DEVELOPMENT OF A RAPID AND INEXPENSIVE PLASMA GLUCOSE ESTIMATION BY TWO-POINT KINETIC METHOD BASED ON GLUCOSE OXIDASE-PEROXIDASE ENZYMES

Anjan Basak

Department of Biochemistry, Jawaharlal Nehru Medical College, Sawangi (Meghe), Wardha-442 004, India

ABSTRACT

A rapid and inexpensive plasma glucose estimation by two-point kinetic method based on glucose oxidase and peroxidase enzymes has been developed. It takes only 1½ minutes of time and validity of the method has been discussed.

KEY WORDS

Glucose estimation, Glucose oxidase – peroxidase, Two-point kinetic method.

INTRODUCTION

Plasma glucose estimation based on Glucose Oxidase (E.C. 1.1.3.4) – Peroxidase (E.C. 1.11.1.7) (GOD-POD) method for the colorimetric determination was first introduced by Worthington in 1956. It was subsequently modified by many workers and Trinder (1), and soon it became very much popular replacing the cumbersome and time consuming multistep chemical methods of sugar estimation. The enzymatic method is specific for glucose, but it takes 10-15 minutes of time in endpoint chemistry. Hence, it is not suitable for emergency cases. Glucometer, though it gives very quick result of blood glucose estimation, is not at all accurate and reliable (author's unpublished data)

Now-a-days in India, kits for glucose estimation by kinetic method based on Hexokinase-Glucose-6-phosphate dehydrogenase (G-6-PD) enzymes(2) are available for rapid estimation, but this is costly. Hence, there is a need of a rapid and inexpensive, yet accurate method of glucose estimation, especially, in hypoglycaemic patients.

The present work has been carried out with the aims and objectives of developing a rapid and inexpensive, yet accurate, method of plasma glucose estimation by kinetic modification

based on GOD-POD enzymes to use it, especially, in emergency cases.

MATERIALS AND METHODS

A. Materials & Equipments : Glucose Oxidase (GOD) : 166.5 U/mg activity (Sigma, USA), Horseradish Peroxidase (POD) : 80 U/mg activity (HiMedia) were used. Other chemicals like, phenol, 4-aminoantipyrine etc. were of analytical grade obtained from s.d. fine-chem. Ltd., India. Glucose estimations were done by GOD-POD based end-point method by the kit of Span Diagnostics Ltd., Surat. Semi-autanalyser used in the study was of Bayer's Diagnostics, Model : RA – 50 and Electronic balance was of Shimadzu Company, Japan. Autopipettes were of Labsystem's Finnpiptette. Blood samples were collected in sugar bulb from healthy persons as well as from Diabetic patients attending O.P.D. of our hospital with prior written consent. Plasma were separated for glucose estimation.

B. Principle of Reactions :

- β -D-Glucopyranose + GOD enz-FAD $\xrightleftharpoons{\quad} \text{GOD enz-FADH}_2$ + D-glucono- δ -lactone
- $\text{GOD enz-FADH}_2 + \text{O}_2 \rightarrow \text{GOD enz} - \text{FAD} + \text{H}_2\text{O}_2$
- $\text{H}_2\text{O}_2 \xrightarrow{\text{POD}} \text{H}_2\text{O} + [\text{O}]$
- $[\text{O}] + \text{phenol} + 4\text{-aminoantipyrine} \rightarrow \text{Quinoneimine dye}$
(Reddish colour)

Rate of intensity of reddish colour formation is directly proportional to the glucose concentration in the sample and change of absorbance per minute ($\Delta A/\text{min}$) was measured at

Address for Correspondence:

Dr. Anjan Basak
Professor & Head, Department of Biochemistry,
Jawaharlal Nehru Medical College,
Sawangi (Meghe), Wardha-442 004, India
E-mail : medical_wda@sancharnet.in

500 nm. The change in absorbance per minute of unknown plasma samples were compared with that of standard glucose solution (100mg/dL) to find out plasma glucose concentrations.

C. Reagents Preparation :

- I. PHOSPHATE BUFFER, pH 7.0, 200 mM, containing 0.15% (v/v) hypophosphorous acid : Na_2HPO_4 , 200 mM, solution was prepared to which 500 μL of 30% hypophosphorous acid solution was added and mixed. Then, KH_2PO_4 , 200 mM solution, was added to it gradually and mixed. pH was adjusted to 7.0 by pH meter.
- II. GLUCOSE COLOUR REAGENT : to 100 ml of above phosphate buffer solution the following chemicals, one by one, sequentially were added and mixed.

Phenol (redistilled)	10 mM (94mg/dL)
4-Aminoantipyrine	6.0 mM (122mg/dL)
Tween-20	50 μL

If slight pink colour develops at this stage or during 06 months of storage in refrigerator then, the solution is to be discarded. Purity of hypophosphorous acid and phenol is to be checked for preparation of this colourless reagent which will keep well at least for 06 months.

Glucose oxidase (GOD)	1800 I.U. (10.8 mg)
Peroxidase (POD)	300 I.U. (3.7 mg)
Sodium azide	10 mg

- III. COLOUR REAGENT OF SPAN DIAGNOSTICS : it contains phosphate buffer of pH 7.5, glucose oxidase, peroxidase, phenol, 4-aminoantipyrine, stabiliser and preservative. Concentration of individual chemicals were not mentioned in the kit's literature.
- IV. GLUCOSE SOLUTIONS : analytical grade of glucose was dried at 80°C for 2 hours. 1000 mg/dL glucose stock solution was prepared in saturated solution of benzoic acid. Then, different concentrations of glucose solutions were prepared starting from 5 mg/dL to 900 mg/dL of which 100 mg/dL solution was used as standard.

D. Autoanalyser Programming : The semi-autoanalyser was programmed for two-point kinetic with standard with reagent blank with concentration / absorbance mode separately and as well as for endpoint method (as per literature of Span Diagnostics' kit) as follows :

	Two-point kinetic assay	Endpoint assay
Temperature	37°C	37°C
Sample vol.	20 μL	10 μL
Reagent vol.	1000 μL	1000 μL
Std. Conc.	100 mg/dL	100 mg/dL
Reaction type	Two-point kinetic with std. with reagent blank Individual progression	Endpoint with std. with reagent blank
Units	mg/dL or Absorbance	mg/dL
Wavelength	500 nm	500 nm
Slope of reaction	Increasing	—
Time T_1	30 secs.	—
Time T_2	90 secs.	—
Result resolution	0.1 mg/dL or 0.0001A	0.1 mg/dL
Aspiration volume	950 μL	950 μL

E. Study Design :

- I. First to check the linearity of the kinetic method, known concentration of glucose solutions were measured in the semi-autoanalyser in absorbance mode taking 100 mg/dL glucose solution as standard. Absorbance vs concentration of glucose solutions were plotted on graph paper to find out the range of linearity. Glucose estimation in different solutions were done by running in semi-autoanalyser in concentration mode. Graph was plotted for estimated glucose values against the values of prepared glucose solutions to find out colinearity.
- II. Glucose in the plasma samples were measured by endpoint method of Span Diagnostics' kit as well as by the two-point kinetic method in concentration mode. Graph was drawn to find out colinearity.
- III. In each plasma samples and glucose solutions, glucose concentrations were estimated in triplicate by the kinetic method as well as by the endpoint method (as per study design) and mean values were taken for results analysis.
- IV. Experimental data were analysed for drawing conclusions subjecting to appropriate statistical methods.

F. Assay Procedure :

- I. GOD-POD endpoint assay :
- Colour reagent 1000 μ L
- Plasma/std.glucose soln.(100mg/dL) 10 μ L
- Mix and keep at 37°C x 10 mins., then aspirate in semi-autoanalyser
- II. GOD-POD based two-point kinetic assay :
- a) Glucose colour reagent 1000 μ L
- Plasma/std.glucose soln. 20 μ L (100mg/dL)
- Mix & aspirate immediately
- b) If glucose concentration was found to be more than 340 mg/dL in kinetic assay or 400 mg/dL in end point assay, then a repeat assay was done with appropriate dilution of plasma with normal saline.

RESULTS

By our two-point kinetic method, in the prepared glucose solutions, the lower limit of glucose estimation was found to be 5.1 mg/dL (less than 5.0 mg/dL solution was not prepared) and the upper limit was found between 300 mg/dL & 400 mg/dL (Table 1) and the upper limit of linearity can be taken upto 340 mg/dL (Figure 1). There was very good linearity ($r=0.9998$) between the glucose values of prepared solutions and the corresponding obtained experimental values by this kinetic method (Table 1 & Figure 2).

A very good correlation ($r=0.9926$) was found between plasma glucose values estimated by this kinetic method and the corresponding values by the endpoint method. (Table 2 & Figure 3). Serial number 9 (Table 2) was Diasys (Germany)

Table 1 : Shows changes of absorbance per minute ($\Delta A/\text{min}$) and the corresponding experimental glucose values.

Conc. of prepared glucose sol. (mg/dL)	$\Delta A/\text{min}$.	Obtained experimental glucose values (mg/dL)
5.0	0.0073	5.1
10	0.0146	10.3
15	0.0223	15.7
20	0.0301	21.2
30	0.0445	31.3
50	0.0700	49.2
75	0.1043	73.4
100	0.1430	100.6
200	0.2817	198.1
300	0.4267	300.1
400	0.5459	383.9
500	0.5711	401.6
600	0.5732	403.1
700	0.5726	402.7
800	0.5743	404.6
900	0.5720	405.8
1000	0.5729	402.9

quality control serum where target value of glucose was 102 mg/dL. We have found very close values by both the methods. Likewise, serial number 19 was Randox (England) quality control serum where target value of glucose was 279 mg/dL. Our measured glucose values were very close to it by both the methods. Difference of intra-assay and inter-assay values was found to be statistically insignificant (P value > 0.10) by this kinetic method (Table 3).

Fig. 1. Shows the changes of absorbance per minute ($\Delta A/\text{min}$) corresponding to concentrations of Glucose solutions

Fig 2. Correlation graph of glucose estimation by Two-point kinetic method. Glucose solutions of known values Vs experimental values

Table 2 : Shows plasma glucose values in same sample by endpoint and two-point kinetic methods.

Sr.No.	Endpoint method (mg/dL)	Kinetic method (mg/dL)
1	25.8	26.3
2	34.1	34.9
3	44	43.7
4	59.2	60
5	63.8	53.1
6	77.7	78.3
7	86	87.2
8	95.5	95
9	104.5	103.6
10	114.6	115.2
11	126.1	126.9
12	132.3	134
13	148.4	147.1
14	167.2	168.6
15	183.3	182
16	202.7	200.8
17	223.1	224.1
18	244.3	246.3
19	275.8	272.2
20	298.5	300.1
21	327.4	328
22	370	371.2
23	442.8	441.1
24	562.6	560.8
25	608.3	611.5
26	646.7	646
27	682.1	679
28	732.4	735.1
29	745.9	748.7
30	778.4	781.8

Sr.No. 9Diasys (Germany) Quality Control Serum

Sr.No. 19 Randox (England) Quality Control Serum

Table 3 : Shows intra-assay and inter-assay variations of plasma glucose estimation by kinetic method.

Sr.No.	Intra-assay values (mg/dL)	Inter-assay values (mg/dL)
1	735.1	736.2
2	734.8	734.4
3	735.6	735.6
4	735.2	737.1
5	736.4	735.7
6	735.6	733.5
7	734.9	736.3
8	735.3	736.2
9	736.2	735.8
10	735.7	738.3
Mean	735.5	735.9
% C.V.	± 0.07	± 0.19

P value > 0.25

DISCUSSION

Addition of hypophosphorous acid to colour reagent is very important. It prevents oxidation of phenol by atmospheric oxygen and light to p-benzoquinone and phenoquinone which react with 4-aminoantipyrine to give reddish colour. Hypophosphorous acid prevents oxidation of phenol getting itself oxidised to phosphoric acid and increases the stability of colour reagent. Without addition of hypophosphorous acid, the colour reagent develops pink colour within 01 month in refrigerator. Different concentrations of reagents were tried to develop the colour reagent. A rough idea was developed on the basis of few companys' literatures which were end-point based. The two enzymes' concentrations were fixed as per its turnover number keeping in mind that peroxidase enzyme concentration should never be a limiting factor during the reactions. Programme development for two-point kinetic assay was tried with various combinations. The programme parameters which we described here have been designed to work for the concentrations of reagents of the Colour solution that we devised. Always sample volumes were fixed to 20 μ L which was double that of endpoint method. Idea behind this was to make glucose estimations more sensitive at very low concentrations.

By this method, linearity of glucose assay is upto 340 mg/dL. In routine laboratory practice, plasma glucose levels in maximum number of patients, remain within this range. So, this procedure of assay can be followed routinely. As the lower

limit of linearity of glucose assay is 5.1 mg/dL, the hypoglycaemic patients can be diagnosed accurately and quickly to initiate prompt treatment. When plasma glucose level in a patient exceeds 340 mg/dL then, a repeat assay is to be done with appropriate dilution of plasma.

This method has a very good correlation with the endpoint method. One good finding is that both the methods of glucose estimation could estimate very accurately the target glucose values of two quality control sera of foreign companies (table 2). This validates our kinetic method as per international standard. The method also validates glucose estimations in other body fluids (author's unpublished data).

Decreasing the sample volume to 10 μ L instead of 20 μ L during the assay procedure, the upper limit of linearity of glucose estimation is raised to 750 mg/dL, instead of 340 mg/dL, but lower limit of detection suffers i.e. 20 mg/dL instead of 5 mg/dL (author's unpublished data). If in a patient, glucose level was found to exceed 340mg/dL (upper limit of linearity), the sample is to be re-run with 10 μ L of sample volume avoiding sample dilution and the result is to be multiplied by two. This is very helpful in estimation of glucose levels in hyperglycaemic patients.

The developed two-point kinetic assay for glucose estimation

has a wide linearity and accurate, as it has a very good correlation with that of endpoint method. By this method, glucose levels in quality control sera were accurately estimated. This is a rapid method i.e. takes only 1½ minutes of time as compared to minimum 10 minutes of time by endpoint method. This method will be of tremendous use in emergency cases i.e. in the suspected hypoglycemic patients where prompt initiation of therapy is must for saving life.

ACKNOWLEDGEMENT

This work has been funded by Smt.Radhibabai Meghe Memorial Medical Trust (R.M.M.M.T.), Nagpur, for which the author is very grateful.

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