International Journal of Clinical Biochemistry and Research 2020;7(3):367–373

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International Journal of Clinical Biochemistry and Research

Journal homepage: www.ipinnovative.com

Original Research Article

# Method verification procedure of boric acid affinity method against high performance of liquid chromatography for glycated haemoglobin

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ARTICLE INFO

*Article history:*
Received 23-07-2020
Accepted 07-08-2020
Available online 28-09-2020

*Keywords:*
Glycated Haemoglobin (HbA1c)
High performance liquid chromatography (HPLC)
Boric acid affinity (BAA)
Precision study

ABSTRACT

**Aim**: The aim of the study is to estimate the glycated hemoglobin (HbA1c) in Boric acid affinity method, (Nycocard reader II) compare and correlate these values with those values of DCCT standardized method, High Performance Liquid Chromatography (D-10, Biorad). As well as to check the boric acid affinity may or not work as an alternative to the costly high performance liquid chromatography method for glycated hemoglobin.

**Materials and Methods**: People visit the clinical laboratory of S.V.S medical college for their HbA1c, to know their glycemic status. A total of40 subjects were included in this cross sectional study irrespective of their glycemic condition after exclusion criteria. Subjects with either anaemia (Haemoglobin less or equal 10% was considered as anaemia)or underlying abnormal haemoglobinopathies or addiction of alcohol or opiates or medication on salicylates and those not willing to give consent were not included. HbA1c levels were measured by both the techniques on the same blood sample of individual participants.

**Results**: The mean ± SD age of the study group was 52 ±17.16 years. The HbA1c values of individuals by HPLC method were between 4.7% - 14.3% were taken in this study. Their whole blood HbA1c levels estimated by HPLC and boric acid affinity in terms of mean ± SD were 7.57 ± 2.64 and 7.67 ± 2.48 respectively. This difference was not statistically significant which indicates no value differences in these two methods under standard conditions and trained techniques. A highly positive correlation was established between these two methods as 0.98, which was statistically significant ($p < 0.05$).The Passing Bablok regression analysis demonstrated similar performance of HPLC and BAA methods for estimating HbA1c with Intercept close to zero and slope close to one. The precision of the boric acid affinity method is not better than HPLC, but it is within IFCC suggested limits.

**Conclusion:** The study revealed that the Boric acid affinity and the HPLC methods show very strong correlation and good agreement. Boric acid affinity performed acceptable precision. Though the HPLC method is established as the gold standard for estimation of HbA1c, Boric acid affinity method can serve as an alternative and suitable in minimal facility laboratories as it is cheaper and easier to analyse.



## 1. Introduction

After diagnosis of diabetes, patients fail to get correct guidance related to laboratory tests, unavailability of testing centres with affordable methods, and wrong reporting by technicians because of lack of performance skills and knowledge related to challenges in interpretation of results of HbA1c are cumulatively misleading the patients and doctors regarding glycemic status and making the diabetes earlier worsen with life threatening complications. There are a number of methods related to HbA1c measurement, but every method has its own advantages and disadvantages. Among all procedures, HPLC method is a standardized method, but to establish this method in clinical laboratories with minimum facilities in rural areas is not possible. The laboratories with basic facilities never encourage procedures like HPLC because of cost, lack of facilities

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https://doi.org/10.18231/j.ijcbr.2020.079

for reagent storage, results interpretation and technical skills. It is time to establish the alternative methods to HPLC without any compromise. As well as these alternatives should be affordable, available, easy to perform analysis, easy to interpret, report, correlate with patient clinical glycemic status, overcome the interference from the underlying abnormal haemoglobinopathies, easy to set up and reproducibility results will be there. It might boost the rural area labs and helps in screening, diagnosis and therapeutic monitoring of diabetes mellitus. While evaluating the glycemic control in patients with diabetes, HbA1c is considered the gold standard method.[1,2]

In the routine clinical setting, several methods have been developed to measure the blood levels of HbA1c. We can conclude all these methods based on the underlying principle as follows. Ion – exchange chromatography, electrophoresis, isoelectric focusing methods, all these work by identifying the charge differences between Hb A1c and Hb A0.[3] Affinity chromatography method's basic principle is identifying haemoglobin glycol groups stereo changes and immunoassay method use either mono or polyclonal antibodies, which are directed against the N-terminal beta chain haemoglobin.[4–7] Electrosprayionization- Mass spectrometry[8] and photometric methods purely based on chemical properties.

HPLC has been considered as the reference method by the Diabetes Control and Complications Trial Research Group (DCCT)[9–11] as well as the anchor method by the National Glycohemoglobin Standardization Program.[12] When considered the advantages of HbA1c over the other diabetic investigations are irrespective of the time in a day patient would request the test, pre-analytical long life of HbA1c (stability), not require fasting state, no need to request repeatedly and helps in assess the average plasma glucose levels of last 3-4 months in subjects because of minimal day to day changes. The drawbacks are cost, need skilled technicians, lack of availability, influence of erythrocyte turnover, iron deficiency anaemia and splenectomy[13] conditions, interference from haemoglobin variants,[14] challenges in reporting as well as in interpretation of values, no recommended national guidelines / non adherence to recommended guidelines. DCCT established the relation between diabetic complications and HbA1c levels. As per IMPROVE Control India (ICI) study, most of the doctors accepted the HbA1c important role in management of diabetes, but it was requested only in 79% of diabetes patients by them because they do not believe the standardization of HbA1c in diagnostic laboratories. Only 33% of diabetes patients know about the HbA1c test. Patients were done HbA1c tests only once or twice in a year rather than recommended.[15]

Based on IMPROVE study outputs, we understand that there are some barriers between the advice of HbA1c test to patients by physicians, the implementation of tests by laboratories and patients willing to test as per each time of recommendation by physicians. On the way to make the HbA1c test available, cheaper and easier to standardize procedure we look into alternative methods over the HPLC method. Hence, we tried to check if the BAA method might act as a substitute to the HPLC method in this study.

## 2. Materials and Methods

This cross sectional study was performed in the Clinical biochemistry department of the central lab of S.V.S Medical College & Hospital. Subjects were selected based on the following inclusion and exclusion criteria.

### 2.1. Inclusion criteria

1. Haemoglobin > 10%.
2. No medical and family history of abnormal haemoglobinopathies.
3. Irrespective of duration and diagnosis of diabetes volunteers came for their regular checkups.

### 2.2. Exclusion criteria

1. Anaemia (Haemoglobin less or equal 10% was considered as anaemia).
2. Abnormal haemoglobinopathies if detected in HPLC method.[14]
3. Alcoholics and opiates addiction individuals.
4. Medication on salicylates.
5. Not willing to give consent.

The study was carried out in the month of January 2020, during this period, after excluding the subjects with the alcoholic addiction, medication on salicylates and explanation of the academic purpose of this study only forty nine volunteers got eligibility and shown interest in participation. These were enrolled in this study after taking written informed consent. 2 ml of whole blood sample was collected under aseptic conditions in K2EDTA tubes from all these volunteers. After that, the precautions were taken to prevent haemolysis and clot formation. Samples were analyzed in haematoanalyzer (sysmex 5 parts) for Haemoglobin estimation. Total 7 volunteers were rejected from this study because of their Hb $\leq$ 10%, 2 volunteers were excluded because of the variant window in the HPLC graph. Then all the samples were analysed in HPLC method (BIORAD - D10) followed by BAA method (Nycocard reader II). The study was approved by the Institutional Ethical Committee.

HbA1c by HPLC principle: The samples were injected into the cation charged particles filled analytical column after dilution in the analyzer. The D-10 analyzer delivers different gradient buffers differ in ionic strength as per program into the analytical column, then the haemoglobins

are separated based on their ionic interactions with the column material. After separation of the haemoglobins then pass through the photometer, where the absorbance changes are measured at 415 nm. A chromatogram with values and retention times is generated after every sample analysis.

HbA1c by BAA principle: The kit contains a porous membrane fitted in a device, washing solution and a test tube prefilled with erythrocytes lysing reagent and blue colour conjugating reagent is blue boric acid. After adding the whole blood sample to the reagents test tube, RBCs are getting into lyse and all haemoglobins will be precipitated. Then Boric acid has affinity towards making bonds with cis – diol configures of glycated haemoglobin. When aliquot this reaction mixture to the test device, the precipitated hemoglobin, boric acid – bound and unbound forms, remains on top of the porous membrane. Any excess of blue colour boric acid is removed with the washing solution. The glycated haemoglobin (blue colour intensity) and total haemoglobin (red colour intensity) were evaluated with the reader, the ratio between these two, proportional to the HbA1c of sample.

19 (47.5%) normal subjects and 21 (52.5%) abnormal subjects with the lowest value 4.7% and 14.3% as the highest value were included in this study. Individuals with HbA1c > 6.5% by the HPLC method were considered as diabetes and those with $\leq$ 6.5% by HPLC method were considered as non – diabetes.[16]

### 2.3. Precision studies

Five abnormal HbA1c samples are mixed up and made a high pool. Established the mean $\pm$ SD as 11.28 $\pm$ 0.08% by running this pooled sample 5 times. Similarly low pool mean $\pm$ SD as 5.5 $\pm$ 0.12% from normal HbA1c samples. These are used to determine precision of assays. 5 times ran these two pools for within run study and between day study on subsequent days in the two methods of this study. Followed by the calculation of mean, standard deviation (SD) and coefficient variation (CV) from obtained values.

### 2.4. Statistical analysis

The continuous variables were summarized by mean and standard deviation (SD) and categorical variables by percentages (%). For correlation the variables are tested for normal distribution and Pearson or Spearman Rank Correlation coefficients are calculated for normally and non-normally distributed variables, respectively. Passing Bablok Regression analysis between HbA1c estimated by the two methods was done to measure the equality and to estimate the slope and intercept. Receiver Operating Characteristic (ROC) Curve was plotted for HbA1c by both the methods to identify the best cut-offs and Area Under Curve (AUC). The data was entered in Microsoft Excel and statistical analysis was done by using R version 3.6.2 (2019-12-12)[17] and the R packages R Commander (Rcmdr),[18] mcr[19] and pROC.[20]

## 3. Results

Finally, 40 members were enrolled in this study. The mean age $\pm$ SD was 52$\pm$17.16 years.

The mean HbA1C estimated by the two methods was similar and paired t- test found that the difference was statistically not significant (p= 0.2644) as shown in Table 1. The correlation between HbA1c estimated by the two methods was very high and positive with Pearson Correlation Coefficient (r) of 0.989, which was found statistically significant (p value < 0.05).

Then the correlation established between these two methods in each group. HbA1c values are distributed normally among diabetes mellitus type 2 patients in both Boric acid affinity and HPLC methods. Hence Pearson Correlation Coefficient ($\rho$) was used for Diabetes mellitus type 2 patients which was very high positive and statistically significant ($\rho$ = 0.97; p value < 0.05). But, HbA1c by both Boric acid affinity and HPLC methods is not normally distributed among non diabetics. Hence Spearman's Rank Correlation Coefficient (rho) is used for this group, which was moderately high positive and statistically significant (rho = 0.67; p value < 0.05). Correlation of HbA1c in Non DM in less compared to DM group. However, in both the groups there is statistically significant positive correlation between these two methods.

Considering HPLC method as the gold standard method, the study subjects were classified into two groups based on estimated HbA1C i.e. Group 1 (Diabetes Mellitus), Group 2 (Non diabetes mellitus), by the two methods as summarized in Table 2. The proportions of patients categorized into groups 1 and 2, respectively, were 52.5% and 47.5% by HPLC method and 62.5% and 37.5% by the BAA method. As shown, 4 (10%) of 40 subjects were overly classified as diabetes and 4 (10%) of 40 participants were not included under non diabetes by the BAA than HPLC method. Among 21 diabetics by HPLC method, 1 was considered as non diabetes and out of 19 non diabetes by HPLC method, 5 were considered as diabetes by BAA (Nycocard reader II). Taken together, the total proportion of patients who were misclassified by the BAA method was 15% (6/40).

The diagnostic accuracy of the BAA method was thus estimated as follows.

Sensitivity: 95.23%

Specificity: 73.68%

Positive predictive value: 80%

Negative predictive value: 93.33%.

Correlation of HbA1c in non diabetes (group 2) is less compared to diabetes (group1) between these two methods.

We further grouped the participants based on cut off age 40 years, percentage of participants are shown in Table 3.

As HbA1C was normally distributed in these two groups, Pearson Correlation Coefficient was used for both BAA and

**Table 1:** Mean and SD values of HbA1c in two different methods

| | HPLC (BIORAD - D10) | BAA(NYCOCARD) | p- value |
|---|---|---|---|
| Mean | 7.57 | 7.67 | 0.2644 |
| SD | 2.64 | 2.48 | |

**Table 2:** Classification of subjects into Diabetes and Non – Diabetes groups by HPLC and BAA methods

| BAA | | HPLC | | |
|---|---|---|---|---|
| | | Group 1 (Diabetes) | Group 2 (Non-Diabetes) | Total |
| | Group 1(Diabetes) | 20 | 5 | 25 (62.5%) |
| | Group 2 (Non Diabetes) | 1 | 14 | 15 (37.5%) |
| | Total | 21 (52.5%) | 19 (47.5%) | 40 |

**Table 3:** Participants were classified into two groups as $\leq$ 40 years and > 40 years

| | $\leq$ 40 years | > 40 years |
|---|---|---|
| Number | 11 | 29 |
| Percentage | 27.5% | 72.5% |

HPLC methods, among both the age groups. In $\leq$ 40 yrs age group, positive correlations have been established between these two methods ($\rho$ = 0. 97) and it was statistically significant (p < 0.05). In > 40 yrs age group, positive correlation has been established between two methods ($\rho$ = 0. 98) and it was statistically significant (p < 0.05).

As can be seen from Table 4, out of 40 members 19 females (47.5%) and 21 males (52.5%) were enrolled in this study. Among both males there was very high positive correlation between the two methods with Pearson Correlation Co-efficient $\rho$ =0.96, which was statistically significant (p < 0.05). Among females there is almost perfect positive correlation, $\rho$ =0.99, and it was statistically significant (p< 0.05).

**Table 4:** Gender wise distribution of participants

| | Females | Males |
|---|---|---|
| Number | 19 | 21 |
| Percentage | 47.5% | 52.5% |

In our study, the analytical performance of the boric acid affinity method for HbA1c was evaluated compared with HPLC using Passing Bablok Regression Analysis. Figure 1 shows that there was astatistically significant highly positive correlation between the values measured by the HPLC and the BAA methods, with 95% CI forthe y-intercept close to zero and that of slope inclusive of one.

The precision study extracted within-run CVs lower than 2.6% and between-run CVs lower than 2.8% as shown in Table 5.

## 4. Discussion

This study compared the boric acid affinity method with the HPLC method. BAA has a very high positive correlation (r =0.98) with HPLC and is specific for HbA1c estimation.

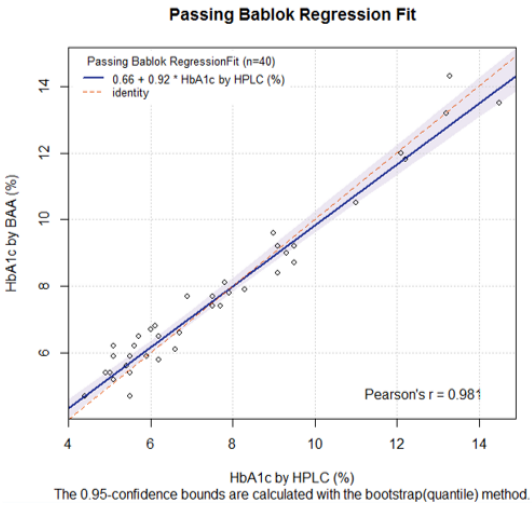


**Fig. 1:** Passing Bablokregression analysis yielded a coefficient of determination$r^2$ = 0.96 (p < 0.001). Values of the y-intercept and slope were 0.66(95% confidence interval [CI], 0.06-1.14) and 0.92 (95% CI, 0.86 - 1), respectively. Hb= haemoglobin

Previous studies with similar targets are summarized in Table 6. All these studies did compare the alternative methods of HbA1c with the HPLC method. All these have proven the significant difference between mean± SD of variant methods of HbA1c. Malekmahmoodi et al.[21] reported a statistically significant correlation coefficient of 0.633 for boric acid affinity with HPLC techniques. As well as Razi et al[22] study also found statistically significant correlation (r = 0.945, p= < 0.05). But in this study the mean ± SD value of HPLC method HbA1c (7.57±2.64) was almost same compared to BAA (7.67±2.48, p = 0.2644)

**Table 5:** Precision study. Within day and between day coefficient variation (CV%) of HbA1c levels measured by the two comparative methods using the whole blood pool at high level (11.28±0.08%) and low (5.5 ± 0.12%).

| | High Pool | | Low Pool | |
|---|---|---|---|---|
| | **Within day CV%** | **Between day CV%** | **Within day CV%** | **Between day CV%** |
| HPLC | 0.6 | 0.6 | 1.3 | 2.0 |
| BAA | 1.8 | 1.6 | 2.6 | 2.8 |

**Table 6:** Comparison of study results mean ± SD with other study results

| **Study name** | **Mean± SD** | | **Other Method name** | **p- value** |
|---|---|---|---|---|
| | **HPLC** | **Other methods** | | |
| Malekmahmoodi et al[21] | 8.3% ± 2.4% | 7.1% ±1.9% | BAA | < 0.05 |
| Razi et al[22] | 7.59% ±1.43% | 6.87%±1.17% | BAA | <0.05 |
| Rukmini MS et al[23] | 7.44%± 2.02% | 6.53%± 1.58% | IEC | 0.01 |
| Hector Garcia Alcala et al[24] | 8.01%± 3.01% | 7.63% ± 2.17% | IT | <0.05 |
| Present study | 7.57%± 2.64% | 7.67% ±2.48% | BAA | 0.2644 |

and our study results (p = 0.2644) not obeying the previous studies output. It indicates similar values obtained in these two comparative methods but this study proved better correlation than the previous studies (r= 0.989 and p < 0.05). Malekmahmoodi et al.[21] study, divided the subjects into normal and abnormal based on HbA1c value 6.5% and by considering HPLC method as a reference, they established the sensitivity and specificity of the boric acid affinity method as 100% and 58.6% subsequently. Positive predictive value (PPV) and negative predictive value (NPV) of the baronet affinity method in comparison with HPLC method was 63.6% and 100%, respectively. Another similar study, demonstrated sensitivity and specificity of boric acid affinity as 82.9% and 100% respectively at 6.5% HbA1c level.[22]

This study observed that the BAA method has sensitivity and specificity as 95.23% and 73.68% respectively at HbA1c 6.5%. Medium level performance response was evaluated, compared to previous studies[21,22] results. All these studies summarized in Table 7 . As per Razi et al[22] study, BAA (Nycocard reader II) was less sensitive than HPLC at cut point value HbA1c 6.5%, because BAA method misclassified 17.1% of patients as the control group. But this study revealed the better performance of BAA, because only 4.76% of subjects were misclassified as non diabetes at a similar cut point. Total precision in terms of CV% was less than 2% and 2.8% for HPLC and BAA respectively. The positive predictive value for the evaluated assay is 80% which means the results more than diagnostic level are most reliable.

Rukmini MS et al[23] established positive and strong correlation between HPLC and IEC in 40 -60 years more than 60 years groups respectively. In this study based on age, participants were divided into two groups; group I: below or equal 40 years and group II: more 40 years. Among both the age groups the correlation between BAA and HPLC is statistically very high. Rukmini MS et al[23] drew the result that men showed significant correlation between HPLC and IEC than women. This study found no significant difference in the HbA1c values of comparative methods in gender based groups (men p=1 as shown in Figure 2 and women p=0.379 as shown in Figure 3. Hector Garcia Alcala et al.[24] established the total proportion of patients who were misclassified by the immune-turbidimetric inhibition method was 17.4% (31/178) in comparison with HPLC. This study has drawn a little better result, that 15% (6/40) by BAA method. It is indicating that BAA is a better method than Immuno-turbidimetric inhibition method. Most of the previous studies tried to establish, the methods of HbA1c other than HPLC, are better alternatives without performing the precision exercises and vice versa.[25] In this study, we tried to include the precision exercise and compare. Genc S et al.[25] studied the precision performance of boric acid affinity, HPLC methods and compared with capillary zone electrophoresis (CE). At high pool (mean value 11.5 ± 0.06%) as well as at low pool (5.2 ± 0.05%) the boric acid affinity method had shown very good performance than the HPLC method. But in our study we got a reverse response that is, the HPLC precision is better than the boric acid affinity at both pools. However boric acid affinity method is not a good performer in high as well as low pool, but these findings are within target goals of IFCC (< 2.8%).[26]

The most advantage of boronate affinity method than the other methods, is able to produce the HbA1c values, those are correlating the glycemic status in diabetes with underlying unknown hemoglobinopathies.[27,28]

## 5. Conclusion

Most of the previous studies showed that the automated HPLC method is a standardized method which measures accurate HbA1c levels within a short time. But considering

**Table 7:** Different studies of comparison between HPLC and BAA and their result outputs

| Study name | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| Malekmahmoodi et al[21] | 100% | 58.6% | 63.6% | 100% |
| Razi et al[22] | 82.9% | 100% | 100% | 64.9% |
| Present study | 95.23% | 73.68% | 80% | 93.33% |

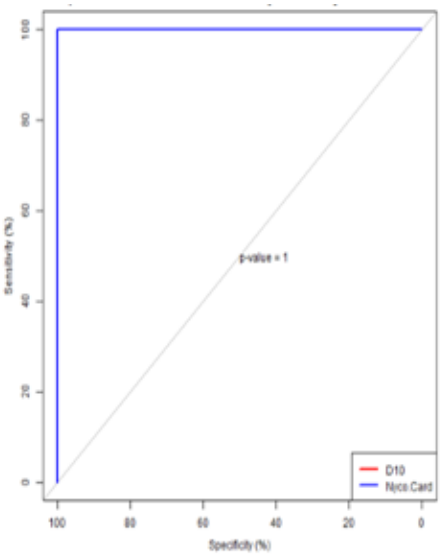


**Fig. 2:** ROC for males

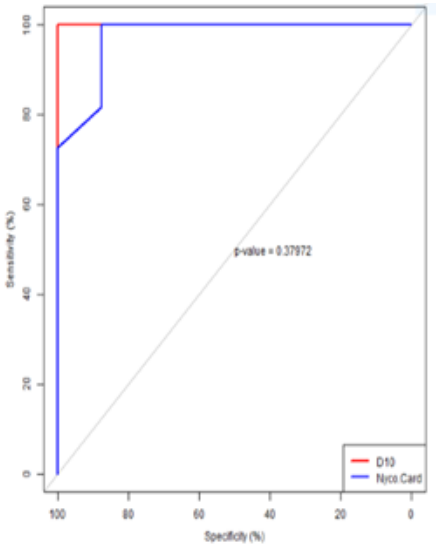


**Fig. 3:** ROC for females

the limitations of HPLC in its process and to overcome the practical problems associated with HPLC, we tried the boric acid affinity method as an alternative. Comparatively boric acid affinity method is cheap, affordable, easy to set up at minimal facilities available labs and easy to perform. By comparing the analytical performance between these methods, this study shows that BAA has a strong correlation and agreement with the HPLC and hence can be used as a substitute.

## 6. Limitations of the study

It is a single centre study, so this study attained a small sample size. This sample size is sufficient for method verification procedure in the laboratory.[29] For subgroups analysis such as gender and age wise comparisons, a higher sample size would have a better result. Also we compared only one method, i.e., BAA with a standardized HPLC method and not suggested other than BAA method. This study has not considered the interfering substances such as triglycerides, urea, bilirubin at high levels, Vitamin C and E, which act on glycation of haemoglobin.

## 7. Sources of Funding

Nil.

## 8. Conflict of Interest

None.

## 9. Acknowledgements

The author thanks S.V.S Medical College, Telangana for their essential support.

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**Cite this article:** Chikine RK, Bhumi MA. Method verification procedure of boric acid affinity method against high performance of liquid chromatography for glycated haemoglobin. *Int J Clin Biochem Res* 2020;7(3):367-373.