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COMPARATIVE EVALUATION OF HPLC, ENZYMATIC, AND IMMUNOTURBIDIMETRIC METHODS FOR HbA1c MEASUREMENT IN PREDIALYSIS CHRONIC KIDNEY DISEASE

Endocrinology

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ABSTRACT

Background: HbA1c is a key marker of long-term glycaemic control; however, its interpretation in patients with chronic kidney disease (CKD) is challenging due to potential analytical interferences such as carbamylated haemoglobin (CarbHb). High-performance liquid chromatography (HPLC) is the reference method for HbA1c estimation, but its cost and technical requirements limit widespread use. This study aimed to compare HPLC with enzymatic and latex agglutination inhibition immunoturbidimetric methods for HbA1c measurement in predialysis CKD patients. **Methods:** This cross-sectional study included 200 participants categorized into healthy controls (n = 40), non-diabetic predialysis CKD (n = 80), and diabetic predialysis CKD (n = 80). HbA1c was measured using HPLC, enzymatic, and immunoturbidimetric methods. Method comparison was performed using correlation analysis, Bland–Altman plots, and Passing–Bablok regression. Analytical performance was evaluated as per CLSI guidelines. **Results:** HbA1c levels were significantly higher in diabetic predialysis CKD patients compared to controls and non-diabetic CKD patients across all three methods ($p < 0.001$), while no difference was observed between controls and non-diabetic CKD patients. HbA1c values obtained using enzymatic and immunoturbidimetric methods showed strong correlation with HPLC ($r > 0.98$). Bland–Altman and Passing–Bablok analyses demonstrated good agreement among methods, with minimal mean bias. Although statistically significant differences were observed between immunoturbidimetric and HPLC measurements, these were not clinically relevant and did not affect patient classification. Estimated CarbHb levels showed no significant correlation with HbA1c by any method. All methods met NGSP quality goals, with coefficients of variation below 2%. **Conclusion:** Enzymatic and immunoturbidimetric methods demonstrate reliable analytical performance and strong agreement with HPLC, supporting their use as cost-effective alternatives for HbA1c measurement in predialysis CKD patients, particularly in resource-limited settings.

KEYWORDS

HbA1c, Chronic kidney disease, High-performance liquid chromatography

INTRODUCTION

In healthy adults, haemoglobin (Hb) predominantly consists of HbA ($\alpha_2\beta_2$), HbA_2 ($\alpha_2\delta_2$), and foetal haemoglobin HbF ($\alpha_2\gamma_2$), accounting for approximately 97%, 2.5%, and 0.5% of total haemoglobin, respectively. HbA1c is generated by a non-enzymatic glycation reaction between glucose and the N-terminal valine of the β-chain of HbA [1]. The International Diabetes Federation and the American College of Endocrinology recommend maintaining HbA1c values below 6.5%, whereas the American Diabetes Association considers levels below 7% as indicative of adequate glycaemic control. HbA1c values exceeding 8% reflect poor glycaemic regulation [2]. An optimal method for HbA1c estimation should demonstrate high analytical precision and accuracy, with minimal susceptibility to interference [3]. Several clinical conditions, including haemolytic anaemia, haemoglobinopathies, iron deficiency anaemia, and uraemia resulting from haemoglobin carbamylation, may produce misleading HbA1c results [4–13]. Interpretation of HbA1c is further complicated in diabetic patients with renal failure. A major source of analytical interference in this setting is carbamylated haemoglobin (CarbHb), [14] which is formed by the non-enzymatic attachment of carbamoyl groups ($-CONH_2$) to amino acid residues or protein functional groups.

High-Performance Liquid Chromatography (HPLC), endorsed by the National Glycohemoglobin Standardization Program (NGSP) and validated in the Diabetes Control and Complications Trial (DCCT), is considered the reference method for HbA1c estimation [15]. This technique separates haemoglobin fractions based on differences in ionic charge. Despite its accuracy, HPLC is resource-intensive, requiring sophisticated instrumentation, skilled personnel, and increased processing time and cost. Consequently, there is a need for more economical and practical alternatives. Comparative data evaluating enzymatic and latex agglutination inhibition methods in patients with uraemia remain limited [16]. In this context, the present study was undertaken to compare these three methodologies in individuals with chronic kidney disease (CKD) and to identify a reliable and appropriate method for HbA1c estimation in this population.

MATERIAL AND METHODS

This cross-sectional study was carried out in the Department of Biochemistry of a tertiary care teaching hospital in South India over a three-year period from January 2022 to December 2024. Consecutive patients attending the outpatient services of the Department of Nephrology who met the inclusion criteria were enrolled. The study was initiated after obtaining approval from the Institutional Ethics Committee (IEC No: 1122/dated 20.11.21).

A total of 200 participants were included in the study, based on a population reliability value of 0.97 derived from a previous study [17]. Although the Clinical & Laboratory Standards Institute (CLSI) guidelines recommend a minimum sample size of 40 subjects per group for method comparison studies, [18] a larger sample was chosen to enhance statistical robustness. The study population was categorized into three groups: Group 1 comprised healthy controls (n = 40), Group 2 included non-diabetic CKD patients (n = 80), and Group 3 consisted of diabetic CKD patients (n = 80) with type 2 diabetes mellitus as the underlying aetiology. Both diabetic and non-diabetic CKD groups were further stratified according to CKD stages: stage 1 (GFR > 90 mL/min/1.73 m^2), stage 2 (GFR 60–89 mL/min/1.73 m^2), stage 3a (GFR 45–59 mL/min/1.73 m^2), stage 3b (GFR 30–44 mL/min/1.73 m^2), stage 4 (GFR 15–29 mL/min/1.73 m^2), and stage 5 (GFR < 15 mL/min/1.73 m^2), excluding patients on dialysis [19].

The biochemical parameters assessed included plasma glucose, serum urea, serum creatinine, and HbA1c. HbA1c estimation was performed using Latex Agglutination Inhibition Assay and Enzymatic Assay on a Beckman AU480 autoanalyzer, while HbA1c by ion-exchange high-performance liquid chromatography (HPLC) was carried out using a dedicated HPLC system. Complete haemogram analysis was performed using a Mindray hematology analyzer. Method evaluation was conducted in accordance with established standard guidelines [20].

HbA1c estimation for all samples was performed on the same day using the three methods-HPLC, Latex Agglutination Inhibition Assay,

and Enzymatic Assay-to avoid analytical variability. Agreement of the enzymatic and latex agglutination inhibition methods with the reference HPLC method was evaluated using appropriate statistical techniques. Quality goals for method comparison were defined based on previously published criteria [21].

Statistical Analysis

Continuous variables are presented as mean ± standard deviation (SD). Baseline characteristics among the study groups were compared using one-way analysis of variance (ANOVA), followed by post-hoc multiple comparison tests. Paired comparisons between methods (HPLC vs enzymatic assay, HPLC vs latex agglutination inhibition assay, and enzymatic assay vs latex agglutination inhibition assay) were performed using Wilcoxon's signed-rank test. Correlations between methods were assessed using Spearman's rank correlation analysis. Agreement between methods was further evaluated using Bland–Altman plots. Passing–Bablok regression analysis was employed to derive regression equations along with 95% confidence intervals (CI) for the intercept and slope.

All statistical analyses were performed using MedCalc statistical software (Version 12.1, Ostend, Belgium) and Microsoft Excel spreadsheets (Microsoft, Redmond, WA, USA). A p-value of less than 0.05 was considered statistically significant.

RESULTS

Demographic details were recorded for all participants, and biochemical parameters including fasting plasma glucose, serum urea, serum creatinine, and haemoglobin were measured. HbA1c estimation was performed using three different methodologies: high-performance liquid chromatography (HPLC), enzymatic assay, and immunoturbidimetric method. Comparison of biochemical parameters among the three study groups-healthy controls, non-diabetic CKD patients, and diabetic CKD patients-was carried out using one-way analysis of variance (ANOVA), followed by post hoc analysis (Table 1).

Table-1: Comparison Of Baseline Clinical And Biochemical Characteristics Of Controls Vs Patients Followed By Post HOC Analysis.

Baseline parameters	Controls (n=40)	NDM predialysis CKD (n=80)	DM predialysis CKD (n=80)	ANOVA p-value	Control group vs NDM predialysis CKD group	Control group vs DM predialysis CKD group	NDM predialysis CKD vs DM predialysis CKD groups
Age (years)	43.28±1.93	52.24±1.96	51.79 ± 15.49	0.008	0.011*	0.017*	1.000
Serum Creatinine (mg/dL)	0.97±0.06	3.52±0.36	3.82±0.38	<0.001	<0.001*	<0.001*	1.000
S.Urea (mg/dL)	27.38±1.37	89.85±7.02	96.46±69.79	<0.001	<0.001*	<0.001*	1.000
FPG (mg/dL)	92.15±1.49	94.13±1.06	187.09±4.67	<0.001	1.000†	<0.001*	<0.001*
Hb (gm %)	13.96±0.24	11.92±0.25	12.29±0.20	<0.001	<0.001*	<0.001*	0.669†
HbA1c (HPLC) (%)	4.93±0.063	4.97±0.05	8.10±0.14	<0.001	1.000	<0.001*	<0.001*
HbA1c (Enzymatic) (%)	4.89±0.063	4.97±0.05	8.03±0.14	<0.001	1.000	<0.001*	<0.001*
HbA1c (Immunoturbidimetry) (%)	5.03±0.06	5.07±0.06	8.15±0.14	<0.001	1.000	<0.001*	<0.001*

Post hoc analysis demonstrated that both non-diabetic (NDM) and diabetic (DM) predialysis CKD patients were significantly older than the control group ($p = 0.011$ and $p = 0.017$, respectively), while no age difference was observed between the two CKD groups ($p = 1.000$). Serum urea and creatinine levels were markedly higher in both NDM and DM predialysis CKD patients compared to controls ($p < 0.001$ for both), with no significant difference between the two CKD groups ($p = 1.000$).

HbA1c levels were significantly elevated in DM predialysis CKD patients compared to controls and NDM CKD patients across all three analytical methods ($p < 0.001$). In contrast, HbA1c values did not differ between the control and NDM CKD groups ($p = 1.000$). Pairwise comparison of HbA1c measurements across all study participants ($n = 200$) showed no significant difference between the HPLC and enzymatic methods ($p = 0.396$; mean difference −0.0055, 95% CI −0.018 to 0.007). However, the immunoturbidimetric method yielded slightly higher HbA1c values compared with both HPLC (mean difference −0.1220, 95% CI −0.141 to −0.103; $p < 0.001$) and the enzymatic method (mean difference −0.1165, 95% CI −0.138 to −0.095; $p < 0.001$).

Strong positive correlations were observed between HbA1c values measured by HPLC and the enzymatic method ($r = 0.998$, $p < 0.001$), and between HPLC and the immunoturbidimetric method ($r = 0.989$, $p < 0.001$). Similarly, HbA1c values obtained using the enzymatic and immunoturbidimetric methods were highly correlated ($r = 0.987$, $p < 0.001$).

Direct measurement of carbamylated haemoglobin (CarbHb) was not performed. To assess its potential influence on HbA1c estimation, estimated CarbHb levels were calculated using the formula indicating that 0.063% of total haemoglobin is carbamylated for every 1 mmol/L increase in serum urea. [22] Correlation analysis revealed no significant association between estimated CarbHb levels and HbA1c values measured by any of the three methods ($p > 0.05$).

Agreement between methods was evaluated using Bland–Altman analysis (Figure 1). Comparison between HPLC and enzymatic methods (Figure 1A) showed excellent agreement with a mean bias of −0.01 (95% CI −0.18 to 0.17), with approximately 90% of values lying within ±1.96 SD. Similar agreement was observed between HPLC and the immunoturbidimetric method (Figure 1B), with a mean bias of −0.12 (95% CI −0.39 to 0.14), and between enzymatic and immunoturbidimetric methods (Figure 1C), with a mean bias of −0.12 (95% CI −0.42 to 0.19). In all comparisons, around 90% of values fell within acceptable limits of agreement. Notably, two non-diabetic patients with HbA1c values close to the diagnostic threshold (6.0–6.5%) were correctly classified by all three methods.

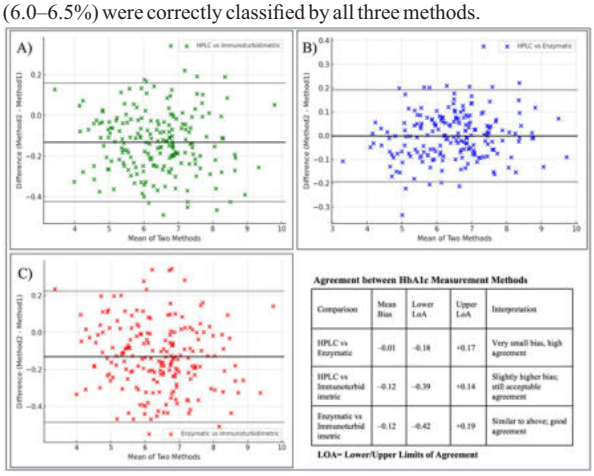

Figure-1: A) Bland–Altman difference plot comparing HbA1c results by HPLC and enzymatic methods, B) Shows the Bland–Altman difference plot comparing HbA1c results by HPLC and immunoturbidimetric methods, C) Bland–Altman difference plot comparing HbA1c results by enzymatic and immunoturbidimetric methods.

Regression Analysis

Passing–Bablok regression analysis is presented in Figure 2. Comparison between the HPLC and immunoturbidimetric methods (Figure 2A) yielded a regression intercept of 0.10, with a 95% confidence interval (CI) ranging from −0.045 to 0.10. The slope was 1.00, with a 95% CI of 1.00 to 1.03, indicating good agreement between the two methods, as the confidence intervals for the intercept and slope included the ideal values of 0 and 1, respectively.

Similarly, regression analysis comparing HPLC and enzymatic methods (Figure 2B) showed an intercept of 0.00 (95% CI: −0.092 to 0.00) and a slope of 1.00 (95% CI: 1.00 to 1.02). These findings further confirm excellent agreement between these two methods.

Comparison between the enzymatic and immunoturbidimetric methods (Figure 2C) demonstrated an intercept of 0.10 (95% CI: −0.10 to 0.10) and a slope of 1.00 (95% CI: 1.00 to 1.00). The inclusion of 0

within the intercept confidence interval and 1 within the slope confidence interval again indicates strong agreement between the methods.

All the three methods studied satisfy the quality goals as per NGSP guidelines i.e imprecision <2% and TE<6.7%. Commercial control material with target of 5.2% (normal range) and 9.2% (pathological range) yielded precision of <2.0% for all the three methods studied. Hence, all the methods can be used for patient care.

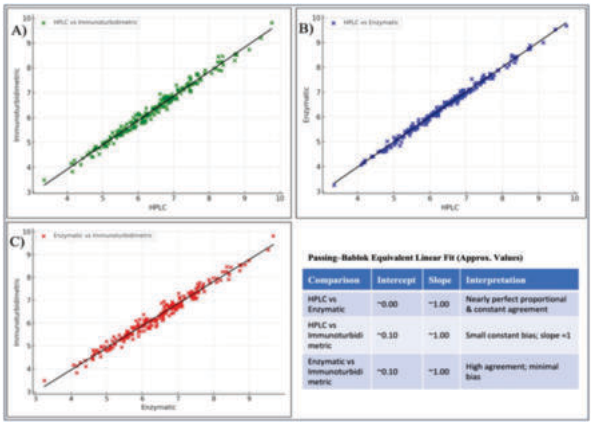

Figure-2: A) The regression equation between the HPLC and immunoturbidimetric methods, B) regression equation between the HPLC and enzymatic methods, C) regression equation between the enzymatic and immunoturbidimetric methods.

DISCUSSION

In the present study, HbA1c estimation was performed using three different methodologies: HPLC, enzymatic assay, and latex agglutination inhibition immunoturbidimetric method. Predialysis CKD patients showed significantly higher HbA1c levels than healthy controls when measured using the HPLC method ($p < 0.001$). Similar trends were observed with the enzymatic and immunoturbidimetric methods (Table 4). Among the study groups, patients with diabetic predialysis CKD demonstrated significantly higher HbA1c values across all three methods compared to both the control group ($p < 0.001$) and the non-diabetic predialysis CKD group ($p < 0.001$). In contrast, no significant difference in HbA1c levels was noted between healthy controls and non-diabetic predialysis CKD patients ($p = 1.000$). These findings are consistent with previous reports, [23] indicating that elevated HbA1c is primarily observed in diabetic CKD patients, while non-diabetic CKD patients may maintain HbA1c levels comparable to healthy individuals.

Direct estimation of carbamylated haemoglobin (CarbHb) was not performed in this study. To explore its potential impact on HbA1c estimation, correlation analysis was carried out between estimated CarbHb levels and HbA1c values obtained using all three methods. Estimated CarbHb levels were calculated using the established formula. No significant correlation was observed between CarbHb and HbA1c values across the three analytical methods ($p > 0.05$), suggesting minimal influence of CarbHb on HbA1c estimation in the present cohort. However, Chachou [24] reported interference from CarbHb and other haemoglobin derivatives in HbA1c estimation by HPLC, with marginally higher values observed in patients with elevated CarbHb levels (>60 µg) compared to those with lower levels (<45 µg), although this difference did not reach statistical significance. Other factors known to affect HbA1c measurement include abnormal haemoglobins and labile HbA1c. In the present study, no abnormal haemoglobin variants were detected on HPLC chromatograms. Previous studies have shown that labile HbA1c can influence HbA1c estimation, particularly when measured by HPLC. [25] The analytical performance of the enzymatic and immunoturbidimetric methods was evaluated against HPLC in terms of precision, bias, and recovery, following CLSI EP5-A guidelines. [20] All three methods met the recommended analytical quality goals, [21] supporting their suitability for routine clinical use.

Method comparison using Bland–Altman plots and Passing–Bablok regression analysis demonstrated good agreement among the three HbA1c measurement techniques. The mean differences between enzymatic and immunoturbidimetric methods compared with HPLC were minimal. No statistically significant difference was observed between HPLC and the enzymatic method, whereas small but statistically significant differences were noted between HPLC and immunoturbidimetric methods, and between enzymatic and immunoturbidimetric methods. Importantly, these differences were not clinically meaningful, as they did not alter patient classification with respect to diabetic or non-diabetic status. These findings align with previous studies supporting the reliability of enzymatic and immunoturbidimetric methods as alternatives to HPLC. [26,27] Analytical performance characteristics-including precision, linearity, recovery, and agreement-were further assessed in accordance with CLSI EP5-A recommendations. [18] Performance was evaluated against established quality goals. [21]

The within-run and between-run coefficients of variation (CV) ranged from 1.2% to 1.6% for HPLC, 1.5% to 1.9% for the enzymatic assay, and 1.7% to 1.8% for the immunoturbidimetric method. Overall imprecision for HbA1c estimation using all three methods (within-run, within-day, and between-day) remained below 2%. These findings are consistent with earlier studies employing similar methodologies. [16,26,28,29] The observed imprecision satisfies NGSP quality criteria of CV < 2%, [21] indicating acceptable analytical performance for routine clinical application.

All three methods demonstrated excellent linearity across the clinically relevant range of HbA1c values, in agreement with previous reports. [16,26,28,29] Recovery studies showed values ranging from 97% to 99% at the two HbA1c levels tested, further confirming analytical accuracy. Taken together, these results suggest that enzymatic and latex agglutination inhibition immunoturbidimetric methods represent accurate, reliable, and cost-effective alternatives to HPLC, particularly in resource-limited settings and smaller laboratories lacking HPLC infrastructure.

CONCLUSION

All three methods evaluated in this study were found to be effective for HbA1c estimation. The enzymatic assay offers the advantage of directly reporting HbA1c percentages without the need for separate total haemoglobin measurement, making it user-friendly, economical, and easily adaptable to routine clinical chemistry analysers. Although the latex agglutination inhibition immunoturbidimetric method requires total haemoglobin estimation, this step is simple to perform, and the method remains cost-effective and compatible with standard analysers. In contrast, HPLC requires specialized instrumentation, skilled personnel, longer processing times, and higher costs.

Based on the findings of this study, enzymatic and latex agglutination inhibition immunoturbidimetric methods can be considered accurate and economical alternatives to HPLC, particularly in resource-poor countries and peripheral healthcare settings. Wider availability of these methods at the community level may facilitate timely diagnosis and improved long-term monitoring of patients with diabetes.

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