Original Article

Comparative Analytical Performance of Various HbA1c Assays in Iran

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Abstract

Introduction: Hemoglobin A1c (HbA1c) measurement devices are widely used to evaluate glycemic control in diabetic patients. The aim of this study was to investigate the comparability of various HbA1c instruments used in Iran.

Methods: In the present study, 154 fresh whole blood samples from diabetic patients, with different HbA1c levels (4.0%-10%) and no types of hemoglobinopathy were analyzed by six HbA1c assays including one high performance liquid chromatography (HPLC) method (D10 HbA1c), two immunoassay methods (COBAS INTEGRA 400 and Pars Azmoon kit), one Boronate affinity method (Nycocard Reader II), and two ion exchange methods (Biosystems and DS5). The two National Glycohemoglobin Standardization Programs (NGSP) certified system, D10 and COBAS INTEGRA 400 which are certified as secondary reference measurement procedures, were considered as reference methods. The CLSI document (EP9-A2) - Method comparison and Bias estimation using patient samples, approved guideline - was used to compare the performance of different HbA1c instruments.

Results: The mean of HbA1c in all four types of assays was less than the reference methods (*P*-value < 0.01).The mean of absolute difference between the reference methods was the least (0.11%). Among the other four tests, Biosystems had the smallest mean of difference (-0.21%), while Pars Azmoon had the highest (-1.18%). Pars Azmoon showed the greatest difference (95% confidence interval) when compared to D10 [-15.5%(-5.7%to -25.3%)] and COBAS INTEGRA [-17% (-9.16% to -24.84%)]. The highest regression slope (B) was found in DS5 method (0.96) in regression model with both reference methods.

Conclusion: It can be concluded that although HbA1c standardization programs have resulted in great improvements in the comparability of HbA1c assays, unacceptable errors still exist and further national and international projects are required for standardization of HbA1c measurement. In this situation, it is recommended to use the same laboratory for HbA1c measurement to monitor diabetic patients.

Keywords: Diabetes mellitus, hemoglobin A1c, HPLC, standardization

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Introduction

Diabetes is a widespread disease in both developed and developing countries which imposes great financial burden on health systems. The prevalence of diabetes mellitus (DM) is alarmingly increasing worldwide and has reached an epidemic state; it also consumes a considerable proportion of healthcare budgets all across the world.[1] It is predicted that the number of diabetic patients will rise from 171 million in 2000 to 366 million in 2030.[2,3]

Monitoring and keeping regular track of patients' blood glucose level is of utmost importance for clinicians; as they should determine whether there is a need for drug dosage and timing alteration. It is documented that measures of metabolic control, particularly glycated hemoglobin in the blood, reflect the circulating blood glucose over the preceding three months.[4]

Moreover, assessment of glycated hemoglobin and timely intervention can prevent a variety of diabetes-related complications. Consequently, frequent measurement of HbA1c levels has been increasingly favored by clinicians as an indicator of long-term glycemic control in diabetic patients.[1] Furthermore, recent studies have demonstrated that high HbA1c blood levels correlate with an increased risk of gestational diabetes.[5,6]

Various methods are adopted for measurement of blood HbA1c levels, and to date, more than 30 different HbA1c assays are used worldwide.[7] These methods can be generally classified into two different categories based on the technology used: charge difference (HPLC and electrophoresis) and structural differences (boronate affinity chromatography and immunoassay).[2,3,8]

Frequent measurement of HbA1c blood levels using the same method can provide a reliable guide for clinicians to evaluate diabetes control; nonetheless if multiple assays are employed, the results may not be reliable as different devices can report different levels.[9] This could distort physicians' diagnosis and prescription of appropriate treatments for the patients. As a result of the implementation of National Glycohemoglobin Standardization

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Program (NGSP) and development of reference materials by the International Federation of Clinical Chemistry (IFCC), consistency of assays has increased significantly and the overall validity of HbA1c assays has improved remarkably.[8,10–13] Nonetheless, there is evidence to indicate that difference among NGSP-certified methods is still significant and this can reduce the diagnostic value of HbA1c measurements.[14] Moreover, use of non-NGSP-certified methods in developing countries such as Iran, where both non-NGSP-certified and certified devices are employed, makes it very difficult to monitor diabetic patients. The current study was designed to compare the results of HbA1c measurements obtained from six commonly used HbA1c assays in Iran.

Materials and Methods

Sample collection

In the current multi-centric study, 154 fresh whole blood samples were assessed from diabetic patients (who were referred for Hba1c measurement). The samples were included if they covered a wide range of HbA1c levels (4.0%–10%) based on D10 results, and also did not have any type of hemoglobinopathy. It should be noted that according to the NGSP protocol and CLSI document EP9-A2 (Method comparison and Bias estimation using patient samples), the minimum number of samples needed for comparative studies is 40, but increasing the number of samples can improve the certainty of statistical analysis.After sample collection, whole blood was quickly split into four vials and sent to four nationally certified laboratories in Tehran, the Iranian capital, where they were analyzed within 2 days of sample collection. Before analysis, they were kept at a temperature of 2–8°C.This study was approved by the Ethics Committee of the Endocrinology and Metabolism Research Institute (EMRI) affiliated with Tehran University of Medical Sciences and all participants signed informed consent letters.

HbA1c Assays

The evaluated HbA1c assays, principles and laboratories in charge of performing are as followed:

1. D-10HbA1c (Bio-Rad Laboratories, Hercules, CA), Ion exchange HPLC method- Reference Health Lab (Ministry of Health);
2. COBAS INTEGRA 400 (Roche Diagnostics, Mannheim, Germany), Immunoassay-Massoud Laboratory;
3. NycoCard Reader II (Axis-Shield, Oslo, Norway), Boronate affinity chromatography-Diabetes and metabolic center- EMRI;
4. DS5 (Drew Scientific, Le Rheu, France), Ion exchange chromatography- Biochemistry laboratory –EMRI;
5. Biosystems (BioSystems S.A, Barcelona, Spain), Ion exchange chromatographic (manual)-Diabetes and metabolic center- EMRI;
6. Pars Azmoon kit (Pars Azmoon Inc., Tehran, Iran), Immunoassay-Diabetes and metabolic center- EMRI.

D10 HbA1c and COBAS INTEGRA, two NGSP certified secondary reference measurement procedures, were considered as the reference methods in our study.

Statistical analysis

The CLSI document (EP9-A2) was used to compare the conformance of various HbA1c instruments. Details are available on the methods used to compute mean and SD of difference, mean and SD of relative difference, as well as regression slope B, intercept and bias. Briefly, the data was examined by outlier tests on within-method duplicates. For each method, absolute values of difference and relative difference between duplicates were computed. If both differences exceeded the appropriate limit value (4 times the mean of differences), they were deleted (maximum 2.5% of results could be deleted). The next step was to visually check for between-method outliers. Scatter plots of data of test and reference methods were designed to check for possible linear relationship. If visual outliers existed, absolute and relative values of the difference were calculated. Data exceeding either limit value (4 times the mean of difference) were deleted (maximum 2.5% of results could be deleted).

The correlation coefficient, r, was used to test the adequacy of the data range. As r was less than 0.975, we used partitioned bias protocol. Data was sorted in an increasing order and then divided into three subgroups (low, medium and high levels of HbA1c) where each subgroup contained approximately the same number of data points. Partitioned bias procedures were then applied to estimate the average bias. Regression slope B and intercept were also calculated for the paired observations by linear regression.

CLSI EP 15-A protocol - User Verification of Performance for Precision and Trueness; Approved Guideline- was also used to investigate the imprecision of the assays (four times measurement on 3 fresh whole blood samples with different levels of HbA1c during five days).

All statistical analyses were performed with STATA software version 11, and graphs were prepared using R software.

Results

The results indicated that COBAS INTEGRA showed the highest mean of HbA1c (7.6%), and the lowest was found in Pars Azmoon method (6.3%). All four types of devices had lower means of HbA1c in comparison with the reference methods (*P*-value < 0.01). D10 and COBAS INTEGRA had the smallest mean of absolute difference (0.11%). Among the other four tests, Biosystems had the smallest mean of difference (-0.2%), while Pars Azmoon had the highest (-1.18%). Table 1 (A and B) outlines the mean of total HbA1c and that broken down by three subgroups (1: low level, 2: medium level and 3: high level of HbA1c) and SD.

Figure 1 illustrates the Bland-Altman plot examined for absolute difference between the methods studied.

Figure 2 clearly shows the bias percentage (with 95% confidence interval) in all of the studied measurement methods. Pars Azmoon shows the greatest difference (95% confidence interval) when compared to D10 [-15.5%(-5.7% to -25.3%)] and COBAS INTEGRA [-17% (-9.1% to -24.8%)]. Although other assays show smaller bias percentage, unfortunately none of them meet the NGSP acceptance criteria for bias (±6% of reference values).

Table 2 summarizes the regression coefficients of reference methods on test methods. All slope coefficients were highly significant regarding *P*-value (<0.001). Coefficient values near 1.00 showed the strong linear relation between two methods. On the other hand, all the coefficients were reported to be less than 1.00, suggesting that all the studied test methods underestimated the HbA1c levels. The DS5 method had the highest regression slope level (0.96) when both D10 and COBAS INTEGRA were considered as reference.

It should be noted that in all assays, the coefficient of variation (CV %) was less than 3.4% for different HbA1c levels (Table 3).

Table 1 (A). The total mean, mean of the three subgroups, mean of absolute difference (total and subgroups) in the six different methods when D10 is the reference method.

Reference Method: D10						
Variable	**D10**	**COBAS INTEGRA**	**Nycocard**	**Biosystem**	**DS5**	**ParsAzmoon**
Mean-total	7.5	7.61	6.81	7.29	6.64	6.32
Mean-group1(low level)	5.82	6.09	5.59	6.18	5.03	5
Mean-group2(medium level)	7.6	7.59	6.86	7.32	6.74	6.42
Mean-group3(high level)	9.12	9.19	8	8.38	8.18	7.58
Mean of difference -total(SD)	—	0.11(0.46)	-0.69(0.57)	-0.21(0.81)	-0.87(0.46)	-1.18(0.49)
Mean of difference-group1 **(SD)**	—	0.27(0.4)	-0.23(0.42)	0.36(0.64)	-0.79(0.43)	-0.82(0.31)
Mean of difference-group2 **(SD)**	—	-0.01(0.47)	-0.74(0.45)	-0.28(0.63)	-0.86(0.48)	-1.18(0.43)
Mean of difference-group3 **(SD)**	—	0.06(0.47)	-1.12(0.43)	-0.74(0.74)	-0.94(0.44)	-1.55(0.42)

Table 1 (B). The total mean, mean of the three subgroups, mean of absolute difference (total and subgroups) in the five different methods when Roche COBAS INTEGRA is the reference method.

Reference Method: Roche- COBAS INTEGRA					
Variable	**COBAS INTEGRA**	**Nycocard**	**Biosystem**	**DS5**	**ParsAzmoon**
Mean-total	7.61	6.81	7.29	6.64	6.32
Mean-group 1(low level)	6.04	5.59	6.13	5.05	4.99
Mean-group 2(medium level)	7.64	6.9	7.35	6.8	6.44
Mean-group 3(high level)	9.27	8.02	8.47	8.17	7.63
Mean of difference -total (SD)	—	**-0.81(0.56)**	**-0.32(0.7)**	**-0.98(0.52)**	**-1.29(0.39)**
Mean of difference- group 1**(SD)**	—	-0.5(0.38)	0.09(0.58)	-0.98(0.45)	-1.1(0.23)
Mean of difference-group 2**(SD)**	—	-0.74(0.46)	-0.27(0.58)	-0.84(0.58)	-1.17(0.32)
Mean of difference-group 3**(SD)**	—	-1.18(0.59)	-0.8(0.63)	-1.1(0.48)	-1.61(0.4)

Discussion

Monitoring HbA1c levels as a diabetes control indicator requires standardized HbA1c assays with the ability of producing reliable results, which is hard to obtain by the instruments currently used. Although the analytic quality of HbA1c assays has improved thanks to NGSP standardization programs,[9] our results showed considerable inconsistency among different NGSP-certified HbA1c assays. In addition, according to our findings, non-NGSP certified devices that are widely used in Iran reported significantly different results when applied to the same sample. Therefore, it can be concluded that using non-NGSP certified assays in developing countries can further hinder the use of HbA1c measurement as an indicator of diabetes control. In this study, the smallest difference was reported between the reference methods, D10 and COBAS INTEGRA; however, the difference between the reference methods and other methods including Nycocard, Biosystem, DS5 and Pars Azmoon was higher.

Our findings are consistent with previous reports. In two separate studies, Terrenia and Twomey assessed the comparability of two different DCCT-aligned HbA1c HPLC analyzers, TOSOH HLC G7 and Biorad Variant II; they concluded that despite the strong correlation between the results, TOSOH reported lower values in comparison with Biorad Variant II.[17,18]

In another study, Karami *et al.* investigated the concordance of the results of HbA1c measurement in 58 samples from diabetic patients assayed using Diazyme (enzymatic assay), Nycocard (boronate-affinity binding), Biosystem (micro column chromatography) and Knauer-HPLC; they reported a considerable difference between all of these methods and HPLC, as the gold standard.[19]

To date, several studies have been carried out to evaluate the performance of point of care testing (POC) instruments. Lenters-Westra investigated the comparability of the results of six HbA1c POC instruments using two lot-numbers of the same kit (including Nycocard reader) with four secondary reference measurement procedures (Roche, Integra 800, Primus Ultra2, and TOSOH G7) by comparing the results of 40 patient samples. Their results indicated that Afinion and DCA Vantage were the only methods that fulfilled the NGSP criteria. Moreover, it was observed that the Nycocard reader did not meet the NGSP criteria. This is while the calculated total error was lower in comparison with our results.[9]

In the Hawkins study, the precision and analytical inaccuracy of five systems (including Nycocard and DS5) were assessed and the results were compared with those of the Roche Tinaquant. They concluded that DS5 has low precision with a significantly positive bias in comparison with other methods. Their findings also demonstrated that the Nycocard system had the lowest precision.[20] Notwithstanding these results, in our study, both mentioned systems demonstrated high precision yet with unacceptable bias. It is noteworthy that the findings of a study with similar design by Lenters-Westra demonstrated that Afinion, DCA Vantage, Cobas B101, and B-analyst instruments met the generally accepted performance criteria for HbA1c. However, their results indicated that although Quo-Test, Quo-Lab, and InnovaStar met the criteria for

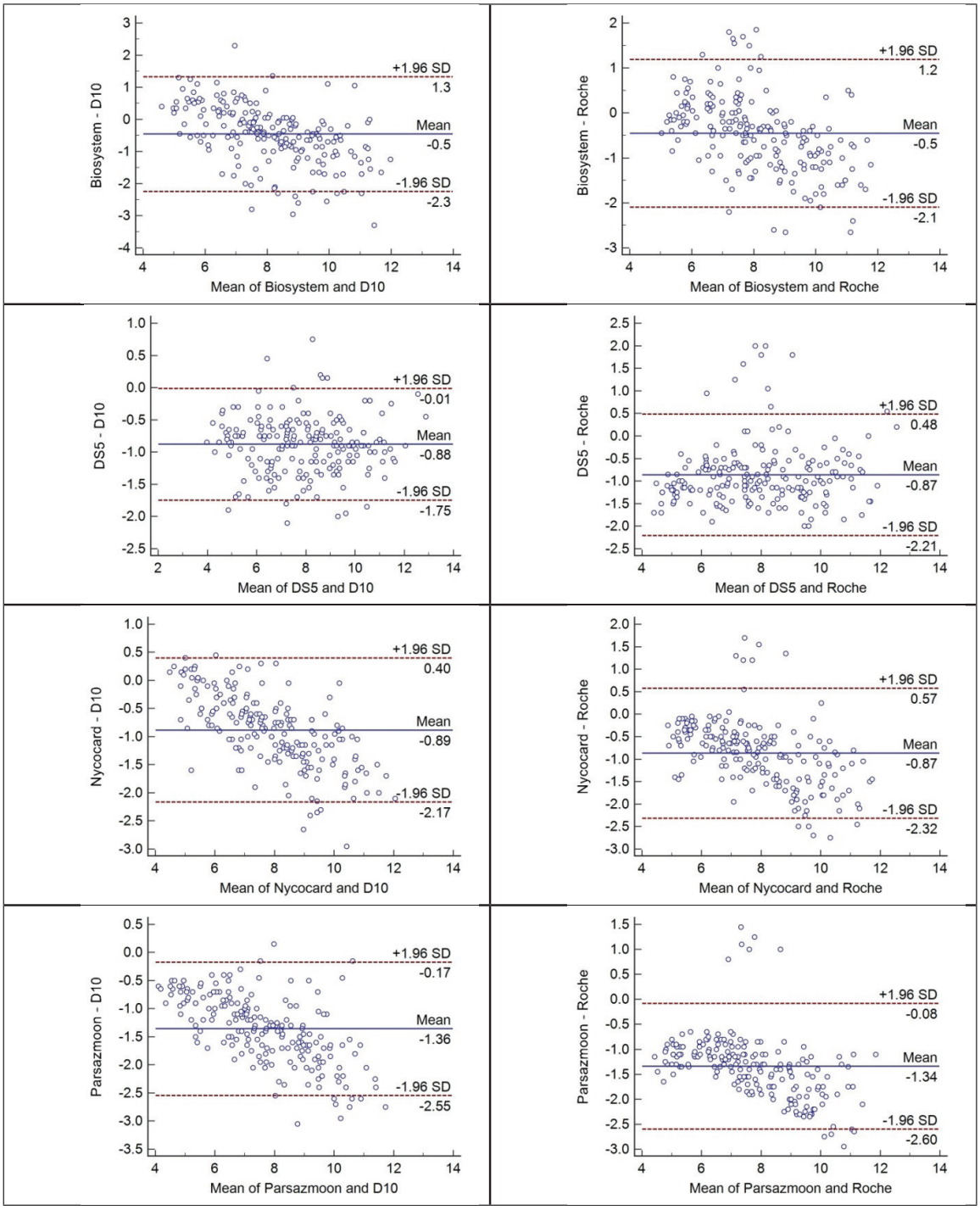

Figure 1. *Bland-Altman* plot examination for calculating absolute difference between methods.

precision, their bias was unacceptable.[21]

In the study by Holmes *et al.,* the analytical bias of seven POC instruments and lab assays were investigated for 33 months. They observed the overtime drifts, reporting a bias increase from 0.4% to -0.9%. It was also reported that the magnitude of relative bias was considerably large and potentially misleading.[22] It is noteworthy, both the abovementioned studies and the Lenters-Westra investigation evaluated different results obtained from various lot numbers. We, however, did not focus on such potential differences.

The different results among NGSP certified assays if used interchangeably can lead to the confusion of clinicians.[17–18] The reason for the dissimilar outcomes could rise from factors interfering with HbA1c measurement. Genetic variants (e.g., HbS trait, HbC trait), elevated fetal hemoglobin (HbF) and chemically modified derivatives of hemoglobin (e.g., carbamylated Hb in patients with renal failure) can affect the accuracy of HbA1c measurements. The effects vary depending on specific Hb variants or derivatives,

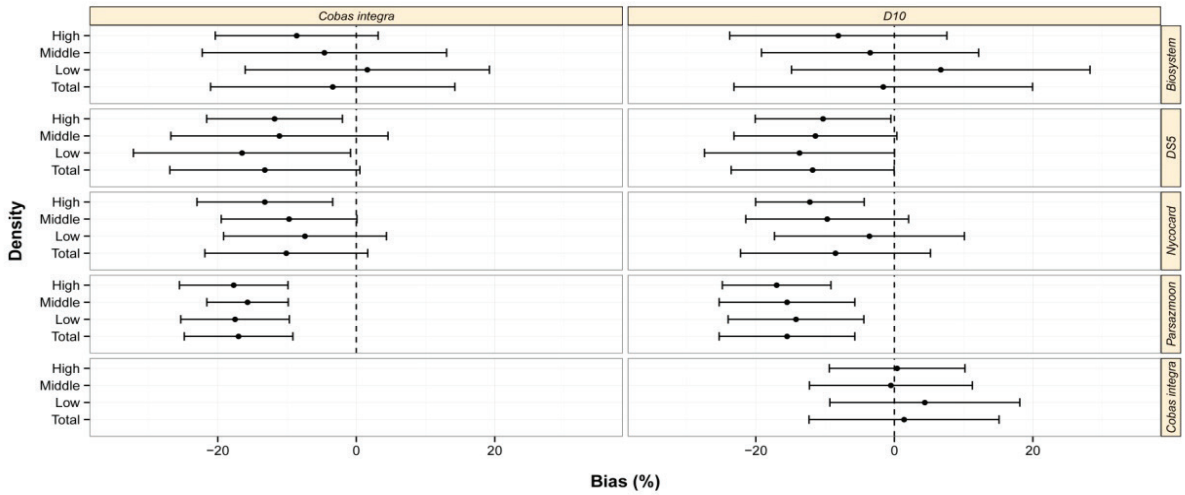

Figure 2. The percentage of bias with upper and lower bound in the different methods.

Table 2. R-squared, regression slope (Beta), *P*-value and intercept in different methods.

Reference Method: D10					
Variable	**COBAS INTEGRA**	**Nycocard**	**Biosystem**	**DS5**	**Pars Azmoon**
R square-total	0.9	0.88	0.69	0.9	0.9
Beta-total	0.93*(<0.001)	0.73*(<0.001)	0.67*(<0.001)	0.96*(<0.001)	0.79*(<0.001)
Intercept-total	0.62*(<0.001)	1.36*(<0.001)	2.22*(<0.001)	-0.54*(<0.001)	0.4*(0.001)
Reference Method: Roche- COBAS INTEGRA					
R square-total	—	0.87	0.76	0.88	0.94
Beta-total	—	0.74*(<0.001)	0.72*(<0.001)	0.96*(<0.001)	0.82*(<0.001)
Intercept-total	—	1.21*(<0.001)	1.79*(<0.001)	-0.65*(<0.001)	0.07*(0.43)

Table 3. Total imprecision levels based on coefficient of variation (CV%) from different assays.

HbA1c Levels / Assays	(5.5%)	(7.5%)	(9%)
D-10 HbA1c	0.94	1.56	0.83
COBAS INTEGRA	0.95	0.74	0.59
NycoCard Reader II	3.12	2.32	2.62
DS5	1.94	3.35	2.08
Pars Azmoon	2.28	1.9	1.9
Biosystems	3.41	2.6	3.27

HbA1c method as well as assay calibration.

Recently, several biological variables have been introduced and pertinent indices are derived for the clinical application of HbA1c measurements. On the basis of biological variation, allowable total error is considered 2.7%.[23,24] As a requirement for the NGSP certification, it is necessary that the results of test methods fall within ±6% (relative) of the NGSP Secondary Reference Laboratories (SRL) results.[25] Our results did not meet any of the above criteria.

The strengths of our study are as follows. First, in the context of available literature, this was the first such study to assess the major HbA1c measurement devices in Iran. The large sample size was another advantage of the current study (154 samples). Finally, it was the first time that statistical model CLSI document -EP9-A2 (Method comparison and Bias estimation using patient samples) was used in Iran. The limitation of our study was that the samples were examined within two days of blood collection.

Based on our findings, it can be concluded that the difference between values measured by various HbA1c assays used in Iran is so large that values cannot be used interchangeably; this can lead to confusing clinical interpretations. Moreover, major assays used in Iranian laboratories show various bias in comparison with the assays used as the reference.

In conclusion, currently, more than 2000 laboratories in different parts of Iran perform HbA1c measurement using different methods. Thus, to minimize the variation between methods, exclusive utilization of NGSP certified methods and reinforcement of nationally endorsed assays based on a standard reference laboratory is required. In this situation, it is recommended to use the same laboratory for HbA1c measurement to monitor the diabetic patients.

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