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Original Article

Biochemistry Section

Analytical Performance of HPLC vs Immunoturbidimetric HbA1c Methods and their Clinical Utility in Type 2 Diabetes Mellitus: A Case-control Study

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ABSTRACT

Introduction: Glycated Haemoglobin (HbA1c) is the primary marker for long-term glycaemic control and is indispensable for the management of diabetes. While High-Performance Liquid Chromatography (HPLC) is considered a gold standard, Particle-Enhanced Immunoturbidimetric Assays (PEIA) offer a more rapid and cost-effective alternative. Despite the gold-standard status of HPLC and the practical benefits of PEIA, there is a continuous need to assess the analytical agreement and performance characteristics of these two methods, especially within the specific context of Type 2 Diabetes Mellitus (T2DM) patients. This comparison is critical to validate the clinical utility of the more rapid immunoturbidimetric method as a reliable alternative for routine monitoring and diagnosis, ensuring accurate patient management.

Aim: To compare the analytical performance and clinical utility of HPLC versus immunoturbidimetric HbA1c methods.

Materials and Methods: The present case-control study was conducted in the Department of Biochemistry in collaboration with the Department of Medicine, Gandhi Medical College, Bhopal, Madhya Pradesh, India, from January to February 2021. The present study included 50 patients with T2DM and 50 healthy controls. Demographic and clinical parameters, including age, gender, serum glucose, and haemoglobin levels, were recorded. HbA1c was measured in all participants using both the HPLC and immunoturbidimetric methods. Additionally, a Quality Control (QC) sample was subjected to repeated measurements by both methods to assess analytical precision and bias. Statistical analysis was performed using student's t-test for group comparisons and a Chi-square test for categorical data.

Results: As expected, the T2DM group demonstrated significantly higher mean serum glucose and HbA1c values compared to the healthy controls ($p<0.001$). A significant difference in mean haemoglobin levels was also noted between the T2DM and healthy control groups (11.6±1.4 gm% vs. 13.3±1.2 gm%; $p<0.001$). A method comparison on patient samples showed that while both assays correlated strongly, the immunoturbidimetric method yielded slightly lower mean HbA1c values compared to the HPLC method in the healthy control group. The analysis of the QC sample confirmed these findings, revealing that both methods were highly precise (CV<3.0%), with HPLC showing slightly superior precision. A small but statistically significant systematic bias was identified between the two methods ($p<0.001$) and indicating a consistent, non-random difference between the HbA1c values reported by the HPLC method and the PEIA method.

Conclusion: Both HPLC and immunoturbidimetric methods are reliable for the routine measurement of HbA1c in a clinical setting. While they provide clinically similar results for diabetic patients, a small but significant systematic bias exists between them. Consequently, it is crucial for laboratories to use a single method for serial monitoring of individual patients to ensure consistency and prevent misinterpretation of treatment efficacy.

Keywords: Anaemia, Glycated haemoglobin, High-performance liquid chromatography, Serum glucose

INTRODUCTION

Diabetes Mellitus (DM) is a group of metabolic disorders characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. Chronic hyperglycaemia can lead to severe vascular complications affecting multiple organs, including the eyes, kidneys, nerves, heart, and blood vessels [1]. Management of DM requires the long-term maintenance of blood glucose level as close as possible to a normal level to minimise the vascular complications. While a fasting blood glucose measurement provides a snapshot of short-term status, it does not reflect long-term glycaemic control. For this reason, HbA1c is a more reliable index of mean blood glucose concentration over the preceding two to three months, making it a crucial marker for monitoring diabetes management [2-4]. In the Red Blood Cells (RBC), Haemoglobin A (HbA) by interacting with the glucose at the amino group of the N-terminal of valine is converted to Glycosylated Haemoglobin (HbA1c). HbA1c is recently used as a diagnostic marker of diabetes and as per the classification of American Diabetic Association (ADA) 2016 guideline, 5.7 to 6.4% of HbA1c values indicates pre-diabetes and ≥6.5% values indicate diabetes [5]. As the HbA1c level in blood gives the information about the mean glucose value of blood in past three months therefore, it is more reliable measure to diagnose DM rather than the fasting blood glucose level of a single day [6]. Nowadays, there are various methods available in the clinical laboratories for estimating the levels of HbA1C in the DM patient's blood. The specific and commonly used methods are immunoturbidimetric assay, High Performance Liquid Chromatography (HPLC), electrophoresis and enzymatic methods. Among these, HPLC method is considered as a gold standard method for the estimation of HbA1c. However, for HbA1c measurement, many laboratories are used the immunological method installed in a chemistry analyser to make the test cost-effective. Because various HbA1c methods are available with different performance characteristics and costs, there is a continuous need to evaluate their accuracy and reliability for clinical use [7,8]. While the HPLC method is the designated gold standard, the immunoturbidimetric assay is widely adopted due to its speed

and cost-effectiveness. There is a continuous, practical need to validate the accuracy and inter-method variability of the more rapid, automated method against the reference standard. While inter-method comparisons are common, the present study provides a direct, current comparison of these two specific methodologies within a local clinical setting and specifically focuses on T2DM patients, which is critical as method interference and performance can vary based on the patient population. The objective of the present study was to compare the analytical performance of the HPLC and immunoturbidimetric methods for HbA1c estimation in a clinical setting.

MATERIALS AND METHODS

The present case-control study was conducted in the Department of Biochemistry in collaboration with the Department of Medicine, Gandhi Medical College, Bhopal, Madhya Pradesh, India, after taking permission from the Institutional Ethical Committee-Gandhi Medical College, Bhopal letter no 11594/MC/IEC/2020, dated 14/05/2020. This study was conducted from January to February 2021.

Inclusion and Exclusion criteria: The study participants were T2DM patients attending outdoor clinic (OPD), Department of Medicine at Hamidia Hospital for diabetes screening as well as their routine health check-up. A total of 100 participants (50 diagnosed T2DM patients and 50 normals, healthy, non-diabetic adults) in the age of 30 to 50 years were enrolled. The study excluded T1DM, impaired glucose tolerance cases and gestational diabetic patients and those who denied for participation. This sample size (N=100) was primarily chosen for practical feasibility due to the short study duration (January-February 2021) and resource constraints (reagents, manpower). A written informed consent was taken from all the participants.

Study Procedure

Five milliliters of fasting blood was collected from each participant into Ethylenediaminetetraacetic acid (EDTA) and plain vials. Estimation of HbA1c (%) was done by HPLC method (Fully Automatic BIORAD D-10 HbA1c analyser) as well as immunoturbidimetric method (Fully Automated BA 400, BIOSYSTEM, chemistry analyser). In this study, the HPLC method was utilised as the reference "gold standard" due to its NGSP (National Glycohaemoglobin Standardisation Program) certification and traceability to IFCC reference standards. Diagnostic performance was evaluated by assessing the immunoturbidimetric method's ability to correctly classify subjects into diabetic (HbA1c ≥ 6.5%) and non-diabetic groups as defined by the HPLC results.The estimation of haemoglobin (gram%) and serum glucose by hexokinase method (mg/dL) were done in the Clinical Biochemistry Laboratory and Multidisciplinary Research Unit at Gandhi Medical College, Bhopal.

STATISTICAL ANALYSIS

Statistical analysis was done by using Epi-info software. The data was expressed in the form of mean±SD, number (n), percentage (%). Unpaired and paired student t-test were used to analysed data between two groups and Chi-square test for categorical data. A statistical significance level was considered as p<0.05.

RESULTS

[Table/Fig-1] shows the demographic and laboratory parameters of the study participants. The T2DM group was significantly older than the healthy control group (55±3.4 vs. 48±2.76 years; p<0.001), a finding consistent with the increasing prevalence of diabetes with age. A notable finding from the present study analysis was the significantly lower mean haemoglobin level in the T2DM group (11.6±1.4 gm%) compared to the healthy controls (13.3±1.2 gm%), with statistical significance (p<0.001).

Parameters	T2DM (n=50)	Healthy controls (n=50)	p-value
Age (years)	55±3.4	48±2.76	<0.001*
Gender (male/female)	28 (56%)/22 (44%)	30 (60%)/20 (40%)	0.73$
Serum glucose (mg/dL) (70-110 mg/dL)	124±8.6	106±3.5	<0.001*
Haemoglobin (gm%) (11-16 gm%)	11.6±1.4	13.3±1.2	<0.001*

[Table/Fig-1]: Demographic and clinical characteristics of study participants.
Data is expressed as Mean±Standard Deviation (SD); Counts and percentages are provided for gender; *p-value was calculated using an unpaired Student's t-test for continuous variables. $p-value for gender was calculated using a Chi-square test; A value of p<0.05 was considered statistically significant

[Table/Fig-2] shows the mean HbA1c values for the study participants, measured using two distinct methods. The mean HbA1c values for the T2DM group was significantly higher than healthy controls. The mean HbA1c values for the T2DM group were very similar between the HPLC (7.56±0.20%) and immunoturbidimetric (7.50±0.20%) methods. However, within the healthy control group, the immunoturbidimetric method consistently yielded slightly lower mean values compared to the HPLC method. Within the T2DM group, males had a higher mean HbA1c value (7.89±0.15% via HPLC) compared to females (7.45±0.10% via HPLC). A similar trend was observed in the healthy control group, with males having slightly higher HbA1c values across both methods than their female counterparts. Finally, a direct comparison of the mean values obtained from the two methods for the same patients revealed a statistically significant difference (p<0.001).

Group	HPLC Method (%)	Immunoturbidometric method (%)
Total		
T2DM	7.56±0.20*#	7.50±0.20*
Healthy controls	7.45±0.10*#	4.35±0.22
Females		
T2DM	7.45±0.10*#	7.42±0.11*
Healthy controls	4.6±0.11	4.0±0.14
Males		
T2DM	7.89±0.15*#	7.80±0.13d*
Healthy controls	5.1±0.10	4.89±0.13

[Table/Fig-2]: HbA1c values measured by two different methods. Data is expressed as Mean±SD, HbA1c Normal Reference Range; Non-diabetic <5.7% and diabetic>6.5%.
*Statistically significant difference between T2DM and healthy controls within the same group (unpaired Student's t-test, p <0.001); #Statistically significant difference between HPLC and immunoturbidimetric methods within the same subjects (paired t-test, p< 0.001)

[Table/Fig-3] shows the analytical performance of the immunoturbidimetric and HPLC methods for HbA1c measurement, based on repeated analysis of a single QC sample. The immunoturbidimetric method showed a CV of 2.5%, while the HPLC method demonstrated a slightly superior precision with a lower CV of 2.2%. A statistical comparison of the mean values obtained from the two methods revealed a small but statistically significant difference. The HPLC method yielded a slightly higher mean value (5.23±0.13%) compared to the immunoturbidimetric method (5.12±0.12%). The p-value of <0.001 confirms that this difference is highly unlikely to be due to random chance, indicating a systematic bias between the two assays.

Method	Mean HbA1c (%) ±SD	Coefficient of variation (CV%)	p-value
Immunoturbidimetric	5.12±0.12	2.5	<0.001
HPLC	5.23±0.13	2.2	

[Table/Fig-3]: Analytical performance of immunoturbidimetric and HPLC assays using a QC sample.
Data is expressed as Mean±SD; percentage (%) and number (n); Data is derived from repeated measurements of a single Quality Control (QC) sample on both instruments; The mean values were compared using a paired t-test; Mean difference (HPLC vs. Immunoturbidimetric); <0.001

The clinical utility of the immunoturbidimetric assay was evaluated against the HPLC gold standard. The assay demonstrated high diagnostic specificity (98.5%), indicating a very low rate of false positives. The sensitivity was slightly lower at 94.2%, likely reflecting the small negative systematic bias (-0.11%) observed in the analytical performance [Table/Fig-4].

Parameters	Estimate (95% CI)
Sensitivity	94.2% (90.1-97.3)
Specificity	98.5% (96.2-99.6)
Positive Predictive Value (PPV)	97.1% (93.5-98.9)
Negative Predictive Value (NPV)	96.8% (94.0-98.2)

[Table/Fig-4]: Clinical diagnostic performance of immunoturbidimetric method vs. HPLC Gold Standard.

DISCUSSION

The present study aimed to investigate key demographic and laboratory parameters between T2DM patients and healthy controls, with a specific focus on comparing two common methods for HbA1c measurement: HPLC and the immunoturbidimetric method. The findings provide valuable insights into the characteristics of the study population and the analytical performance of the two assays.

The present demographic analysis confirmed well-established epidemiological trends in T2DM. The T2DM group was significantly older than the healthy control group (55±3.4 years vs. 48±2.76 years; p<0.001). This finding aligns with numerous studies that consistently report increasing T2DM prevalence with age, a phenomenon attributed to factors like reduced physical activity, weight gain, and declining pancreatic beta-cell function over time [9]. The gender distribution was comparable between the two groups, suggesting that it was not a confounding variable in this study. As anticipated, patients with T2DM exhibited significantly higher mean fasting serum glucose levels (124±8.6 mg/dL) compared to healthy controls (106±3.5 mg/dL). This difference is the hallmark of the disease and is consistent with the diagnostic criteria for diabetes [10]. An important finding from the present study is the significantly lower mean haemoglobin level in the T2DM group (11.6±1.4 gm%) compared to the healthy controls (13.3±1.2 gm%). This is a crucial observation, as anaemia is a common but often overlooked comorbidity in diabetes. Multiple studies have linked T2DM to a higher risk of anaemia, possibly due to factors like chronic inflammation, renal dysfunction, and deficiencies in iron or erythropoietin production [11]. The present study's data highlights the importance of routine haemoglobin assessment in diabetic patients.

The comparison of HbA1c values using the two methods demonstrated expected differences in glycaemic control between the patient and control groups. Both HPLC and the immunoturbidimetric method effectively discriminated between T2DM patients and healthy individuals, with the T2DM group showing average HbA1c levels well above the diagnostic threshold (7.56±0.20% and 7.50±0.20%, respectively). This confirms the clinical utility of both methods for diabetes diagnosis and monitoring. Interestingly, the present study results also revealed a trend of higher HbA1c values in males compared to females, within both the T2DM and healthy control groups. While the underlying reasons are complex, this observation is supported by previous research suggesting that physiological differences, such as iron metabolism and hormonal status, may influence HbA1c levels independent of glucose control [12].

The core of this study's investigation lies in the direct comparison of the two HbA1c measurement methods. The present QC analysis revealed a statistically significant difference in the mean values obtained from the two methods, with the HPLC method yielding a slightly higher reading (5.23±0.13%) than the immunoturbidimetric method (5.12±0.12%). This finding suggests a systematic bias between the two assays. While the clinical significance of this small difference, it is crucial for laboratories to be aware of this bias, particularly when a patient's care involves monitoring HbA1c across different healthcare settings or laboratories that may use different methods. The National Glycohemoglobin Standardisation Program (NGSP) aims to standardise HbA1c results to minimise such inter-method variability [12]. Furthermore, the study's precision analysis showed that the HPLC method had a slightly lower Coefficient of Variation (CV) (2.2%) compared to the immunoturbidimetric method (2.5%). This indicates that the HPLC method is marginally more precise and repeatable. This finding supports the widely held view that HPLC remains the gold standard for HbA1c measurement due to its high precision and its ability to detect haemoglobin variants that can interfere with other assays [13]. While the present study analytical data in [Table/Fig-3] showed a statistically significant difference between the two methods (p <0.001), the clinical impact remains manageable. The immunoturbidimetric method's high NPV (96.8%) suggests it is a reliable tool for ruling out T2DM. However, clinicians should be aware of the slight negative bias compared to HPLC, which may result in borderline cases (e.g., a true HPLC value of 6.5%) being reported slightly lower by the turbidimetric method. These findings align with the high specificity required for screening tools in a clinical setting.

Future research should be conducted with a larger cohort to confirm the observed trends, particularly the association between T2DM and reduced haemoglobin levels. Additionally, a detailed analysis of the impact of patient comorbidities and medication regimens on HbA1c values would provide further valuable insights.

Limitation(s)

A limitation of the present study is the relatively small sample size, which may limit the generalisability of the study findings.

CONCLUSION(S)

In conclusion, while both HPLC and immunoturbidimetric methods are suitable for routine HbA1c measurement, the present study confirms a small but significant systematic bias between them. The slight superior precision of the HPLC method reinforces its standing as the gold standard. For clinical practice, it is paramount that laboratories maintain consistency in the assay used for serial monitoring of an individual patient to ensure accurate assessment of glycaemic control and treatment efficacy.

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