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A practical and reliable of determination of erythrocyte hexokinase and glucose-6-phosphate dehydrogenase

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Abstract
The erythrocyte enzymopathies are among diseases affecting red blood cell enzymes and several of them are known worldwide: Glucose-6-Phosphate Dehydrogenase deficiency, Pyruvate Kinase deficiency, Hexokinase deficiency, etc... The Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency is most common and most widespread erythrocyte enzymopathy worldwide. In the case of enzyme deficiency, the red blood cells will not be able to properly perform their duties. Enzymes are currently assayed in clinical in order to highlight the enzymopathies cases. Nowadays, the evaluation of the enzymatic activity involved to highlight clinically enzymopathies. This evaluation requires the use of reliable methods. We have conducted this work with the objective of establishing a practical and reliable spectrophotometric assays of hexokinase (HK) and G6PD making an automated method used by the chemistry laboratory of the hospital manual Erasmus. Some validation criteria including repeatability, linearity and intermediate precision were evaluated and showed that the methods are reliable. Comparison of the results from analyzed samples using both the developed method and the reference one by the statistical Student-test hasn't shown a significant difference between the two methods.

Keywords: hexokinase, G6PD, 6PGD, erythrocytes, enzymopathies

1. Introduction

The enzymology is a branch of biochemistry that studies enzymes which are natural proteins endowed of catalytic properties [1, 2]. However, various kinds of enzymopathies are likely caused by deficiency in enzymes activities. In some erythrocytes, enzymes such as Glucose-6-Phosphate Dehydrogenase (G6PD), Pyruvate kinase (PK), Hexokinase (HK), etc., may have deficiency in activity likely to cause hemolytic syndromes, previously unexplained, nowadays recognized being due to an inherited enzyme deficiency of erythrocytes [3]. Compared to other medically known enzymopathies, G6PD deficiency remains the most frequent and the best known worldwide. It affects about 400 million people in different world regions which the most concerned are Tropical Asia, Middle-East, and tropical Africa [4-5]. In Democratic Republic of Congo (DRC), a country in the Tropical Africa region, local statistics show that some tribes of Congolese population have very high frequencies of cases with G6PD deficiency (≈20%). These patients are mainly boys since the coding gene for the biosynthesis of G6PD in humans is located on X chromosome [6-8]. Their erythrocytes are fragile and they are proned to develop hemolytic anemia in various situations labeled altogether oxidative stress. On the other hand, Hexokinase (HK) is the first acting enzyme of glycolysis. Its activity deficiency often leads to a rare form of enzymopathy. Individuals suffering from this type of deficiency produce little hexokinase glucose-6-phosphate and thus also have low levels in glycolytic intermediates and low energy production [9-11].

The diagnostics of these enzymopathies mainly resorts to the activities assays of the aforementioned enzymes (G6PD and HK). For these diagnostics, there exist precise and reliable methods [3, 12-13]. However, these methods have some drawbacks. The G6PD dosage resort to methodsin which no erythrocytes washing which allows no erythrocyte enzymes cleaning after a blood sample centrifugation and plaquet layer elimination susceptible to hide an eventual G6PD deficiency case in addition to the evaluation of NADPH production from the reaction catalyzed by both the erythrocyte G6PD et the 6PGD (6-phosphogluconate-déshydrogénase) instead of that is produced by the G6PD alone. In addition to these drawbacks, other erythrocyte enzymes are not commonly measured throughout tests worldwide especially in developing countries. However, these enzymes are responsible of other enzymopathies which can also cause hemolytic anemia like G6PD deficiency does. This is particularly the case of pyruvate kinase and hexokinase (HK) which activity evaluation is important in the diagnosis of certain erythrocyte enzymopathies [13-15].

Currently, for determining erythrocyte enzymes, several Western laboratories use automated methods which are very expensive to the point that most laboratories in developing countries cannot afford. Hence, they manifest reduced interests to these kinds of analysis, while the needs are real to face the problem posed by enzymopathies.

To address this problem, we made manual, the automated method used by the chemistry laboratory of the Erasmus Hospital Belgium for the determination of erythrocyte enzymes.

The spectrophotometric methods thus developed will enable laboratories with just a spectrophotometer to assess the problem without G6PD and HKenzymatic activity evaluation. These methods will be able to meet the demand of analysis prescribers. The most the questioning purpose of our study was to determine to what extent our method could be reliably compared to others, while a particular emphasis to the reference method.

The G6PD catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate with NADPH production. The 6PGD catalyzes the conversion 6-phosphogluconate to ribose with production of the reduced NADPH. The G6PD activity is evaluated by difference of NADPH which is measured at 340 nm [12, 16].

2. Materials and Methods

2.1 Materials

2.1.1 Biological materials

The biological materials used in this study were whole blood samples collected in EDTA tubes from patients in consultation at Erasmus Hospital:

2.1.2 Laboratory materials

A spectrophotometer (Perkin Elmer, model Lambda 25) was used to perform the G6PD and HK enzymatic activity evaluations as well as a water bath incubator (Analis Anatherm II) working 37 °C. The balance (Mettler Toledo AT261 Delta model range) was used to weigh chemicals for reagents preparations. Conservation of reagents and samples was done in a refrigerator (Liebherr premium model) at 4°C.

2.1.3 Reagents

Reagents were prepared according to the procedure for the dosageof erythrocyte enzyme describe in Erasme Hospital Chemistry Laboratory manual [14].

G solution used as hemolyzing solution

EDTA 0.2 mM solution (1.35 ml) + 2-Mercaptoethanol (0.05 ml) + $NADP^+$(1mg) to 100 ml distilled water.

Reagents for hexokinase

The mixture solution was composed of distilled water (14 ml); TRIS EDTA pH 8 (60 ml); 0.1 M $MgCl_2$solution (60 ml); 0.05 M glucose solution (60 ml); 2 mM $NADP^+$solution (60 ml). This solution was distributed into tubes (6.35 ml per tube) and stored at -20 °C for not more than 6 months. The working solution was prepared by mixing before use: each tube contain (6.35 ml) + 30 mM ATP solution (3.75 ml) + 0.1 mg/ml G6PDsolution of G (150 µL).

Reagent for G6PD

- Reagents mixture : the working solution consisted of TRIS EDTA pH 8 (50 ml); 40 mM glucose 6-phosphate (50 ml); 10 mM 6-phosphogluconate (50 ml); 0.05 M solution $MgCl_2$(5 ml); 5 mMsolution $NADP^+$(50 ml); distilled water (190 ml). The prepared reagent is stored at -20 ° C. This reagent evaluates the activity of total reduced NADPH.
- Reagents for assay 6GPD: This reagent consisted of the same to the precedent reagents mixture except the substrate is glucose-6-phosphate. The water content becomes 240 ml to supplement it.

Reagents for filtration

Cellulose-microcrystalline cellulose mixture: microcrystalline cellulose (5g) and cellulose (5g) have been mixed and placed in a graduated cylinder or in a flask, then brought to 125 ml with saline solution (NaCl 0.156 M) and put under magnetic stirring for 2 minutes. The mixture was kept for 3 days at 0-4 °C [15]. This mixture is used to remove the leuco-platelet layer. This layer may also be removed using a vacuum pump.

2.2. Methods

2.2.1 Pre-analytical phase

Determination of reaction volumes

Using cuvettes at least 1 ml, we have increased four times the volumes using the procedure described in the chemistry laboratory book of the Erasme Hospital for PLC PENTRA automate. The following volumes were used: Hexokinase (working solution 1000 µl; 80 µl distilled water; samples 40 µl); G6PD and 6PGD (working solution 1000µl; distilled water 80µl; sample 20 µl)

Determination of calculation factor

Determining calculation factor takes into account the following parameter: dilution factor, extinction coefficient, different conversions (molto micromol; IU/ l to IU/g Hb), reading time [16, 17]. We found this factor equals450.16 IU/g Hbfor hexokinase and 884.24 IU/g Hb for G6PD.

Choice of working parameter

The catalytic activity of an enzyme is affected by several factors including pH, temperature, the quality of the samples, the quality of the reactants, the contact time between the enzyme and the substrate, buffer capacity assessment, etc. The catalytic activity evaluation requires careful control of these parameters. The following conditions were used: buffer system: Tris- EDTA pH 8; Temperature: 37 °C; Storage of Reagents: 2-8 °C; Conservation of working solutions: - 20 °C; Samples collected in EDTA and stored at 4 °C for not more than 4 days [16-17]. We maintained the conditions used by the reference method as defined in the quality control laboratory book.

Determination of the contact time and reading

Several trials have led us to determine the duration of contact between the enzyme and the reaction medium to 4 minutes before reading. The spectrophotometer was program to read the optical density every 30 seconds for 10 minutes.

2.2.2 Analytical phase

Preparation of hemolysis

Place 5 ml syringes into a device which maintains the vertical position with the bottom downwards and in the centrifuge tubes, then removes the pistons. Put at the bottom of the syringe a four-folded Whatman paper. Check that leaves no openings. Firstly, add saline solution (1 ml) for the proper spreading of the bed, then cellulose mixture (4 ml) with a Pasteur pipette. After passage of the saline solution, add extra saline solution (3 ml) and directly well mixed blood (1 ml). Bring to 10 ml with saline solution and centrifuged at 1500g for 10 minutes. Remove the supernatant. Repeat twice, each time with 10 ml of saline [15]. Hemolysateis made of packed red blood cells (50 µl) + the solution G (950 µl) [14].

Dosage of l'hexokinase and the G6PD activity

- **Assay principle**

Hexokinase catalyzes the phosphorylation of glucose at the position 6. The glucose-6-phosphate so obtained is converted

into 6-phosphogluconate upon the action of G6PD with $NADP^+$ as cofactor. In fact, a coupling is achieved. It is the kinetics of the NADPH production which is run and observed at 340 nm [12, 16].

The G6PD catalyzed the transformation of glucose-6-phosphate into 6-phosphogluconate with NADPH production. The 6PGD catalyzes the transformation of 6-phosphogluconate into ribose with NADPH production as well. Thus, the G6PD activity is obtained by the difference of activity of the mixture reagent and that of the reaction catalyzed only by 6PGD.It is the kinetics of the NADPH production which is run and observed at 340 nm [14, 16, 17].

➢ **Procedure**

a) Assay of hexokinase and G6PD in hemolysates

- Wavelength-340 nm
- Zero the instrument with the reagent blank

	HK	G6PD	
		G6PD Mixture	6PGD
Working solution	1000 µl	1000 µl	1000 µl
Distilled water	80 µl	80 µl	80 µl
Mixture and Incubation for 5 minutes at 37 °C.			
Échantillons	40µl	20µl	20µl

Mix and read the optical density in a spectrophotometer after 4 minutes of latency. The spectrophotometer was programmed to read the optical density after 4 minutes of contact. After the 4 minutes, the optical densities were taken initially at time T0 and then every 30 seconds for 10 minutes. The kinetics of formation of NADPH is measured.

b) Determination of Hemoglobin in hemolysates

We assayed the hemoglobin in the hemolysate by the method of DRABKIN expressing activities IU/g Hb [3].

c) Calculations

The activities were obtained by multiplying the absorbance differences by hemoglobin concentration of the hemolysate and by the factors 450.16 and 884.24 respectively for calculation HK and G6PD.

Determination of reactions kinetics parameters

We wanted to reassure that the developed method was under control regarding the factors with notorious influences on the catalytic activity of assayed enzymes. In fact, the reaction kinetics should be linear during the activity evaluation. This analysis was performed on each parameter. We started from a sample on which was applied the aforementioned procedure. The sample was followed for 10 minutes by its kinetics with the optical density reading of sample every 30 seconds to note the linearity of the evolution of the reaction kinetics.

Evaluation of the method

Some validation criteria were checked to assess the reliability of the method. The objective was to characterize the best possible performance under optimal conditions and to verify the proper functioning of the system (instrument/reagent) for the concerned parameter. The following validation criteria were evaluated: The repeatability, intermediate precision, linearity, limit of detection, limit of quantification [18-19].

Comparison with the reference method

We used the automated reference method "Pentra" which is capable of determining several enzymes such as hexokinase, G6PD, the pyruvate kinase, phosphofructoisomérase and 6-dehydrogenase phosphogluconate. The results of implemented methods should be compared with those of the reference method by the statistical test to conclude whether or not there is a significant difference between the two methods. The student test was applied after verification homocedacity. We had to use 7 samples for HK, 5 for each G6PD and 6PGD mixtures [20].

3. Results and Discussion

3.1 Results

Result of reaction kinetics

The results of the reaction kinetics of the determination of hexokinase, G6PD mixture and 6PGD.

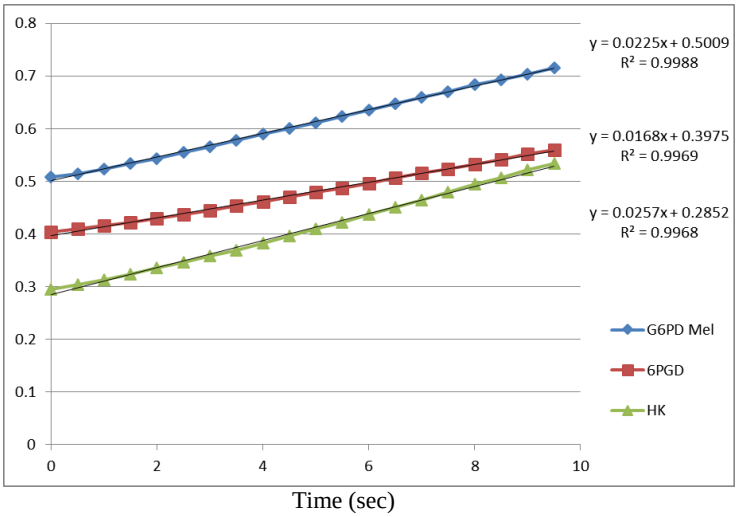

Fig 1: Evaluation of the reaction kinetics of Hexokinase

Repeatability

The repeatability was performed on three samples assayed three times on the same day. The results of the repeatability are shown in Table I.

Table I: Results of the repeatability

Mean	16,754	17,873	9,193
Standard deviation	0,559	0,297	0,232
CV(%)	3,47	5,18	2,52

Intermediate precision

The intermediate precision was determined from three samples assayed for three consecutive days. Means, standard deviations, CV% daily are shown in Table II.

Table II: Results of intermediate precision

Parameters	G6PD			6PGD			Hexokinase		
Mean	15,8	17,28	8,18	8,51	6,77	9,17	1,17	1,61	1,12
Standard Deviation	0,117	0,472	0,656	0,500	0,326	0,584	0,028	0,077	0,065
CV (%)	0,074	2,73	8,02	2,38	4,81	6,37	2,38	4,81	5,78

Linearity

Linearity determined five concentration levels led to results shown in Figure 2 that the assay results of these three parameters are linear.

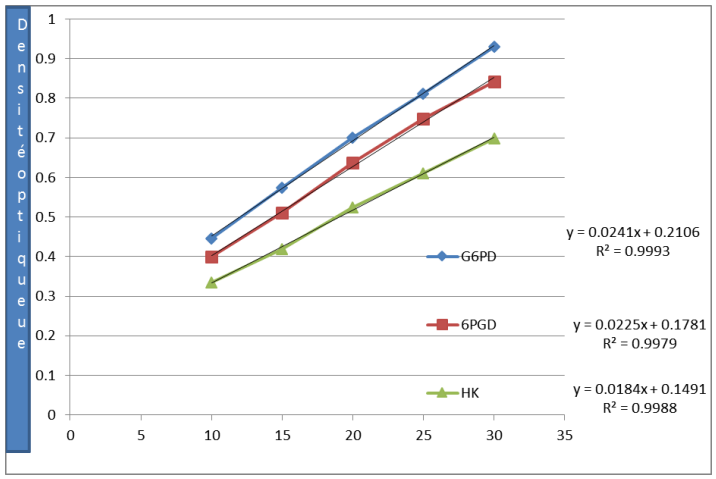

Fig 2: Linearity assay Hexokinase, G6PD and 6PGD

Detection and quantification limits

The detection andthe quantification limits were determined from the method that uses the blank solutions means.The calculated standard deviation is 0.0177 to 0.06 HK and G6PD. The limit of quantification was 0.18 IU / g Hb for HK and 0.6 IU / g Hb for G6PD.

Comparison the method "Manual" with the reference method "Pentra"

The results found by the method were compared with the reference one to check if there was no significant difference between the two methods. The statistical test applied is the student test. The test gives the results shown in Table III.

Table III: Comparison of the manual method and Pentra Method (hemolysate prepared by filtration through cellulose-microcrystalline cellulose mixture)

	Activité de Hexokinase en UI/g d'Hb		Activité de G6PD en UI/g d'Hb	
Parameters	**Méthode manuelle**	**Méthode automatisée**	**Méthode manuelle**	**Méthode automatisée**
Mean	2,13	2,87	8,95	10,86
Standard deviation	1,484	2,272	4,433	4,81
Variance	2,202	5,162	19,651	23,136
Calculated T	0,668		0,498	
Tabulated T (α = 0,05)	1,782 (DL= 12)		1,860 (DL=8)	

T is less than the calculated quartile Qt for both methods; we do not reject the null hypothesis. So, the two methods developed have average equal to that of the reference method. We can conclude that there are no significant differences between the developed methods and the reference method.

The developed methods were compared with the reference method using this time the hemolysate used for the reference method. The developed test is the Student t. The test gives the results shown in Table IV.

Table IV: Results of the assay of hemolysate Pentra G6PD by PENTRA and Manual Method

	Activité de G6PD e en UI/g d'Hb	
Parameters	**Méthode manuelle**	**Méthode automatisée**
Mean	8,2	11,87
Standard deviation	5,479	7,193
variance	30,019	51,739
Calculated T	1,468	
Tabulated T (α = 0,05)	1,706(DL= 26)	

T is less than the calculated quartile Qtin, we do not reject the null hypothesis. So the average developed method is equal to that of the reference method. We can conclude that there are no significant differences between the developed method and the reference method.

3.2 Discussion

Our objective was to develop a facile accessible method to assess the hexokinase and G6PD activities. The procedure is applied directly or hexokinase, but it is not the case for the G6PD. Indeed, the evaluation of the activity of G6PD passes through two steps: evaluation of the activity of the total reaction and that of the second reaction, which is an evaluation of the activity of the 6-phosphogluconate dehydrogenase. The G6PD activity is obtained by subtraction [17].

Above all, we started with a pre-analytical phase objectives to master various parameters that influence the enzymes kinetics, to define the reaction volumes, the working temperature and calculating factors for both assayed enzymes. The kinetics of reactions was checked for three reactions: Reaction hexokinase reaction mixture G6PD and 6PGD.

The analytical phase allowed us to verify the reliability of the developed method by evaluating a number of validation criteria including repeatability, intermediate precision, limit of detection, limit of quantification and to compare the results of the new method with the reference automated method "Pentra" used by the Erasmus Hospital for the determination of various erythrocyte enzymes.

The reaction kinetics was linear with respect to the three reactions as shown in the graphs 1-3. The absorbances increased linearly regarding the time. This is equivalent to the number of micromoles of the appearing product as a function of time. The substrate concentration in excess is not a limiting factor because the reaction has a zero-ordered kinetics, thus, the curves have a linear shape. This means that the rate of appearance of the reduced cofactor rises linearly with time [12]. This implies that the parameters that influence the enzymatic activity such as temperature, pH were controlled [16].

The methods developed are reliable given the results obtained after evaluation of some validation criteria. The coefficients of variation of repeatability and intermediate precision are less than 9%. This shows that random errors are minimized. This explains why the precision of the method is good especially since the coefficient of variation is less than 15% organic methods [18-19].

Checking the linearity of the method showed that the method is linear especially as the R^2 is close to unity [20]. The calculated detection limit should not be too high so that the first significant difference with the actual test, which defines the detection limit of the method, being in a relatively low area to be interesting. It was set at 0.2 IU / g Hb for G6PD [12].

Comparison of the results obtained by the new methods showed, after treatment by statistical methods, there was no significant difference between the developed methods and the reference method. Any time the developed methods give low values compared with those of the reference method. In practical terms this is not a drawback because the purpose of the determination of enzymes is not missed the screening of an individual with the enzyme deficiency.

4. Conclusions

The purpose of this work was to develop a simple and easily accessible method for the determination of hexokinase and glucose 6-phosphate deshydrogenase activities to be applied in hospitals to determine the enzymatic activity of these enzymes implicated indeficits causing some enzymopathies in the body.

After checking some criteria for validating analytical methods, it was found that the developed methods reliable in terms of evaluation results obtained.

Comparison of the results of the developed method with those from the reference method by the statistical test concluded that there are no significant differences between the two methods. Therefore, the developed method can be used concurrently by laboratories without any fear of false results. Thus, for a complete reliability of the developed method, the complete validation process is in progress.

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