

COMPARATIVE STUDY OF NBT REDUCTION METHOD FOR ESTIMATION OF GLYCATED PROTEIN (SERUM FRUCTOSEAMINE) WITH GLYCATED HbA1c ESTIMATED ON DCA 2000+ANALYZER (IMMUNOAGGLUTINATION INHIBITION)

ASHOK SAHU* AND PURNIMA DEY SARKAR

*Department of Biochemistry,
N.S.C.B. Medical College,
Jabalpur – 480 023*

(Received on June 27, 2008)

Abstract : Glycated protein estimation is a diagnostic tool, used for the long term and short term monitoring of the glycemic status of diabetic patients. The present study is designed to compare and correlate modified NBT reduction method for the estimation of Glycated protein (serum fructosamine) with HbA1c estimated on DCA+2000 Analyzer. Glycated protein (serum fructosamine) reduces Nitro Blue Tetrazolium (NBT) reagent in alkaline medium to tetrazinoyl radical NBT[•] which forms a highly colored monoformazen compound, absorbance of which is directly proportional to the concentration of glycated protein (serum fructosamine) present in the sample and is recorded as $\Delta A/\text{min}$. The results of modified NBT were then compared with HbA1c estimated by immunoagglutination inhibition method. Correlation coefficient between HbA1c with serum fructosamine was found to be $r=0.739$ using Shimadzu CL-750 spectrophotometer and $r=0.731$ using colorimeter. Results of this study were found to be statistically significant $P<0.001$. Hence this method could be used for routine monitoring of blood glucose control in diabetics as HbA1c estimation.

Key words : glycated protein
HbA1c

serum fructosamine
nitro blue tetrazolium (NBT)

INTRODUCTION

Glycated proteins are recognized as a biochemical marker for the complication of diabetes (1, 2). 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 (3), which directly depends upon prevailing glucose concentrations (4). Diabetics 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 (5, 6, 7, and 8). Glycated protein concentration

*Corresponding Author : E-mail : asahu888@gmail.com

reflects an average of blood glucose level over a period time; their determination provides a reliable means of monitoring diabetic control (9, 10). Glycated protein Serum fructosamine being one of them, can 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 (11) developed and modified NBT reduction method which requires simple equipments and chemicals and has significant advantage over a sophisticated clinical laboratory. The present study is conducted to assess the specificity of NBT reduction method by comparing and correlating it with HbA1c estimated on DCA+2000 Analyzer which has been regarded as a gold standard (12, 13).

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, Serum fructosamine, Glycated HbA1c was estimated. Blood samples were collected in potassium EDTA vial for Glycated HbA1c, in fluoride oxalate vial for FPG, PPG and in plain sterile vial for fructosamine test (Blood samples were kept at room temperature; centrifuged and serum was separated).

Plasma glucose was estimated by glucose oxidase method (Trinder 1969) using Reagent Kit supplied by Qualigens Diagnostics [Sigma Diagnostic (India) Pvt. Ltd. Baroda].

Serum fructosamine was estimated by NBT reduction method (B.L. Somani et al) (12) using Shimadzu CL-750 Spectrophotometer and Colorimeter. Fresh 0.2 ml of serum was taken in 1 ml of 9 gm/l sodium chloride 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 Shimadzu CL-750 Spectrophotometer. The $\Delta A (A_2 - A_1)$ was calculated and the results were expressed as $\Delta A/\text{min}$. The same procedure was followed for serum fructosamine estimation using Colorimeter.

RESULTS

Patients were divided into two groups according to the standardized protocol (13).

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

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

TABLE I: The mean value \pm SD of fasting plasma glucose, postprandial plasma glucose, HbA1c, serum fructosamine (n=35).

Parameters	Value
Fasting plasma glucose(mg/dl)	131.91 \pm 56.90
Postprandial plasma glucose(mg/dl)	183.62 \pm 63.00
HbA1c (%)	7.54 \pm 1.93
Serum fructosamine using Colorimeter	0.0188 \pm 0.004
Serum fructosamine using CL-750 ($\Delta A/\text{min}$)	0.0210 \pm 0.005

TABLE II: Correlation between HbA1c and Serum fructosamine using two different instruments.

Parameters	HbA1c (%)	P value
Serum fructosamine using colorimeter ($\Delta A/\text{min}$) (Fig. 1)	$r=0.731$	$P<0.01$
Serum fructosamine using CL-750 ($\Delta A/\text{min}$) (Fig. 2)	$r=0.739$	$P<0.01$

Correlation, r between serum fructosamine using Colorimeter and CL-750 is 0.816 ($P<0.01$) (Fig. 3).

TABLE III: Mean value \pm SD of HbA1c and Serum fructosamine using Colorimeter and CL-750 for two groups ($n=35$).

	Group-1	Group-2
Fasting plasma glucose	89 ± 17.34	164.10 ± 56.55
Post prandial glucose	126.13 ± 29.25	226.75 ± 43.64
HbA1c	6.10 ± 1.39	8.61 ± 1.55
Serum fructosamine using Colorimeter ($\Delta A/\text{min}$)	0.015 ± 0.003	0.031 ± 0.046
Serum fructosamine using CL-750 ($\Delta A/\text{min}$)	0.018 ± 0.004	0.023 ± 0.004

On perusal of the above data, it is observed that group-2 shows distinctly higher values than group-1 for HbA1c and Serum fructosamine. Based on above analysis it is inferred that all parameters are significantly higher ($P<0.001$) in Group-2 than Group-1.

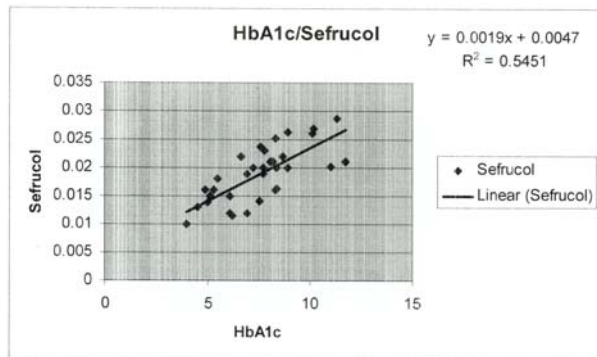


Fig. 1: HbA1c = Glycated hemoglobin A1c
Serfrucol = Serum fructosamine using Colorimeter.
Correlation between HbA1c and Serum fructosamine on Colorimeter is $r=0.74$.

On perusal of the above data, it is observed that group-2 shows distinctly higher values than group-1 for HbA1c and Serum fructosamine. Based on above analysis it is inferred that all parameters are significantly higher ($P<0.001$) in Group-2 than Group-1.

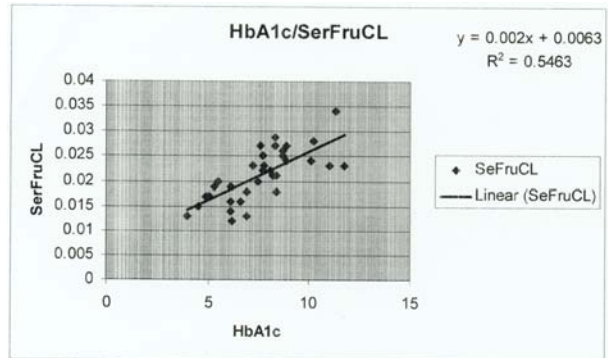


Fig. 2: HbA1c = Glycated hemoglobin A1c
Serfrucol = Serum fructosamine using CL 750 Spectrophotometer.
Correlation between HbA1c and Serum fructosamine on CL 750 Spectrophotometer is $r=0.74$.

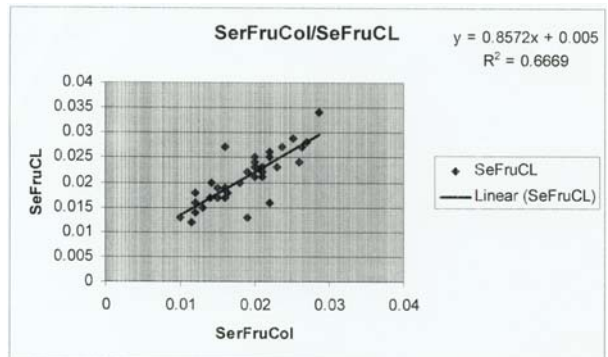


Fig. 3: SerFruCol = Serum Fructosamine Colorimeter
SerFruCol = Serum Fructosamine using CL 750 Spectrometer.
Correlation between serum fructosamine on Colorimeter and CL 750 Spectrophotometer is $r=0.81$.

DISCUSSION

DCA 2000+Analyzer based on immunoagglutination inhibition assay is a

sensitive, specific and reproducible method in determination of glycated hemoglobin (HbA1c). 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. The NBT method does not require any sample preparation and not affected by any chemical interference (15). The result of present study shows significant correlation ($P < 0.001$) between HbA1c on DCA+2000 Analyzer and serum fructosamine (NBT) by using Colorimeter and Spectrophotometer. The modified NBT method shows statistically significant correlation ($r = 0.816$) between serum fructosamine using Colorimeter and CL750 Spectrophotometer. This shows its feasibility to replace a costly and complicated test.

Other analytical methods available like phenol sulphuric acid (16, 17) and (Thio barbituric acid) TBA (18) were studied for glycated protein estimation. In TBA there is lack of absolute proportionality between carbohydrate release (and estimation) and haemoglobin concentration and therefore, there is non linear relation between haemoglobin concentration and amount of sugar released. In phenol-sulphuric acid method there is higher value for sugar bound hemoglobin than TBA, as well as interference of heme. 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 such limitations.

Results of our study suggest that modified NBT reduction method is reliable and suitable for routine use. Statistically significant correlation of HbA1c with serum fructosamine 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 gly Hb less correlation coefficient found was due to serum fructosamine having short half life of (15–20 days) and is a short term glycemic control indicator. Statistically significant ($P < 0.001$) correlation ($r = 0.816$) was found between two instruments with same method and procedure. Therefore simple colorimetric instruments can be used for estimation of GHb in place of 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 diabetics. However, the method needs further independent evaluation.

ACKNOWLEDGEMENTS

I feel myself to be fortunate to work in the Department of Biochemistry, AFMC, Pune, for this work. With deep gratitude I would like to take the opportunity to express my sincere thanks to Professor and Head of Department, Guide and all Faculty Members of Department of Biochemistry, AFMC, Pune.

REFERENCES

1. Goldstein DE, Parker KM, England JD. Clinical application of glycosylated hemoglobin measurements. *Diabetes* 1982; 31(Suppl 3): 70-78.
2. KOG, Chan J, So Wy et al. Combined use of a fusing plasma glucose concentration and hemoglobin A1 or fructosamine predicts the likelihood of having diabetes in high risk subjects. *Diabetes Care* 1998; 21: 1221.
3. Monitoring of Diabetes Mellitus; Kor Medima (Ph.D); Ejifcc, Vol. 13; No. 5.
4. Dyjf, Thrope SR, Baynes JW. Non enzymatic glycosylated albumin. *J Biol Chem* 1979; 254: 295.
5. Miller JA, Bunn HF. Non-enzymatic glycation of RBC membrane proteins relevance to diabetic subjects. *J Clin Invest* 1980; 65: 896-901.
6. Nathan D, Singer DE, Hurxthal K, Goodson JD. The clinical information values of the glycosylated hemoglobin assay. *N Eng J Med* 1984; 310-346.
7. Jain SK, Mc Vie R, Duett J, Herbst JJ. Erythrocyte membrane lipid peroxidation and glycosylated hemoglobin in diabetes. *Diabetes* 1989; 38: 1539-1543.
8. National Glycohemoglobinization Programme <http://www.missouri.edu>.
9. R Phill. Semi automated colorimetric method for measuring Glycated hemoglobin with reduction of NBT evaluated. *Clin Chem* 1990; 36/12, 2131-2133.
10. Somani BL, Sinha R, Gupta MM. Fructosamine assay modified for the estimation of glycated hemoglobin. Let. to Editor. *Clin Chem* 1989; 35/3: 497.
11. Somani BL, Sinha R, Gupta MM. Dept. of Biochemistry to develop a new method for the estimation of glycated hemoglobin/protein and its comparison with other methods. *AFMRC Project No. 1335/85*.
12. Koieng et al. Correlation of glucose regulation and hemoglobin A1c in diabetes mellitus. *N Eng J Med* 1976; 295: 230-232.
13. Defination, Diagnosis and classification of diabetes mellitus and its complications. *World Health Organization* 1999.
14. American Diabetes Association. Tests of glycemia in diabetes in clinical practice recommendation 2000. *Diabetes Care* Vol. 23 (January 2000): Supl. 1.
15. Somani BL, Ambade V, Bulakh PM, Sharma YV. Elimination of superoxide dismutase interference in fructosamine assay. *Clin Biochem* Vol. 32, No. 3; 1999: 185-188.
16. Nayak SS, Pattabiraman TN. A new colorimetric method for the estimation of glycosylated hemoglobin. *Clin Chem Acta* 1981, Feb. 5; 109(3): 267-274.
17. Nayak SS, Pattabiraman TN. Determination of glucosylalbumin by phenol-sulphuric acid reaction in hyperglycemia. *Indian J Med Res* 1982 Aug; 79: 305.
18. Pecovaro RE, Graf RJ, Halter JB, Beiter H, Porter, D. Jr. Comparison of a colorimetric assay for glycosylated hemoglobin with ion exchange chromatography. *Diabetes* 1979; 28: 1120-1125.