

# Comparative evaluation of three different methods for HbA<sub>1c</sub> measurement with High-performance liquid chromatography in diabetic patients

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## Abstract

**Background:** The global prevalence of diabetes mellitus is increasing rapidly. Measurement of glycosylated hemoglobin, predominantly HbA<sub>1c</sub>, is fundamental to the management of patients with diabetes. HbA<sub>1c</sub> is used to monitor long-term glycemic control, adjust therapy, assess the quality of diabetes care and predict the risk for the development of complications. While HbA<sub>1c</sub> is the standard method for long-term glycemic control in diabetic patients, there are different methods for measurement of HbA<sub>1c</sub> and all laboratories do not use the reference method (high-performance liquid chromatography [HPLC]). The objective of this study is comparison of three different methods with HPLC to find out which method has an acceptable concordance and correlation with the reference method.

**Materials and Methods:** Fifty-eight diabetic patients were assessed in this study. The blood sample of each patient was checked with Diazyme (enzymatic assay), Nycocard (boronate-affinity binding) and Biosystem (micro column chromatography). The values of HbA<sub>1c</sub> of each method were compared with the Knauer-HPLC results.

**Results:** The means of the differential values between each method and HPLC in the ANOVA test are as follows: M = 1.8, SD = 1.09 for Nycocard-HPLC; M = 1.5, SD = 1.08 for biosystem-HPLC; M = 1.3, SD = 1.2 for Diazyme-HPLC. Pearson's correlation coefficient between HPLC and Nycocard; 0.76, HPLC and Diazyme; 0.75 and between HPLC and Biosystem was 0.68. Linear regression parameters for each method with HPLC were also determined.

**Conclusion:** Diazyme had a better performance and showed a greater concordance with HPLC among others, although it was not an ideal alternative for HPLC.

**Key Words:** Column chromatography, diabetes mellitus, enzymatic assay, HbA<sub>1c</sub>, High-performance liquid chromatography

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## INTRODUCTION

Metabolic disorders accompanied with diabetes result in pathophysiological changes due to hyperglycemia in various systems in the body.<sup>[1-4]</sup> Because the complications of diabetes mellitus are related to glycemic control, normoglycemia is an appropriate goal for most of the patients.<sup>[2-4]</sup> Measurement of HbA<sub>1c</sub> is a

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gold standard to check long-term glycemia in patients with diabetes mellitus.<sup>[5-7]</sup> There are various methods to measure glycohemoglobin,<sup>[1,3,8]</sup> but the difference in reported values by these methods is high, making the comparison of these values very difficult.<sup>[5,9]</sup> In addition, various methods are under the influence of different factors such as types of anemia, pregnancy, splenectomy, transfusion and intake of medications (salicylates).<sup>[1,3,10]</sup> An economical method is defined as a precise, cost-effective, functional and convenient method.<sup>[11]</sup> High-performance liquid chromatography (HPLC) is a reference method to standardize other routine methods with long-term validity, accuracy and stability.<sup>[6,12-14]</sup> In addition, calibration based on HPLC has been proven to enhance comparability among the various methods.<sup>[11,15]</sup> Many specialists are not well satisfied due to the inconsistency of HbA<sub>1c</sub>, reported through various methods, with patients' values attained by a reference method (HPLC). On the one hand, the HPLC device is very expensive, difficult and time consuming to work with; therefore, it needs professional personnel to work with, consequently making it impossible and not cost-effective for all laboratories. On the other hand, diabetic patients need HbA<sub>1c</sub> frequent check, and most of them cannot afford the cost of HbA<sub>1c</sub> by the HPLC method. Numerous studies have compared different methods; therefore, with regard to the above reasons, we decided to compare three routine methods: boronate affinity binding (Nycocard), enzymatic (Diazyme), column chromatography (Biosystem), with HPLC in order to declare which method reports are consistent and correlated with those of HPLC so as to replace that in clinical laboratories.

## MATERIALS AND METHODS

This is an analytical correlation prospective study. The population studied included diabetic patients referred to the laboratory in Al-Zahra hospital in 2010, selected through simple sampling, who filled a consent form and the research questionnaire. The exclusion criteria were pregnancy, splenectomy, anemia, any type of blood transfusion in the past 3 months and intake of medication (salicylates).

### Research design

A total of 58 diabetic patients were selected (31 female and 27 male). Firstly, after taking a blood sample from fasting patients (8 cc), the blood was collected in EDTA anticoagulant tubes. Next, 3/4 of the samples were sent to laboratory to measure HbA<sub>1c</sub> with Diazyme, Nycocard and Biosystem instruments in Al-Zahra hospital, and the rest of the samples (1/4) was kept in the refrigerator for sending to another reference laboratory for HPLC measurements. Samples were

transferred using a special ice bag. The HbA<sub>1c</sub> level of each sample was separately measured by each device after calibration and giving the devices quality control samples in identical conditions. Our licensed level was considered to be 4%, which was under the coefficient variation percentage (CV%) (4.3%), based on the biological variation theory.<sup>[16]</sup>

### Statistical analysis

The variance analysis test was employed for comparison of mean interval of attained values through all three methods with HPLC, and the Pearson correlation test and Regression analysis test were employed to determine the correlation values obtained by the three methods and the HPLC value. The data were analyzed through SPSS ver 15.5.

### Procedure

Knauer-HPLC Germany (advanced scientific instruments) is a device designed based on affinity chromatography with high function.

The needed sample was 4 µL of blood, which was centrifuged after addition of the lysing solution. The supernatant was used to be injected into the device. HbA<sub>1c</sub> measurement was indirectly done based, on the following formula:

$y = 0.58x + 1.75$ ,  $x$  = glycosilated Hb (glycosilated hemoglobin),  $y$  = HbA<sub>1c</sub>

Each test needs professional personnel, and lasts for 30 min.

Nycocard is a small device with a Nycocard reader kit, which is the base for the Boronat affinity binding test. Whole blood sample was mixed with chemical reagent based on kit instructions and the final product was poured on a test device. Next, rinsing liquid was added and, finally, the result was read by the Nycocard reader. Working with the device is convenient, and each test lasts for 10 min.

Biosystem is a kit containing chromatographic columns accompanied with chemical reagent, which should be used at room temperature. It functions based on spectrophotometer ion exchange. According to the kit instructions, we used chemical reagents with a separate column for each sample and, finally, collected the rinsed liquid from the column (HbA<sub>1c</sub>). We mixed the hemolysate and a chemical reagent to attain total Hb. Finally, the spectrophotometer was accessed by a device with a wavelength of 415 nm. HbA<sub>1c</sub> was calculated using the following formula:

$$\frac{AHbA_{1c}}{AHbTotal} \times \frac{100}{3} = \%HbA_c$$

$A$  = absorbance

This is a very time consuming (about 1 h) and temperature-sensitive method, and should be administrated very carefully.

Diazyme is a kit containing chemical reagents and buffers made to be used in autoanalyzers based on enzyme reactions.

Whole blood is mixed with the lysate liquid based on the kit instruction and put into the autoanalyzer, Hitachi 717, shortly afterwards, and the optical density of the samples is assessed at a wavelength of 430 nm.

The result is reported in percentage, and working with this test is very convenient, needing 15 min for each test. It should be indicated that all four employed methods in this research are traceable to the DCCT/NGSP standards.

## RESULTS

The obtained HbA<sub>1c</sub> from each of the four methods include the min, max and mean values as well as the standard deviation presented in the following table [Table 1]

Among the administrated methods, the mean value of Diazyme was closer to HPLC. Then, the parallel mean difference absolute value obtained by each method was calculated by that of HPLC to reach its mean as the following:

HPLC-Nycocard: Mean  $1.8 \pm 1.09$ .

HPLC-Biosytem: Mean  $1.5 \pm 1.08$ .

HPLC-Diazyme: Mean  $1.3 \pm 1.2$ .

The variance analysis test through repetitive observations showed a significant difference in the

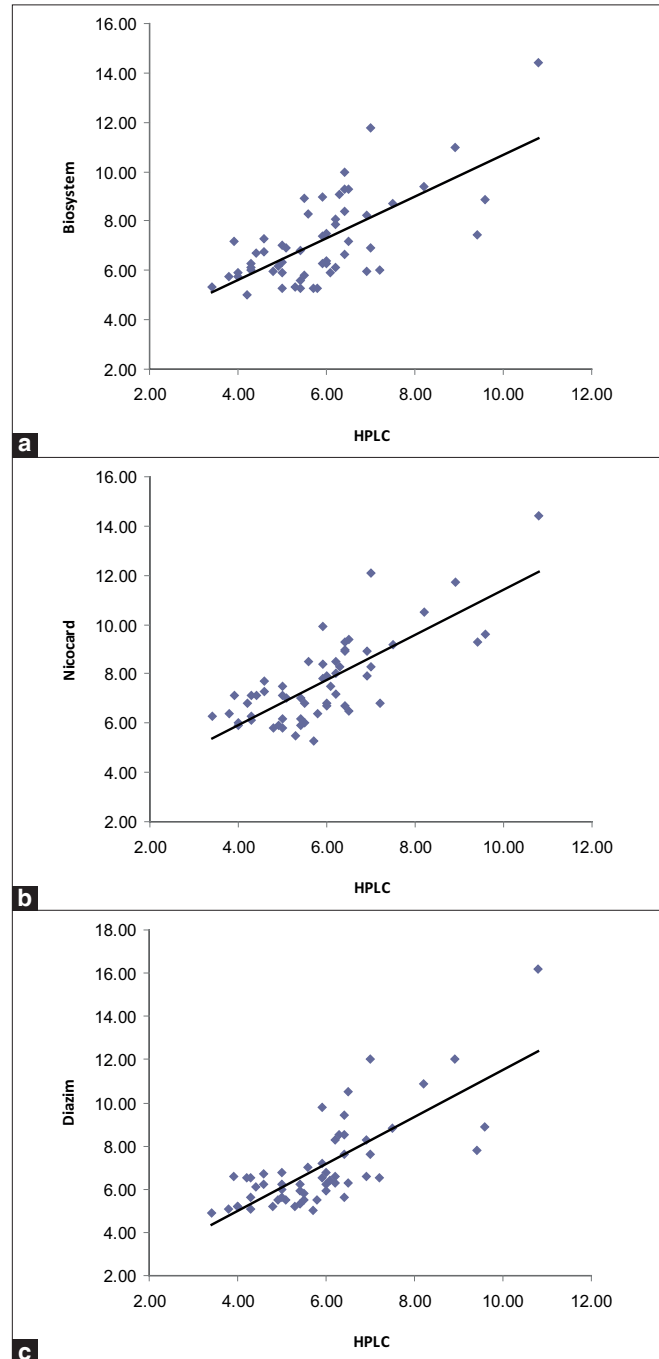
**Table 1: HbA<sub>1c</sub> values obtained through various methods**

Measurement method	HbA <sub>1c</sub> value	
	Min-Max	Mean $\pm$ SD
HPLC	3.4-10.8	5.8 $\pm$ 1.4
Nycocard	5.3-14.4	7.6 $\pm$ 1.76
Biosystem	5.01-14.3	7.2 $\pm$ 1.8
Diazyme	4.9-16.2	7.03 $\pm$ 2.1

**Table 2: Regression line parameters for  $y = ax + b$  and Pearson correlation coefficient for comparison of the measurement methods**

y	X	A (slope)	B (intercept)	R
Nycocard	HPLC	0.908	2.316	0.76
Biosystem	HPLC	0.836	2.295	0.68
Diazyme	HPLC	1.081	0.685	0.75

three obtained means ( $P < 0.001$ ). The lowest mean was for HPLC-Diazyme, such that parallel values obtained by the Diazyme device were closer to HPLC compared with the other two methods. The Pearson correlation test showed a significant linear association between HbA<sub>1c</sub> obtained values in each method with that of HPLC ( $P < 0.001$ ). In addition, the regression line parameters obtained by each method based on HPLC have been presented in Table 2, accompanied



**Figure 1:** Comparison of the HbA<sub>1c</sub> results obtained by the three new methods (y) versus Knauer-high-performance liquid chromatography (HPLC) (X). (a) Biosystem versus HPLC, (b) Nycocard versus HPLC, (c) Diazyme versus HPLC

with the value of correlation (r). The regression line diagram has been presented in Figure 1.

The Pearson correlation coefficient (r) is much closer to 1 in Nycocard and HPLC compared with the two other methods, showing a tighter correlation between Nycocard and HPLC compared with the two other methods.

## DISCUSSION

Based on statistics, the diabetic patients' population is growing. Microvascular complications of diabetes, including nephropathy, neuropathy and retinopathy, impose a great cost on the patients and the health system.<sup>[17-23]</sup>

The incidence of these complications is associated with patients' long-term glycemia. HbA<sub>1c</sub> is a marker for patients' glycemic history in the past 2-3 months. Therefore, glycated Hb measurement is a standard method to investigate the long-term glycemic control of the patients.<sup>[1,5,24]</sup> Thus, its precise measurement by laboratory methods to follow-up the patients and treat them is essential. Because employing a reference method (HPLC) is not affordable for all laboratories, the necessity for replaceable methods whose reports are, as much as possible, closely and strongly correlated to those of HPLC is clarified. Various studies have been conducted in this field.

Halwachs–Baumann *et al.* compared variant HPLC, Roche immunoassay and Hi-auto A<sub>1c</sub> analyze systems with the reference method of Diamat HPLC, and reported the Roche immunoassay to have the closest mean to that of the reference method (correlation of the employed methods with the reference method was 0.970, 0.977 and 0.972, respectively, showing an appropriate correlation with Diamat).<sup>[25]</sup>

Turpeinen *et al.* compared three devices. The Pearson correlation coefficient between poly CAT A (a HPLC based on column chromatography) and Diamat (an autoanalyzer based on ion exchange chromatography) was  $0.9 \pm 0.3$ . In addition, the correlation index between poly CAT A and IMX (based on Boronate affinity binding) was obtained as  $0.85 \pm 0.04$ . Restrictions of the Diamat method as a reference method were revealed by this study. It was also declared that there may be serious problems in clinical follow-ups in switching from one method to another.<sup>[26]</sup>

Hawkins *et al.* compared four point of care methods with the Roche tinaquant, and obtained the Pearson correlation coefficient of over 0.9 for all the four methods: DCA 2000, Nycocard, Diastat and D55.

Diastat and DCA 2000 showed the best function and correlation with the central laboratory. He concluded that these two methods can be an appropriate replacement for each other, and also for the Roche method.<sup>[27]</sup>

In none of the above studies, was the mean value interval of each method with a reference method assessed. In the present study, the correlation index of Nycocard with HPLC and Diazyme with HPLC were obtained as 0.76 and 0.75, respectively, although, generally, Diazyme had a better function and closer mean values to those of HPLC compared with the other two methods. It also had the least value interval with HPLC compared with the other two methods.

However, because the Pearson correlation coefficient was 0.75 (so far from 1 and not counted as a complete correlation), this method cannot be an ideal method to replace HPLC.

In the present study, regression line formulas were obtained for all three methods, which can be employed to convert the obtained values to that of HPLC. It is recommended to conduct further studies with a higher sample size and on the other routine methods and devices used in clinical laboratories to facilitate patients' follow-up and treatment and to amend the existing problems.

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