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Comparison of high performance liquid chromatography and turbidimetric inhibition immunoassay methods for measurement of hemoglobin A1c

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ABSTRACT

Aim: Hemoglobin A1c is a valuable parameter for the diagnosis and follow-up of its diabetes mellitus since its biological variation is low, does not require preparation before the test, is not affected by acute stress, and has high preanalytical stability. HbA1c measurement by HPLC has been determined as the reference method by National Glycohemoglobin Standardization Program (NGSP) in USA; after that The International Federation of Clinical Chemistry (IFCC) defined another reference method which could be related with NGSP. In our study, we aim to compare the two NGSP-certified methods of HbA1c, which are high-performance liquid chromatography (HPLC) and turbidimetric inhibition immunoassay (TINIA).

Material and Method: HbA1c levels of the patients were measured using two HPLC and one TINIA method in three different hospitals (Lab A, Lab B (Both are HPLC), and Lab C (TINIA), in which Lab A was served as a reference). Because of the lower precision values of LabB, we firstly conducted a method comparison study of 40 volunteers (Group 1). After that, corrective and preventive activities carried out and the precision values in LabB reached the desired range. Following this, another method comparison study consisting of 60 new volunteers (Group 2) was conducted. The statistical flow of this study complied with Clinical Laboratory Standards Institute (CLSI) EP09-A3; Precision studies, Blant-Altman and Passing Bablok regression analysis were performed.

Results: The percentage of the mean difference between the two HPLC methods (LabA and LabB) was 3.1%. After corrective and preventive actions had been taken, the mean difference between the two HPLC methods decreased to 2.0%. A decrease in systematic bias was found in our study. Two HPLC methods can be used interchangeably in both Group 1 and Group 2. In Group 1; 95% CI of intercept and slope were found as (-1.41 to -0.30) and (1.03 to 1.22), respectively. In Group 2; 95% CI of intercept and slope were found as (-1.33 to -0.31) and (1.01 to 1.17), respectively. HPLC and TINIA methods could not be used interchangeable without affecting patient results and outcome in both Group 1 and Group 2.

Conclusion: Our study concluded that TINIA and HPLC methods could not be used interchangeably without affecting patient results and outcome. Because of the methodology that clinical laboratories are used to, clinicians and clinical biochemists should collaborate on managing diabetes mellitus regarding diagnosis, treatment, and follow-up.

Keywords: Diabetes mellitus, HPLC, immunoturbidimetry, HbA1c

INTRODUCTION

HbA1c (Hemoglobin A1c) molecule is formed by Maillard reaction where N-terminal valine of the β chain reacts with glucose to the aldimide (Schiff base) and performs an Amadori rearrangement to the stable ketoamine (1). Measurement of HbA1c is essential for the evaluation of retrospective glycemia. It is well known that the measurement of HbA1c reflects the mean value of blood glucose for 6-8 weeks and is also related to late complications of diabetes mellitus (2).

HbA1c is a valuable parameter for the diagnosis and follow-up of its treatment since its biological variation is lower compared to fasting plasma glucose and/or 2 hour plasma glucose in both within (CVI) and between subject (CVG) variation (CVI and CVG values for HbA1c and plasma glucose are 1.6% and 5,0% and 7.1% and 8.1%; respectively) (3), does not require preparation before the test, is not affected by acute stress, and has high preanalytical stability (4).

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High-performance liquid chromatography (HPLC) is a reference method to standardize other routine procedures with long-term validity, accuracy, and stability (5,6). Other methods for measurements of HbA1c are immunologic methods, affinity chromatography, capillary electrophoresis, and enzymatic methods (7,8).

HbA1c was defined as a diagnostic criteria of diabetes mellitus, after the International Expert Committee's report that was published in 2009 (4). HbA1c measurement by HPLC has been determined as the reference method by National Glycohemoglobin Standardization Programme (NGSP) in USA; after that The International Federation of Clinical Chemistry (IFCC) defined another reference method which could be related with NGSP (9,10).

In our study, we aim to compare the two NGSP-certified methods of HbA1c, which are high-performance liquid chromatography (HPLC) and turbidimetric inhibition immunoassay (TINIA).

MATERIAL AND METHOD

This study was approved by the Ordu University Clinical Researchers Ethics Committee (Date: 17.06.2022, Decision No: 2022/149). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

Study Design

One hundred patients from Ordu University Hospital diagnosed with prediabetes or diabetes were admitted to the study. HbA1c levels of the patients were measured using two HPLC (Adams HA8180V (Arkray Inc, Kyoto, Japan)) and one TINIA method (Cobas, Roche Diagnostics GmbH, Mannheim, Germany) in three different hospitals (Lab A, Lab B, and Lab C, in which Lab A was served as a comparative laboratory) in Ordu, a city in Turkey. Lab A and Lab B used the HPLC method with the same kit, Lab C used the immunoturbidimetric method). The study group was grouped into two subgroups; Group 1 consisted of 40 patients, and Group 2 consisted of 60 patients. Main reason of forming Group1 and Group 2 is to evaluate the corrective and preventive actions. LabB were facing problems regarding both internal quality control (recorded lower precision values (3.5%) than we expected) and patient results. As a result of this, Group 1 was formed to show that whether a systematic and/or random error in LabB; so method comparison study which was taken place with 40 patient samples were completed . According to the EP09-A3 guideline, 40 patient samples were enough to perform for method comparison study. In the group 1, because of the method comparison study's results were not efficient, preventive and corrective actions (implementing new calibration, new kit and requested service care) were performed. After the preventive and corrective actions had been taken, another method comparison study (Group 2) was planned to observe the effects of preventive and corrective actions.

Procedures

HPLC (Adams Arkray 8180T)

This system is a fully automated HbA1c analyzer using reverse-phase cation exchange chromatography. The system can handle both whole blood and manually diluted samples. Four microlitres of whole blood are automatically sampled, hemolyzed, and injected into the column. Hb molecules elute with inorganic phosphate buffers (80A, 80B, and 80CT) from low to high polarity. Upon elution, sample components pass through the spectrophotometric detector, where fractions are monitored with dual-wavelength detection (420 and 500 nm). (11) This autoanalyzer complies with the latest Diabetes Control and Complications Trial (DCCT) reference method, which is stated by National Glycohemoglobin Standardization Programme (NGSP).

TINIA (Cobas c501, Roche Diagnostics)

In this method, sample pretreatment to remove labile HbA1c is not necessary. The HbA1c determination is based on the TINIA for hemolyzed whole blood. HbA1c in the sample reacts with anti-HbA1c antibody to form soluble antigen-antibody complexes. Since the specific HbA1c antibody site is present only once on the HbA1c molecule, formation of insoluble complexes does not take place. The polyhaptens react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex which can be determined turbidimetrically. % hemoglobin A1c is measured using a ratio (12).

Medical decision limit was stated as 6,5%, and desirable imprecision and bias for HbA1c were determined as 0,6% and 1,2%, respectively; according to the EuBIVAS (3)

Lab A and Lab B used the HPLC method, with the coefficient of variation (CV%) <1%; Lab C used the immunologically based method, with the <3%CV. Lab A and B's linearity range is 4-15% and 4.2-20.2% for Lab C.

Statistical Methods

Statistical analyses were performed using IBM SPSS Statistics Ver.22, MedCalc® Statistical Software version 20.009 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021) and GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com".

Evaluation of normality of HbA1c values were performed with Kolmogorov-Smirnov test.

The statistical flow of this study, which has complied with Clinical Laboratory Standards Institute (CLSI) EP09-A3,

has three steps; Precision studies which were found within biological variation limits were performed in the first step, and comparisons of mean and the differences of the mean values that were performed with Blant-Altman analysis in the second step, Passing Bablok analysis were performed in the last step.

RESULTS

Mean HbA1c and SD (Error Bars) of the laboratories in Group 1 and Group 2 are shown in **Figure 1**.

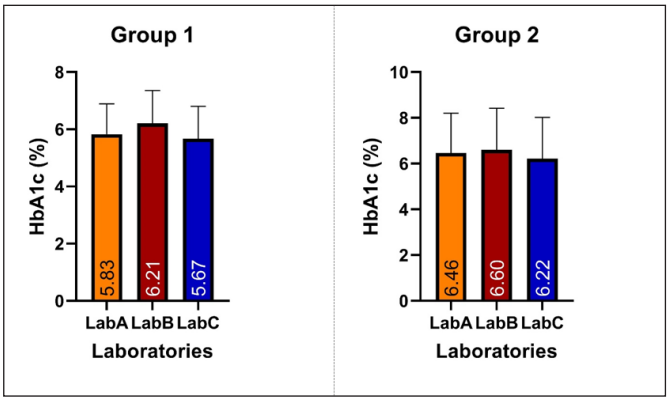

Figure 1. Mean HbA1c and SD Values of the Laboratories in Group 1 and Group 2

Comparisons of the median HbA1c values of the laboratories in Group 1 and Group 2 are shown in **Table 1**

In the **Table 1**, median values of LabB and LabC are compared with the LabA, which is selected as "comparative laboratory"

Table 1. Comparisons of the median HbA1c values of the laboratories in Group 1 and Group 2

	LabA Median (Minimum-Maximum)	LabB Median (Minimum-Maximum)	LabC Median (Minimum-Maximum)	p*	p**	p***
Group 1 (n:40)	5.80 (5.30-11.00)	5.40 (4.60-10.40)	5.50 (4.90-10.20)	<0.01	<0.01	<0.01
Group 2 (n:60)	5.80 (4.60-12.40)	5.80 (4.70-13.00)	5.55 (4.00-12.20)	0.30	<0.01	<0.01

p*: Mann Whitney U test of LabA and LabB, p**: Mann Whitney U test of LabA and LabC, p***: Mann Whitney U test of LabB and LabC

In Group 1; median HbA1c value of LabC is significantly lower than LabA and LabB (p<0.01 for both, respectively).

In Group 2; median HbA1c value of LabC is significantly lower than LabA and LabB (p<0.01 for both, respectively).

In Group 2, there was no difference between median values of LabA and LabB (p:0.30)

In group 1, there is a positive significant correlation between LabA vs Lab B (r: 0.99, p<0.001 and 95% CI: 0.99 to 0.99) and LabA vs LabC (r:0.99, p <0.01 and 95% CI: 0.98 to 0.99).

In group 2, there is a positive significant correlation between LabA vs Lab B (r: 0.99, p<0.001 and 95% CI: 0.99 to 0.99) and LabA vs LabC (r:0.99, p <0.01 and 95% CI: 0.98 to 0.99).

Blant-Altman analysis of LabA vs LabB and LabA vs LabC in Group 1 are shown in **Figure 2A and 2C**, respectively. Scatter plots of LabA vs LabB and LabA vs LabC in Group 2 are shown in **Figure 2B and 2D**, respectively.

As it is shown in **Figure 2A-D**; Blant-Altman plot demonstrating that confidence interval of the mean bias which is shown as the green line in the **Figure 2A-D** do not include zero value, that is indicative of systematic bias.

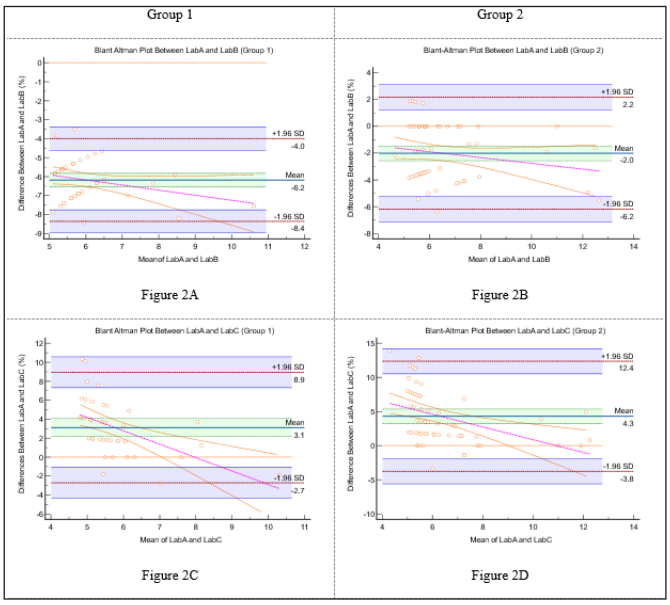

Figure 2. Blant Altman Plot for the Laboratories in Group 1 and Group 2

Passing-Bablok Regresyon analysis and equations of laboratories are shown in **Figure 3**.

Regression analysis of LabA vs LabB and LabA vs LabC in Group 1 are showed in **Figure 3A and 3C**, respectively. Regression analysis of LabA vs LabB and LabA vs LabC in Group 2 are showed in **Figure 3B and 3D**, respectively.

In Group 1;

Regression equation of LabA and LabB is found as y=-0.02 +1.06 x. 95% CI of intercept and slope were found as (-0.24 to 0.40) and (1.00 to 1.10), respectively.

Regression equation of LabA and LabC is found as y=-0.07 +1.16 x. 95% CI of intercept and slope were found as (-1.41 to -0.30) and (1.03 to 1.22), respectively.

In Group 2;

Regression equation of LabA and LabB is found as y=-0.06 +1.03 x. 95% CI of intercept and slope were found as (-0.25 to 0.15) and (1.00 to 1.06), respectively.

Regression equation of LabA and LabC is found as y=-0.75 +1.08 x. 95% CI of intercept and slope were found as (-1.33 to -0.31) and (1.01 to 1.17), respectively.

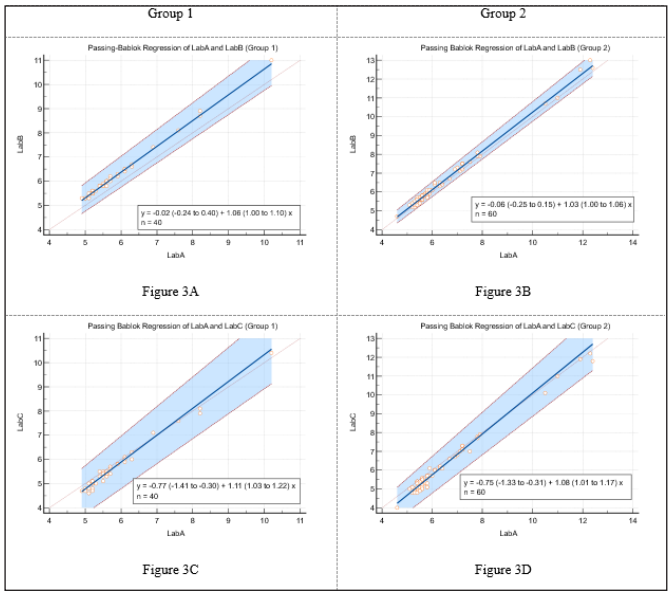

Figure 3. Passing-Bablok Regression of Laboratories in Group 1 and Group 2

DISCUSSION

It is widely stated that complications of diabetes are related to patients' long-term glycemia. Therefore, the measurement of HbA1c is a standard method to evaluate the long-term glycemic control of the patients (5, 13). Due to the relatively higher cost of implementing a reference method, like HPLC in the clinical laboratory, method comparison studies that aim to compare HPLC and the other methods should be performed.

Our study concluded that TINIA and HPLC methods could not be used interchangeably without affecting patient results and outcome in both Group 1 and Group 2. In the literature, there are studies that TINIA is a reliable method with high imprecision and accuracy (14-16). In this article, the authors used correlation analysis to evaluate the method comparison. However, according to the CLSI EP09-A3 guideline (17), Blant-Altman analysis and Passing Bablok or Deming Regression analysis could be performed for the method comparison study. Contradictive findings can be attributed to differences in sample preparations, internal quality control rules, etc.

In the passing-bablok analysis, significance testing is performed by examining the confidence intervals for a and b in the equation of y=ax+b. Null hypothesis H0 is accepted if confidence intervals for a and b's include 0 and 1, respectively (18).

Our first hypothesis was that lower precision in the HPLC method in LabB affected the results shown in Group 1. The percentage of the mean difference between the two HPLC methods (LabA and LabB) was 3.1%. The maximum allowable bias for HbA1c is 1.2% (3). After corrective and preventive actions had been taken, the mean difference between the two HPLC methods decreased to 2.0%. A decrease in systematic bias was found in our study. To summarize, it can be concluded that harmonization among hospitals within defined periods can be helpful for the management of patients with diabetes mellitus.

According to the Passing Bablok analysis, two HPLC methods can be used interchangeably in both Group 1 and Group 2. In Group 1; 95% CI of intercept and slope were found as (-1.41 to -0.30) and (1.03 to 1.22), respectively. In Group 2; 95% CI of intercept and slope were found as (-1.33 to -0.31) and (1.01 to 1.17), respectively. Because of the fact that 95% CI of intercept values did not included 0 and slope values did not included 1; we could conclude that However, HPLC and TINIA methods could not be used interchangeable without affecting patient results and outcome in both Group 1 and Group 2.

In our study, mean HbA1c values with HPLC methods are relatively higher than with TINIA. These results are consistent with the literature (15, 19-21). This finding is also essential, because the TINIA method is widely used in clinical laboratories due to its relatively lower cost and is easily applicable. Clinicians should keep in mind this conclusion for the management of patients with diabetes. Furthermore, turnaround time is also important parameters that should be kept in mind. Turnaround time of HPLC method is much faster than immunoturbidimetric method. Therefore, clinical biochemists should not be validate of the HbA1c results without evaluating related analytes (Glucose etc).

Cost is an important parameter in the clinical biochemistry laboratories. According to the social security instution communique on healthcare practices, HPLC method is approximately 4.5x expensive, comparing the TINIA method (22).

Our study aims to show the importance of preventive and corrective actions and harmonization steps for evaluating the HbA1c levels in three hospitals. This is the main advantage of our study. However, we could not analyze repeated measurements for each method and could not perform appropriate sampling. These are the main drawbacks of our study.

CONCLUSION

Clinicians and clinical biochemists should collaborate on managing diabetes mellitus regarding diagnosis, treatment, and follow-up.

ETHICAL DECLERATIONS

Ethics Committee Approval: This study was approved by the Ordu University Clinical Researchers Ethics Committee (Date: 17.06.2022, Decision No: 2022/149).

Informed Consent: All patients signed the free and informed consent form.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All the authors declare that they have all participated in the design, statistical evaluation and writing and that they have approved the final version.

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