ESTIMATION OF GLYCATED HEMOGLOBIN BY 2,6-DIMETHYLPHENOL: SULPHURIC ACID CONVENTIONAL METHOD

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ABSTRACT

Glycated hemoglobin levels in hemolysate of normal and diabetic patients were determined by the 2,6-dimethylphenol:57.5% sulphuric acid conventional method and the values were 0.39 ± 0.025 and 0.69 ± 0.21 moles of hydroxymethylfurfural(HMF)/mole of globin, respectively. The mean increase in glycated hemoglobin values in diabetics (1.8 fold) was highly significant ($p < 0.001$). A good correlation ($r = 0.95$) was found between the glycated hemoglobin values obtained by this method and the phenol:sulphuric acid method. The values obtained by former method were about 1.2-1.4 times the values by the phenol:sulphuric acid method. This study indicates that conventional 2,6-dimethylphenol:57.5% sulphuric acid method is more sensitive for the estimation of glycated hemoglobin than any other method based on the same principle. It is less time consuming, reliable and hence can be employed for the routine laboratory estimation of glycated hemoglobin for the assessment of glycemic control.

KEY WORDS : Diabetes Mellitus, Estimation of glycated proteins, Glycated hemoglobin.

INTRODUCTION

The hallmark of diabetes mellitus is hyperglycemia, which leads to glycation of proteins, which in turn, is thought to contribute to the long-term complications of diabetes. A number of proteins like hemoglobin, serum albumin, globulins, collagen, lens crystallins, calmodulin, osteocalcin and lipoproteins have been reported to be prone to glycation (1,2). The measurement of glycated hemoglobin has been suggested as an alternative to oral glucose tolerance test for diabetes screening (3,4). A number of methods have been developed for the estimation of glycated proteins. The principle of one group of methods is based on their property of undergoing dehydration in acidic medium to form hydroxymethylfurfuraldehyde and its condensation with different developers, which include thiobarbituric acid (5) and phenol (6).

The various steps involved in the assay and the non-stoichiometric nature of the assay due to the

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destruction of hydroxymethylfurfuraldehyde at elevated temperature, remain as the drawbacks of the thiobarbituric acid method, which was used for the estimation of glycated hemoglobin and other proteins (7,8). Phenol : sulphuric acid method which was used for the estimation of glycated hemoglobin, involved long, time-consuming dialysis and protein precipitating steps (9). Subsequently, elimination of dialysis step by precipitation of globin by HCl-acetone mixture treatment, whereby both heme and glucose were removed, shortened the assay considerably (10). The values obtained in the direct phenol:sulphuric acid assay were higher than the values obtained in the thiobarbituric acid assay. Further, the modified phenol:sulphuric acid method (11) which significantly improved the sensitivity over the original method, was also employed for the estimation of glycated hemoglobin (12). The assay gave low absolute values compared to original phenol:sulphuric acid method and there was no overlap of individual values between normals and diabetics (12). 2,6-Dimethylphenol was shown to be a better prochromogen for the estimation of hexoses and use of 57.5% sulphuric acid avoided

the complication of interfering sulphonation of the prochromogen (13,14). 2,6-Dimethylphenol:57.5 % sulphuric acid conventional method (14) was employed for the estimation of glycated hemoglobin in normal and diabetic hemolysate samples. The values obtained by this method were compared with those obtained by modified phenol:75% sulfuric acid method by the student's paired 't' test.

MATERIALS AND METHODS

5-Hydroxymethyl-2-furaldehyde(HMF) and 2,6-dimethylphenol were obtained from Aldrich chemical company, Milwaukee, Wis, USA. Phenol was redistilled before use. Analar grade sulphuric acid (98%, specific gravity 1.84) used throughout the study, was from Ranbaxy Laboratories, Punjab, India.

Blood samples used in the study were collected from 22 confirmed diabetic persons (age: 20-60 years) admitted to or attending the out-patient clinics of the Department of Medicine, Kasturba Medical College, Manipal. Healthy volunteers comprised the control group (age: 20-40 years, n=20). All the specimens obtained by venepuncture were collected into blood collection tubes containing 2 mg potassium oxalate and two drops of 6% sodium fluoride. Three ml post-prandial blood sample was collected in all the cases.

The glucose level in different post-prandial blood samples was estimated using the autoanalyser (glucose oxidase method) in the clinical laboratory, Kasturba Medical College, Manipal.

The hemolysate for globin analysis was prepared as follows: Blood sample which was drawn in anticoagulant tube was transferred to graduated centrifuge tube. It was centrifuged at 2000 rpm for 5 minutes. Plasma was separated. Pellet was washed with normal saline and the liquid part was drained. To 1 ml packed cells left behind, 1.5 ml of deionized water was added with constant stirring in a vortex mixer for 5 minutes and centrifuged at 3000 rpm for 5 minutes. The supernatant hemolysate was preserved for analysis.

Preparation of globin was carried out by Diorio's

method (15), at laboratory temperature. Biuret reaction was employed to estimate the protein content in different globin solutions (16) and hemoglobin in hemolysate samples was estimated by cyanmethemoglobin method (10).

Carbohydrate content in globin solutions was estimated by: i) Phenol:sulphuric acid method: To one ml of aqueous globin solution, 3 ml concentrated sulphuric acid was added and vortexed. The solutions were allowed to cool for 30 minutes at room temperature before the addition of 0.025 ml of 80% phenol. The optical density values were measured at 485 nm, 30 minutes after the addition of phenol to the system. ii) 2,6-dimethylphenol:57.5% sulphuric acid method: To 1.7 ml of aqueous globin solution, 0.1 ml of 20% 2,6-dimethylphenol solution in ethanol was added, followed by the rapid addition of 2.3 ml of concentrated sulphuric acid using a dispensette, freely and continuously. The tubes were allowed to cool at room temperature for 30 minutes and optical density values were measured at 505 nm.

In both the methods HMF was used as a standard. The absorbance values were measured using a Uvicon 810P double beam spectrophotometer, equipped with one cm quartz cuvettes.

RESULTS

The absorption spectra of the chromogens formed by globin prepared from a pooled diabetic hemolysate sample and HMF by 2,6-dimethylphenol : 57.5% sulphuric acid method are shown in Fig 1.

The spectral patterns were almost similar with absorption maximum in the range 495 - 505 nm. A sharp increase in absorbance with globin sample at the lower end of the spectrum (at 400 nm) may be due to interference by traces of heme present in the globin samples.

Table I shows the glycated hemoglobin levels (expressed in terms of moles of HMF per mole of globin) in diabetic and normal individuals, as estimated by the conventional 2,6-dimethylphenol :57.5%

Fig. 1. Spectra of chromogens formed by globin and 5µg HMF by 2,6-dimethylphenol:sulphuric acid conventional method

sulphuric acid method and the modified phenol:75% sulphuric acid method. The glycated hemoglobin values in diabetics were higher when compared to those in normal subjects by both methods. The levels in diabetics were nearly twice (2,6-dimethylphenol:sulphuric acid method-1.78, phenol:sulphuric acid method-2.0) compared to those in normals and the increase in values obtained in diabetics by both the methods was highly significant ($P < 0.001$). The scatter dot diagram of glycated hemoglobin values obtained by the above two methods for diabetics and normal individuals is shown in Fig 2.

Fig. 2. Glycated hemoglobin levels in normal individuals and diabetics by 2,6-dimethylphenol : 57.5 % sulphuric acid (DMPSA) conventional method and phenol : 75% sulphuric acid (PSA) modified method.

Table 1. Glycated hemoglobin levels (moles of HMF/mole of globin) in normal and diabetic subjects by conventional 2,6-dimethyl phenol:57.5% sulphuric acid method (2,6-DMP SA) and modified phenol:75% sulphuric acid method (PSA)

Method	Diabetics (n=22) Mean±S.D (range)	Normals (n=20) Mean±S.D. (range)
2,6-DMP SA	0.69±0.21* (0.473-1.173)	0.39±0.024 (0.345-0.428)
PSA	0.57±0.17* (0.367-0.944)	0.28±0.026 (0.230-0.326)

* $p < 0.001$ compared to normal by both methods

All the diabetic patients had glycated hemoglobin value more than 0.45 moles of HMF/mole of globin and normals had less than the above value by 2,6-dimethyl phenol : 57.5% sulphuric acid method. By phenol:75% sulphuric acid method, the cut-off value was 0.35 moles of HMF/ mole of globin. In terms of mg of HMF/gram of hemoglobin, the cut-off levels for glycated hemoglobin to distinguish diabetics from normals were 0.84 and 0.64 respectively by the above methods.

A good correlation ($r=0.95$) was found between the glycated hemoglobin values obtained by the two methods (Fig 3). The glycated hemoglobin values obtained by 2,6-dimethylphenol:sulphuric acid method were nearly 1.2 to 1.4 fold more when compared to those obtained by phenol:sulphuric acid method. The regression was: $y=0.85x-0.03$, where "y" and "x" respectively represent the glycated hemoglobin values by modified phenol:75% sulphuric acid method and conventional 2,6-dimethylphenol:57.5% sulphuric acid method.

However a poor correlation between post-prandial blood glucose (mg%) and glycated hemoglobin (moles of HMF/mole of globin) by 2,6-dimethylphenol:57.5% sulphuric acid method was observed ($r=0.48$, $p < 0.001$).

DISCUSSION

The mean glycated hemoglobin values in control

Fig. 3. Correlation between glycated hemoglobin values by 2,6-DMPSA and PSA method

and diabetic subjects estimated by conventional 2,6-dimethylphenol:57.5% sulphuric acid assay were 0.39 ± 0.025 and 0.69 ± 0.21 moles of HMF/mole of globin (0.72 ± 0.047 and 1.3 ± 0.39 mg of HMF/gram of hemoglobin), respectively. The mean increase in glycated hemoglobin values in diabetics (1.8 fold) was highly significant ($p < 0.001$). This value confirms the values reported by the earlier workers. Nayak and Pattabiraman (9) reported 1.82 fold increase in glycated hemoglobin levels in diabetics compared to normals (normals 2.32 ± 0.36 and diabetics 4.21 ± 0.66 mg fructose/gram of hemoglobin). Similarly 1.79 fold increase was reported by Rai and associates (normals 0.286 ± 0.057 and diabetics 0.513 ± 0.081 mole of fructose/r.mole of hemoglobin) (10).

It has been shown that modified phenol:75%

sulphuric acid method accurately reflects HMF levels (11) and effectively discriminates diabetics from normal individuals (12). A significant correlation ($r=0.95$, $p < 0.001$) between the glycated hemoglobin values obtained by this method and 2,6-dimethyl phenol:57.5% sulphuric acid method was observed showing that the latter method can also be employed for the estimation of glycated hemoglobin in assessing the glycemic status. However, the latter method is more sensitive than the modified phenol:75% sulphuric acid method.

Glycated hemoglobin values by thiobarbituric acid assay as reported by Subramaniam and coworkers (17) were 1.67 ± 0.23 for normals and 2.93 ± 0.95 for diabetics in terms of micromoles of HMF per gram of globin. Our mean values of glycated hemoglobin in these units were 4.5 for normals and 8.9 for diabetics by modified phenol:sulphuric acid method. However, by conventional 2,6-dimethylphenol : sulphuric acid method the mean values were 6.0 for normal individuals and 10.8 for diabetics. The data shows that, in thiobarbituric acid method there is considerable destruction of HMF formed.

This study shows that conventional 2,6-dimethylphenol:57.5% sulphuric acid method is more sensitive for the estimation of glycated hemoglobin than other methods based on the same principle. It clearly discriminates diabetics from normal control group. Since it is less time consuming and reliable, it can be employed for routine laboratory estimation of glycated hemoglobin for the assesment of glycemic control.

REFERENCES

1. Dolhofer, R. and Wieland, O.H.(1979) Glycosylation of serum albumin; Elevated glycosyl-albumin in diabetic patients. FEBS. Lett. 103,282-286.
2. Lopalla, A., Poli, T., Meneghini, F., Zucchetto, M., Franchin, A., Barison, A. and Fedele, D.(1988) Glycated serum proteins and glucose tolerance Acta. Diabetologica Latina 25,325-332.
3. Santiago, J.V., Davis, J.E. and Fisher, F.(1978) Hemoglobin A_{1c} levels in diabetes detection program. J. Clin. Endocrinol. 47, 578-581.
4. Dods, R.F. and Bolmey, C.(1979) Glycosylated hemoglobin assay and oral glucose tolerance test compared for detection of diabetes mellitus. Clin.Chem. 25, 764-768.

5. Fluckiger, R. and Winterhalter, K.H. (1976) In vitro synthesis of hemoglobin A_{1c}. FEBS.Lett. 71, 356-360.
6. Du Bois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric method for the determination of sugar and related substances. Anal.Chem. 28, 350-356.
7. Parker, K.M., England, J.D., Dacosta, J., Hess, R.L. and Goldstein, D.E. (1981) Improved colorimetric assay for glycated hemoglobin. Clin.Chem. 27, 669-672.
8. Ahmed, N., Malik, N.S. and Furth, A.J. (1992) A colorimetric micro assay for glycated collagen based on the thiobarbituric acid method. Clin.Chim.Acta. 212, 133-139.
9. Nayak, S.S. and Pattabiraman, T.N. (1981) A new colorimetric method for the estimation of glucosylated hemoglobin. Clin.Chim.Acta. 109, 267-274.
10. Rai, K.B., Sharma, K.K. and Pattabiraman, T.N. (1984) A short duration colorimetric method based on phenol-sulfuric acid reaction for the estimation of glycosylhemoglobin. Biochem.Med. 31, 65-72.
11. Rao, P. and Pattabiraman, T.N. (1990) Further studies on the mechanism of phenol: sulfuric acid reaction with furalddehyde derivatives. Anal. Biochem. 189, 178-181.
12. Raghavendra, D.S. and Pattabiraman, T.N. (1993) Evaluation of phenol and O-cresol as prochromogens in glycated hemoglobin estimation. Ind.J.Clin. Biochem. 8, 59-62.
13. Pattabiraman, T.N. and Mallya, M. (1993) Efficiency of substituted phenols in phenol:sulfuric acid reaction. Biochem.Arch. 9, 327-334.
14. Mallya, H.M. and Pattabiraman, T.N. (1997) Effect of acid concentration on chromogen formation from hexoses in sulfuric acid based reactions. Anal.Biochem. 251, 299-301.
15. Ascoli, F., Fanelli M.R.R. and Antoneni, E. (1981). Preparation and properties of hemoglobin and reconstituted hemoglobins. In: Methods in Enzymology Eds. Antoneni, E., Bernardi L. R. and Chiancone, E. Academic Press, New York. Vol. 76 (Hemoglobins), p-72-94.
16. Kumar, A., Rao, P. and Pattabiraman, T.N. (1988) A colorimetric method for the estimation of glycated proteins based on differential reduction of free and bound glucose by sodium borohydride. Biochem.Med. Metab.Biology. 39, 296-304.
17. Subramaniam, C.V., Radhakrishnamurthy, B. and Berenson, G.S. (1980) Photometric determination of glycosylation of hemoglobin in diabetes mellitus. Clin.Chem. 26, 1683-1687.