JMMIHS Vol 7 issue 1 2021, 49 - 59

Original Article

STANDARDIZATION OF VISIBLE KINETIC ASSAY FOR THE ESTIMATION OF PLASMA GLUCOSE BY GLUCOSE-OXIDASE AND PEROXIDASE METHO

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DOI: https://doi.org/10.3126/jmmihs.v7i1.43150

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ABSTRACT

Objective: In the clinical laboratory, glucose is the most frequently analyzed test in blood which plays a vital role in the diagnosis and management of patients suffering from diabetes mellitus and metabolic disorders. The glucose oxidase and peroxidase (GOD-POD) method is an end point reaction method for glucose estimation, which is cheap and readily available in routine laboratories with significant time consumption. However, Glucose estimation by hexokinase is available for rapid estimation that may cost comparatively higher for the routine laboratories. Thus, our study is designed to standardize rapid and convenient method of plasma glucose estimation with modification by kinetic mode based on GOD-POD reaction for the rapid and high through put analysis of glucose estimation using semi-automated or autoanalyzers.

Method: Photometric linearity of the kinetic method is compared with that of end point reaction methods. Furthermore, correlation between an endpoint and kinetic method was determined using Pearson correlation using plasma from normal and diabetic patients (n=32) visiting Manmohan Memorial Teaching Hospital, Kathmandu.

Result: Our study showed, significant positive correlation between the end point and the kinetic method (r=0.99) The linearity of modified kinetic (GOD-POD) method is up to 400 mg/dl in comparison to that of existing end point method (500 mg/dl), which covers the

normoglycemic to pathological hyperglycemic range of glucose estimation in routine laboratories.

Conclusion: The significant positive correlation of our visible kinetic method with end point reaction method shows possibilities for the high through put and rapid analysis of the glucose estimation in 3 minute using semi-automated and autoanalizers.

Key Words: *GOD-POD, Kinetic Method, End Point Method*

INTRODUCTION

Glucose in the blood is the most frequently analyzed parameter in a clinical biochemistry laboratory. Detection and quantification of glucose in blood plays a vital role in the diagnosis and management of patients suffering from disorders of carbohydrate metabolism and blood glucose homeostasis in various diseases[1]. Most essentially, measurement of blood glucose plays vital role in diagnosis, management and prevention of diabetes mellitus (DM) and associated organ specific complications and prevents hyperglycemic metabolic memory[2,3]. Basically, blood glucose homeostasis is regulated by the hormones insulin and glucagon and these hormones are secreted by the islet of Langerhans of the pancreas affecting the blood glucose levels[4] . In absorptive stage, increase in blood glucose influence the beta cells and increase the release of insulin into the blood to stimulate cellular uptake of glucose from the blood by muscle and adipose tissue. After post-absorptive state, insulin levels decrease along with blood glucose and this results in the glucagon being released by the alpha cells of the pancreas[5]. Diabetes mellitus is a common non-communicable disease worldwide, which is characterized by hyperglycemia due to defective insulin production causes type-1 DM, and due to insulin resistance results in type-2 DM[2,6] leading to macrovascular and microvascular complications[7]. So, laboratory analysis of blood sugar levels are an important parameter for the diagnosis and management of diabetes and its complications[8].

Among various method for the detection of blood glucose levels, enzymatic methods are currently in practice including glucose oxidase and peroxidase (GOD-POD) and most recently the hexokinase method. However, chemical methods for glucose detection such as Folin-Wu method and O-Toluidine method having risk of chemical hazards and have been replaced in

the laboratories due to their low sensitivity and specificity[9,10]. The GOD-POD method is comparatively cheaper, commercial kits are easily available and used by the routine laboratories and can be adopted using colorimeter, spectrophotometer in manual as well as in automated systems with linearity up to 500 mg/dl in visible spectrum (the absorbance to be read between 505 nm to 550 nm)[11]. Glucose oxidase is an enzyme highly specific for glucose. The enzymatic, GOD-POD method is specific for glucose, but it takes 10-15 minutes of time with end point chemistry. So, it may consume time for high through put and in emergency cases. Glucometer as a point of care gives very quick result of blood glucose estimation with compromised accuracy and reliability. Method for glucose estimation by kinetic method based on Hexokinase and Glucose-6-phosphate dehydrogenase (G-6-PD) enzymes are available for rapid estimation with high cost [9] . Although, GOD-POD and Hexokinase methods both have linearity up to 500mg/dl, hexokinase require UV (340 nm) range for the measurement[11]. GOD–POD end point reaction method is specific for the glucose estimation in routine laboratories but it take longer time i.e10-15 minute in comparison to hexokinase UV- kinetic method. Thus, there is still a need of a rapid and convenient, accurate and high throughput method of glucose estimation.

This study aims to standardize a rapid and accurate method of plasma glucose estimation by kinetic modification based on GOD-POD enzymatic reaction for high through put analysis within short turnover time. Linearity of the method and assay time is determined and validated and correlated with the conventional method using normal and hyperglycemic patient's samples.

MATERIALS AND METHOD

Chemicals and equipment: This study was laboratory-based cross sectional study conducted in the Department of Laboratory Medicine at Manmohan Memorial Institute of Health Science (MMIHS). All the chemicals including glucose and buffer reagents were purchased from Sigma Aldrich, India. Glucose kit with known calibrator standard and well defined reagent composition were purchased from Agappe diagnostics Ltd., Switzerland, and glucose concentration was estimated by GOD-POD method following manufacturer's guideline. Internal control materials were supplied by Meril Diagnostics, India.

Photometric measurements: Measurement of glucose was done using known concentration of glucose standard solution 1000mg/dl prepared in phosphate buffer (pH 7.0) and serial dilution was made for the calibration of the visible kinetic method and compared with existing end point reaction method. All tests were analyzed in semi auto analyzer (Bio analyzer 100, China). Glucose is measured in plasma or standard solutions by GOD-POD reaction as described by manufacturer. The sample (10ul) when mixed with reagent (1000ul), glucose present in sample is oxidized by the enzyme glucose oxidase (GOD) to gluconic acid with the liberation of hydrogen peroxide, which is converted to water and oxygen by the enzyme peroxidase (POD), 4-aminophenazone an oxygen acceptor together with phenol forms a pink colored chromogen which was measured at 515nm.

The incubation time for the end point reaction method recommender by manufacturer (Agappe Diagnostics, Switzerland) was 10 minutes at 37^{o}C. For the kinetic assay, we performed time course experiment for the determination of assay time. Known concentration 100mg/dl standard solution of glucose is measured thrice using increasing time intervals and mean glucose concentration with standard deviation and coefficient of variance was used to determine correct assay time interval in two point kinetic mode. For the determination of linearity of the methods, primary glucose standard solution of 1000mg/dl was prepared and made serial dilution for the analysis of absorbance and mean Δ absorption for end point as well as kinetic method, respectively. The lowest calibration standard which produced a peak response corresponding to the analyte was measured 8 times. The average response (X) and standard deviation (SD) was calculated and limit of detection (LOD) or sensitivity was obtained by the formula: LOD= X + (3 x SD).

Specimen collection: Kinetic method was correlated with end point method using patient's plasma as a sample. Performa was used as sample collection tool. Random blood sample was collected in fluoride vials using Standard Operating Procedure (SOP) from the normoglycemic and hyperglycemic with total of 32 (n=32) patient visiting Manmohan Memorial Teaching Hospital, Kathmandu. Plasma was separated by centrifugation at 3000 rpm for 5 minutes and was analyzed for blood glucose by semi-automated method using manufacturer's guidelines. Internal and external quality control was maintained in the laboratory.

Data analysis: The laboratory work was performed following standard operating procedures under manufacturer guideline using standard operation procedure approved by Institutional Review Committee of MMIHS. Entry of data was manually recorded and entered into SPSS and Microsoft excel for the analysis. Ethical approval was taken from Institutional Research Committee (IRC) of Manmohan Memorial Institute of Health Sciences. The informed consent was taken from the patients.

RESULTS

Photometric measurement of glucose was done using known concentration of glucose standard solution 1000mg/dl prepared in phosphate buffer (pH 7) and serial dilution was made for the calibration of the visible kinetic method and compared with existing end point reaction method. Glucose was measured using GOD-POD reagent kit supplied by Agappe Diagnostics, Switzerland.

Calibration of kinetic assay:

We calibrated the method of glucose estimation by kinetic assay with two point visible mode using semiautomatic analyzer in which we determined linearity of method and compared with that of end point method. Limit of detection and assay time were also determined for the analytical sensitivity and correlation of results using human plasma from normal and diabetic subjects were analyzed and tests were validated with control sera. The incubation time for the end point reaction method recommender by manufacturer was 10 minutes at 37^{o}C. For the kinetic assay, we performed experiment for the determination of assay time. Known concentration 100mg/dl standard solution of glucose is measured thrice using increasing time intervals and mean glucose concentration with standard deviation and coefficient of variance was used to determine correct time 3 minute for the kinetic assay with two point mode (Table 1). For the assay sensitivity, limit of detection is measured by using average response (X) and standard deviation (SD) was calculated and LOD was obtained by the formula: LOD= 4.92+3x0.7=5.1.

Assay time for the kinetic method was determined using mean of triplicate test results of 100mg/dl glucose standard measured immediately and reading time started from 1 to 5

minutes. In end point method, mean and CV% was 98.8±0.48 and 0.45% respectively in 10 minutes. The least CV %(0.8416) was found in the shortest time 180 seconds (3 minutes) indicates the suitable reading time for the kinetic assay.

Table 1: Calibration of assay time for the kinetic method: mean of triplicate test results of 100mg/dl glucose standard was analyzed 3 times for the determination of photometric assay time.

Test Method	**Time** (minutes)	**Glucose, mg/dl** (Mean±SD)	**%CV**
End point reaction method	**10**	**98.8±0.447**	**0.45**
Kinetic method	1.5	97.72±3.42	3.51
	2.0	97.78±2.93	2.93
	2.5	97.58±4.26	4.37
	3.0	**98.52±1.03**	**0.8416**
	3.5	94.2±3.76	4
	5.0	98.16±1.03	1.0664

Measurement of linearity of the kinetic method in comparison to end point assay:

For the measurement of linearity of the test method, we used known concentration of glucose standard solution 1000mg/dl freshly prepared in phosphate buffer (pH 7) and serial dilutions: 5ml, 10ml, 15ml, 25ml, 50ml, 100ml, 200ml, 300ml, 400ml, 500ml, 600ml, 700ml, 800ml, 900ml and 1000ml was made and glucose is estimated by both methods three times for the calibration curve of the visible kinetic method and compared same with existing end point reaction method.

Graph of the glucose concentration versus absorbance of end point reaction method shows the straight line up to 500mg/dl, that indicates the linearity of this method up to 500mg/dl (Figure 1). When we measured the rate of reaction in two point kinetic mode, the glucose concentration versus average change in absorbance per unit time (Δ absorbance/min) of kinetic method graph shows the straight line up to 400mg/dl indicates the linearity of method is 400mg/dl, (Figure 2). Although, similar study performed the comparison of two point assay of

glucose estimation, we standardized and verified more clearly the time linear rate of reaction with limit of detection of the kinetic assay[11].

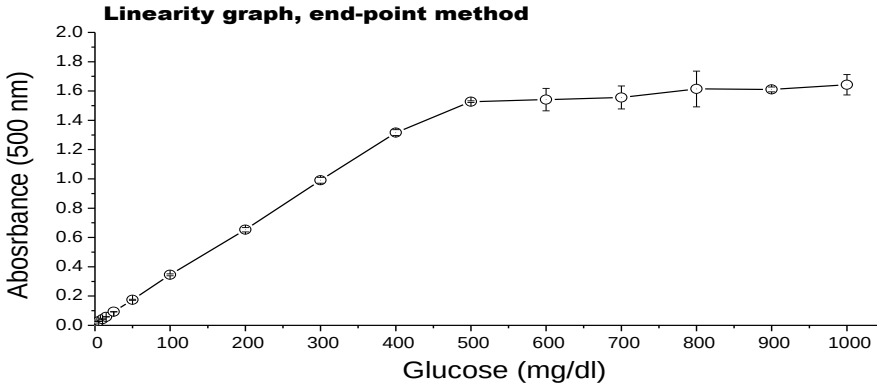

Figure 1: Absorbance versus concentration of the glucose in mg/dl by endpoint method in 10 minute

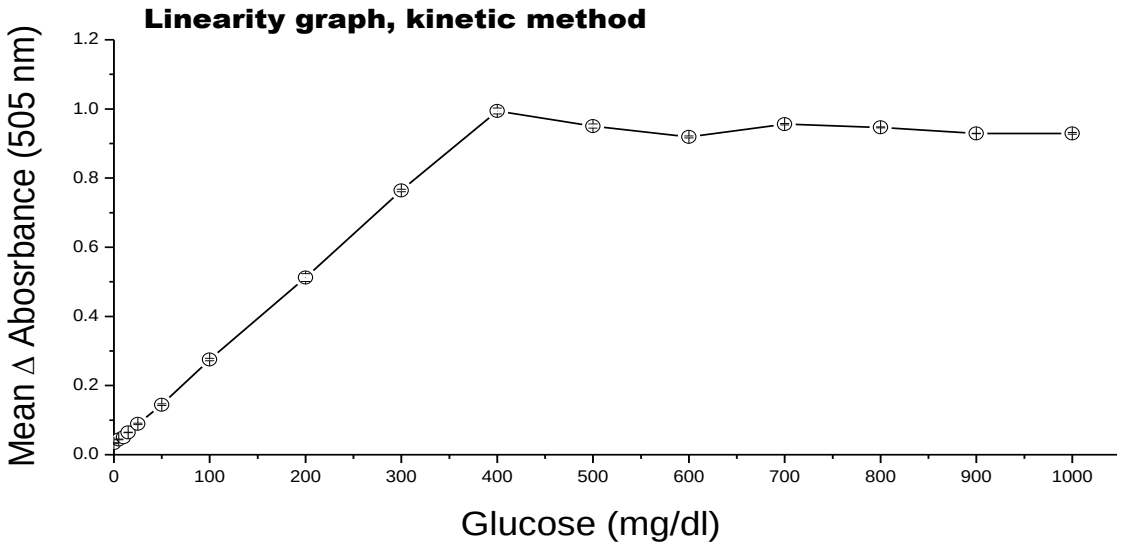

Figure 2: Rate of reaction, change in absorbance in 180 seconds (Mean Δ absorbance) versus concentration of the glucose in mg/dl by kinetic method.

Correlation and validation of kinetic method with end point reaction method:

Plasma specimen from 32 individual, 7 were male and 25 were female among them 12(38%) were hyperglycemic and 20 (62%) were normoglycemic were analyzed by both end point reaction method and kinetic method (Figure 3). Pearson correlation is performed in order to observe linear relation. Results show significant positive correlation between end point and kinetic method with $p<0.001$ in both methods (Table 2 and 3). For the validation of our results of glucose measured by both methods, we maintained external as well as internal quality control as shown in Table 4.

Table 2: Correlation between end point and kinetic method.

Methods	**Pearson(r)**	***p* -value**
End –point	**0.99****	**<0.001**
Kinetic method	**0.99****	**<0.001**

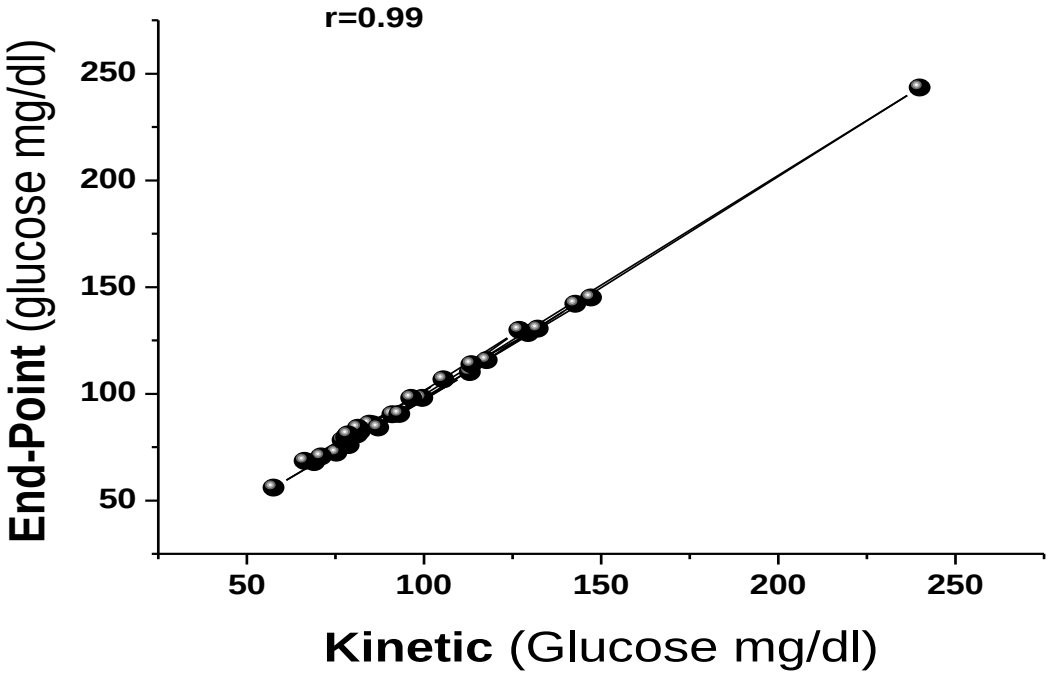

Table 3: Two-tailed Pearson correlation between end point and kinetic method.

2-tailed Pearson correlation		**Kinetic method**	**End point method**
Kinetic method	Pearson Correlation	1	.99**
	Sig. (2-tailed)		00
	N	32	32
End point method	Pearson Correlation	99**	1
	Sig. (2-tailed)	00	
	N	32	32

Table 4: Validation of test methods using internal quality control sera

Quality control sera	**Methods:**		**Normal range**
Pathological sera	End point	Kinetic method	
Normal sera	243.60	242.0	220.28-310.32
	93.06	90.45	77.61-109.40

DISCUSSION

Key themes emerged around: the conceptualisation of CPD in Nepal; provision and funding; barriers – fiscal, political and geographical; and future priorities which included a discussion around basic skills versus advanced practice. Our study is focused on analytical method for the diagnosis of diabetes and metabolic syndrome in which glucose measurement plays crucial role.

Glucose estimation is very important for screening, diagnosis and monitoring of glucose status in healthy as well as diabetic mellitus patients with or without complications. Although for the rapid estimation blood glucose we can use glucometer as point of care testing, accurate laboratory method is required for the evidence based practice with high sensitivity and specify.

Standard time for the end point is 10-15 minutes in different preparations. Hexokinase methods are available as a kinetic measurement which may be rapid but require UV spectrum and also cost high for the routine laboratories and patients. Linearity of glucose assay by kinetic method is up to 400mg/dl while by end point method is up to 500mg/dl, however, Basak *et al*. have found linearity up to 340mg/dl[11]. According to the commercially available protocol the linearity

is up to 600 mg/dl it may be due to that we use the aqueous standard solution. Linearity may vary according to the instrument that we use. So we should establish our own linearity curve in the lab. If result obtained above the linearity we option to dilute the sample to achieve result under linear range and multiplied by dilution factor. In our study, positive correlation among kinetic and endpoint method was found by measurement of normal and hyperglycemic plasma, which is consistent with the result of Basak *et al* described earlier using standard glucose solution[11]. Additionally, we described measurement of our kinetic assay with reading time determination using time course experiment, linearity measurement as well as correlation study data, limit of detection and results are validated using normal as well as patient's samples and quality control sera.

CONCLUSION

Our study of glucose estimation by visible kinetic method can be the right choice of method in routine laboratories for the rapid assay time and high throughput analysis of plasma glucose estimation. Although, linearity of the end point method is higher than that of the kinetic method, sample dilution can be followed in case of hyperglycemia beyond linear range. Thus, our kinetic method is faster than the end-point method, so this method can be installed in automation and semi automation in routine laboratories.

RECOMMENDATION

Our study recommends the opportunity of newly established visible kinetic method of glucose estimation is cost effective, rapid and can be suitable for high through put analysis in routine laboratories.

CONFLICT OF INTEREST: Authors declared, there is no conflict of interest.

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