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Research Article

ESTIMATION OF GLYCATED HAEMOGLOBIN IN TYPE 2 DIABETES MELLITUS

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ABSTRACT

The study on measurement of glycated haemoglobin levels serves as an important index in the diagnosis of diabetes and also monitoring of diabetes patients to prevent the complications.

The cause of diabetic complication is not known. Major emphasis has been placed on the polyol pathway where in glucose is reduced to sorbitol by the enzyme aldose reductase with reduced NADPH as the electron donating coenzyme. Sorbitol molecule is oxidised to fructose by the enzyme sorbitol dehydrogenase and reduced NAD+. Sorbitol appears to function as a tissue toxin and has been implicated in pathogenesis of retinopathy, neuropathy, cataract and aortic diseases.

A second mechanism of potential pathogenetic importance is glycation of proteins. Glycation is non enzymatic addition of hexoses to proteins and glycosylation is enzymatic addition. This chemical reaction is the linkage between a reducing sugar and a receptive amino acid. It was first described in relation to food proteins (Millard, 1913). It has been shown that the difference in ionic charge between HBA_{1c} and ordinary adult haemoglobin was due to addition of a glucose molecule to the amino terminal valine of the beta chain via an aldimine linkage which then underwent an Amadori rearrangement to form a more stable and virtually irreversible ketamine product (Bookchin, 1968; Bunn, 1975). The logical culmination of these findings was the hypothesis that in diabetes the level of glycosylated haemoglobin HBA_{1c} or HBA_1 would be proportional to the time averaged blood glucose level in the previous 7-8weeks, a period approximating to the half life of the average red blood cells. Normal glucose produces a normal amount of glycated haemoglobin. In diabetes mellitus higher amount of glycated haemoglobin indicates poorer control of blood glucose level.

Fasting plasma glucose and glycated haemoglobin was estimated and based on the results the subjects were classified into three groups.

Group I: Normal control non diabetic subjects
Group II: DM with good glycaemic control
Group III: DM with poor glycaemic control

Group II and group III patients were evaluated for complications by including lipid profile, tests for protein in urine and fundus examination. It was found that one or more complications were more predominant in Group III than in group II subjects.

Keywords: *Haemoglobin, Type 2 Diabetes*

INTRODUCTION

It has been estimated that on the average the expected life span of diabetics is only $2/3^{rd}$ that of non diabetics. In the past, knowledge about diabetic complications was largely limited to description of morphological changes and clinical manifestations. However in the last decade, physiological and biochemical studies have provided a great deal of new information about micro-angiopathy and diabetic complication (Brownlee, 1981; Albert 1982).

Retinal capillary damage resulting in edema new vessel formation and haemorrhage makes blindness 25 times more prevalent than in normal population. Cataract appears early in life and seems to progress more rapidly in diabetics than in non diabetics. Chronic renal failure with proteinuria resulting from glomerular capillary damage secondary to basement membrane thickening is 17 times more prevalent (Crafford, 1977). Focusing on prevalence of CAD amongst diabetics in India starting with data of multicentric study

conducted by ICMR (1984-1987) to recent publication from Ahmedabad, there has been visible rise of prevalence from 5-8% to 20-30% amongst diabetics over the period of time. This is an alarming situation and needs introspection with reference to quantum increase in prevalence as well as risk factors.

MATERIALS AND METHODS

Selection of subjects:

The study is conducted in three groups of subjects selected from out patients as well as in patients from the Department of Medicine at Owaisi Hospital & Research Centre and Princess Esra Hospital, Hyderabad.

The following parameters were studied on fasting blood sample.

1. Fasting plasma glucose
2. HBA_{1c}

Based on the results the subjects were classified into three groups.

Group-I:

Consists of (10) normal adult males and females between the age group of 30 – 50 years selected as control group with no history of diabetes mellitus.

Group – II:

Consists of (15) subjects of similar age group with controlled Diabetes Mellitus.

Group – III:

Consists of (15) subjects of similar age group with uncontrolled diabetes mellitus.

Method: Glucose oxidase- Peroxidase method

Principle: This enzymatic method employed in the clinical laboratory for the estimation of glucose. Glucose is oxidised by glucose oxidase to gluconic acid and hydrogen peroxide is liberated. The colorimetric indicator, quinonemine is generated from 4-amino antipyrene and phenol by hydrogen peroxide under the catalytic reaction of peroxidise intensity of colour generated is directly proportional to glucose concentration.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + 4\ \text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinonenmine} + 4H_2O$$

Reagents

Reagent 1:	Phosphate buffer pH 7.0	100mmol/l
	Phenol	5mmol/l
	4- Aminoantipyrine	0.5 mmol/l
	Glucose oxidase	$\geq$15 KU/l
	Peroxidase	$\geq$1 KU/l

Reagent 2: Glucose standard 100mg/dl

Sample Material

Serum, heparin- Plasma or fluoride – plasma

The stability in serum and plasma is 1day at 2-8 degree centigrade, serum or plasma must be separated from erythrocyte within 60 minutes of collection.

Assay Procedure

Wave length	Hg 546mm 500-540nm
Light path	1cm
Temperature	37 degree centigrade
Measurement	Against reagent block

Reference Range

Serum/Plasma	70-110 mg/dl

Dilution limit

If the concentration exceeds 500mg/dl samples should be diluted 1+4 with NaCl solution (9g/l) and the result multiplied by 5.

Interferences

No interferences were observed by bilirubin upto 10mg/dl. Fructose, galactose, reduced glutathione, ascorbic acid and xylose do not interfere in the measurement.

REAGENT	BLANK	STANDARD	TEST
Standard	-	10uL	-
Sample	-	-	10uL
Reagent 1	100uL	1000uL	1000uL

Mix, incubate for approximately 15minutes at 37 degree centigrade and read the absorbance against reagent blank within 30minutes.

Table 1: Fasting Plasma Glucose (Mg/Dl)

S No.	Group I Normal Control	Group II Controlled DM	Group III Uncontrolled DM
1.	88	130	194
2.	96	140	194
3.	96	111	186
4.	92	142	182
5.	90	118	188
6.	100	139	166
7.	98	114	169
8.	89	120	192
9.	94	124	192
10.	101	160	170
11.		148	188
12.		148	182
13.		162	230
14.		118	226
15.		126	200
Mean	94.40	133.33	189.00
± SD	4.57	16.45	18.70
±SE	1.44	4.25	4.82

ANOVA:

Table 1.1: Fasting Plasma Glucose

Source	DF	Sum of Squares	Mean Squares	F	Significance
Between Groups	2	56674.24	28337.12	118.208	$P < 0.01$
Within Groups	37	8889.73	239.72		
Total	39	85543.97			

Then glycated haemoglobin was estimated in the above three groups

Test Use

Recombigen Glycosylated Hemoglobin (GHb) kits are intended for in vitro quantitative determination of GHb% in whole blood.

Summary and Explanation of the Test

Glycosylated hemoglobin (GHb) is normal adult hemoglobin (HbA1) which is covalently bonded to a glucose molecule. GHb concentration-is dependent on the average blood glucose concentration. It is formed progressively and irreversibly over a period of time and is stable till the life of the RBC. A single glucose determination gives a value, which is true only at the time the blood sample is drawn.

GHb on the other hand is unaffected by diet, insulin or exercise on the day of testing and thus reflects the average glucose level over the last several weeks. Hence, it reflects on the long term metabolic control of glucose in individuals.

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Principle
Whole blood is mixed with lysing reagent to prepare a hemolysate. This is then mixed with a weakly binding cation-exchange resin. The non-glycosylated hemoglobin binds to the resin leaving GHb free in the Supernatant. The GHb percentage is determined by measuring the absorbance of the GHb fraction and of the total Hb.

Reagents and Materials Provided
1. Resin Reagent: 8 mg/ml Cation-exchange Resin buffered at pH 6.9.
2. Lysing Reagent: 10mmPotassium Cyanide surfactant added.
3. Glycohemoglobin Standard: 10% Glycohemoglobin.
4. Serum separators.

Preparation of Reagents
1. Glycohemoglobin Lysing Reagent: Bring Contents to room temperature.
2. Glycohemoglobin Cation- Exchange Resin: Bring contents to room temperature, swirl and gently inverts a minimum of 10 times, swirl the bottle after addition of each tube.

Reagents Storage and Stability
All reagents are stable at 2-8º C till the expiry date mentioned on the label. Do not freeze.

Physical or Chemical Indications of Instability
Alternations in the physical appearance of the reagents or values of control sera outside the Manufacturer's acceptable range may be indications of reagent instability.

Instruments
Use a spectrophotometer or colorimeter set at 415 nm,

Specimen Collection and Preparation
Special preparation of the patient is unnecessary. Fasting specimens not required. No special additives or preservatives other than the anticoagulants are required. Collect venous blood with EDTA Using aseptic technique.

Interfering Substances
Samples that are severely lipemic may cause elevated results. Fetal hemoglobin (HbF) has resin binding characteristic similar to Glycohemoglobin value if present. Glycosylated HbS and HbC bind more tightly than HbA1 and produce lower values. Other hemoglobino pathies (e.g. beta-thalassemia and haemolytic anemia) also produce lowered results.

Materials Required by Not Provided
1. 20 µl and 100 ul micropipettes.
2. 500 µl, 3ml and 5ml pipettes or dispensers.
3. 13x100mm glass tubes.
4. Glass or plastic test tubes to hold 0.6 ml and 5ml.
5. Rocker or rotator.
6. Glycohemoglobin controls: Normal Level Elevated Level.

Procedural Outline
A. Hemolysate Preparation:
Dispense 500 µl Lysing Reagent into tubes
Labelled: Standard, control, sample 1 etc.
Place 100 µl of the well-mixed blood sample
Standard or control into the appropriately labelled tube. Mix well.
Allow to stand for 5 minutes.
Plastic or glass tubes of appropriate size are acceptable.
B. Glycohemoglobin Preparation:
1. Dispense 3.0 ml of Glycohemoglobin Cation-exchange Resin into 13x100 mm glass tube labelled: Standard, Control, Sample 1 etc.
Note: Before use, mix the resin by inverting at least 10 times, swirl the bottle after addition to each tubes.
2. Add 100 µl of the hemolysate (from Step A).

3. Position the Filter Separators in the tubes so that the rubber sleeve is approximately 1 cm above the liquid level.
4. Place the tubes on the rocker or rotator and mix continuously for 5 minutes.
5. Remove the tubes from the rocker or rotator.
6. Push the filter Separator into the tubes until the resin is firmly packed.
7. The supernatant may be poured into another tube or directly into a cuvette for absorbance measurement.
8. Adjust the instrument to zero absorbance at 415 nm with deionized Water as the blank.
(Wavelength range: 390-420)
9. Read and record the absorbance values for Standard. Control, Sample 1 etc. These reading are for glycohemoglobin.
Do not use plastic tubes.

C. Total Haemoglobin Fraction:

1. Dispense 5.0 ml deionized water into tubes labelled Standard Control, Sample 1, etc.
2. Place 20 µL of the Hemolysate (from Step A) into the appropriately labelled tube mix.
3. Adjust the instrument to Zero absorbance at 415 nm with deionized water as the blank.
4. Read and record the absorbance values for Standard, Control, and Sample 1 etc. these readings are for total haemoglobin.
Plastic or glass tubes of appropriate size are acceptable.

Quality Control

The reliability of test result should be monitored routinely using stable quality control materials and analyzed in the same manner employed for the unknowns. We suggest the use of Glycohaemoglobin.
Control: Normal, Elevated.

Table 2: Glycated Haemoglobin (%)

S.No.	Group I Normal Control	Group II DM with Good Glycemic Control	Group III DM With Poor Glycemic Control
1.	4.6	8.4	10
2.	5.2	8.6	10.6
3.	5.2	8.2	10.4
4.	4.5	8.7	10.2
5.	4.5	7.6	10.4
6.	5.8	8.7	9.8
7.	5.6	8.2	10.2
8.	4.5	8.3	10.8
9.	4.8	8.4	10.5
10.	5.8	9.0	10.1
11.		8.8	1.3
12.		8.9	10.3
13.		9.1	11.4
14.		7.9	11.2
15.		8.4	11.0
Mean	5.05	8.48	10.48
± SD	0.542	0.41	0.44
±SE	0.17	0.10	0.11

ANOVA:

Table 2.2: Glycated Haemoglobin

Source	DF	Sum of Squares	Mean Squares	F	Significance
Between Groups	2	177.187	88.593	418.480	P < 0.01
Within Groups	37	7.833	0.212		
Total	39	185.020			

Calculations
Results for the unknowns and controls are calculated as follows.

$$\text{Absorbance of Std} = \frac{\text{Absorbance of Std GHb}}{\text{Absorbance of Std THb}} = A1$$

$$\text{Absorbance of sample} = \frac{\text{Absorbance of sample GHb}}{\text{Absorbance of sample THb}} = A2$$

$$\%\ \text{GHb in sample} = \frac{A2 \times 10}{A1}$$

Expected values:
Non diabetic: 4.5%- 8.0%
Good control: 8.0%-9.0%
Fair control: 9.0%-10.0%
Poor control: 10.0% and above
Based on the HbA_{1c} results the subjects were divided into group I normal control non diabetic patients with HbA_{1c} between 4.5%-8.0%.
Group II subjects having HbA_{1c} 8.0%-10% with good to fair control of diabetes mellitus.
Group III subjects with HbA_{1c} 10% and above grouped under poor control of diabetes mellitus.

RESULTS AND DISCUSSION
Diabetes mellitus is a complex disorder affecting the metabolism of carbohydrates, proteins and lipids. Thus early detection and treatment of this condition prevents complications and further decreases the morbidity and mortality. Persistent elevation in blood sugar (and therefore HbA_{1c}) increases the risk of long term complications of diabetes such as coronary artery disease, stroke, heart failure, kidney failure, blindness, erectile dysfunction, neuropathy (loss of sensation, especially in the feet) gangrene and gastroparesis. Present studies have been carried out on diabetic patients with good glyacemic control and diabetics with poor glycaemic control. These patients were evaluated for complications by including lipid profile, proteins in urine, fundus examination. It was found that one or more complications were predominant in group III than in group II. Thus diabetic patients are advised to maintain the blood sugar level HbA_{1c} level within normal range by proper diet, drug, exercise, reduced intake of saturated fats and cholesterol. As proper glycaemic control has been suggested to prevent the complication of coronary artery disease (Brown, 1994).

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