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Assessment of Glucose-6-Phosphate Dehydrogenase Activity with a Delay

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ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) is the most common and important enzyme deficiency in red blood cells. Lack of G6PD causes severe hyperbilirubinemia and increase the risk of kernicterus in neonates. A reliable diagnosis of G6PD status by spectrophotometry is costly, has a turn-around time of several hours, and requires good laboratory infrastructure; limitations that render routine G6PD testing by spectrophotometry unsuitable in most remote areas. In this study, we analyze different storage conditions to determine the best sample handling way during the pre-analytical step. G6PD activity was analyzed in blood samples from 10 non-G6PD-deficient adult males by the spectrophotometric quantitative method in different conditions of time and temperature. Results showed that sample hemolysate with glycerol 30% at 4°C (9.00±0.63) and heparin at 4°C (11.48±0.62) stabilize G6PD activity level within normal range 21 days after the initial dosage at t_0. At 30°C, heparin stabilized G6PD activity level within a normal range (10.24±2.30) during 4 days. In sample hemolysate with 30% glycerol stored at -20°C, G6PD activity did not vary significantly during the 3 months of study. In a laboratory where frozen sample at -20°C is possible, our results suggest that the best way of keeping samples for delay G6PD activity measurement was to prepare hemolysate with glycerol 30% and store at -20°C. In a condition where the temperature is high, heparin anticoagulant seems to be more suitable for G6PD enzyme activity determination.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) is a vital protective enzyme present in all body cells [1,2]. G6PD deficiency is the most common and important enzyme deficiency in red blood cells. Lack of G6PD might cause severe hyperbilirubinemia and increase the risk of kernicterus in neonates [3]. According to the World Health Organization, 7.5% of the world's population has one of the G6PD deficiency variants, of which about 3.4% are at risk for potential pathology [4]. In the black population of the United States and the Mediterranean countries, the prevalence in G6PD is 1/40 to 1/10 [5]. In West Africa, the prevalence of G6PD deficiency is estimated at 15 to 26% [6]. The World Health Organization recommends routine G6PD deficiency screening in newborns in areas where the prevalence of G6PD deficiency can be as high as 3-5% or more [7]. Studies have shown that early deficit detection reduces mortality and morbidity in newborns by establishing the etiological diagnosis of jaundice and facilitating therapeutic choices [3]. In Benin, neonates are not routinely screened for G6PD deficiency due to many reasons.

The gold standard for determining G6PD status is through direct measurement of G6PD activity, normalized either by red blood cell count or hemoglobin concentration [8]. The statute is then defined based on where this value lies relative to a normal G6PD value. Unfortunately, as a consequence of poor standardization of G6PD enzyme assay kits, it is hard to ensure the enzyme deficiency diagnosis in remote areas. Alternative approaches have been considered in some countries with the establishment of specialized reference centers for enzymatic G6PD deficiency detection [9].

A reliable diagnosis of G6PD status by spectrophotometry is costly, has a turn-around time of several hours, and requires good laboratory infrastructure; limitations that render routine G6PD testing by spectrophotometry unsuitable in a most remote area in Sub-Saharan Africa countries [10]. Available field applications for G6PD deficient diagnosis provide a qualitative result with reliably identifying only individuals with less than 30% enzyme activity [10]. Because G6PD deficiency results from an X-linked mutation, females can be fully deficient (homozygotes), partially deficient (heterozygotes) or G6PD normal, whereas males are either hemizygote deficient or normal. The degree of deficiency in heterozygous females varies due to random X-inactivation (lyonization) [11]. Current qualitative tests are unsuitable for females heterozygous for the G6PD gene who may have a G6PD normal qualitative result while still being at risk of drug-induced hemolysis [12]. Heterozygous females can only be diagnosed by

either quantitative assays or genetic testing. Diagnosis of patients with moderate G6PD enzyme deficiency requires a safe quantitative assay method with the least possible bias. In this study, we propose an improvement of the pre-analytical phase of the most used G6PD assay method in health centers in Benin. Besides, the stability of the G6PD enzyme activity was studied under different storage conditions and with different time intervals between blood collection and initial testing time.

MATERIAL AND METHODS

The study was carried out from March to June 2019. It was approved by the National Research Ethics Review Boards of Benin and participants provided informed consent. The blood sample was collected from each of ten healthy subjects with normal G6PD activity in tubes containing K2-EDTA and lithium heparin anticoagulants.

A part of the sample collected on K2-EDTA was washed several times and the red cell pellet was lysed by adding hemolysing Solution (Digitonin). The lysate homogenate was centrifuged and glycerol was added to the supernatant at a concentration of 30%. The mixture was aliquoted (100 µl/aliquot) and stored at different conditions. The remind part of K2-EDTA samples and samples collected in lithium heparin tubes were also aliquoted in a volume of 200µl aliquot and stored as described above.

- Heparin, +4°C: Blood taken on lithium heparin and stored at 4°C;

- Heparin, +30°C: Blood was taken with lithium heparin and stored at ambient temperature;

- Glycerol, +30°C: Hemolysate treated with 30% glycerol and stored at room temperature;

- Glycerol, +4°C: Hemolysate treated with 30% glycerol and stored at 4°C;

- Glycerol, -20°C: Hemolysate treated with 30% glycerol and stored at -20°C;

- EDTA, -4°C: Hemolysate without glycerol blood stored at 4°C;

- EDTA, -20°C: Hemolysate without glycerol blood stored at -20°C.

G6PD activity was determined by an enzymatic kinetic assay for the quantitative determination of G6PD enzymatic activity using a commercial kit from Cypress Diagnostics (Cypress Diagnostics, Langdorp, Belgium). The G6PD quantitative spectrophotometric assay is based

on the evaluation of absorbance given by NADPH formation. The quantitative evaluation is made by adding a precise amount of hemolysate to an assay mixture containing the substrate (glucose-6-phosphate) and its cofactor NADP. The rate of NADPH generation is measured at a wavelength of 340 nm. All G6PD measurements were undertaken in duplicate and if there was an error message, a third measure was taken. G6PD activity was subsequently normalized to hemoglobin (Hb) level. The result is expressed as G6PD U/Hb ratios. Hemoglobin was assayed in the hemolysate by the method of Drabkin [13].

Statistical analyses

Data were analyzed by SigmaPlot statistical analysis software 2014 (Systat Software, Inc. San Jose, CA, USA). Means and standard errors on the mean (SEM) of blood parameters were calculated. Student's t-test was used to ascertain any difference between the group characteristics. A *p*-value of <0.05 was deemed significant.

RESULTS AND DISCUSSION

RESULTS

Repeatability

The repeatability study was performed on a control hemolysate taken the same day and using the same batch of reagents. Three blood samples from different subjects were tested. Ten assays were performed for each sample (Table 1). The CV from each assay series was below the recommended CV for validation of assays for G6PD enzymatic activity.

Table No. 1: Intra-essay validation

Sample No	Measurement	Mean (SD)	%CV	Remanded %CV
1	10	14.42 (2.05)	14.22	18.50
2	10	11.69 (1.73)	14.80	18.50
3	10	9.17 (1.06)	11.56	18.50

Results are presented mean (Standard error of the mean); CV, constant of variation

G6PD enzymatic activity stability

The time course of G6PD enzymatic activity level for10adult males with normal G6PD activity was tabulated in Figure1. The first time dosage of G6PD enzymatic activity was determined 12 min after sample collection. In hemolysate without additive and hemolysate with glycerol kept at 30°C, results showed a significant decrease ($p < 0.05$) in G6PD activity 30min after first time dosage (fig. 1). The decrease in G6PD activity was maintained during the time course experience and reached undetectable levels 2 days (2D) and 7 days (7D) later respectively in hemolysate without additive and in hemolysate with glycerol (Fig. 1). In hemolysate without additive kept at -20°C, G6PD activity decreased 4h after first time dosage (4H). In hemolysate isolated from the sample collected on heparin tube and kept at 30°C, G6PD enzymatic activity did not vary significantly during the first 12h (12H) of experience. But the activity decreased significantly at 24h (24H) ($p < 0.05$) to reached undetectable level at day 2 (2D) (Fig. 1). In hemolysate containing glycerol and kept at 4°C, G6PD enzymatic activity was stable during 7 days (7D) but decreased significantly at 14 days (14D) of the time course ($p < 0.05$). In samples collected on heparin tube and kept at 4°C, G6PD enzymatic activity was stable until 14 days (14D) before decreasing significantly at 21 days (21D) ($p < 0.01$) compared to a level of the first dosage at t_0 (fig. 1). G6PD activity decreased to an undetectable level at the 3rd-month dosage (fig. 1) in heparin samples kept at 4°C. G6PD enzymatic activity level showed no significant variation in hemolysate containing glycerol kept at -20°C compared to starting dosage throughout of the experience (fig. 1).

Fig. 1

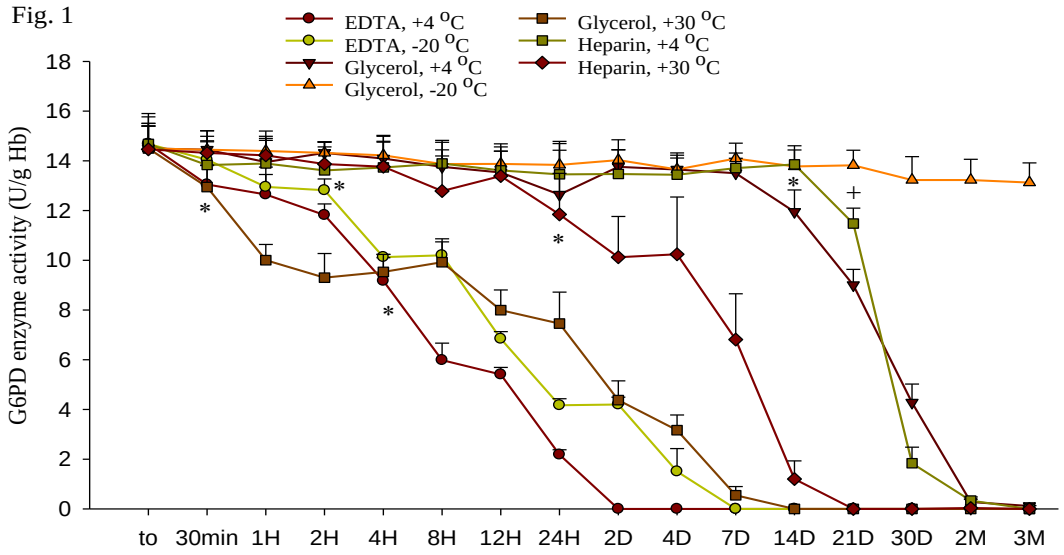

Figure No. 1: The variation of G6PD enzymatic activity is determined over time. For the sample taken on lithium Heparin, whole blood was stored until the time of assay. For assays

performed on EDTA and glycerol, the samples were taken on EDTA and the hemolysate was prepared within 12 minutes of collection. The hemolysates were placed in an aliquot of 100µl and stored under indicated conditions until the assay. The EDTA curves are obtained from hemolysates carried out on EDTA tubes without any additive. Glycerol curves are obtained from hemolysates to which glycerol was added at 30%. The results are expressed as mean±SEM on the mean and represent 10 independent experiments (n=10) (*p <0.05).

G6PD enzymatic activity level was compared in hemolysate isolated from samples collected in EDTA tube to which glycerol 30% was added or not. Samples on heparin tube were kept as a whole and hemolysate was extemporally generated at the time of dosage. All 3 types of sample were kept at 4°C.G6PD enzymatic activity level showed no significant variation in the 3 types of hemolysate during 2 hours (2H). From 4h (4H) however, G6PD enzymatic activity level decreased significantly (p <0.005) in hemolysate without additive compared to the initial dosage at t_0 (Fig. 2). G6PD enzymatic activity level was steady in both hemolysate with glycerol and heparin samples during 7 days (7D) (Fig. 2). At day 14 however, G6PD activity decreased significantly in hemolysate containing glycerol compared to the starting level (p <0.05). In heparin samples, however, the level of G6PD enzymatic activity was unchanged at day 14 (14D) but decreased significantly at day 21 (21D) (p <0.01) (Fig. 2).

Fig. 2

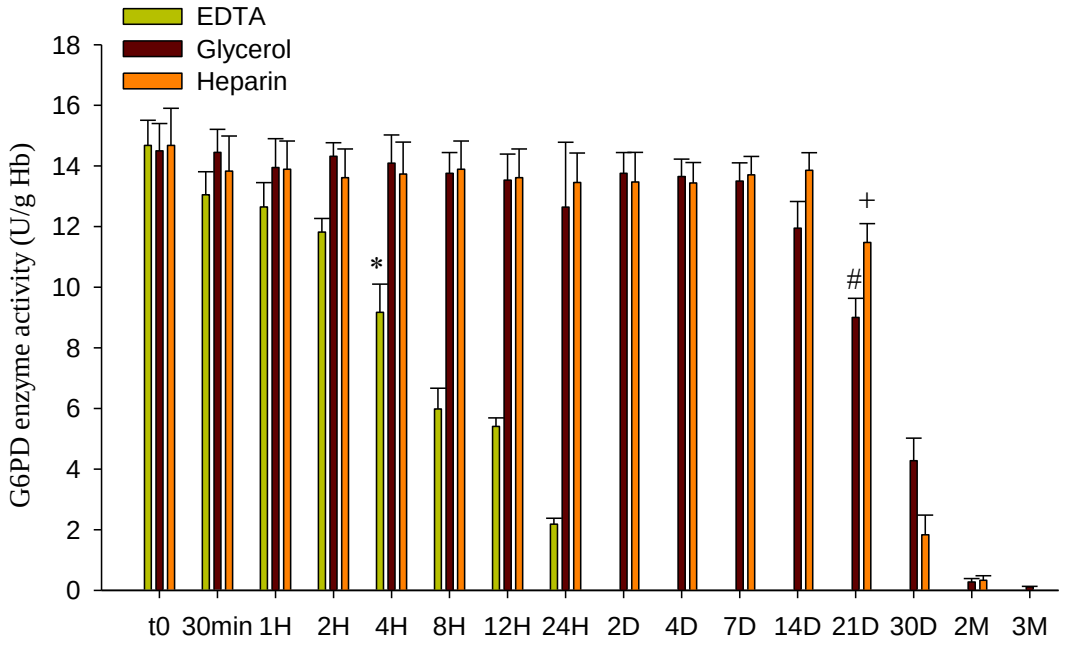

Figure No. 2: The enzymatic activity of G6PD was performed on isolated hemolysates from blood samples taken from EDTA and lithium heparin samples. The results obtained on

hemolysates containing glycerol 30% (Glycerol) were compared with the results obtained on hemolysates of EDTA samples containing no additive (EDTA) and containing 30% glycerol (Glycerol). Heparin specimens, and EDTA, and Glycerol hemolysates were all stored at 4ºC. The results are expressed as mean ± Standard error on the mean and are representative of 5 independent experiments (n=10) (* p <0.05, ** p <0.005, + p <0.01, #p <0.02).

G6PD enzymatic activity stability was analyzed at -20ºC in hemolysate with or without glycerol. The enzymatic activity stayed unchanged in hemolysis with glycerol and without any additive the first 2 hours (2H) of the time course (Fig. 3).G6PD enzymatic activity level decreased significantly at 4 hours (4H) in hemolysate without additive (Fig.3). In hemolysate with glycerol, however, the enzyme level did not vary significantly from the first dosage (t_0) to the end of the study 3 months later (3M) (Fig.3). To determine if there was any significant variation, we compared the all-time course dosage to the initial level at t_0 (Table 2). Results showed no significant variation in G6PD enzymatic activity level compared to the initial level throughout the experience (Table 2).

Fig. 3

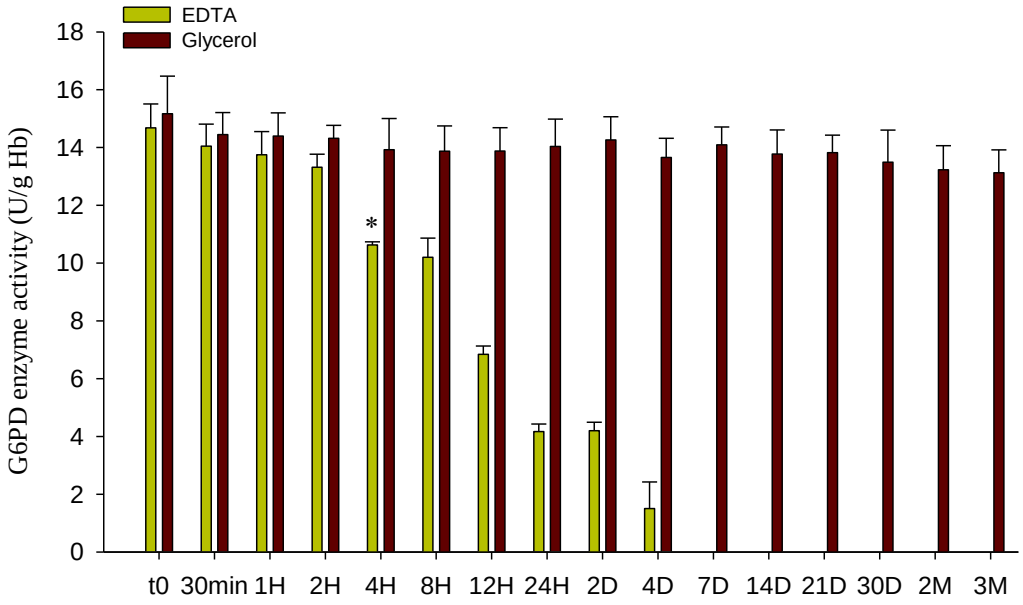

Figure No. 3: The enzymatic activity of G6PD was performed on isolated hemolysates from EDTA blood samples. The results obtained on the additive-free hemolysates (EDTA) were compared with the results obtained from hemolysates to which glycerol was added at 30% (Glycerol). Hemolysates from both groups of samples were stored at -20ºC. The results are

expressed as mean ± Standard error on the mean and are representative of 5 independent experiments (n=10) (*p <0.005).

Table No. 2: Time course mean G6PD activity in samples treated with Glycerol

Variable	Mean (SD)	Mean of score difference (95% CI)	*P-value*
Pair 1 t_0 30min	14.50 (1.56) 14.34 (1.56)	0.157 (0.070 - 0.260)	0.908
Pair 2 t_0 1H	14.50 (1.56) 14.11 (1.28)	0.390 (-0.03 – 1.180)	0.755
Pair 3 t_0 2H	14.50 (1.56) 14.31 (1.68)	0.193 (0.00 - 0.300)	0.891
Pair 4 t_0 4H	14.50 (1.56) 14.22 (1.33)	0.278 (0.032 - 0.64)	0.826
Pair 5 t_0 8H	14.50 (1.56) 13.88 (1.52)	0.626 (0.032 - 1.815)	0.644
Pair 6 t_0 12H	14.50 (1.56) 13.81 (1.40)	0.623 (0.032 - 1.642)	0.634
Pair 7 t_0 24H	14.50 (1.56) 13.84 (1.50)	0.665 (0.075 - 1.815)	0.74
Pair 8 t_0 2D	14.50 (1.56) 14.03 (1.42)	0.470 (0.119 - 1.122)	0.854
Pair 9 t_0 3D	14.50 (1.56) 13.66 (1.15)	0.845 (0.032 - 1.815)	0.492
Pair 10 t_0 1W	14.50 (1.56) 14.09 (1.07)	0.406 (-0,438 - 1.295)	0.729
Pair 11 t_0 2W	14.50 (1.56) 13.78 (1.44)	0.724 (0.345 - 1.468)	0.587
Pair 12 t_0 3W	14.50 (1.56) 13.82 (1.05)	0.677 (-0.125 - 1.468)	0.566
Pair 13 t_0 M1	14.50 (1.56) 12.90 (1.92)	1.605 (0.524 - 3.127)	0.323
Pair 14 t_0 M2	14.50 (1.56) 13.23(1.45)	1.272 (1.127 - 1.524)	0.359
Pair 15 t_0 M3	14.50 (1.56) 13.13 (1.37)	1.605 (0.164 - 2.524)	0.155

The rate of enzymatic activity of G6PD variation was determined at each stage in comparison to the initial dosage at 0H. The variation was deduced on each of the 10 series of assays. Student's t-test was used to determine if the variation was significant (*p-value*) (H, Hour, D, Day, W, Week, M, Month, SD, Standard of Deviation).

The mean G6PD activity from the 10 normal G6PD activity subjects included in the study was 14.50 U/g Hb. The cut-off points for G6PD deficiency were established according to WHO

classification and are as follows: 8.70 U/g Hb (60% of normal mean), 2.90 U/g Hb for severe deficiency (20% of normal mean activity) and 2.90 to 8.70 U/g Hb being the range for moderate deficiency (20% - 60% of normal mean activity) [14]. Results in Table 3 showed the time course moment where G6PD activity went from the normal value of 14.50±0.90 U/g Hb to moderate deficiency range and later to the severe deficient range. In hemolysate with glycerol at -20ºC, G6PD activity level stayed within the normal range until the end of the 3 months of the study (Tab. 3). Hemolysate with glycerol 30% at 4ºC (9.00±0.63) and heparin at 4ºC (11.48±0.62) showed G6PD activity level within normal range 21 days after the initial dosage. At 30ºC, heparin stabilized G6PD activity level within the normal range (10.24±2.30) during 4 days while in hemolysate with glycerol, the delay was 4h (9.92±0.82) (Table 3).

Table No. 3: G6PD activity level varies according to diagnosis ranges

Sample	G6PD Statut		
	Normal (>8.70 U/g Hb)	**Moderate** (2.90 - 8.70 U/g Hb)	**Severe** (<2.90 U/g Hb)
EDTA, 4ºC	4H (9.17±0.93)	12H (5.41±0.28)	24H (2.18±0.20)
EDTA, -20ºC	8H (10.20±0.66)	2D (4.20±0.29)	4D (1.50±0.92
Glycerol, 4ºC	21D (9.00±0.63)	30D (4.28±0.75)	2M (0.28±0.11)
Glycerol, -20ºC	3M (13.13±0.79)	-	-
Glycerol, 30ºC	8H (9.92±0.82)	4D (3.16±0.61)	7D (0.55±0.35)
Heparin,4ºC	21D (11.48±0.62)	-	30D (1.83±0.65)
Heparin, 30ºC	4D (10.24±2.30)	7D (6.81±1.84)	14D (1.20±0.73)

The time course period of G6PD activity measurement and the level of the enzyme activity are shown. Data are the mean±SEM.

DISCUSSION

Neonatal screening for G6PD deficiency is recommended by the WHO in regions where the prevalence of G6PD deficiency is ≥ 3% [7]; unfortunately, this practice is not yet applied in West Africa. Biochemical definitive diagnosis is recommended for quantitative analysis of G6PD activity. The G6PD activity quantitative spectrophotometric assay is based on the evaluation of absorbance at 340 nm given by NADPH formation [15]. In Benin, neonates are not routinely screened for G6PD deficiency because of many reasons: a reliable diagnosis of

G6PD status by spectrophotometry is costly, has a turn-around time of several hours, and requires good laboratory infrastructure; limitations that render routine G6PD testing by spectrophotometry unsuitable in a most remote area [16].

This study was carried out to determine a suitable condition for G6PD stability for better pre-analytic step. The optimal anticoagulant for G6PD stability is k2-EDTA that ensures good conservation of blood cells and in the presence of which G6PD activity is stable at 4°C up to 72h [17]. In this study, we showed that the maximum delay allowed by k2-EDTA anticoagulant was 4h which is much lower than expected. The difference in our result could be explained by the poor quality of EDTA anticoagulant, a matter of bad storage condition of laboratory material [18, 19]. The stability of G6PD in whole blood stored with different anticoagulants is contradictory. Glycerol is known to stabilize enzymes and help keep enzyme normal activity [20]. In this study, we used hemolysate to better explore the stability effect of glycerol. Our results showed that when glycerol was added to hemolysate at a concentration of 30%, G6PD activity level was maintained within the normal range during 21 days at 4°C. This performance is similar to that of heparin anticoagulant witch is recommended for G6PD deficiency test with a delay. In sample hemolysate with 30% glycerol stored at -20°C, G6PD activity was steady during the 3 months of the study. In a remote area, most laboratories lack air conditioning or refrigerator. Since increased temperature decreased the stability of G6PD in both liquid blood and dried blood spots [21], we analyzed the stability potential of glycerol and heparin at 30°C. One of the problems faced by laboratory practitioner is the effect of time and temperature on G6PD activity in samples during transportation [21, 22] which in turn may affect the accuracy and the consistency of the results from outside referred samples. Here, we show that G6PD activity remained normal more than 8h, in hemolysate with glycerol, after collection at 30°C. In heparin, our results showed that the enzyme activity was maintained within the normal range for 4 days at 30°C. In a laboratory where frozen sample at -20°C is possible, our results suggest that the best way of keeping samples for delay G6PD activity measurement was to prepare hemolysate with glycerol 30% and store at -20°C. In a condition where the temperature is high, heparin anticoagulant seems to be more suitable for G6PD enzyme activity determination.

CONCLUSION

The pre-analytical delay of G6PD is of paramount importance because the diagnostic value of the determination of enzyme activity is based on threshold values. Maintaining the enzymatic activity of G6PD is analyzed in whole blood and the hemolysate as a function of time and

temperature. Of the conditions studied, hemolysate with 30% glycerol stored at -20°C represents the best condition for the stabilization of G6PD activity in vitro.

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