzyme activity in blood samples preserved with two commonly used anticoagulants, EDTA and heparin, under different storage conditions (RT, 4°C, and -20°C). The evaluation was conducted using an automated enzymatic UV assay, a reliable and widely used automated technique in hospital diagnostic services.[9, 10] The findings from this research will help determine the optimal anticoagulant, storage duration, and temperature conditions that best preserve red blood cell integrity and G6PD enzyme activity. Establishing these parameters will enhance the accuracy and efficiency of G6PD deficiency screening, ensuring precise patient diagnoses and providing reliable guidelines for laboratory procedures. Ultimately, this will minimize errors caused by enzyme degradation, improving diagnostic outcomes for individuals at risk of G6PD deficiency-related complications.

Materials and methods

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA No. 0870/2024).

Study design

Leftover blood samples from healthy volunteers were collected in EDTA and heparin tubes at King Chulalongkorn Memorial Hospital (KCMH). A total of 22 blood samples (16 females and 6 males) were included in the study. G6PD activity and hemoglobin concentration were first measured on the day of collection (Day 1). For storage experiments, blood samples were centrifuged at 3,000 rpm for 5 minutes to collect packed red cells, which were then aliquoted into microtubes and stored at three different temperatures: RT, 4°C, and -20°C.

Each of the 22 samples was divided into multiple aliquots for repeated measurements across different time points and storage conditions. For each temperature condition, both EDTA and heparin samples were evaluated as follows:

- Room temperature: A total of 220 aliquots each for EDTA and heparin (22 samples x 10 time points). Time points included 0, 4, 8, and 12h on Day 1, and daily from Day 1 to Day 7.
- 4°C: A total of 154 aliquots each for EDTA and heparin (22 samples x 7 time points: Daily from Day 1 to Day 7).
- -20°C: A total of 176 aliquots each for EDTA and heparin (22 samples x 8 time points: Daily from Day 1 to Day 7, and 1-month follow up).

To avoid temperature fluctuations (cool-warm or freeze-thaw cycles), each aliquot was retrieved and analyzed only on its designated assay day.

Hemoglobin and G6PD activity assays

G6PD enzyme activity was assessed using an automated enzymatic UV assay (BS-360E, Mindray Medical International, PRC). A 22 µL packed red cell sample was mixed with 1,000 µL of distilled water in a microtube and was incubated for 5 minutes before measurement. Hemoglobin concentration and G6PD activity were analyzed at 505 nm and 340 nm, respectively. The change in absorbance for G6PD activity was calculated using the formula (ΔA/min sample) – (ΔA/min blank). Results were expressed as units per gram of hemoglobin (U/g Hb) to ensure consistency. Instrument calibration and quality control checks, including positive and negative control tests, were conducted before each analysis. G6PD activity was classified based on predefined cut-off values: <30% of the reference value for G6PD deficiency; 30–80%: intermediate deficiency; and >80% for normal activity.

Statistical analysis

Data from Hb testing and G6PD activity assays were analyzed for statistical differences. The Kolmogorov–Smirnov and Shapiro–Wilk tests were used to assess data normality of the data. Results were presented as mean ± standard deviation (SD). Statistical significance was determined using one-way and two-way repeated measures analysis of variance (ANOVA). All analyses were conducted using SPSS version 29.0.1.

Results

Comparison of G6PD activity in packed red cells stored at RT, 4°C, and -20°C.

For blood samples stored in EDTA at 0h, G6PD activity was 12.7 ± 0.7 U/g Hb. When stored at room temperature, G6PD activity significantly increased to 15.4 ± 1.3 U/g Hb on Day 3 (mean difference = -2.7, 95% CI [-4.1, -1.5], P <0.001), before gradually returning toward baseline levels (13.5 ± 1.0 U/g Hb) by Day 7 (mean difference = -0.8, 95% CI [-3.1, 2.1], P = 0.152) (**Figure 1A**). In contrast, samples stored at 4°C showed stable G6PD activity during the first four days, followed by a significant increase from Day 5 onward, reaching 15.4 ± 0.8 U/g Hb by Day 7 (mean difference = -2.7, 95% CI [-3.9, -1.7], P <0.001). Samples stored at -20°C exhibited a sharp decline in G6PD activity by Day 2 (11.2 ± 0.6 U/g Hb; mean difference = 1.5, 95% CI [0.2, 2.6], P = 0.009) and remained relatively stable thereafter. After one month at -20°C, G6PD activity declined further to 9.6 ± 0.5 U/g Hb (mean difference = 3.1, 95% CI [0.6, 5.4], P = 0.005). A significant decrease in Hb levels was observed only in samples stored at 4°C, with Hb declining from 215.3 ± 5.0 g/L Hb on Day 5 to 196.2 ± 5.5 g/L Hb on Day 7 (mean difference = 19.1, 95% CI [1.7, 36.4], P = 0.019).

Comparing storage conditions, blood stored in EDTA showed a significant difference in G6PD activity between room temperature and -20°C from Day 2 onward, with G6PD activity at room temperature (14.9 ± 0.9 U/g Hb) significantly higher than at -20°C (11.1 ± 0.7 U/g Hb; mean difference = 3.8, 95% CI [0.7, 6.5], P = 0.01). However, Hb levels remained stable across all storage conditions (RT, 4°C, and -20°C, P = 1.0) (**Figure 1B**), suggesting that changes in G6PD activity were likely due to enzyme stability rather than Hb degradation.

For blood samples stored in heparin, initial G6PD activity at 0h was 11.3 ± 0.6 U/g Hb. At room temperature, G6PD activity significantly increased by Day 4 (13.0 ± 1.0 U/g Hb; mean difference = -1.7, 95% CI [-3.0, -0.2], P = 0.019), whereas at 4°C, it remained stable until Day 7 (11.7 ± 0.6 U/g Hb; mean difference = -0.4, 95% CI [-0.9, 0.1], P = 0.133) (**Figure 1C**). In sample stored at -20°C, G6PD activity sharply declined to 9.9 ± 0.6 U/g Hb by Day 2 (mean difference = 1.4, 95% CI [0.4, 2.3], P = 0.002) and remained stable up to one month. Similar to EDTA-stored blood, significant differences in G6PD activity between room temperature and -20°C were

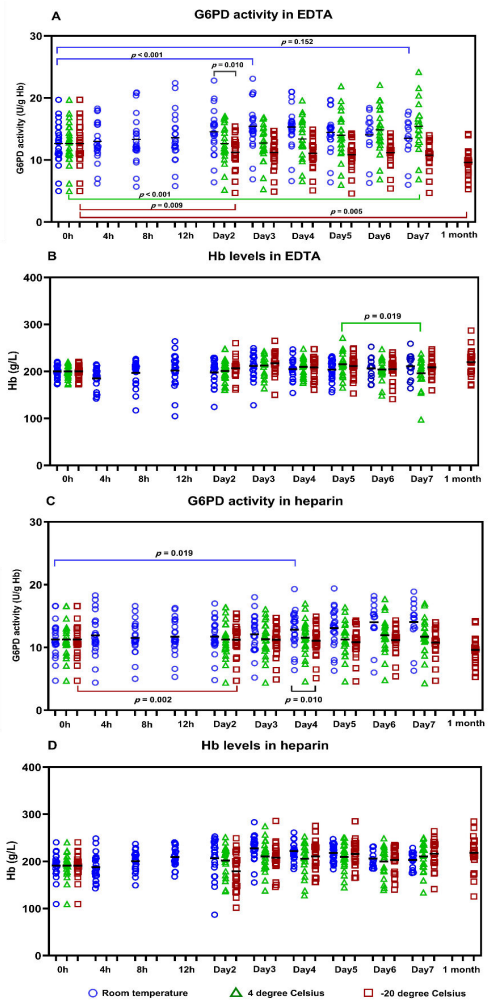

Figure 1. Stability of G6PD activity in packed red cells stored in EDTA and heparin. **(A)** G6PD activity in EDTA **(B)** Hb levels in EDTA **(C)** G6PD activity in heparin **(D)** Hb levels in heparin.

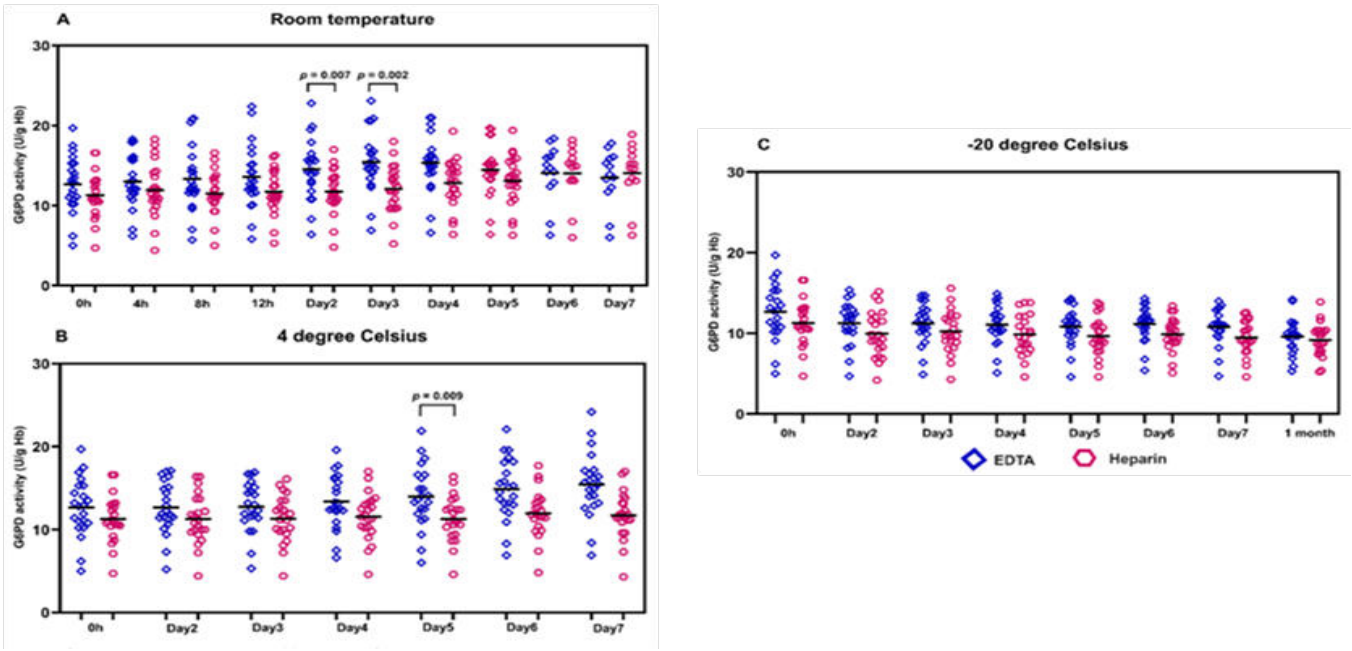

Figure 2. Comparison of G6PD activity in packed red cells stored in EDTA and heparin tubes under the same temperature conditions. **(A)** room temperature **(B)** 4°C **(C)** -20°C.

observed in heparin-stored blood, although these differences appeared later, from Day 4 onward. G6PD activity at room temperature was 13.0 ± 0.8 U/g Hb compared to 9.9 ± 0.6 U/g Hb at -20°C (mean difference = 3.1, 95% CI [0.6, 5.5], P = 0.010). Despite these variations, Hb levels remained stable across all storage conditions (**Figure 1D**).

Comparison of G6PD activity in packed red cells stored in EDTA and heparin

When comparing G6PD activity between EDTA- and heparin-stored samples at the same temperature, a significant difference was observed in room temperature beginning on Day 2. G6PD activity 14.5 ± 0.7 U/g Hb in EDTA samples and 11.7 ± 0.7 U/g Hb in heparin samples (mean difference = 2.8, 95% CI [0.8, 4.8], P = 0.007). This difference persisted on Day 3, with G6PD activity increasing to 15.4 ± 1.3 U/g Hb in EDTA samples and 12.1 ± 0.7 U/g Hb in heparin samples (mean difference = 3.3, 95% CI [1.3, 5.4], P = 0.002) (**Figure 2A**). At 4°C, a significant difference emerged from Day 5 onward, with G6PD activity of 14.0 ± 3.8 U/g Hb in EDTA samples compared to 11.3 ± 2.7 U/g Hb in heparin samples (mean difference = 2.7, 95% CI [0.7, 4.7], P = 0.009) (**Figure 2B**). In contrast, no significant differences in G6PD activity were observed between the two anticoagulants at 20°C (**Figure 2C**). Although G6PD activity was slightly higher in EDTA-stored samples at 0h, the difference was not statistically significant.

Discussion

In this study, we evaluated the impact of anticoagulants (EDTA and heparin), storage temperature (room temperature, 4°C, -20°C), and storage duration on G6PD enzyme activity using the automated enzymatic UV assay. Room temperature was selected in this study due to the logistical challenges of storing and transporting blood samples from malaria patients in remote areas, where electricity-dependent analytical equipment is often unavailable. Since these samples must be transported to a laboratory for screening, refrigeration for preservation is frequently impractical or unavailable during transit. Given these constraints, storing samples at room temperature offers a practical and viable alternative.

Our findings demonstrate an increase in G6PD activity at room temperature by Day 3, despite stable hemoglobin (Hb) levels, suggesting that oxidative stress may contribute to this increase. We propose that elevated ROS levels under room temperature conditions promote oxidative stress, which in turn enhances the interaction between SIRT2 and G6PD enzymes. This interaction increases SIRT2's deacetylation activity, leading to the deacetylation of G6PD at K403 in a SIRT2-dependent manner[11], thereby upregulating G6PD enzymatic activity to maintain cellular NADPH homeostasis. Supporting this proposed mechanism, Preston K et al.[12] reported that oxidative stress accumulation plays a key role in the development of RBC storage lesions, potentially triggering compensatory antioxidant responses, including increased G6PD activity. In contrast, previous studies have reported a continuous decline in G6PD activity at room temperature from Day 1 to Day 3[13], highlighting potential variability depending on study conditions and storage environments.

Storing red blood cells at 4°C reduces their metabolic activity, extending their preservation. In contrast, higher temperatures increase enzyme degradation and accelerate catalyzed reactions. At 4°C, our results showed that G6PD activity remained stable for the first four days, aligning with findings from Pengboon P et al.[13] , who also reported stability during this period. However, we observed a significant decrease in Hb levels in EDTA-stored blood at 4°C, which may have contributed to the increase in G6PD activity on Day 5. In contrast, our findings differ from studies indicating that blood stored at 4! for one week lost approximately 16% of G6PD activity.[14] For temperature at -20°C, where metabolic processes are effectively halt due to freezing, G6PD activity significantly declined, with the most pronounced decrease occurring between Day 1 and 2. This decline may be attributed to rapid freezing, which promotes the formation of small ice crystals and increases protein interaction with the ice-liquid interface, leading to protein denaturation and damage.[15]

When comparing anticoagulants, EDTA and heparin are commonly used in modern laboratory settings for their effectiveness in preventing blood clotting. While both inhibit coagulation effectively, EDTA is primarily preferred for hematological analyses, whereas heparin is commonly used in clinical chemistry tests due to its minimal interference with biochemical reactions, particularly in assays sensitive to metal ions. In contrast, EDTA strongly chelates calcium and other metals, which can affect certain biochemical processes. Our data showed that EDTA better preserved G6PD enzyme activity than heparin when measured using the automated enzymatic UV assay. This may be attributed to EDTA's ability to chelate metal ions such as Ca^{2+}, preventing coagulation and stabilizing enzyme proteins by minimizing metal-ion-induced destabilization. These findings are consistent with those of Jalil N et al.[1], who recommended EDTA as the optimal anticoagulant for preserving G6PD activity in erythrocytes.

Conclusion

This study highlights the impact of anticoagulants, storage temperature, and duration on G6PD enzyme activity in blood samples. EDTA was more effective than heparin in preserving G6PD activity, likely due to its metal ion-chelating properties. Storage at 4°C best maintained enzyme stability, while room temperature led to transient increase in activity, possibly through oxidative stress-induced upregulation. In contrast, freezing at -20°C significantly reduced G6PD activity, likely due to protein denaturation. These findings have practical implications for improving diagnostic accuracy, particularly for G6PD deficiency screening in malaria endemic or resource-limited settings. Limitations include donor variability and unaccounted pre-analytical factors, as well as the absence of oxidative stress marker assessment. Future studies should investigate underlying mechanisms and explore stabilization strategies suitable for field conditions.

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Conflict of interest statement

The authors declare no commercial or financial relationships that could be perceived as potential conflict of interest.

Data sharing statement

The data that support the findings of this article cannot be made publicly available because they are part of a commercially valuable project. The data may be made available upon reasonable request from the authors.

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