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Determination of the intra erythrocyte Glucose-6-phosphate dehydrogenase in human blood by UV-Visible spectroscopy: Development, validation and application

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Abstract
G6PD deficiency is the most common enzymopathy. It would affect more than 400 million people worldwide. It is sometimes called favism due to possible occurrence of hemolysis after ingestion of beans. The clinical manifestations are associated acute hemolytic crises sometimes chronic. These attacks can be triggered by infections, ingestion of oxidant drugs. The diagnosis of this enzymopathy based on the evaluation of the activity of the enzyme implicated. This implies the use of reliable method capable of giving reliable results. In this work a quantitative method of erythrocyte G6PD by UV-Visible spectroscopy was developed and then validated using the strategy of the total error. The method is valid in the range of dosage of 30 µl (1.5 mg / ml) in 50µl (mg / mL). The validated method has been successfully used in Kinshasa (the capital of Democratic Republic of Congo) to assess the activity of G6PD In people with anemia and newborns. The impact of the deficit was 4% in neonates (10/250) and 3.35% for those with anemia (8/239).

Keywords: validation, G6PD, erythrocytes

1. Introduction

The lack of glucose-6-phosphate dehydrogenase (G6PD), also called favism, is a genetic enzyme deficiency disease [1]. This is the most common enzyme deficiency in the world: it concerns 420 million people. It mainly affects populations from Africa, Asia, Middle East and Mediterranean basin [2, 3]. Statistics show that some tribes in the Congolese population can reach very high frequencies even beyond 20% [4].

This deficiency disturbs the regeneration of NADPH, and consequently reduces the exposure of red cells during absorption of oxidizing agents (antimalarials, vitamin K, sulfonamides etc.) causing acute hemolytic crisis. Thus, the G6PD is an essential enzyme for the protection of red blood cells against oxidative stress [5, 6, 7].

The diagnosis of this enzymopathy is based on the evaluation of the enzyme activity [8]. This requires the use of accurate and reliable methods. Currently there are several techniques of qualitative and quantitative diagnosis of the enzyme deficiency among which the fluorescent spot test, the spectrophotometric assay and automated methods [1]. Since qualitative methods have sensitivity problems. Therefore, quantitative methods are not saved and have several drawbacks among which can be quoted the existence of leukocytes coats which may hide G6PD deficiency (leukocytes contain high levels of G6PD), the account of the extra erythrocyte G6PD and of the total NADPH (produced by G6PD and 6 P gluconate) in the activity assessment.

Currently the determination of erythrocyte enzymes such as G6PD is performed by automated methods which use very expensive equipment to the point that poor laboratories cannot afford. In this context, the primary objective of this work was to develop a manual method for the determination of G6PD from an automated reference method used by The Chemistry Laboratory of the Erasmus Hospital. The second objective was to validate the method by applying the strategy of the total error in order to ensure the reliability of routinely obtained results. This strategy uses the accuracy profile as decision tool [9, 10].

These principles apply to all methods developed in the laboratory before its routine use. Different regulations on good practices (GMP, GLP...) as well as normative or regulatory documents (ISO, ICH, FDA) suggest that all assay procedures meet a number of eligibility criteria. This approach therefore requires that these procedures are broadly validated [9]. Once validated method, we applied it assaying G6PD in new borns and people with anemic conditions in Kinshasa / DR Congo.

2. Materials and methods

2.1 Instrumentation

The UV-Visible spectrophotometer used the brand Humalyzer 2000 was purchased Human lab (Gyeonggi-do, korea). Statistical analysis of the data for validation was performed by the software Enoval ® Laboratory of Analytical Chemistry of the University of Liège, Belgium.

2.2 Chemicals and reagents

The glucose-6-phosphate dehydrogenase (Batch Nº1138725), Glucose-6-phosphate (Batch Nº 13,056,928) and Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$) (Batch Nº11409669) were obtained from Roche Diagnostic Gm GH (Mannhem/Germany). 6-Posphogluconate (Batch Nº095K3783) was supplied by Sigma Aldrich. Tris (Batch

N°5277834), Magnesium chloride hexahydrate ($MgCl_26H_20$) (Batch N°6322313), Ethylenediaminetetraacetic acid (EDTA) (Batch N°7492845), 2-mercaptoethanol (2.30339 million) has been provided by Merk (Darmstadt, Germany).

2.3 Working solution

The working solution was prepared from the following reactants: 50ml of 40 mM glucose-6- phosphate, 50 ml of 10 mM 6-Phosphogluconate, 50 ml of 5 mM $NADP^+$, 50 ml of 0.05 M $MgCl_2$, 50 ml Tris-EDTA at pH 8 and 190 ml of distilled water for the first reaction. For the second reaction, the glucose-6-phosphate is replaced by 50 ml of distilled water [11].

The solution G was prepared as follows:

To 1.35 ml of 0.2 M EDTA pH 7.0 was added 0.05 ml of mercaptoethanol and 1 mg of $NADP^+$ to make 100ml of solution. This solution was used as hemolysate, calibration matrix and dilution solvent because the enzyme is stable in solution G once outside the red blood cell [11].

2.4. Assay procedure

Test samples consisted of the standard solutions. Incubation of the working solution (1000µl reagents and 80 µl of distilled water) at 37 °C for 5 minutes. Insert 20 µl of the sample in the working solution. Mix and place the sample in the spectrophotometer which measures the activity of the enzyme for 10 minutes after a latent time of 4 minutes. This indeed measures the kinetics of production of NADPH. Each level of concentration was determined by three times a day for three successive days. The assay is carried out both on the validation standards as well as calibration standards. The results obtained were analyzed by using the strategies that uses the total error of the accuracy as a decision tool profile.

2.5. Standard solutions

2.5.1 Validation Standard

Blood missing Glucose-6-phosphate dehydrogenase was used for the preparation of standard validation. We first washed erythrocytes three times by centrifugation at 2000 rpm for 5 minutes in physiological saline followed by decanting each time and this was done three times. The erythrocyte pellet was hemolyzed by G. The hem lysate solution was used to prepare standard validation: 50 microliters of packed red blood cells obtained after washing the red blood cells were added to 950 microliters of solution G. We have first determined the total activity and then that of the 6- phospho gluconate. By difference, we obtained the activity of glucose-6-phosphate.

2.5.2 Calibration Standard

We used a matrix other than the one used for the validation standard. In this case, given the stability of the enzymes in the solution G, which is the haemolyzing solution, it was used for the preparation of calibration standards. We have determined directly the activity of glucose-6-phosphate dehydrogenase without going through one of the 6-Phosphogluconate dehydrogenase which is not found in the reaction mixture.

We first prepared a stock solution containing 10 for each standard µl of the enzyme (2mg/10ml) supplemented with 990 µl of the hemolysate for validation and the standard 990 µl of solution G for the calibration standard. These two standards have enabled us to prepare for each 5 standard concentrations using the solution G as a diluting solvent.

Each concentration levels was repeated three times daily and that for 3 days (see table 1).

Table 1: Concentration level of G6PD

Concentration level (Volume of test concentrations relative to a concentration of G6PD) (µL)	Concentration of G6PD (µg/mL)	
	Calibrations standards	Validations standards
10	0,50	0,50
15	0,75	0,75
20	1,00	1,00
30	1,50	1,50
50	2,50	2,50
Total	Sample day	15 samples/day

2.6 Development of the method

Principle

Glucose 6-phosphate + $NADP^+$ → G6PD 6-Phosphogluconate + NADPH + H^+ Eq. 1

6-Phosphogluconate + $NADP^+$ → 6PGD Ribulose - 5-phosphate + NADPH + H^+ + CO_2 Eq.2

Where G6PD is glucose-6-phosphate dehydrogenase and 6PGD is 6-phosphogluconate dehydrogenase.

The G6PD activity is equal to the difference between the mixture of the two enzymes activity (total activity) and the activity of the second reaction. The kinetics of the formation of NADPH was monitored at 340 nm [12, 13].

2.6.1 Reaction volumes

Using cuvettes at least 1 ml, we multiplied by 4 the volumes used by the Automate Pentra (reference method). The following volumes were used: working solution 1000 µl; distilled water (diluent) 80 µl; sample 20 µl; a total volume of 1100 µl.

2.6.2 Parameter setting

- Tris-EDTA buffer system was chosen for its efficiency since catalytic activity of an enzyme is a function of pH. Each enzyme has an optimum pH and of the G6PD is 8 [14].

The temperature was stabilized by using a thermostated spectrophotometer because the change in temperature affects the catalytic activity. We chose to work at 37 °C

- A contact time of the enzyme and the working solution was 4 minutes which was estimated after several attempts before evaluation of the activity. We estimated to evaluate the activity of the enzyme for 10 minutes.
- The preservation of reagents should be done in 2-8 °C temperature range while the working solution should be stored at -20 ° C once established. Samples collected with EDTA should be stored at 2-8 °C not more than 4 days. The analysis should be done if not the same day of collection the next day for not more than 4 days since the activity decreases [11, 14].

A factor estimated to 884.24 was introduced into the machine to calculate the enzyme activity. This factor takes into account the dilution factor, conversion mole to micromoles, duration and evaluation of the expression of the activity used IU/g Hb unit. We then measured the hemoglobin in the hemolysate.

2.7 Method validation

In this process, we used the strategy based on estimation of the total error which reflects the systematic error and random error. This strategy is widely used in several studies [15, 16].

3. Results and discussion

The developed method was validated before use to demonstrate its reliability. Thus, in this part of this work, we will discuss results related to criteria involved in the validation of a quantitative method in bioassay. The limit of acceptance was set at ± 30%.

We selected the simple linear regression model during calibration to determine concentrations. These concentrations are then used to evaluation different validation criteria for the method.

3.1 Specificity

An analysis procedure is called specific when it ensures that the measured signal is provided unequivocally by the analyte. The developed method is an enzymatic method, it operates with one of the important characteristics of enzymes which is specificity. Enzyme attacks only its substrate. In our method, the glucose-6-phosphate is the substrate of G6PD. This is confirmed by the fact that in the reaction catalyzed by 6PGD in the absence of glucose-6-phosphate reaction G6PD does not react. This is related to the substrate specificity, which is glucose-6-phosphate and not to its group [13, 14].

3.2 Trueness

The results of the Trueness of this method are shown in Table 2. As shown in Table 2, we observe well that the greatest value of the relative bias is 45.34 and the highest absolute bias is 6.249. High values of relative biases are observed during use of small volumes such as 10 to 20 µL. The method shows good Trueness between the volume of 30 to 50 µl according to the acceptance set limit [15, 16].

Table 2: Trueness for the determination of glucose-6-phosphate dehydrogenase in human blood

Theoretical volume (µL)	Observés mean volume (µL)	Absolute bias (µL)	Relative bias (%)
10	14.53	4.534	45.34
15	20.30	5.297	35.31
20	26.25	6.249	31.25
30	34.50	4.499	15.00
50	51.62	1.615	3.230

3.3. Precision

The results of Precision are shown in Table 3. Looking at Table 3, we find that the largest value is 2.425 Repeatability and the intermediate precision is 5.804. The method is faithful to all levels of concentration despite the high value of coefficient of variation of the intermediate precision to 50 µL, this value is less than the acceptance set limit [16, 17].

Table 3: Precision for the determination of glucose-6-phosphate dehydrogenase in human blood

Theoretical volume (µL)	Repetability (RSD%)	Intermediaite fidelity (RSD%)
10	1.528	4.694
15	1.869	3.577
20	0.9160	5.892
30	2.425	3.276
50	2.390	5.804

3.4. Accuracy

According to the strategy of the total error that takes into account the random error and systematic error, the accuracy of the method taking into account the accuracy and precision is usually expressed by the accuracy profile shown in Figure 1.

As shown in Figure 1, the tolerance intervals Beta is out in the acceptance level set at the lower concentration 27 µL which confirms the fact that low-concentration method does not show a good accuracy limit. The method is accurate only 27 to 50 µL [17, 18, 19].

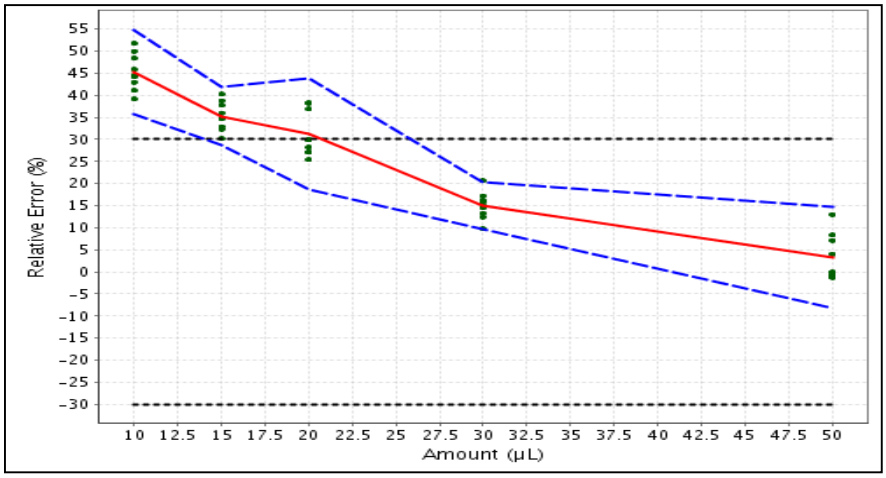

Fig 1: Accuracy profile for the determination of glucose-6-phosphate dehydrogenase in human blood

In order to determine the values likely to go outside the limits of acceptability and the risk profile was determined and shown in Figure 2.

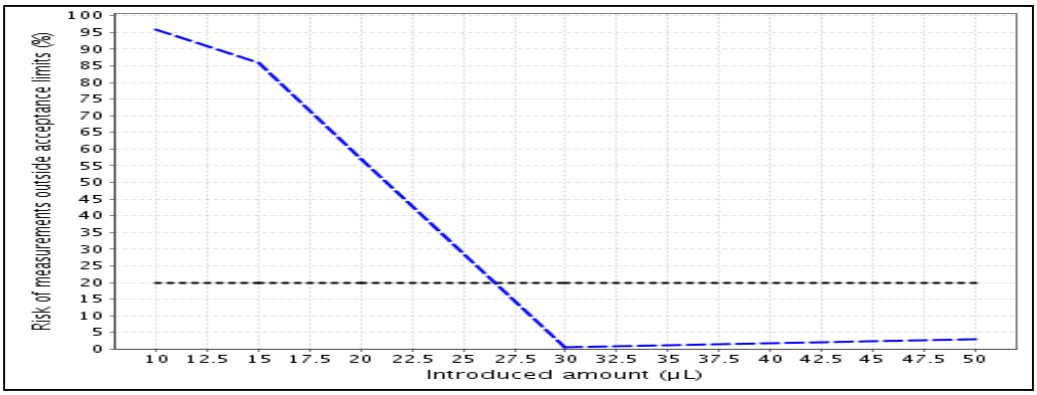

Fig 2: Risk profile for the determination of glucose-6-phosphate dehydrogenase in human blood

As shown in Figure 2, there is more likely to find values outside the acceptance limit when working with low volumes (10 to 29 µL). While in the validated interval, this risk is not more than 5%, which is a good thing for the routine application of the method [18, 19].

3.5 Linearity

The linearity of an analytical method is the ability to obtain a certain range of results directly proportional to the amount of analyte in the sample [18, 19]. The linearity of the method is shown in Figures 3.

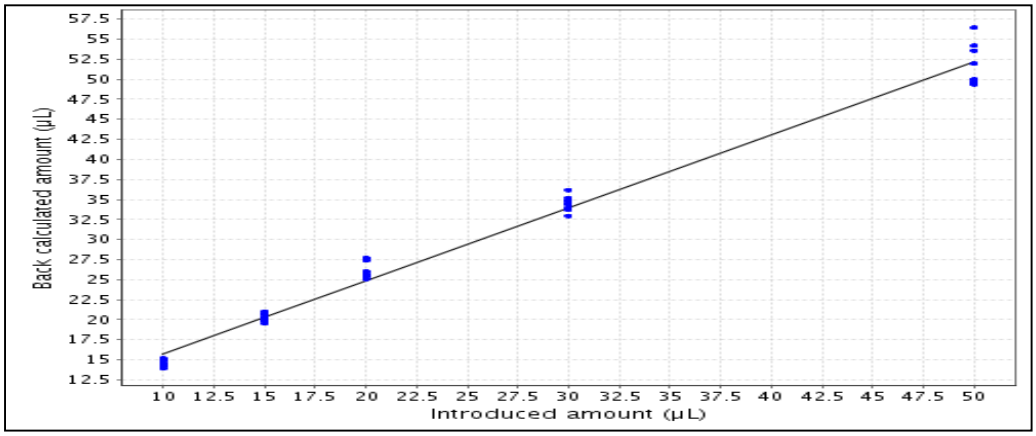

Fig 3: Relationship between the introduced concentration and the result

The equation of this linearity is as follows: Y = 6.672 X + 0.9107 with a slope of 0.910 close to unity, hence showing a good sensitivity of the method. The coefficient of determination (R^2) is equal to 0.9857, which explains the good agreement between the introduced concentrations and the calculated concentrations. Since this indicator (R^2) is not sufficient to confirm the linearity of a method, we used the profile of linearity to confirm this correlation among concentrations. As shown in Figure 4, the tolerance intervals (in blue) and the line of identity (in gray) are included within the limits of acceptance (in black) with respect to the approved range (30 to 50 µL). The method is linear over the validated part [17, 18, 19].

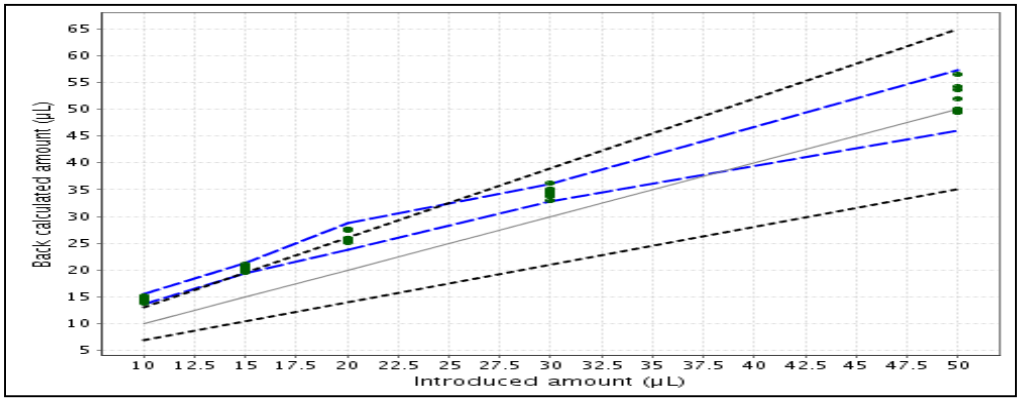

Fig 4: Profile of linearity for the determination of glucose-6-phosphate dehydrogenase in human blood 14

3.6 Limit of quantification and the dosing interval

According to the strategy for validating this method, the lower limit of quantification (lower LOQ) and higher (higher LOQ) are obtained by the intercession of tolerance interval beta and acceptance limit. As can be seen in the Figure 1, the lower LOQ is 25.9 µL and the higher LOQ is 50.0 µL. [17, 18, 19]. The dosage range is from 25.9 to 50.0 µL. The method is thus validated and can be used routinely with accuracy.

3.7 Applications

In the following, the validated method has been successfully applied by assaying G6PD in 250 newborns and 239 people with anemia in Kinshasa / DRC. The results of this assay are shown in Table 4.

Table 4: Results of assay G6PD in newborns and people with anemia in Kinshasa

Subjet	Sex	Number	G6PD Activity (UI/gHb) (Mean ± Standard-Deviation)
Newborns	Boys	147/ 250	8,141 ± 5,643
	Girls	103/ 250	9,771 ± 4,159
People With Anemia	Men	117/239	8,887 ± 5,986
	Women	122/239	9,705 ± 5,329

The frequency of G6PD deficiency in neonates is 4% or 10/250 which 3.2% of boys and 0.8% girls. The frequency of G6PD deficiency seen in people suffering from anemia is 3.35% or 8/239 boys 2.93% and 0.42% girls. We considered deficient in G6PD, people who had G6PD activity less than 0.5 IU/g Hb. The anomaly is much more severe in men than in women because the gene for G6PD is carried by the X-chromosome. Male carriers of the disease variant are actually homozygotes. Conversely, women except in rare cases of homozygosis can compensate the anomaly due to the existence of a gene for a normal variant on the other X-chromosome [8, 20]. Oxidizing drugs should be prohibited to persons deficient in G6PD. This would avoid them unpleasant and sometimes fatal hemolysis events [7.21].

4. Conclusion

The purpose of our work was to develop and validate a method of quantifying the activity of in traery throcyte G6PD to allow a proper diagnosis of G6PD deficiency. The method developed evaluates the G6PD activity after removal of the extra RBC washing G6PD and without taking into account the activity of the reaction such as 6PGD which produces NADPH which is the measured parameter.

This method was validated following the strategy of the total error. The method is accurate (both fair and faithful) in the dosing interval from 30 to 50µ L. It showed good linearity in the assay range. Then applied in Kinshasa / DRC by assaying G6PD in 250 newborns and 239 people suffering from anemia. This simple method can be used with warranty in laboratories for biochemical analysis.

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