Comparative Study of Nitro Blue Tetrazolium (NBT) Reduction Method for Estimation of Glycated Haemoglobin with Glycated HbA1C Estimated on DCA2000+Analyzer (Immunoagglutination Inhibition)

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Abstract

Glycated haemoglobin is a diagnostic tool, used for the monitoring of the glycemic status among diabetic patients. The present study is designed to compare and correlate modified NBT reduction method for the estimation of Glycated protein (Glycated Haemoglobin) with HbA1C estimated on DCA+2000Analyzer. Glycated protein reduces Nitro Blue Tetrazolium (NBT) reagent in alkaline medium to tetrazinolyl radical NBT⁺ which is disproportional to yield a highly colored formazan dye (MF⁺) (monoformazen), absorbance of colored compound was measured which gives the concentration of glycated proteins present in the sample. Heme free globin (glycated hemoglobin) was extracted out and dissolved immediately in 1ml normal saline. Dissolved globin was treated with modified NBT reagent, absorbance of color developed was recorded in milli ΔA/min. The results of modified NBT were then compared with HbA1c estimated by immunoagglutination inhibition method. Correlation coefficient between Glycated hemoglobin and HbA1c was found to be r=0.926 using Schimadzu CL-750 spectrophotometer and r=0.902 using colorimeter. Results of this study were found to be statistically significant p < 0.001. Thus the present study concludes that Glycated hemoglobin testing by modified NBT reduction method is as sensitive as HbA1c estimated by DCA2000+Analyzer (immunoagglutination inhibition). Hence it could be used for routine monitoring of blood glucose control level in diabetic subjects.

Introduction

Glycated proteins are recognized as a biochemical marker for the complication of diabetes. Glycated hemoglobin HbA1c is widely accepted as a single most reliable indicator of metabolic control in diabetes mellitus. But the specific method used for its determination is too costly, requiring expensive reagents and sophisticated dedicated instrumentation. Glycation of proteins can occur as a non-enzymatic posttranslational modification, which directly depends upon prevailing glucose concentrations. Diabetic patients tend to have elevated concentration of glycated proteins, therefore the degree of glycation of hemoglobin and serum protein has been correlated with indices of glycation. Glycated protein concentration reflects an average of blood glucose level over a period time; their determination provides a reliable means of monitoring diabetic control. Glycated protein Serum fructosamine is one of them, could be used as a biochemical marker. Investigators conducted studies to develop routine assays for determination of glycated proteins but method used by them is cumbersome, time consuming and difficult to follow. So there is need to find out a feasible, viable and cost effective method to be used in a developing country like India. In this direction B L Somani et al developed and modified NBT reduction method that could be used to quantify the serum glycated protein fructosamine as well as glycated hemoglobin, which requires simple equipments and chemicals and has significant advantage over a sophisticated clinical laboratory. The present study is conducted to assess the reproducibility of NBT reduction method for estimation of Glycated hemoglobin by comparing and correlating it with HbA1c estimated on DCA+2000Analyzer which has been regarded as a gold standard.

Material and Methods

The present study was conducted in the Department of Biochemistry, Armed Forces Medical College, Pune. 35 Diabetic patients referred for glycated hemoglobin testing from Diabetic Clinic of Medicine OPD were selected for the study.

Fasting plasma glucose, postprandial plasma glucose, GHb, HbA1c were estimated. Blood samples were collected in potassium EDTA vial for HbA1c testing; in fluoride oxalate vial for FPG, PPG and Glycated hemoglobin.

Plasma glucose was estimated by glucose oxidase method (Trinder 1969) using reagent kit supplied by Qualigen Diagnostics, Manufactured by Sigma Diagnostic (India) Pvt. Ltd. Baroda.

Glycated hemoglobin was estimated by NBT reduction method (Somani et al) using Schimadzu CL-750 Spectrophotometer and Colorimeter. Fresh extracted globin was taken in 1 ml of normal saline and incubated at 37°C for 5 to 10 min. 1.0 ml of pre warmed NBT reagent was added and absorbance was measured at 530 nm at interval of 5 min (A1) and 10 min (A2) using Schimadzu CL-750 Spectrophotometer. The ΔA=A2-A1 was observed, the results were expressed as ΔA/min. The same procedure was followed for Glycated hemoglobin estimation using Colorimeter.
Results

A total of 35 diabetic patients were studied, the mean value of Fasting plasma glucose is found to be 131.91±56.90 (mg/dl), Postprandial plasma glucose 183.62±63.00 (mg/dl), HbA1c 7.54±1.93 (%), GHb using colorimeter 0.015±0.004 (milli ∆A/min), GHb using CL-750 Spectrophotometer 0.014±0.003 (milli ∆A/min).

Patients (n=35) were divided into two groups13 (Table 1).

Group-1 Diabetic patients having fasting plasma glucose (n=9) ≤126 mg/dl and
Postprandial plasma glucose ≤140 mg/dl.

Group-2 Diabetic patients having fasting plasma glucose ≥126 mg/dl (n=26) and
Postprandial plasma glucose ≥140 mg/dl.

On perusal of the above data, it is observed that group -2 showed distinctly higher values for HbA1c, GHb and Plasma glucose.

On applying statistics for comparison of above two methods; NBT reduction method using colorimeter, CL 750 Spectrophotometer and HbA1c estimation using DCA2000+Analyzer. The correlation coefficient between GHb was found to be r = 0.902, p<0.001, n = 35 (Fig. 1). Between GHb estimated by NBT reduction method using CL 750 Spectrophotometer and HbA1c correlation coefficient is r = 0.926, p<0.001, n = 35 (Fig. 2). Both r values are statistically significant. Correlation between GHb estimated by NBT reduction method using colorimeter and CL750 Spectrophotometer that is on two different instruments is r = 0.860, P<0.001, n = 35 (Fig. 3).

Discussion

DCA 2000+Analyzer based on immunoagglutination inhibition assay are a sensitive, specific and reproducible method in determination of Glycated hemoglobin (HbA1c). It does not require any sample preparation and it is not affected by chemical interference. The use of immunoagglutination inhibition assay is recommended due to its high analytical sensitivity and specificity.14 Therefore it is used as a gold standard but this method is very costly, not available in all laboratories due to high equipment and reagents cost in comparison NBT reduction method. As NBT reduction method does not require any sample preparation and not affected by any chemical interference15 NBT reagent is easily available in labs its reaction mixture preparation is not cumbersome, simple and easy to store for 1-6 months. Only 1 ml of prepared NBT reagent is required for 1 test that cost very less, when performing on simple instrument like colorimeter and spectrophotometer.

The result of present study shows significant correlation (p<0.001) between HbA1c on DCA+2000 Analyzer and Glycated hemoglobin (NBT) by using Colorimeter and Spectrophotometer. The modified NBT method shows statistically significant correlation r=0.860 between Glycated hemoglobin using Colorimeter and CL750 Spectrophotometer. This shows its low instrumentation cost and feasibility.

Other analytical methods available like phenol sulphuric acid16,17 and TBA (Thio barbituric acid)18 were studied for glycated protein estimation. In TBA there is lack of absolute proportionality between carbohydrate release (and estimation) and haemoglobin concentration thus there is non linear relation between haemoglobin concentration and amount of sugar released. In phenol-sulphuric acid there is higher value for sugar bound hemoglobin than TBA, as well as interference of heme is also there. Both methods require use of corrosive reagent and long cumbersome hydrolysis process for sample and chemical preparation but modified NBT method used for the present study does not have any such factors.

Results of our study suggest that modified NBT reduction

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<td>Fasting plasma glucose (mg/dl)</td>
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method is reliable and suitable for routine use. Statistically significant correlation of HbA1c with Glycated Hemoglobin suggests that this method can be used for assessment of short and long term blood glucose control on simple colorimetric equipments. Hence, it could be an alternative method for estimation of GHB. Statistically significant (p<0.001) correlation r=0.860 were found between two instruments with same method and procedure. Therefore simple colorimetric instruments can be used for estimation of GHB in place of using sophisticated and high cost instruments and methods. Hence, NBT reduction method can be used widely and extensively for routine monitoring of short and long term blood glucose control in diabetic subjects. However, the method needs further standardization.

References


Dr. J C Patel and Dr. B C Mehta Best Papers Awards

1st Prize for Best Original Article entitled “The Malaria Severity Score : A Method for Severity Assessment and Risk Prediction of Hospital Mortality for Falciparum Malaria in Adult” – MK Mohapatra, SP Das – Professor, Medicine, VSS Medical College and Hospital, Burla, Orissa.
1st Prize for Best Case Report entitled “Multifocal Idiopathic Fibrosclerosis Mimicking Tuberculosis of the Abdomen” – A Chriupal, S Sathyendra, Elsa Mary Thomas, H Boorugu, KP Mathews – Dept. of Medicine Unit 2; Department of Medicine Unit 3; Dept of Radiology, Christian Medical College and Hospital, Vellore 632 004. Tamil Nadu. J Assoc Physicians India 2009; 57 (04):301 -304.
1st Prize for Best Case Report entitled “Quinolone – Resistant Salmonella Enterica Serovar Typhi Presenting as Acute Fulminant Hepatitis” – S Mitra, R Karthik, V Balaji, Ige Abraham George, Arti Kapil, OC Abraham – Department of Medicine Unit 1 and Infectious Diseases, Department of Microbiology, Christian Medical College and Hospital, Vellore 632 004; Dept. of Microbiology, All India Institute of Medical Sciences, New Delhi. J Assoc Physicians India 2009; 57 (01):72 - 75.
1st prize for Best Correspondence entitled “Primary Antiphospholipid Antibody Syndrome with Optic Neuritis” – D Rajasekaran, P Jayapandian, G Subbaraghavalu – Addl. Professor of Medicine; Postgraduate; Asst. Professor of Medicine, Institute of Internal Medicine, Madras Medical College, Chennai. - J Assoc Physicians India 2009; 57: (09) 665.
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