Research Article

Comparative Analysis of HbA1c Estimation Using Immunoturbidimetry and High-Pressure Liquid Chromatography Methods in Non-Dialysis Chronic Kidney Disease Patients

Chanchal Dhingra[1], Namrata Rao[2], Shefali Singh[1], Anumesh K Pathak[1], Vandana Tiwari[1], Manish R Kulshrestha[1*], Juhi Verma[1]

[1*, 2]*Department of Biochemistry, Dr Ram Manohar Institute of Medical Sciences, Lucknow, Uttar Pradesh*

Article Info

Author of correspondence:
Dr. Manish Raj Kulshrestha
Additional Professor, Department of Biochemistry
E-mail: drmrkul@gmail.com
Tel.: +91-8607184136
Address:
Dr Ram Manohar Lohia Institute of Medical Sciences
Lucknow-226010, Uttar Pradesh, India

Keywords

Chronic Kidney Disease, HbA1c, Turbidimetric Inhibition Immunoassay, High-Pressure Liquid Chromatography, Carbamylated Hemoglobin.

Abstract

Background

Chronic kidney disease (CKD) concomitant with diabetes mellitus (DM), anemia and uremia. Thus, monitoring HbA1c levels presents a complex clinical challenge.

Methods

This analytical cross-sectional study was conducted from May 2022 to April 2023 at Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow. We compared HbA1c values obtained by the turbidimetric inhibition immunoassay (TINIA) and high-pressure liquid chromatography (HPLC) methods among non-dialysis CKD patients (n=127).

Results

HbA1c was not detectable among 27 patients by TINIA but measurable with HPLC, all being's anemic. The remaining 100 patients, it was detectable by both the methods. Among these 100 patients, linear regression analysis showed a very strong positive correlation between TINIA-HbA1c and HPLC-HbA1c ($R2=0.861$; $p<0.0001$). The agreement between methods was substantial (Cohen's kappa 0.657; $p<0.0001$). However, HbA1c levels were detected significantly higher with HPLC (Median 7.9, IQR 2.7) than that of TINIA (Median 7.0, IQR 2.9;$p=0.025$) in diabetics while the difference was not significant in non-diabetic group with both HPLC (Median 5.4,IQR 0.8) and TINIA (Median 5.1,IQR 1.1). Carbamylated Hb (CHb; as detected by HPLC as a side product) was correlated to both HbA1c by HPLC ($r=0.299$;$p=0.007$) and TINIA ($r=0.336$;$p=0.006$) as well as to serum urea levels ($r=0.439$;$p<0.0001$).

Conclusion

HPLC estimates all HbA1c patients in our study group while TINIA failed to do so in around 21.26% cases. The very low hemoglobin levels and high carbamylated hemoglobin were apparent as two most common causes. Also, the values with TINIA are significantly lower in comparison to HPLC among diabetics with CKD.

Introduction

Chronic kidney disease (CKD) is a significant health concern, impacting approximately 27% of the population, with a heightened occurrence among individuals with diabetes [1,2]. Glycosylated hemoglobin (HbA1c) serves as a crucial biomarker for managing diabetes mellitus (DM), as it reflects long-term glucose control. Accurate HbA1c measurements are vital for reducing vascular complications by maintaining glycemic control [1]. However, stringent HbA1c targets may pose risks [2], and the measurement methods—chromatographic or immunochemical measurement methods can yield different results [3]. In CKD patients, carbamylated hemoglobin (CHb), produced when hemoglobin reacts with urea-derived isocyanate [4,5], can interfere with HbA1c readings [6,7]. Despite advancements in analytical methods, elevated CHb levels in CKD patients continue to challenge the accuracy of HbA1c measurements, complicating the assessment of glycemic control in patients with uremia. Addressing this issue necessitates a nuanced understanding of the biochemical interactions between CHb and HbA1c, as well as the implementation of mitigation strategies to ensure reliable HbA1c assessments in this patient population. To measure HbA1c, various analytical techniques have been employed, with chromatography being the most prevalent technique because of its effectiveness in detecting total glycated hemoglobin [8]. The National Glycohemoglobin Standardization Program (NGSP) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) have been instrumental in enhancing the precision of HbA1c measurement [9,10]. Despite advancements in quality control, variability still exists among the methods certified by the NGSP for HbA1c testing. Additionally, one can employ various laboratory techniques to determine HbA1c levels in the blood. Studies have consistently shown notable discrepancies between these analytical methods. Biological variation sets the permissible total error for HbA1c at 3.0%, while NGSP standards allow up to 6.0% [11,12]. Based on the above facts, this study aims to compare the efficacy of the immunoturbidimetric inhibition immunoassay (TINIA) with that of high-pressure liquid chromatography (HPLC) in the analysis of HbA1c. In addition, there is no correlation between CHb, iron, and creatine levels.

Methodology

Study design and participants

This analytical cross-sectional study was conducted at the Department of Biochemistry in collaboration with the Department of Nephrology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India. HbA1c values obtained by HPLC and TINIA were compared in CKD patients [HbA1c-D (n = 100) and HbA1c-ND (n = 27)]. These patients were confirmed to have CKD by a nephrologist and divided into two groups: those with diabetes mellitus (DM) (n = 40) and those without DM (n = 60) (Table 1). The severity of the disease was divided by the KIDGO guidelines. The repetition was performed with fresh calibration and controls to validate the results. Exclusion criteria included patients undergoing routine hemodialysis or peritoneal dialysis, renal transplant recipients, those unwilling to participate, or individuals under 18 years of age. The Institutional Ethics Committee approved the study (reference number: IEC-48/22), and informed consent was obtained from all participants in accordance with the principles of the Declaration of Helsinki [13] and institutional ethical guidelines.

Table 1: On the basis of detectable and not-detectable HbA1c by TINIA method, status of demographical, biochemical, and hematological variables in chronic kidney disease (CKD) patients.

Variables	Detectable (n=100) N(%)	Not-detectable (n=27) N(%)	p-value
Gender			
Male	63(63.0)	16(59.3)	0.115
Female	37(37.0)	11(40.7)	
Anemia			
Yes	82(82.0)	27(100.0)	0.013*
No	18(18.0)	0	
HTN			
Yes	63(63.0)	08(29.6)	0.002*
No	37(37.0)	19(70.4)	
Severity			
Stage 3	25(25.0)	8(29.6)	0.023*
Stage 4	37(37.0)	16(59.3)	
Stage 5	38(38.0)	03(11.1)	

	Median (IQR)	**Median (IQR)**	
Urea (mg/dL)	81.0 (64.68)	157.5(63.2)	<0.0001*
Creatinine (mg/dL)	3.4 (3.55)	7.9(6.7)	<0.0001*
Iron (µg/dL) Male Female	48.0(22.0) 50.1(21.8) 48.0(16.8)	24.0(12.9) 23.9(11.5) 15.0(12.2)	<0.0001* <0.0001* <0.0001*
HPLC- HbA1c (%)	5.9 (1.9)	5.7(1.4)	0.040*
TINIA- HbA1c (%)	5.6 (1.6)	-	-
CHb	1.8 (0.7)	2.5(1.8)	<0.0001*
CHb/Hb ratio	0.18 (0.10)	0.30 (0.22)	<0.0001*
eGFR	23.8 (16.1)	25.4(18.5)	0.165
UACR	8.2 (45.5)	7.4(5.7)	0.232
Hb(g/dL)	10.4 (3.3)	6.6(0.8)	<0.0001*
RBC Count (million/mm3)	3.8(1.0)	3.4(1.0)	0.500
MCV (fL)	89.2(8.0)	85.3(11.0)	0.208
MCH (pg)	28.4(3.0)	27.6(4.0)	0.424
MCHC (g/dL)	31.8(1.0)	32.3(2.0)	0.582
RDW (%)	14.0(2.0)	12.0(1.0)	0.493
PCV (%)	34.5(8.0)	30.6(12.0)	0.596

HbA1c: glycated hemoglobin, DM: Diabetes mellitus, NDM: No diabetes mellitus. HTN: Hypertension. IQR: Interquartile range, HPLC-Hba1c: High pressure liquid chromatography- Glycated hemoglobin, TINIA: Turbidimetric inhibition immunoassay, CHb: Carbamylated hemoglobin, eGFR: Estimated glomerular filtration rate, UACR: Urine albumin creatinine ratio, Hb: Hemoglobin, RBC: Red blood cells, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDW: Red blood cell distribution width, PCV: Packed cell volume. The Man-whitney test was used to calculate the p-value. * p-value <0.05 was considered as statistically significant.

Sample collection

Venous blood (2 mL) was collected from each vial, plain and EDTA, after information regarding age, sex, comorbidities, and patient consent was obtained. The samples were then centrifuged at 3000 rpm for 10 minutes to separate plasma and serum for biochemical estimation. Blood samples collected for the study were stored at -20 °C for six months (free only once) [14].

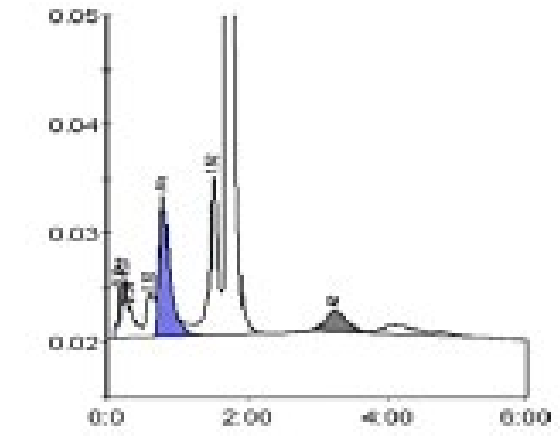
Biochemical estimation

Urea and creatinine levels were analyzed using commercial reagents on a fully automated analyzer (Cobas 6000; Roche Diagnostics).

HbA1c and carbamylated Hb estimation using the HPLC technique

HbA1c levels were measured using ion-exchange HPLC (Bio-Rad D10 Laboratories, Inc., Hercules, California, USA). The three-minute short program (HbA1c mode) was primarily utilized for HbA1c quantification. Carbamylated hemoglobin is represented as the area percentage (Figure 1.).

Figure 1: Representative chromatogram for HbA1c and CHb on HPLC.



Peak table - ID: 110

Peak	R.time	Height	Area	Area %
A1a	0.39	4963	14263	0.8
A1b	0.37	5382	21376	1.3
Unknown	0.34	2922	19643	1.1
LA1c/CHb-I	0.62	3968	29071	1.7
A1c	0.89	12686	154030	9.0
P3	1.52	14526	116873	6.8
A0	1.72	266323	1334732	78.1
A2	3.24	1795	28369	2.5
Total Area:			1708356	

Concentration:	%
A1c	9.0
A2	2.5

Estimation of HbA1c using the TINIA technique

HbA1c was estimated using the immunoturbidimetry technique using commercial reagents on a fully automated integrated analyzer (Cobas 6000, Roche Diagnostics). This method involves analyzing HbA1c levels without needing to measure total hemoglobin levels. The absorbance of HbA1c bound to the particles was measured and found to be proportional to the percentage of HbA1c in the samples.

Data analysis

The baseline characteristics of the participants are summarized as numbers, percentages, and medians (IQR). The chi-square and Mann-Whitney test were used for group comparisons. Pearson's correlation coefficients were used to assess the correlations between urea, creatinine, HbA1c, CHb, and hemoglobin levels. The two methods (HPLC-HbA1c and TINIA-HbA1c) were compared using Bland-Altman plots. Scatter plots of the test data and reference methods were created, and their linear relationship was calculated using a linear regression model (OLR may be applied when the correlation coefficient exceeds 0.9 or 0.99 (slope (b) and y-intercept (a)). All analysis was performed using SPSS software version 24 (Chicago, IL, USA). Statistical significance was set at $p<0.05$.

Results

Glycated hemoglobin (HbA1c) detectability and its association with renal markers in CKD patients

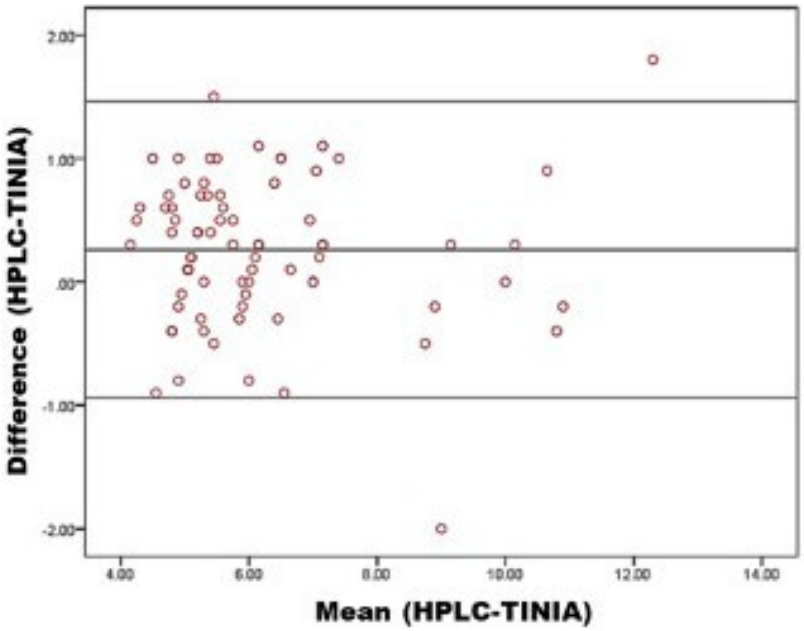
The gender distribution showed a slight male predominance in both HbA1c detectable (HbA1c-D) and non-detectable (HbA1c-ND) groups (p=0.115). Anemia and hypertension were significantly more prevalent in the HbA1c-D group, with 82% (p=0.013) and 63% (p=0.002) of the subjects affected, respectively. CKD severity was also significantly higher in the HbA1c-D group (p=0.023). Biochemical analysis revealed significantly lower urea levels in the HbA1c-D group (81.0 mg/dL, IQR: 64.68) compared to the HbA1c-ND group (157.5 mg/dL, IQR: 63.2, p<0.0001). Creatinine levels also showed a similar pattern, with lower levels in the HbA1c-D group (3.4 mg/dL, IQR: 3.5) than in the HbA1c-ND group (7.9 mg/dL, IQR: 6.7, p<0.0001). Conversely, iron levels were significantly higher in the HbA1c-D group (48.0 µg/dL, IQR: 22.0) compared to the HbA1c-ND group (24.0 µg/dL, IQR: 12.9, p<0.0001). HPLC-HbA1c levels were significantly higher in the HbA1c-D group (5.9%, IQR: 1.9) than in the HbA1c-ND group (5.7%, IQR: 1.4, p=0.040). Hb levels were also significantly higher in the HbA1c-D group (10.4, IQR:3.3) than in the HbA1c-ND group (6.6, IQR: 0.8, p<0.0001). CHb and CHb/Hb ratios were significantly lower in the HbA1c-D group (1.8, IQR:0.7; 0.18, IQR:0.1) compared to the HbA1c-ND group (2.5, IQR:1.8; 0.3,

IQR:0.2, p<0.0001, respectively). No significant differences were observed in UACR, eGFR, RBC count, MCV, MCH, MCHC, RDW, and PCV (p> 0.05) (Table 1).

Correlation and agreement of HbA1c levels measured using HPLC and TINIA methods

The Bland-Altman method was used to calculate the mean difference (bias) between the two techniques. The plot from this analysis indicates agreement between the two methodologies. As shown in Figure 2, 95% of the values were within the range of the mean ± 2 standard deviations (SD) around the study mean. The data revealed no significant differences in HbA1c values measured using HPLC and TINIA methods (Figure 2).

Figure 2: Bland Altman plot for HPLC-HbA1c- and TINIA- HbA1c method.



Further, patients were classified into two categories: those with HbA1c levels between 5.7% and 6.4%, indicating an increased risk of developing diabetes, and those with HbA1c levels of ≥6.5, considered confirmed diabetic. Both methods demonstrated substantial agreement with Cohen's kappa (κ) values of 0.657 (p<0.0001) (Table 2).

Table 2: Concordance of HbA1c between two methods HPLC and IT.

HPLC-HbA1c	IT-HbA1c		Cohen's kappa (κ)	p-value
	≥6.5%	≥6.5%	0.657	<0.0001*
≥6.5%	65	2		
≥6.5%	12	21		

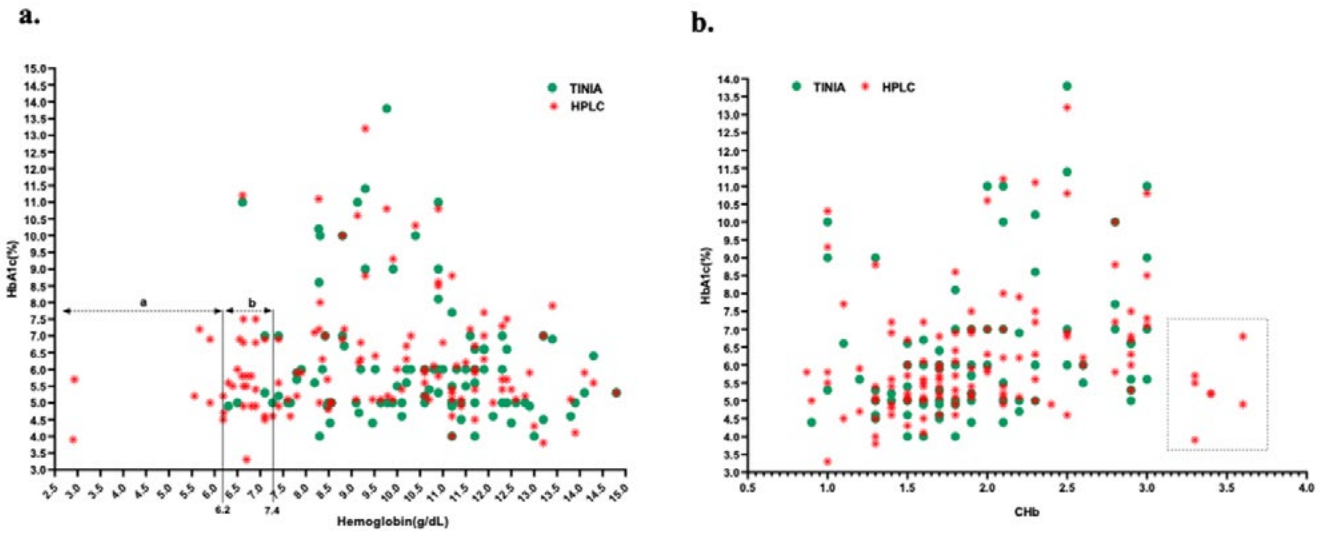
HbA1C; glycated hemoglobin; HPLC, high-performance liquid chromatography; IT, immunoturbidimetry. *p-value <0.05 was considered as statistically significant.

TINIA detection limit compromise with Hb and CHb levels in CKD patients

Figure 3a illustrates that in patients with CKD who had hemoglobin (Hb) levels below 6.2 g/dL, the TINIA method failed to detect HbA1c in 22.2% of cases. Conversely, for patients with Hb levels ranging from 6.2 to 7.4 g/dL, TINIA was able to detect HbA1c in 29.2% of cases, and detection was successful for all patients with Hb levels above 7.4 g/dL.

In contrast, the HPLC method successfully detected HbA1c in all CKD cases. Particularly, with an increased level of CHb above 3.0, TINIA failed to detect HbA1c. Similarly, the ratio of CHb to Hb was higher in patients where TINIA detection failed (Figure 3b). Interestingly, TINIA-HbA1c and HPLC-HbA1c have a very strong positive correlation (r=0.928, p<0.0001). Also, there was a significant concordance, with 87.4% of the variance in the TINIA result explained by HPLC results (R2 = 0.861, p<0.0001) (Figure 4d). A positive correlation was observed between CHb and HPLC-HbA1c (r=0.299, p=0.003) and TINIA-HbA1c (r=0.336, p=0.002) (Figure 4a and 4b). The urea level also demonstrated a positive correlation with CHb (r=0.439, p<0.0001) (Figure 4c). CHb and creatinine also showed a weak positive correlation (r=0.30, p=0.003). Hb and creatinine showed a strong negative correlation (r =-0.492, p<0.0001) (Table 3).

Figure 3: Scattered plot represents the status of HbA1c versus Hemoglobin and b. carbamylated hemoglobin (CHb).



Doted box (a) represents the cases with <6.2 g/dL Hb; Horizontal line (b) donated the cases had Hb: 6.2 to 7.4 g/dL and square box denoted the cases with CHb >3.0 detected by HPLC method.

Table 3: Pearson correlation among the biochemical parameters

Variables	CHb	Hb (g/dL)	TINIA-HbA1c (%)	HPLC-HbA1c (%)	Creatinine (mg/dL)
CHb	1	r=-0.383 p<0.0001*	r=0.336 p=0.002*	r=0.300 p=0.003*	r=0.222 p=0.027*
Hb (g/dL)		1	r=0.063 p=0.579	r=0.051 p=0.625	r=-0.492 p<0.0001*
TINIA-HbA1c (%)			1	r=0.928 p<0.0001*	r=-0.081 p=0.472
HPLC-HbA1c (%)				1	r=-0.096 p=0.340
Creatinine (mg/dL)					1

HbA1c: glycated hemoglobin; HPLC: High-pressure liquid chromatography, TINIA: Turbidimetry inhibition immunoassay, Hb: Hemoglobin, CHb: Carbamylated hemoglobin. *p-value <0.05 was considered as statistically significant.

Clinical and laboratory parameters analysis in diabetic versus non-diabetic CKD patients with detectable HbA1c levels Anemia was observed in 82.5% of the diabetic group (p=0.758) and hypertension in 72.5% (p=0.108), with no significant difference in CKD severity (p=0.846). Urea levels averaged 87.3 mg/dL (IQR: 71.9) in diabetics and 79.4 mg/dL (IQR: 58.9) in non-diabetics (p=0.207). Creatinine was similar for both groups, with diabetics at 3.3 mg/dL (IQR: 2.7) and non-diabetics at 3.4 mg/dL (IQR: 3.8) (p=0.332). Iron levels were higher in non-diabetics, averaging 47.0 µg/dL (IQR: 31.7) versus 33.7 µg/dL (IQR: 19.5) in diabetics (p=0.042). HPLC-HbA1c levels were lower in non-diabetics at 7.0% (IQR: 2.9) compared to 7.8% (IQR: 2.7) in diabetics (p=0.001). Similarly, HbA1c-TINIA levels were lower in non-diabetics at 5.0% (IQR:1.05) versus 6.7% (IQR: 2.2) in diabetics (p<0.0001). CHb levels were also lower in non-diabetics at 1.7 (IQR:0.5) compared to 2.3 (IQR: 1.2) in diabetics (p=0.018). The eGFR was higher in non-diabetics at 22.0 (IQR: 18.6) versus 10.9 (IQR: 7.8) in diabetics (p=0.029). Other parameters, including CHb/Hb ratio, UACR, Hb, RBC count, MCV, MCH, MCHC, RDW, and PCV, showed no significant differences (p> 0.05) (Table 4).

Figure 4: Pearson correlation graph: a. Carbamylated Hemoglobin vs. HbA1c-HPLC, b. Carbamylated Hemoglobin vs. HbA1c-TINIA, c. Carbamylated Hemoglobin vs. Urea, d. A linear regression scattered plot by comparing using two different methods: HbA1c-HPLC and HbA1c-TINIA.

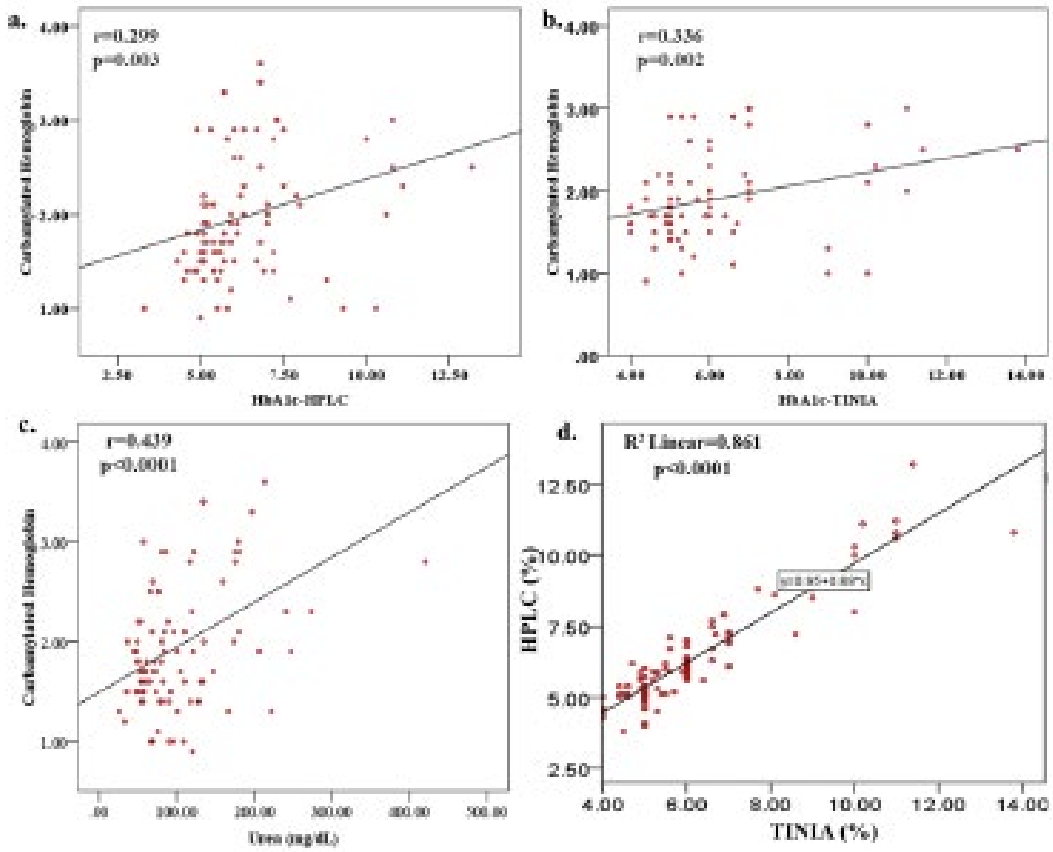


Table 4: On the basis of diabetic and non-diabetic in detectable HbA1c by TINIA method, status of demographical, biochemical, and hematological variables in chronic kidney disease (CKD) patients.

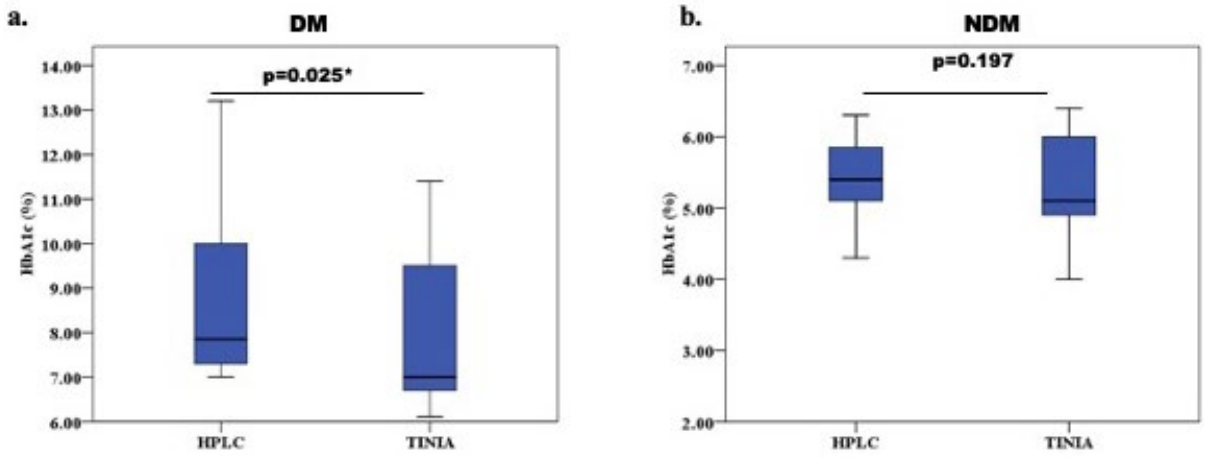
Variables	DM (n=40)	NDM (n=60)	p-value
Gender Male Female	 30(75.0) 10(25.0)	 33(55.0) 27(45.0)	0.025*
Anemia Yes No	 33(82.5) 07(17.5)	 49(81.7) 11(18.3)	0.758
HTN Yes No	 29(72.5) 11(27.5)	 34(56.7) 26(43.3)	0.108
Severity Stage 3 Stage 4 Stage 5	 25(25.0) 37(37.0) 38(38.0)	 8(29.6) 16(59.3) 03(11.1)	0.846
	Median (IQR)	**Median (IQR)**	
Urea (mg/dL)	87.3(71.9)	79.4(58.9)	0.207
Creatinine (mg/dL)	3.3(2.7)	3.4(3.8)	0.332
Iron (µg/dL) Male Female	33.7(19.5) 56.5(19.7) 53.0(47.7)	47.0(31.7) 49.7(26.7) 45.0(12.7)	0.042* 0.048* 0.034*
HPLC- HbA1c (%)	7.8 (2.7)	7.0 (2.9)	0.001*
TINIA- HbA1c (%)	6.7 (0.1)	5.0 (1.1)	<0.0001*
CHb	2.3(1.2)	1.7(0.5)	0.018*
CHb/Hb ratio	0.19 (0.1)	0.16(0.1)	0.512
eGFR	10.9(7.8)	22.0(18.6)	0.029*
UACR	31.0(13.4)	29.3(14.5)	0.056
Hb(g/dL)	8.54(1.4)	9.55(1.3)	0.128
RBC Count (million/mm3)	3.4(1.1)	3.3(1.0)	0.541
MCV (fL)	88.1(9.0)	89.9(9.0)	0.801
MCH (pg)	28.4(2.0)	27.9(3.0)	0.984
MCHC (g/dL)	32.0(2.0)	31.7(1.0)	0.528
RDW (%)	14.9(1.0)	14.5(1.0)	0.773
PCV (%)	31.4(9.0)	30.4(8.0)	0.972

HbA1c: glycated hemoglobin, DM: Diabetes mellitus, NDM: No diabetes mellitus. HTN: Hypertension. IQR: Interquartile range, HPLC-Hba1c: High pressure liquid chromatography- Glycated hemoglobin, TINIA: Turbidimetric inhibition immunoassay, CHb: Carbamylated hemoglobin, eGFR: Estimated glomerular filtration rate, UACR: Urine albumin creatinine ratio, Hb: Hemoglobin, RBC: Red blood cells, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDW: Red blood cell distribution width, PCV: Packed cell volume. The Man-whitney test was used to calculate the p-value. * p-value <0.05 was considered as statistically significant.

Comparison of HbA1clevels estimated in diabetic and non-diabetic patients by HPLC and TINIA methods

The box-and-whisker plot compares the HbA1c levels estimated using HPLC and TINIA in diabetic and non-diabetic patients. In DM group exhibited; HPLC-HbA1c%was (median: 7.9, IQR: 2.7) significantly higher than TINIA-HbA1c% (median: 7.0, IQR: 2.9, p=0.025), while in NDM group the HPLC-HbA1c and TINIA-HbA1c were not found significant (Figure 5. a, b).

Figure 5: Comparison of HbA1c by two different methods: HPLC and TINIA. a. Among DM, b. Among NDM.



DM: Diabetes mellitus, NDM: No diabetes mellitus, HPLC: High pressure liquid chromatography, TINIA: Turbidimetric inhibition immunoassay

Discussion

HbA1c has been a preferred tool for diabetes monitoring, but its accurate estimation in CKD patients remains a challenge. With anemia, iron deficiency, and uremia common among these patients, the selection of an appropriate method for HbA1c estimation is essential. While HPLC is preferred method worldwide, alternative methods such as the immunoturbidimetry (TINIA in our study) are gaining attention for their cost-effectiveness and operational simplicity, making them a promising solution for resource-limited settings as it does not require a separate instrument in contrast to HPLC. Studies have shown that the correlation between the immunoturbidimetric method and HPLC suggests that the former is a reliable substitute for HbA1c measurement in diabetic patients. The literature's comparison of both techniques in the CKD group is limited, especially given its complexity [15-19]. The present study aimed to evaluate the agreement and efficacy between HPLC and TINIA methods in CKD patients as well as the correlation between CHb, urea, Hb, and HbA1c levels. The present study revealed that TINIA-HbA1c and HPLC-HbA1c have a very strong positive correlation ($r=0.928$; $p<0.0001$) signifying 87.4% concordance among the two techniques. The Bland-Altman analysis indicates a strong concordance between the HPLC and TINIA methods for measuring HbA1c levels. This agreement suggests that either method can be used interchangeably without compromising accuracy. The categorization of patients based on HbA1c levels, and the substantial agreement indicated by Cohen's kappa values ($\kappa=0.657$; $p<0.001$) offers a strong foundation for stratifying patients according to diabetes risk, which is crucial for glycemic monitoring and treatment. Our findings, which are in line with the advocacy of Genc S et al. [20], compare the HbA1c values obtained by TINIA and HPLC to assess the concordance between these methods. Their results showed that the mean HbA1c values were 7.789% (± 2.106%) for TINIA and 7.797% (± 2.552%) for HPLC. However, on further comparison, the two methods did not show significant differences in the non-diabetic group, suggesting that both methods are equally suitable for estimating HbA1c levels in non-diabetic individuals. Conversely, within the diabetic CKD cohort, HPLC significantly overestimates HbA1c among diabetics (median; 7.8) in comparison to TINIA (median 6.7).

Most importantly, HbA1c estimation was not possible with TINIA in 27 patients (21.25%), all these patients were severely anemic (Hb<7.4 g/dl). However, HPLC provided value to all the patients. This may be owing to the dependence of the TINIA method on hemoglobin estimation. The manufacturer's insert mentions that Hb<4 g/dl can't be determined. In our study, we found that TINIA failed to calculate HbA1c in 100% of cases with Hb below 6.2. This highlights that TINIA should not be preferred among severely anemic patients. This limitation is less pronounced but still present (29.2%) in patients with Hb levels between 6.2-7.4 g/dL, indicating the presence of some additional interfering factor such as carbamylated Hb, and iron deficiency.

Our findings demonstrated that hemoglobin and iron levels are significantly higher in non-diabetic patients with chronic kidney disease (CKD) as compared to diabetic CKD patients. This observation aligns with other studies highlighting the prevalence of anemia in CKD patients [21,22]. According to a meta-analysis, iron deficiency did not affect the HbA1c levels [23]. In addition, patients with iron deficiency anemia have been found to have a higher glycation rate, which may be due to the higher malondialdehyde levels, a lipid peroxidation metabolite, observed in this population, thus enhancing Hb glycation [24,25]. Furthermore, anemia affects hemoglobin metabolism, thereby impacting HbA1c levels [26]. In our study 85.8% (n=109 out of total 127) of the CKD patients were anemic. The HPLC method gives different peaks for the HbA1c and CHb, but the literature suggests that CHb levels interfere with HbA1c estimation in HPLC as well as TINIA. In our study, we observed that in all the patients with CHb >3.25 by HPLC, TINIA failed to detect

the HbA1c levels. CHb was positively correlated with HPLC-HbA1c (r=0.299; p=0.003), TINIA-HbA1c (r=0.336;p=0.002) and urea (r=0.439;p<0.0001). The reaction of Hb with urea-derived isocyanate forms CHb, which may spuriously cause high values of HbA1c by interfering with the estimation method [5]. Thus, precise assessment of glycemic control in patients with uremia remains problematic. In CKD patients, urea is often dissociated into isocyanate in vivo, reacts with hemoglobin, and forms CHb in a process called carbamylation [27]. However, studies have shown that high blood urea levels interfere with the estimation method, leading to spuriously high HbA1c values [28]. The CHb formation may represent a possible interference in HPLC during HbA1c measurement because the chemical modification at the N-terminal valine results in both molecules co-eluting almost simultaneously, subsequently producing an overlapping peak [29]. Naresh et al. (2018) studied 60 patients and divided them into three groups: acute kidney injury (AKI), CKD, and controls. CHb was highest in CKD patients, intermediate in AKI patients, and lowest in normal patients [30] and it was concluded that CHb can be used to differentiate between AKI and CKD patients. They also reported that carbamylated hemoglobin levels are more directly related to urea than creatinine levels [30]. Wynckel et al. conducted a similar study and stated that the longer the duration of exposure of proteins to high urea concentrations, the higher the amount of CHb formed [31]. According to Stim et al., the relationship between CHb and blood urea nitrogen was linear, but in the case of renal failure patients, it was exponential [32]. Sabrinathan et al. 2020 estimated HbA1c levels in 50 patients with diabetes by comparing the same methods and showed a good positive correlation (r =0.992) [33]. Conversely, the strong negative correlation between Hb and creatinine (r=-0.492) highlights an inverse relationship, affirming lower erythropoiesis in renal failure. Although we did not measure other interfering factors like vitamin (B12, B9, C and E) levels or drug history (aspirin, dapsone, sulfasalazine) levels, the failure of TINIA in HbA1c calculation in 21.25% of cases clearly depicts the superiority of HPLC. Both techniques show minimal interference with CHb. Further studies comparing upcoming methods for HbA1c estimation, like capillary electrophoresis (CE) with HPLC, may be performed by fixing the above limitations in the study design.

Conclusion

This study provides valuable insight into glycemic monitoring by comparing HPLC and TINIA for HbA1c estimation. HPLC detects the HbA1c peak directly, while TINIA is dependent on the biochemical estimation of Hb. TINIA fails to estimate HbA1c in both anemia and high CHb levels (owing to uremia), which are integral to CKD. Thus, HPLC should be preferred among CKD for HbA1c estimation. Alternatively, non-Hb-based tests, such as GA (glycated albumin) or serum fructosamine, may be used.

Declaration

This original article has not been previously published and is not currently being considered for publication elsewhere. All authors read and approved the study.

Financial support

Indian Council of Medical Research (ICMR), New Delhi, India (Ref No.: STS/2022-08152).

Declaration of Conflict of interests

The authors of this article declare that there is no conflict of interest with regard to the content of this manuscript.

References

1. Fenta, E.T., Eshetu, H.B., Kebede, N. et al. Prevalence and predictors of chronic kidney disease among type 2 diabetic patients worldwide, systematic review and meta-analysis. DiabetolMetab Syndr 15, 245 (2023). https://doi.org/10.1186/s13098-023-01202-x
2. C, Millart H, Chanard J, Gillery P. Influence of in vivo hemoglobin carbamylation on HbA1c measurements by various methods. Clin Chem Lab Med. 2000:321-326.
3. Szymezak J, Lavalard E, Martin M, Leroy N, Gillery P. Carbamylated hemoglobin remains a critical issue in HbA1c measurements. Clin Chem Lab Med. 2009;47(5):612-613.
4. Chen Z, Shao L, Jiang M, Ba X, Ma B, Zhou T. Interpretation of HbA1c lies at the intersection of analytical methodology, clinical biochemistry, and hematology. Exp Ther Med. 2022;24(6).
5. Ashcroft RE. The declaration of Helsinki. The Oxford textbook of clinical research ethics. 2008;21:141-148.
6. Liyanage T, Toyama T, Hockham C, Ninomiya T, Perkovic V, Woodward M, Fukagawa M, Matsushita K, Praditpornsilpa K, Hooi LS, Iseki K. Prevalence of chronic kidney disease in Asia: a systematic review and analysis. BMJ Glob Health. 2022;7(1):e007525.
7. Lin CY, Hsieh MC, Kor CT, Hsieh YP. Association and risk factors of chronic kidney disease and incident diabetes: a nationwide population-based cohort study. Diabetologia. 2019;62:438-447.
8. Klenk DC, Hermanson GT, Krohn RI, Fujimoto EK, Mallia AK, Smith PK, England JD, Wiedmeyer HM, Little RR, Goldstein DE. Determination of glycosylated hemoglobin by affinity chromatography: comparison with colorimetric and ion-exchange methods, and effects of common interferences. Clinical Chemistry. 1982;28(10):2088-2094.
9. Little RR, Rohlfing C, Sacks DB. The national glycohemoglobin standardization program: over 20 years of improving hemoglobin A1c measurement. Clinical chemistry. 2019;65(7):839-848.
10. Lenters-Westra E, English E. Evaluating new HbA1c methods for adoption by the IFCC and NGSP reference networks using international quality targets. Clinical Chemistry and Laboratory Medicine (CCLM). 2017;55(9):1426-1434.

11. English E. Monitoring Diabetes. Textbook of Diabetes. 2024:404-13.
12. Åsberg A, Odsæter IH, Carlsen SM, Mikkelsen G. Using the likelihood ratio to evaluate allowable total error–an example with glycated hemoglobin (HbA1c). Clinical Chemistry and Laboratory Medicine (CCLM). 2015;53(9):1459-1464.
13. Dal-Ré R. Waivers of informed consent in research with competent participants and the Declaration of Helsinki. European journal of clinical pharmacology. 2023;79(4):575-8.
14. Braae UC, Johansen MV, Ngowi HA, Rasmussen TB, Nielsen J, Uttenthal Å. Detection of African swine fever virus DNA in blood samples stored on FTA cards from asymptomatic pigs in Mbeya Region, Tanzania. Transboundary and emerging diseases. 2015;62(1):87-90.
15. Little RR, Rohlfing CL, Tennill AL, Hanson SE, Connolly S, Higgins T, Wiedmeyer CE, Weykamp CW, Krause R and Roberts W: Measurement of Hba(1C) in patients with chronic renal failure. Clin Chim Acta. 418:73–76. 2013
16. Dolscheid-Pommerich RC, Kirchner S, Weigel C, Eichhorn L, Conrad R, Stoffel-Wagner B and Zur B: Impact of carbamylation on three different methods, HPLC, capillary electrophoresis and TINIA of measuring HbA1c levels in patients with kidney disease. Diabetes Res Clin Pract. 108:15–22. 2015.
17. Ng JM, Cooke M, Bhandari S, Atkin SL and Kilpatrick ES: The effect of iron and erythropoietin treatment on the A1C of patients with diabetes and chronic kidney disease. Diabetes Care. 33:2310–2313. 2010.
18. Kilpatrick ES and Atkin SL: Using haemoglobin A(1c) to diagnose type 2 diabetes or to identify people at high risk of diabetes. BMJ. 348(g2867)2014.
19. Galindo RJ, Beck RW, Scioscia MF, Umpierrez GE, Tuttle KR. Glycemic monitoring and management in advanced chronic kidney disease. Endocrine reviews. 2020;41(5):756-774.
20. Genc S, Omer B, Aycan-Ustyol E, Ince N, Bal F, Gurdol F. Evaluation of turbidimetric inhibition immunoassay (TINIA) and HPLC methods for glycated haemoglobin determination. Journal of clinical laboratory analysis. 2012;26(6):481-485.
21. Kidanewold A, Woldu B, Getie A, Enawgaw B. Anemia and its predictors among adult non-dialysis chronic kidney disease patients in Southern Ethiopia: a cross-sectional study. Current Medical Research and Opinion. 2022;38(3):393-400.
22. Guedes M, Muenz D, Zee J, Lopes MB, Waechter S, Stengel B, Massy ZA, Speyer E, Ayav C, Finkelstein F, Sesso R. Serum biomarkers of iron stores are associated with worse physical health-related quality of life in nondialysis-dependent chronic kidney disease patients with or without anemia. Nephrology Dialysis Transplantation. 2021;36(9):1694-1703.
23. Cavagnolli G, Pimentel AL, Freitas PA, Gross JL and Camargo JL: Factors affecting A1C in non-diabetic individuals: Review and meta-analysis. Clin Chim Acta. 2015;445:107–114.
24. Sundaram RC, Selvaraj N, Vijayan G, Bobby Z, Hamide A and Rattina Dasse N: Increased plasma malondialdehyde and fructosamine in iron deficiency anemia: Effect of treatment. Biomed Pharmacother. 2007;61:682–685.
25. Guo W, Zhou Q, Jia Y and Xu J: Increased levels of glycated hemoglobin A1c and iron deficiency Anemia: A review. Med Sci Monit. 2019;25:8371–8378.
26. Isa SB, Hami R, Alma'Norliana JA, Noordin SS, Ismail TS. Glycaemic Monitoring in Diabetic Kidney Disease–Is HbA1c Reliable?. J Health Sci Med Res. 2022;40(2):229-237.
27. Jaisson S., Pietrement C., Gillery P. Protein carbamylation: chemistry pathophysiological involvement and biomarkers… Adv Clin Chem. 2018;84:1-38.
28. Gorisse L., Jaisson S., Piétrement C., Gillery P.Carbamylated proteins in renal disease: aggravating factors or just biomarkers?. Int J Mol Sci. 2022;23(1):574.15.
29. Roy S., Schweiker-Kahn O., Jafry B., Masel-Miller R., Raju RS., O'Neill LM., Correia CR., Trivedi A., Johnson C., Pilot C., Saddemi J. Risk factors and comorbidities associated with diabetic kidney disease. J Prim Care Community Health. 2021;12:21501327211048556.
30. Naresh Y, Srinivas N, Vinapamula KS, Pullaiah P, Rao PS, Sivakumar V. Carbamylated hemoglobin can differentiate acute kidney injury from chronic kidney disease. Indian journal of nephrology. 2018;28(3):187.
31. Wynckel A, Randoux C, Millart H, Desroches C, Gillery P, Canivet E, Chanard J. Kinetics of carbamylated haemoglobin in acute renal failure. Nephrology Dialysis Transplantation. 2000;15(8):1183-1188.
32. Stim J, Shaykh M, Anwar F, Ansari A, Arruda JA, Dunea G. Factors determining hemoglobincarbamylation in renal failure. Kidney international. 1995;48(5):1605-1610.
33. Sabarinathan M, Ananthi N, Ashwin H. Comparative study of various methods in the estimation of blood HbA1c and its outcome. Int J Res Pharm Sci. 2020;11(SPL2):43