Lectin-Based Estimation of Glycated Hemoglobin in Diabetes Mellitus

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This study was undertaken to distinguish between normal and diabetic subjects by lectin–glycated hemoglobin interaction. The quantitative precipitin method was performed for the interaction between glucose-specific lectin Concanavalin A (Con A) and the glucose-containing RBC-lysate for the estimation of calculated HbA1c% from a standard curve. The standard curve was prepared by plotting the optical density of the precipitin for the interaction of standard HbA1c concentration with Con A against HbA1c reference standard. The absorbance range of the precipitate was 0.14–0.20 in normal subjects and the corresponding calculated HbA1c% along with plasma glucose (mg/dl) levels was 4.1–5.8% and 82–101 mg/dl respectively. Higher absorbance values, 0.22–0.42, were obtained in diabetic patients when the calculated HbA1c% was 6.3–12.2% and plasma glucose level was

120–292 mg/dl. Almost similar results were observed for HbA1c% (6.1–11.9%) of the same diabetic samples measured by conventional ion-exchange high performance liquid chromatography (HPLC). Excellent correlation coefficients of the two methods from regression analysis graph for normal ($r = 0.98$) and diabetic patients ($r = 0.99$) were observed. Furthermore, nondiabetic and diabetic hemoglobin variant subjects showed similar HbA1c% by our lectin-based assay when compared with standard HPLC method. We conclude that this lectin-based assay may be adopted to estimate glycated hemoglobin level in differentiating between normal and diabetic patients. This assay offers a good correlation with standard HPLC method. Moreover, the method is convenient, cheap, and needs no sophisticated instruments. *J. Clin. Lab. Anal.* 26:45–48, 2012. © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

Diabetes is a disease characterized by abnormal metabolism, most notably hyperglycemia and an associated risk for relatively specific long-term complications affecting the eyes, kidneys, heart, and nervous system. Diabetes Control and Complications Trial and United Kingdom Prospective Diabetes Study (1, 2) conclusively showed that a reduction in blood sugar levels close to normal range decreased the risk of complications of diabetes. Historically, the measurement of glucose has been the means of diagnosing diabetes. Glucose in the blood binds irreversibly to a specific part of hemoglobin in red blood cells (RBC), forming glycated hemoglobin (Hb1a, HbA1b, and HbA1c). HbA1c binds more glucose than HbA1a and HbA1b (3). The American Diabetes Association recommends routine glycosylated hemoglobin testing to measure long-term control of blood sugar (4). The

amount bound reflects how much glucose has been in the blood during the previous 6 weeks. Several methods (5–7) are used to measure the amount of bound glucose to hemoglobin. The superiority of ion-exchange high performance liquid chromatography (HPLC) method (8) for estimation of HbA1c has been recognized by experts for many years for monitoring the long-term control of diabetes (9–11). Although HbA1c assay serve a better biochemical marker of diabetes, the expert committee criticized the lack of standardization in the assay system (12).

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It was observed from our earlier studies that a variety of glycoproteins react with lectin, a sugar binding protein (13, 14) in which interaction takes place between the glycan part of glycoprotein and lectin. The binding of Concanavalin A (Con A), a glucose-specific lectin with glycosylated hemoglobin in RBC, prompted us to explore the possibility of estimation of glycosylated hemoglobin in normal and diabetic patient.

MATERIALS AND METHODS

Chemicals

Con A was purchased from M/S Sigma-Aldrich Co. (St. Louis, MO) and HbA1c standard was obtained from M/S Bio-Rad Laboratories (Haryana, India). All other chemicals were of analytical grade.

Sample

Sixteen patients of both sexes with type-2 diabetes (35–60 years) and 4 patients with newly diagnosed type-1 diabetes (15–41 years) attended the diabetic and endocrinology outpatient clinic of Park Medical Research Society, Kolkata, and were selected for this study. Twenty sex-matched normal healthy subjects (33–65 years) were also included as controls. Patients with type-2 diabetes had fasting C-peptides >3.5 ng/ml (normal 0.6–3.2 ng/ml) and serum GAD-antibodies were below the normal range of 1.0 u/ml. Type-1 diabetes had serum C-peptides <0.6 ng/ml and GAD-antibodies >2.5 u/ml. In addition, five nondiabetic and four diabetic hemoglobin variants subjects were also recruited from the hematology clinic of Park Medical Research Society for HbA1c estimation by HPLC and our lectin-based assay systems. Hemoglobin variants consisted of HbE- β -thalassaemia, β -thalassaemia carrier state, HbE trait, and HbS trait. Informed consent was obtained in all study subjects.

Plasma Glucose Estimation

Plasma glucose was estimated from the diabetic and nondiabetic subjects by glucose oxidase method using phenol 4-aminophenazone (12) and HbA1c% were measured by ion-exchange HPLC method with Bio-Rad D-10 analyzer.

RBC Isolation From Blood Sample and Preparation of Lysate

Anticoagulant (EDTA) used 2 ml of human blood and was centrifuged in cold at 2,000 rpm for 10 min for RBC suspension. The packed RBC was washed twice with nor-

mal saline at same rpm and at the same time after removing the plasma supernatant.

RBC lysate was prepared by adding 2 ml double distilled water to the packed (0.2 ml) washed RBC for 60 min and then centrifuged at 4,000 rpm for 10 min in cold centrifuge. Supernatant was used as RBC-lysate.

Precipitation Test

Precipitation reaction of 0.35 ml PBS buffer (pH 7.2), 0.25 ml Con A (2 mg/ml), and 0.40 ml standard HbA1c of different concentrations (2.5, 5.0, 8.0, and 10.0%) and 0.40 ml experimental RBC-lysate of both diabetic and nondiabetic samples were taken to perform the method, as described by So and Goldstein (16). The blank experiments for both standard and experimental cases were done with the reaction mixtures as above, but without Con A. The reaction mixtures were incubated at 0°C for 60 min, and then centrifuged at 4,000 rpm for 10 min to sediment the precipitate. Precipitates thus obtained were washed by PBS (pH 7.2). Supernatant was decanted and tubes were kept upside down on the filter paper to remove the last drop. The precipitate was estimated spectrophotometrically at 480 nm in terms of carbohydrate by the method of Dubois et al. (17) using 5% phenol solution, conc. sulphuric acid, and glucose as standard. Each precipitate value is the average of five times estimation of the same sample.

RESULTS

The O.D. value of the interaction between a definite volume (0.4 ml) of lysate of nondiabetic and diabetic RBC sample with an exact volume of glucose-specific Con A (0.25 ml of 2 mg/ml) was obtained by the analysis of carbohydrate content in the precipitate and this absorbance value signifies total glycosylated hemoglobin content of RBC-lysate. A standard graph was plotted against absorbance of the precipitate from the interaction between HbA1c of reference standard and Con A with standard HbA1c concentrations (Fig. 1). The quantity of Con A (0.25 ml of 2 mg/ml) was sufficient for the precipitation of all glycosylated hemoglobin in 0.40 ml lysate. It was observed that the absorbance of the precipitate of diabetic samples was higher than those of nondiabetic samples. The absorbance of carbohydrate content in the precipitate, percentage of calculated glycohemoglobin calHbA1c%, and plasma glucose level in mg/dl of the same diabetic and same nondiabetic samples were observed. The respective values of normal healthy persons were 0.14–0.20, 4.1–5.8%, and 82–101 mg/dl, whereas diabetic patients showed 0.22–0.42, 6.3–12.2.0%, and 120–292 mg/dl, respectively. The calHbA1c% of nondiabetic and diabetic patients obtained from our lectin-based method was

Fig. 1. Standard curve for calculated HbA1c%. Optical density of the precipitation for the interaction between standard HbA1c% and Con A against standard HbA1c%.

compared with the glycated hemoglobin observed by the currently used ion-exchange HPLC method. The results in both cases were found to be almost similar. A regression analysis graph (Fig. 2A and B) of glycated hemoglobin values by our method against the corresponding data of the conventional HPLC method for same normal subjects and same diabetic patients was plotted. Excellent correlation of glycated hemoglobin values from the graph by the two methods was observed between nondiabetic persons and diabetic patients. There was a very good correlation coefficient (r) by the above two techniques and r -values for normal subjects and diabetic patients were 0.98 and 0.99, respectively. Further glycated hemoglobin of nine hemoglobin variant subjects (five nondiabetic and four diabetic) were also analyzed by these two methods where no unique differences were observed.

DISCUSSIONS

Glucose attachment to hemoglobin has been used to monitor glycemic control in the diabetic patient. Three minor hemoglobins-HbA1a, HbA1b, and HbA1c are formed by the addition of sugars to HbA and this glycosylation is a slow, continuous, and nonenzymatic process. Most laboratories use microcolumn ion-exchange technique, which isolate the glycohemoglobins as a group. The ion-exchange HPLC method currently used in the hospital showed HbA1c%, because most of the unused glucose in blood binds to HbA1c fraction of the erythrocyte cell (3). Total glycated hemoglobin was measured in our precipitation method in RBC-lysate of both normal and diabetic patients. The cut-off value of the absorption for precipitate in our precipitin method for normal person is 0.20, along with the corresponding calHbA1c% and plasma glucose are 5.8% and 101 mg/dl, respec-

Fig. 2. A regression analysis graph. Correlation of glycated hemoglobin by lectin-based method (Y-axis) and by HPLC technique (X-axis) for normal subjects. (A) Correlation coefficient $r = 0.98$. (B) Correlation of glycated hemoglobin by lectin-based method (Y-axis) and by HPLC technique (X-axis) for diabetic patients. Correlation coefficient $r = 0.99$.

tively. In the routinely used HPLC method, the nondiabetic reference range of the HbA1c values is 4–6%. In diabetic patients, the optical density of the carbohydrate binding precipitin was increased along with calHbA1c% and plasma glucose concentration. The regression analysis graph (Fig. 2A and B) indicated that CalHbA1c%

values of nondiabetic and diabetic patients showed almost similar results of respective HbA1c values estimated by HPLC method. Excellent correlation coefficient value between the two methods for normal subjects was 0.98 and for diabetic patients, it was 0.99. Hence, the absorbance values of our lectin-based precipitin method may be used to differentiate between normal and diabetic patients. Analytical methods for measurement of glycohemoglobin may be divided into three categories: (1) charge-dependent method; (2) immunoassay method; and (3) Ion-exchange HPLC method. Because these different methods measure different proportions of glycohemoglobin and do so in different ways, results are not the same. The National Glycohemoglobin Standardization Program suggested that glycohemoglobin results could be expressed in such a way as to provide essentially identical results regardless of the method used (18). The comparative results between lectin-based carbohydrate precipitin method and ion-exchange HPLC technique showed almost identical data. Hemoglobinopathies may affect the reliability of HbA1c test with HPLC method by altering the process of glycosylation of normal hemoglobin. However, the analyses of glycated hemoglobin percent in both diabetic and nondiabetic hemoglobin variants by conventional HPLC technique and by our method were found to be close to each other.

The above findings suggest that this carbohydrate binding precipitation method is comparatively accurate with the conventional ion-exchange HPLC method to discriminate between normal and diabetic patient. Moreover, the method is convenient, cheap, and needs no sophisticated instrument. This method may be adopted for routine use in the laboratory having modest facility. Further work is in progress to substantiate the result in a large section of normal and diabetic subjects.

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REFERENCES

1. The Diabetes Control and Complications Trial Research Group. *N Eng J Med* 1993;329:977–986.
2. UK Prospective Diabetes Study Group. *Lancet* 1998;352:837–853.
3. Kenneth EB, Suzaune ML, eds. *Carbohydrates. Principles of Clinical Chemistry*. John Wiley & Sons, NY, USA: A Wiley Medical Publication, 1985; p 208.
4. American Diabetes Association: Standards of Medical Care in Diabetes Care. 2007;30:S4–S41.
5. Garlick RL. Affinity chromatography: An improved method for measuring glycosylated hemoglobin (abstract). *Clin Res* 1981;30:A393.
6. Roberts WL, Frank EL, Moulton L, Papadea C, Noffsinger JK, Ou CN. Effect of nine hemoglobin variants on five glycohemoglobin methods. *Clin Chem* 2000;46:569–572.
7. Little RR, England JD, Wiedmeyer HM. Interlaboratory standardisation of glycated hemoglobin determinations. *Clin Chem* 1986;32:358–360.
8. Instruction Manual in Bio-Rad Laboratories, Inc. Hercules., CA 94547, March 2009.
9. Nathan DM, Buse JB, Davidson MB, Heine RJ, Holman RR, Sherwin R. Management of hyperglycemia in type 2 diabetes: A consensus algorithm for the initiation and adjustment of therapy. *Diabetologia* 2009;52:17–20.
10. Sox HC, editor. *Common Diagnostic Tests: Use and Interpretation*, Second Edition. Philadelphia, PA: American College of Physicians. 1990. p 133–134 and 138–140.
11. Perling MA. *Physicians Guide to Insulin-Dependent (Type 1) Diabetes: Diagnosis and Treatment*. Alexandria, VA: American Diabetes Association, Inc. 1988. p 44–45.
12. The Report of the Expert Committee on the diagnosis and classification of diabetes mellitus. 1997. *Diabetes Care* 20:1183–1197.
13. Basu PS, Majhi R, Batabyal SK. Lectin and serum-PSA interaction as a screening test for prostate cancer. *Clin Biochem* 2003;36:373–376.
14. Batabyal SK, Majhi R, Basu PS. Clinical utility of the interaction between lectin and serum prostate specific antigen in prostate cancer. *NEOPLASMA* 2009;56:68–71.
15. Alan HG editor. *Verley's Practical Clinical Biochemistry*. Sixth Edition. London: Heinemann Medical Books. 1988. p 323.
16. So LL, Goldstein J. Application of the quantitative precipitin method to polysaccharide-Concanavalin A interaction. *J Biol Chem* 1967;242:1617–1622.
17. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;28:350–356.
18. Meidema K. Towards worldwide standardization of HbA1c determination. *Diabetologia* 2004;47:143–148.