ORIGINAL ARTICLE

To Compare Blood Glucose Estimation Using the GOD-PAP Technique with the Hexokinase Method

Syeda Sabahat Haider Zaidi[1*], Anber Rahim[2], Sadia Khalid[1], Ume-Farwa[1] and Rabia Mehmood[1]

[1]*Department of Pathology, Sheikh Zayed Hospital, Rahim Yar Khan, Pakistan*

Abstract

Background: Glucose is the major source of energy for all the processes working within the human body and hence it's the major carbohydrate required. During starvation or fasting, its concentration in blood is maintained by glycogenolysis and gluconeogenesis otherwise, a routine diet is sufficient for this task. Routine methods like glucose-oxidase coupled dehydrogenase are most commonly used on semiautomatic instruments with mono reagent. Hexokinase is the definite method to measure glucose concentration during different fed and fasting states but it requires an automated instrument and specific reagent which comparatively affects its cost.

Objective: To compare the GOD-PAP and Hexokinase methods for blood glucose estimation.

Methodology: This study was conducted in the Chemical pathology lab and Diabetic clinic of Sheikh Zayed Medical College and Hospital, Rahim Yar Khan from June to December 2023. 110 samples were obtained. Both genders with hyperglycemia and normoglycemia were selected. Hemolyzed, lipemic, icteric specimens and blood samples with low glucose <50 mg/dl were excluded.

Results: 110 patient specimens were analyzed on both fully automated and semi-automatic instruments for method comparison. Precision determined by replication study in which Random Error was (RE) < Total Allowable Error (TAE). The mean value determined by the Hexokinase method was 106.34 while by GODAP it gave 100.29.

There was a negative bias of -6.14. The linear regression analysis was performed for comparison of methods. The line of best fit was obtained. Linear correlation between the two methods was documented by calculating the correlation coefficient. The accuracy of the test method was measured by conducting a "Recovery study" on the specimen sera. The proportional error was less than the total allowable error. The *P*-value was found to be 0.000.

Conclusion: Our study concluded that the estimation of glucose using the GOD-PAP (glucose oxidase-peroxidase) technique gave acceptable results when compared with the Hexokinase reference method. So, the validated method can be used in small and medium workload laboratories for cost-effective reliable blood glucose test results.

Keywords: *Glucose oxidase, hexokinase, recovery study, proportional error, random error, linearity study, correlation coefficient, regression analysis.*

INTRODUCTION

The principal source of energy which our bodies obtain from diet is the monosaccharide sugar known as glucose. There are several different forms of glucose such as lactose, and fructose which enter our body [1]. Protein, lipids and carbohydrates consumed in daily food ultimately break down to glucose which serves as the basic major metabolic fuel for fetuses and all other mammals. Plants utilize sunlight for the process of photosynthesis to produce glucose from water and carbon dioxide which is then stored as starch [2]. Glucose is the final substrate at the cellular level which is then converted to adenosine triphosphate (ATP) for all the energy processes going on within the body such as nerve impulse conduction, active transport of different molecules across the cell membranes, muscle contraction, cell growth and division *etc.* [3].

Gluconeogenesis, glycogenolysis and intestinal absorption are the major sources of circulating glucose levels. In the fasting state among euglycemic individuals glucose is derived from glycogenolysis under glucagon control, while during the fed state, insulin promotes glycogenolysis and gluconeogenesis in the liver resulting in peripheral deposition of glucose [4]. Insulin given exogenously to diabetics (during fed state) has minimal or almost no effect in suppression of glucagon secretion through the paracrine pathway hence elevating hepatic glucose production. Ultimately the rate of glucose production in circulation surpasses the rate of glucose clearance resulting in postprandial high blood glucose levels [5].

Blood glucose analysis is the most common routine test in every clinical facility. A blood glucose of 126 mg/dl or higher on two separate occasions is required for diagnosis of diabetes by WHO and ADA. Due to the blood glucose wide reference range both hypoglycaemia and hyperglycaemia have significant effects on short as well as long-term mortality and morbidity of diabetic patients [6]. A large randomized international trial (NICE-SUGAR) documented that strict glycaemic control, greater the mortality risk in critically ill diabetic patients while mortality was reduced by

**Corresponding author: Syeda Sabahat Haider Zaidi, Department of Pathology, Sheikh Zayed Hospital, Rahim Yar Khan, Pakistan, Email: sabahattariq@gmail.com*
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keeping the blood glucose value 10mmol/L instead of the target value *i.e*. 4.5 -6.0 mmol/L [7]. Other large studies like DCCT (Diabetes Control and Care Trial) and UKPDS (United Kingdom Prospective Diabetes Study) encompassing adults of type 1 DM and type 2 DM respectively, demonstrate increased risk of death, hypoglycemia and major macrovascular complications by intensive glucose control than less intensive target groups [8].

Glucose concentration in blood is estimated by using methods based on three basic principles of condensation, reduction and enzymatic reaction *i.e*. Endpoint. Among these three basic approaches, the Reduction technique is the oldest which utilizes the reducing capacity of glucose when it is being oxidized. However, these reducing methods are not very specific and the presence of another strong reducing agent can result in a false positive higher test result [9]. Clinical diagnosis of hyperglycaemia, hypoglycaemia, and normoglycaemia weighs on accurate glucose estimation. It takes a lot of technical effort, time and cost to ensure test results are accurate. The technique or method of analysis must undergo a procedure known as quality assurance before being introduced in the lab. Test efficiency can be ensured by selecting a validated method which critically assesses the analytical specificity, sensitivity, limit of detection, and limit of quantification and also documenting the role of interfering substances hence resulting in improvised test results and estimation of imprecision and inaccuracy possible with method comparison [10].

The commonly used laboratory method for the measurement of glucose is mostly considered as a field method so some degree of inaccuracy or biasness may exist making it less specific. In contrast, a reference method is developed by using standard reference material for assay, calibrator and control and the gained value is the real test value which matches the target result with a minimal difference [11]. Our study has presented the comparison of the two most widely used methods for glucose estimation incorporating the valid statistical tool *i.e*. regression analysis and linear correlation coefficient. There are various methods for the measurement of serum glucose on different instruments. Also, there are enzymatic and non-enzymatic methods for blood glucose estimation [12-15].

Various standardized substrates and co-substrates are being used in the enzymatic methods; each has a certain advantage over the others claiming the minimal amount of 20 µl sera required for accurate glucose determination but has certain limitations such as long incubation at 37ºC for ten minutes (minimum) [16]. Moreover, the interferences caused by similar sugars such as mannose, galactose, maltose and fructose are not being addressed while some methods require dedicated sensitive instruments with high technical expertise [17-19].

We aimed this study to observe and determine a simpler method which is not only specific but also accurate and independent of any dedicated instrument making the test cost effective for both patients and clinicians.

METHODOLOGY

We conducted an observational descriptive study over a period of six months after getting approval from the ethical review board of the institution. An informed consent was taken from the participant. A total of 250 fasting blood samples were analyzed but samples with glucose levels less than 50 mg/dl as well as lipemic, hemolyzed or icteric samples were rejected. Subsequently, 110 samples were run using GOD-PAP assay and compared with the Hexokinase method. Glucose measured by GOD-PAP assay (HUMAN) utilizes highly specific glucose oxidase enzyme while Hexokinase assay (BECKMAN) measures glucose at 340nm using the absorbance of NADPH. Both methods used to measure glucose employ spectrophotometric techniques. A sample size of 43 (rounded to 110) was calculated by using a confidence interval of 95%, a level of significance of 1% (two-sided) with the power of test as 98%, the difference in means of both methods 20mg/dl as in the previous study conducted by Kumar *et al*. [20].

Principle

Glucose Oxidase Peroxidase Method

Glucose Oxidase enzyme oxidizes β-D glucose to Gluconic acid while oxygen is reduced to H2O2. Under the influence of the Peroxidase enzyme, H2O2 reacts with 4-amino antipyrine and produces quinoneimine in the presence of Phenol. Quinonamine is a coloured compound that can be analyzed using colourimetric analysis at 505 nm.

Hexokinase Method

The hexokinase method is a highly specific method for determining plasma glucose. This enzymatic method yields NADH through the hexokinase-catalyzed transformation of glucose. In this enzymatic method, glucose is converted to glucose-6-phosphate (G-6-P) by hexokinase in the presence of ATP, a phosphate donor. Glucose-6-phosphate dehydrogenase then converts the G-6-P to gluconate-6-P in the presence of NADP/NAD which is converted to NADPH/NADH. The absorbance

of NADH is then quantified at 340nm and is directly proportional to glucose concentration in a blood sample.

Data Collection and Analysis

Fasting blood specimens were collected in Na-fluoride-Potassium oxalate vials and were centrifuged at 2000 rpm for 10 minutes. Plasma was separated within 40 minutes of sample collection and was saved with labelling. Glucose was analyzed by using a fully automated chemistry analyzer (Atellica CH-930) for the hexokinase method using Atellica CH Glucose Hexokinase_3 assay and a semi-automated chemistry analyzer (ERBA-Chem 7) for GOD-PAP method using HUMAN Glucose liquid color assay. Quality control was ensured on both instruments by running respective third-party QC material (Bio-rad and Serados respectively). We used analytical grade reagents for analysis. Results were obtained on predesigned proforma. Data was collected and entered into the social statistical package. A replication study was done to determine imprecision (random error). Random error was found to be 1/3rd of Total Allowable Error hence acceptable. A recovery study was performed to determine inaccuracy. The results of the recovery study were less than the total allowable error (TAE) for the point estimate of proportional error. The method of comparison was then followed to determine the average bias which was also less than TAE making it acceptable according to CLIA. Linear regression technique is used for evaluating comparison of methods using equation y=mx+b and least square regression gave the line of best fit using SPSS 23. The relationship between the two methods and their agreement is shown by the Bland Altman plot as in **Fig. (1)**. We have studied the linearity by using glucose standards in different concentrations and plotted a standard curve. Method precision was determined by establishing the CV and Std deviation of the samples at normoglycemia and hyperglycemia [21]. P-value less than or equal to 0.05 was considered as statistically significant.

RESULTS

A total of 110 patient specimens were run on both a fully automated chemistry analyzer and semi-automated instrument using Hexokinase and GOD-PAP assay respectively for method comparison. Precision determined by replication study as in Table **1** showed the random error was less than TAE. The mean value determined by the hexokinase method was 106.34 while from GOD-PAP assay it gave 100.29. There was a negative bias of -6.14 shown in Table **2** and **Fig. (2)**.

In Table **3**, the correlation coefficient (calculated parameter) represents the linear correlation between the two methods. The accuracy of the said methods was measured by a recovery study. Table **4** shows that the Proportional Error was less than the Total Allowable Error. Statistical Parameters in method comparison as in

Table 1: Glucose assay quality control.

Method	GOD-PAP Method		Hexokinase Method	
Parameter	Std (100 mg/dL)	Std (200 mg/dL)	QC- L1 (base value)	QC- L2 (base value)
Mean	100.1	200.1	59.55 / 58.29	115.6 / 114
± SD	0.538	0.587	3.069 / 3.385	3.84 / 5.75
CV %	0.537	0.293	5.1 / 5.8	3.32 / 5.04

Table 2: Descriptive statistics.

Test Method	n	Mean	± Std. Deviation	CV%
GOD-PAP	110	100.29	± 41.860	40.58
Hexokinase	110	106.34	± 43.150	41.78
Normoglycemia				
GOD-PAP	99	91.44	± 31.253	34.18
Hexokinase	99	94.24	± 31.288	34.20
Hyperglycemic				
GOD-PAP	11	202.73	± 54.200	26.74
Hexokinase	11	211.09	± 57.735	27.35

Table 3: Pearson correlation.

Instrument	n	Pearson Correlation (Hexokinase)	Pearson Correlation GOD-PAP
Atellica Ch-980 Hexokinase	110	1.000	0.996
ERBA CHEM-7 GOD-PAP	110	0.996	1.000

Table 4: Recovery study.

Specimen (mg/dL)	Baseline Glucose Value	Spiked Value	Glucose Recovered	% Recovery
S- 1	115.6	47.6	161.3	96
S- 2	66	47.6	110.3	93
S- 3	142.3	47.6	190.6	101

Table 5: Method comparison statistics.

M	(mg/dl)	Sy/x	Bias	F	R
1.026	3.44	3.17	-6.14	1.091	0.996

Table **5** show values of slope and intercept. The linear regression analysis was performed for comparison of methods. The line of best fit was obtained by least square linear regression on SPSS as in **Fig. (3)** and total error was calculated manually.

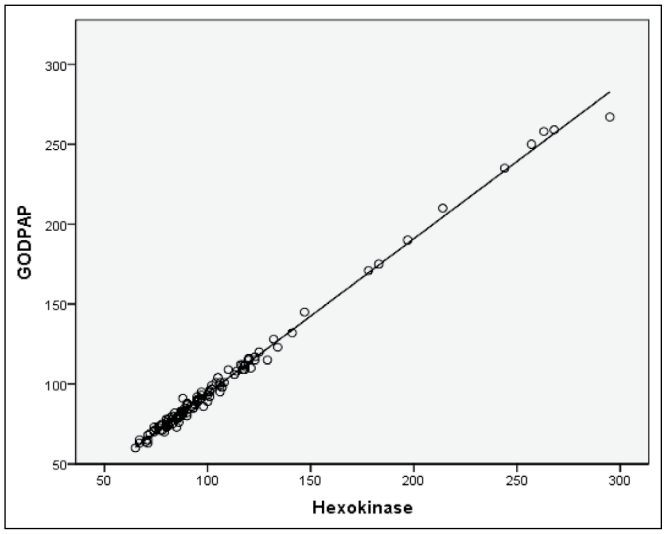

Fig. (1): Bland-Altman difference Plot showing comparing the glucose estimation of samples by GOD-PAP and Hexokinase method.

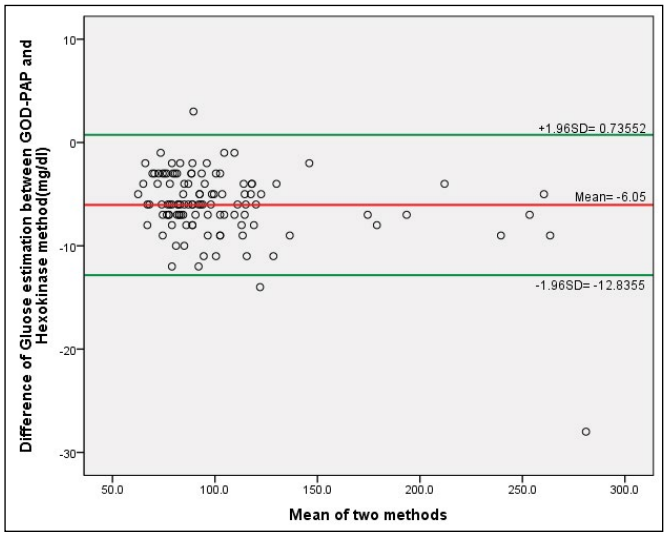

Fig. (2): Means of Hexokinase and GOD-PAP methods.

DISCUSSION

Various methods for glucose estimation have been proposed and developed according to its different

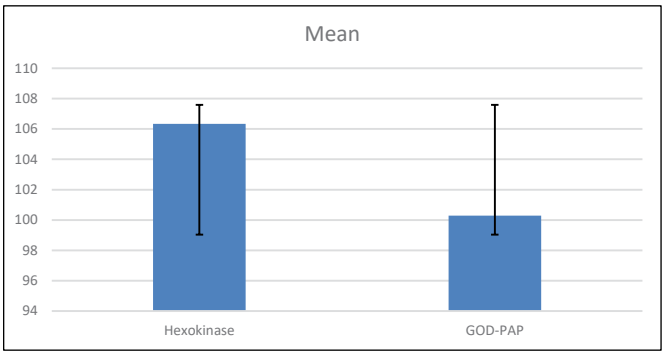

Fig. (3): Line of best fit.

properties. We aimed this study to determine the degree of agreement between a commonly used method and a reference method based on its accuracy, precision and linearity in hyperglycemic and normoglycemic patients. Of the total 110 selected specimens, 23 were fasting and 87 were non-fasting samples. There were 11 hyperglycemic and 99 normoglycemic specimens.

In previous studies, it was found that the GOD-PAP values tend to be lower than the Hexokinase method. In a study by Dickson *et al*., they demonstrated that the mean plasma glucose concentration measured by GOD-PAP gives higher results than that measured by Hexokinase at each point in the testing of OGTT in 1776 pregnant women [22]. Another study by Kumar *et al*. demonstrated that there was a negative bias of -19.49 in her study. She performed experiments on 105 samples. A comparative prospective study of the two methods was performed. Linear regression analysis was more useful than *t*he t-test for evaluating the comparison of method (COM) studies [20]. However previous studies had found a good correlation between the two methods.

We determined precision by repeated analysis. Examining random error is a test that was experimented with replication. The degree of error varies between samples. Two controls were conducted twice a day during a precision investigation for 20 days. Our study estimated the amount of imprecision. Multiple aliquots of the same samples were run repeatedly and the results were monitored and noted. The replication study

showed that the Random error was < Total Allowable Error which was acceptable according to CLIA.

The SD (σ) for the GOD PAP at 100 mg strength was 0.538, with a coefficient of variance of 0.587 and for the 200 mg standard its SD and CV were found to be 0.537 and 0.293 respectively which serve as a measure of a test technique's accuracy. While the reference method quality control L1 and L2 SD was 3.06 and 3.84 while coefficient of variance was 5.1 for L1 and 3.32 for L2. The mean value of 110 samples obtained by Hexokinase was 106.34±43.15 and obtained by GOD-PAP was 100.2± 41.86. In the current investigation, a negative bias (-6.14 mg/dl) was used to establish the test method's accuracy. Our study also determined the constant systematic error by calculating bias against the test technique, glucose oxidase-peroxidase. The test readings always come below the reference value, which is a sign of negative bias. Recovery experiments were intended to gauge accuracy so it was conducted in a replicate of the patient sample and spiked sample and the concentration recovered was calculated by subtracting the baseline value and spiked sample value.

The estimated correlation coefficient (r) shows how linearly the two approaches are correlated. The correlation coefficient demonstrates the connection between the two methodologies under comparison. Both variables were quantitative and normally distributed with no outliers, so we calculated Pearson's r correlation coefficient and it came out as r= -0.996. The value of the correlation coefficient always ranges between 1 and -1 and is treated as a general indicator of the strength of the relationship between variables. These results are by Fiedorova *et al*. [11] and also the research done by Jia and Zhang who evaluated the reference method with GOD-PAP and a close correlation *i.e*. r > 0.99 was found between the two methods [23]. According to Gurung *et al*. [10], and Kumar *et al*. [20] they obtained the correlation coefficient "r" =1.00 between the two methods in their study.

Total Error

"Scatter of the data points" along with the regression line demonstrated the standard error of the estimate. Significant random error between the procedures under comparison is indicated by a high Sy /x number. So, the Total Error is calculated as 14.98 mg/dl. In this study, the proportional error is 2.6 % which is less than the Total Allowable Error and is acceptable.

LIMITATION

Small sample size and the effect of hematocrit and water concentration in plasma could be the limitation of our study which needs to be explored in terms of accuracy with the reference method**.**

CONCLUSION

These results of our study show that both test methods can be used to measure glucose as per "CLIA *88*" [24]. The glucose oxidase-peroxidase technique has a total error (7.49%) which is lower than the 10% of the total allowable error TAE (10%) when compared to the hexokinase method, hence acceptable.

ETHICAL APPROVAL

Ethical approval was obtained from the Institutional Review Board of Sheikh Zayed College/Hospital, Rahim Yar Khan (REF letter No. 721IRB/SZMC Dated: 15-06-2023). All procedures performed in studies involving human participants followed the ethical standards of the institutional and/ or national research committee and the Helsinki Declaration.

CONSENT FOR PUBLICATION

Written informed consent was taken from the participants.

AVAILABILITY OF DATA

The data set may be acquired from the corresponding author upon a reasonable request.

FUNDING

Declared none.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS' CONTRIBUTION

All the authors contributed equally to the publication of this article.

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