

An HPLC Method for Estimation of HbA1c with Variation of HbA1c and Glucose Level among Diabetes Mellitus Patients

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Abstract

In this study, 75 patients with type two diabetes (38 females and 37 males) were divided into two groups. The first composed of patients using oral hypoglycaemic treatment and the others were newly diagnosed not using tabs. Also blood samples were collected from 25 healthy Sudanese subjects represented the control groups. The following assays were carried out for all of them: (a) Haemoglobin A1c using Ion Exchange chromatography. (b) Fasting blood sugar (8 hours fasting). (c) Post prandial blood sugar (2 hours after meal). The overall objectives of our study were to evaluate and introduce the Ion Exchange Chromatography method, and to evaluate the economical cost of introducing the method routinely in the measurement of HbA1c. Also to validate the HbA1c in the patients who were : on oral treatments, the newly diagnosed patients and in the healthy control group and to co-relate the HbA1c and plasma glucose in fasting and post prandial profiles in diabetic patients during the routine follow up and to evaluate the HbA1c as screening tool for diagnosis of diabetes. Our study showed no significance difference in haemoglobinA1c level between females and males in control group (females 5.23 ± 0.483 vs. males 5.40 ± 0.370). The level of haemoglobin A1c was found to be significantly higher in the patients group than in healthy control subjects (patients vs. controls: 7.01 ± 1.44 vs. 5.316 ± 0.433 $p < 0.001$), and the level was higher in female patients than male patients (females vs. males: 7.3 ± 1.493 vs. 6.8 ± 1.356 $P = 0.133$) without significant difference ($P > 0.05$). There were no statistic differences in the level of HbA1c between the patients who were on oral treatment and those who were newly diagnosed ($7.1 \pm 1.5\%$ vs. $6.75 \pm 1.3\%$ respectively $P = 0.416$ Not significant). We found that the mean total fasting blood sugar level was significantly higher ($P < 0.01$) in patients group

(147.75 mg/dl) than in healthy control group (90.45 mg/dl). The mean total post prandial blood sugar level was significantly higher ($P < 0.01$) in the diabetic patients (256.2 mg/dl) than in the healthy control group (118.9 mg/dl). Also fasting blood sugar and post prandial blood sugar were higher in female patients than male patients (FBS females patient vs. male patients: 148.26 vs. 147.21) also (PPBS female patients vs. male patients: 260.13 vs. 252.08). It is to be concluded that the Sudanese patients with type two diabetes who were on oral hypoglycaemic treatments performing an excellent glycaemic control. Their mean HbA1c was 7.1%, this may indicate an excellent glycaemic control and that the Sudanese patients, compared with other populations had higher average medication Adherence. The study also showed that HbA1c has a low predictive value when screening for diabetes. Finally, we thought it is better to measure haemoglobin A1c with the simple, economic, and flexible Ion exchange chromatography method. The introduction of HbA1c in the routine investigations and follow up of diabetes patients will help in proper management and prevention of complications of diabetes that leads to a good glycaemic control.

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Introduction

Haemoglobin A1c is composed in the red blood cells of the diabetic patient by combination of the glucose to the amino acid molecule of the haem, one of the component of haemoglobin so the name glycosylated Haemoglobin. The Diabetes mellitus is caused by an absolute or relative insulin deficiency; severe cases have persistent hyperglycaemia (Philip et al., 2002). The life span of red blood cell is 90 to 120 days, so the measurement of Haemoglobin A1c reflect the levels of the blood sugar for the last four to six weeks. Glycated haemoglobin is produced by non enzymatic condensation of glucose molecules with free amino groups on the globin component of haemoglobin. It is abnormally high in diabetics with chronic hyperglycemia and reflects their metabolic control. The higher the prevailing ambient level of glucose, the higher will be the level of glycated haemoglobin (Tierney et al.,

2003). The non enzymatic glycosylation of proteins including haemoglobin, occurs in hyperglycemic persons. The glycosylation of haemoglobin is thought to be a slow and irreversible process that occurs through the life span of the erythrocyte. Once the sugar is attached, it stays there for the life of the red blood cell, which is about 120 days. The higher the level of blood sugar, the more sugar attaches to red blood cells. The haemoglobin A1c test measures the amount of sugar sticking to the haemoglobin in the red blood cells (Nasrin et al., 1999). The glucose binds irreversibly to haemoglobin molecules within red blood cells; the amount of glucose that is bound to haemoglobin is directly tied to the concentration of glucose in the blood.

HbA1c was first described in the late 1960, since it was first detected and rechromatographed to three fast haemoglobins HbA1a, HbA1b, and HbA1c (Allen et al., 1958). The medical importance arises as it was increased in two diabetes in haematological survey in Tehran (Rahbar, 1968). It was first chromatographed and related to diabetes in 1968 by Rahbar. Haemoglobin may be glycosylated by a post translational, essentially irreversible non-enzymatic process. The resulting modified haemoglobins, haemoglobin A1a, A1b, and A1c are eluted more rapidly than non-glycosylated haemoglobins during ion-exchange chromatography and have therefore been called “fast haemoglobins” or haemoglobin A1 (HbA1). Haemoglobin A1c (HbA1c) is the major fraction of HbA1, representing about 80% (Thomas et al., 1981).

In Sudan glycosylated haemoglobin was measured, by a colorimetric method in 49 patients with sickle cell anaemia attending Khartoum Teaching Hospital. The level obtained (4.9%, SD 1.3) was significantly lower than the control value (5.6%, SD 0.2; $p < 0.0025$) (Atabani et al., 1989).

The use of serum fructosamine as indicator for diabetic complication in Sudanese diabetics by (Nasruddin, 1989) reported that fructosamine is much expedient than glycated haemoglobin in the assessment of glycaemic control in diabetes. Philip and his colleagues (2002), reported that the fructosamine assay has methodological limitations. With easier and cheaper tests now available, the measurement of glycaemic control could be also introduced into regional hospitals in developing countries (Rahlenbeck, 1998).

Patients' knowledge of HbA1c is important, especially among persons with type 2 diabetes. Improvement in patients' understanding of HbA1c, particularly among those with very poorly controlled diabetes with no prior knowledge of HbA1c is associated with improvement in

their glycaemic control. Strategies to engage patients to know and interpret their HbA1c values should be encouraged within routine clinical practice (Nosheen Iqbal et al., 2008). During the last few decades evidence has highlighted the role of elevated levels of Haemoglobin A1c as a risk factor for complication of diabetes especially nephropathy, neuropathy, and cataract of eyes. Early studies on HaemoglobinA1c were done in many ethnic group worldwide and even in Sudan. The current study was undertaken in Sudan where the uncontrolled diabetes due to many economical factors is expected. Determination of HbA1c is an important diagnostic tool for monitoring the efficiency of dietary control and therapy during treatment of diabetes mellitus. Self-monitoring blood glucose gives a snapshot of control at the time of the test, while the haemoglobin A1c test gives the big picture of control over the past 3 months. Glycation of protein in the lens may cause cataracts, glycation of haemoglobin may also occur, and may be assayed to assess long-term diabetic control. Glycated haemoglobin (HbA1c) is most commonly measured for this purpose (Thomas, 2002).

Methodology of estimation of HbA1c

Cation exchange chromatographic techniques were used in separation and identification of HbA1c (Allen et al. 1958). This technique gives false increase in the values of HbA1c due to presence of labile fractions of HbA1c. Glucose concentration and RBC half life influence glycation of Haemoglobin. Lower than expected levels of HbA1c can be seen in people with shortened red blood cell life span, such as with glucose-6-phosphate dehydrogenase deficiency, sickle-cell disease, or any other condition causing premature red blood cell death. Conversely, higher than expected levels can be seen in people with a longer red blood cell life span, such as with Vitamin B12 or folate deficiency. Estimation of HbA1c by High performance liquid chromatography (HPLC) is not affected by the presence of Hb F,C, and S. but the cost of equipment is high (Goldstein et al., 1986). The major form of glycohaemoglobin is termed haemoglobin A1c, which normally comprises only 4 -6 % of the total haemoglobin. The remaining glycohaemoglobins (2-4 % of the total) consist of phosphorylated glucose or fructose and are termed hemoglobin A1a and hemoglobin A1b. The HPLC (High Performance Liquid Chromatography) method gives the percentage for all these three groups but the method Ion exchange liquid chromatography we used is specific and gives the results for only HbA1c.

Materials and Methods

Selection of subjects

Patient group

In this study 75 sudanese patients with type two Diabetes Mellitus were selected (37 women and 38 men). Subjects of this study were randomly selected from Diabetes Mellitus and endocrine gland center (Khartoum North), and from Alwaha Medical Center (Khartoum). Measurements of glycated haemoglobin and glucose were easily accepted by patients with diabetes. The patients we choose have no history of haemoglobinopathy like sickle cells anaemia or other factor that affecting the HbA1c level.

Clinical information and data of these subjects were obtained according to the questionnaire in the proposal that including the name, age, sex, age of onset, duration of the disease and treatment.

Control group

A total number of 25 healthy Sudanese subjects were recruited to represent the control group with no history of diabetes mellitus this group contains 12 women and 13 men. The non diabetic volunteers selected as controls proved to have no complication of diabetes and no history of haemoglobinopathy at the time of investigations.

Blood samples collection and plasma preparation

A minimum of 5 ml of blood were collected by vein puncture using plastic disposable syringe (BKMI. South Korea). 2.5 ml of the blood sample were transferred to tubes containing EDTA for estimation of HbA1c and the other 2.5 ml into tubes containing fluoride oxalate for estimation of blood sugar. For blood sugar estimation the plasma was separated from blood cells by centrifugation at 2000 xg for 10 minutes. Glycosylated HbA1c was detected using Fast Ion-Exchange Resin Separation High performance liquid chromatography Method, (Human, Germany, Catalog Number 10658).Method used for estimation of glucose is Glucose oxidase method (GOD-PAP Method).

Results

Normal population:

Control males vs. females

Age

The mean age was slightly higher in female group (39.00 ± 8.765 years) than in male group (35.50 ± 4.562 years).

HbA1c

The total HbA1c levels were not significantly different between the two healthy control groups (males: $5.40 \pm 0.370\%$, females: $5.23 \pm 0.483\%$). These findings are shown in table 1.

Patients group:

Ages: patients females vs. males

The mean age and age of onset was slightly higher in male group (55.16 ± 11.198 and 47.48 ± 10.72 years) than in female group (53.15 ± 11.672 and 45.00 ± 10.56 years) respectively. These findings are shown in table 2.

HbA1c

The mean total HbA1c levels were statistically significant difference between the diabetic patients ($7.01 \pm 1.44\%$) and the healthy control group ($5.316 \pm 0.433\%$) ($P < 0.001$), these findings are shown in table 3.

There is a statistically significant difference in HbA1c levels between male patients and male controls (6.8 ± 1.4 vs. 5.4 ± 0.4 , $P < 0.001$). This is shown in table 4. There is a statistically significant difference in HbA1c levels between female patients and female controls (7.3 ± 1.5 vs. 5.2 ± 0.5 , $P < 0.001$). This is shown in table 5. There were no statistically differences in HbA1c of patients according to gender, the level of HbA1c was higher in female patients than

male patients (7.3 % vs. 6.8 % respectively) ($P = 0.133$), this is shown in table 6. The mean HbA1c levels were significantly higher in the newly diagnosed patients group than in control group (6.758 ± 1.278 vs. 5.316 ± 0.433 , $P < 0.001$) this is shown in table 7. There were no statistically differences in the levels of HbA1c between the patients who were on oral treatment and those who were newly diagnosed (7.1 % vs. 6.75 % respectively) ($P = 0.416$) this is shown in table 8.

FBS

The mean total fasting blood sugar levels were significantly higher in the diabetic patients (147.75 mg/dl) than in the healthy control group (90.45 mg/dl) $P < 0.001$, this is shown in table 9. The mean total fasting blood sugar levels were slightly higher in the female patients (148.26 mg/dl) than in male patients (147.23 mg/dl) this is shown in table 10.

PPBS

The mean total post prandial blood sugar levels were significantly higher in the diabetic patients (256.2 mg/dl) than in the healthy control group (118.9 mg/dl) $P < 0.001$, this is shown in table 11. The mean total of post prandial blood sugar levels were slightly higher in the female patients (260.2 mg/dl) than in male patients (252.1 mg/dl) this is shown in table 12.

Table 1: Comparison between the mean ages and HbA1c levels of healthy control males and females

	Males (n=12)	Females (n=13)
Age (years)	35.50±4.562	39.00±8.765
HbA1c %	5.40±0.370	5.23±0.483

Table 2: Comparison between the mean ages and ages of onset of patients

	Males (n=37)	Females (n=38)
Age (years)	55.16±11.198	53.15±11.672
Age of onset	47.48±10.72	45.00±10.56

Table 3: Comparison the mean of HbA1c levels between the patients and controls

Group	HbA1c (Mean ±SD)	P Value
Patients (n=75)	7.01 ± 1.4	P < 0.001 Significant
Controls (n=25)	5.3 ± 0.4	

Table 4: Comparison the HbA1c levels between the male patients and male controls

Group	HbA1c (Mean ±SD)	P Value
Male Patients (n=37)	6.8 ± 1.4	P < 0.001 significant
Male Controls (n=12)	5.4 ± 0.4	

Table 5: Comparison the HbA1c levels between the female patients and female controls

Group	HbA1c (Mean \pm SD)	P Value
Female Patients (n=38)	7.3 \pm 1.5	<i>P</i> < 0.001 Significant
Female Controls (n=13)	5.2 \pm 0.5	

Table 6: Comparison the HbA1c levels between the male patients and female patients

Group	HbA1c (Mean \pm SD)	P Value
Male Patients (n=37)	6.8 \pm 1.4	<i>P</i> = 0.133 Not significant
Female Patients (n=38)	7.3 \pm 1.5	

Table 7: Comparison the mean of HbA1c levels between the newly diagnosed patients and controls

Group	HbA1c (Mean \pm SD)	P Value
newly diagnosed patients	6.758 \pm 1.278	<i>P</i> < 0.001 significant
Controls	5.3 \pm 0.4	

Table 8: Comparison the HbA1c levels between the patients who were on oral treatment and those who were newly discovered

Group	HbA1c (Mean \pm SD)	<i>P</i> Value
Patients on treatment	7.1 \pm 1.5	<i>P</i> = 0.416 Not significant
Newly diagnosed patients	6.758 \pm 1.278	

Table 9: Comparison the fasting blood sugar between the patients and controls

Group	FBS (Mean \pm SD)	<i>P</i> Value
Patients (n=75)	147.8 \pm 51.2	<i>P</i> < 0.001 Significant
Controls (n=25)	90.5 \pm 9.5	

Table 10: Comparison the fasting blood sugar between the male patients and female patients

Group	FBS (Mean \pm SD)	<i>P</i> Value
Male Patients (n=37)	147.2 \pm 51.1	<i>P</i> = 0.930 Not significant
Female Patients (n=38)	148.3 \pm 51.9	

Table 11: Comparison the post prandial blood sugar between the patients and controls

Group	PPBS (Mean \pm SD)	P Value
Patients (n=75)	256.2 \pm 85.8	<i>P</i> < 0.001 Significant
Controls (n=25)	118.9 \pm 7.2	

Table 12: Comparison the post prandial blood sugar between the male patients and female patients

Group	PPBS (Mean \pm SD)	P Value
Male Patients (n=37)	252.1 \pm 87.8	<i>P</i> = 0.688 Not significant
Female Patients (n=38)	260.1 \pm 84.9	

Discussion

Healthy Control group

The results obtained in healthy controls were analyzed to see the effect of sex on HbA1c levels. In males the total HbA1c concentrations (5.40 \pm 0.370%) were slightly higher than in females (5.23 \pm 0.483), this is within normal range worldwide of HbA1c. In the US and Japan the HbA1c is usually 4 - 5.9% while at least in some European countries the reference range is 2.8 - 4%. In Thailand it was 2.90-4.90% (Paisooksantivatana et al., 2009). In Asian Indians it is 5.5 \pm 0.4%, in Taiwan it is 4.7 – 6.4%, in Saudi Arabia it is 4.8 – 6%. The level is low compared to (Gomo, 1985) in Zimbabwe who reported that in the volunteer group subjects the ranges found for HbA1c were: men, 4.86-9.78%; women, 4.30-8.22%.

Elizabeth Selvin and her colleagues (2009) reported that the overall prevalence of HbA_{1c} >6% was 3.8%, corresponding to 7.1 million adults without diabetes in the U.S. population. Approximately 90% of these individuals had fasting glucose \geq 100 mg/dl. and that older age, male sex, non-Hispanic black race/ethnicity, hypercholesterolemia, higher BMI (body mass index), and lower attained education were significantly associated with having a higher HbA_{1c} level even among individuals with normal fasting glucose (<100 mg/dl)

Patients on oral hypoglycaemic treatment

The mean levels of HbA_{1c} for the patients who were on oral treatment are 7.1%, According to the method we used, the well controlled metabolism HbA_{1c} is 4.5- 7.0, So the level obtained indicating to an excellent glycaemic control and that the Sudanese patients , compared with other populations had higher average medication Adherence. These levels were low compared to the results obtained by Otieno and his colleagues (2003) in Kenya who reported that patients on OHA (Oral Hypoglycaemic Agent) -only had mean HbA_{1c} = 9.06%.

In this study HbA_{1c} in the patients group is slightly high in females than males (7.3% vs.6.8%) this compared to the results obtained by Boul and his colleagues (2001) Who reported that exercise reducing HbA_{1c} level in type two diabetic patients, which supports the present study, They reported that HbA_{1c} was lower in the exercise groups compared with the control groups (7.65% vs. 8.31%; weighted mean difference, -0.66%; $P < .001$). Depending on the fact that males in Sudan have stronger exercise lifestyle out the houses during different jobs than females. (Nilsson et al., 2004) reported that smoking was associated with elevated HbA_{1c} levels ($p < 0.001$) and microalbuminuria ($p < 0.001$), independently of other study characteristics .smoking is uncommon among Sudanese due to many economical factors in comparison to other populations.

Self monitoring of blood glucose is not available for most of the Sudanese diabetic type two; in our study only 20 % of the patients are having glucometer. (Karter et al., 2001) reported that self-monitoring of blood glucose (at any frequency) had a 0.4 point lower HbA_{1c} level than those not practicing at all ($P < 0.0001$).They concluded that more frequent self-monitoring of blood glucose levels was associated with clinically and statistically better glycaemic control regardless of diabetes type or therapy. Patients' knowledge of HbA_{1c} is important, especially among persons with type 2 diabetes Strategies to engage patients to know and interpret their HbA_{1c} values should be encouraged within routine clinical practice (Nosheen Iqbal et al., 2008).

Newly diagnosed patients

In the group of the newly diagnosed patients when HbA1c was estimated to this group, the mean was 6.758%, it was slightly higher than the level in Asian Indians where HbA1c cut points of 6.1 and 6.4% defined diabetes by 2-hour post load plasma glucose or FPG (fasting plasma glucose) criteria undiagnosed diabetes (Viswanathan Mohan et al., 2010). Also it was slightly higher than the level in Chinese population where HbA1c threshold of 6.3% was highly specific for detecting undiagnosed diabetes in Chinese adults and had sensitivity similar to that of using a fasting plasma glucose threshold of 7.0 mmol/l (Yuqian Bao et al., 2010).

The mean level of HbA1c for the newly diagnosed patients was lower than those found by (Mayer and David, 2009) in U.S.A who reported that the HbA1c tend to be high in blacks compared to other ethnicity, they reported that the American blacks compared with Americans Mexican and non-Hispanic whites had higher HbA1c levels and that the American Blacks at levels of 6.5–6.9% HbA1c, 68% of those 40–74 years old and 87% of those over 64 years old would not have diabetes by current FBS/OGTT criteria. This is supported in U.S.A by (Sequist et al., 2006) in research about (Medication Adherence and Racial Differences in HbA1c Control) they concluded that it have been found that , At initiation of therapy, black patients had higher average HbA1c values compared with whites (9.8% vs. 8.9%, a difference of 0.88; $P < 0.0001$).

Advantages of Ion exchange chromatography method in comparison with other methods

We used Ion Exchange chromatography which is more advanced than Cation exchange chromatography that measure the labile form of HbA1c (resulting from acute changes of blood glucose) producing a false increase in HbA1c levels (Goldstein et al., 1980). The colorimetric method measures the ketoamine linked glucose to the haemoglobin, but not the labile form of HbA1c, this is a good advantage when compared with cation exchange chromatography, but the needing for removal of free glucose by double washing of the cells with normal saline made this a complex, time consuming procedure. The Ion exchange chromatography not needs the removing of free glucose by washing the cells with saline as in colorimetric method. Free glucose if not removed, falsely increases HbA1c in colorimetric method. Ion Exchange chromatography measurement has the advantages of accuracy, sensitivity; low cost (only fifteen Sudanese pounds per test).The colorimetric method, mainly

was used in Sudan has many disadvantages: It is complex, low sensitivity; low accuracy due to many steps procedures needed that lead to error prone . Ion exchange chromatography is expedient when compared with complex, time consuming colorimetric method. It should be introduced in major hospital laboratories. (Thevarajah et al., 2008) reported that patients with clinically silent hemoglobin variant as in Hemoglobin D Punjab, their HbA1c concentration cannot be detected using ion-exchange high performance liquid chromatography (HPLC) method. He concluded that in such cases HbA1c concentrations must be measured with immunoturbidimetry method. But this haemoglobin variant is very rarely cases.

HbA1c in the assessment of glycaemic control

(Nasruddin, 1989) in a study of fructosamine in Sudanese diabetics reported that fructosamine is much expedient than HbA1c in the assessment of glycaemic control in diabetes. (Tierney et al., 2003) reported that Reduction in serum albumin (eg, nephritic state or hepatic disease) will lower the serum fructosamine value. When abnormal haemoglobins or haemolytic states affect the interpretation of glycohaemoglobin or when a narrower time frame is required, such as for ascertaining glycaemic control at the time of conception in a diabetic woman who has recently become pregnant, serum fructosamine assays offer some advantage. This compared to (Philip et al., 2002) who reported that the measurement of plasma fructosamine concentrations may be used to assess glucose control over a shorter time course than that of HbA1c (about two to four weeks), but the assay has methodological limitations.

(Nasruddin, 1989), concluded that fructosamine test is simple, easy, precise and of low cost when compare with the methods of estimation of HbA1c, he used colorimetric method for the estimation of HbA1c, 10 years later, (Rahlenbeck, 1998.) disagree with this he concluded that With easier and cheaper tests now available, the measurement of glycaemic control, (HbA1c) could be also introduced into regional hospitals in developing countries. Feasible methods for clinical laboratories with limited resources is now available. Which strongly confirm the present study. The method we used (Ion exchange chromatography) for estimation of HbA1c is accurate, sensitive and of low cost (fifteen Sudanese pounds per test). Glycated haemoglobin (HbA1c) is now widely routinely used than fructosamine.

Conclusion

From this study we conclude the following:

1. Normal values of HbA1c were established in Sudanese populations with no significance differences between males and females and were within range compared with those found in other populations.
2. The HbA1c levels were slightly above normal in Sudanese patients with type two diabetes patients who were on oral hypoglycaemic treatments. The mean levels of HbA1c for the patients who were on oral treatment are 7.1%, this indicating to an excellent glycaemic control and that the Sudanese patients, compared with other populations had higher average medication adherence.
3. HbA1c has a low predictive value when screening for diabetes, only 47 % of the newly diabetes investigated had shown abnormal values, indicating that diabetes cannot be excluded by a normal value.
4. in the study the mean age of onset of diabetes in male patient is 47.45 year and for female is 45, so after the 40 year the families those with history must have dietary advice and keep a healthy optimum weight.
5. Although the current cost of HbA1c test done with Ion Exchange HPLC method (15 Sudanese pounds) is higher than the cost of FBS test, the additional benefits in predicting complications of Diabetes may make this a good choice to be introduced together with blood sugar.

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