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Evaluation of Preanalytical Variables and the Use of Alternative Anticoagulants in HbA1c Testing

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KEYWORDS

HbA1c, pre-analytical errors, Anticoagulants, EDTA, Lithium Heparin, Diabetes Mellitus

ABSTRACT:

Introduction: Preanalytical variables significantly influence laboratory testing accuracy, and their impact on glycated haemoglobin (HbA1c) measurements remains a critical area of study. HbA1c is widely used for diagnosing and monitoring diabetes mellitus, with EDTA being the standard anticoagulant. However, challenges such as sample underfilling and alternative anticoagulant use remain underexplored.

Objectives: This study examines the effects of preanalytical factors, including underfilled sample volumes and alternative anticoagulants, on HbA1c reliability.

Methods: We analysed 150 blood samples collected from adult patients. Samples were collected in tubes containing K3-EDTA, K2-EDTA, and lithium heparin at varying fill volumes. HbA1c concentrations were measured using a standardized immunoturbidimetric assay on the Cobas c 6000 platform. Results were analysed using paired t-tests, Wilcoxon signed-rank tests, and bias analysis. The clinical impact was assessed against the diagnostic cutoff of HbA1c ≥6.5%.

Results: Underfilled EDTA tubes (<50%) showed statistically significant deviations in HbA1c measurements ($P < 0.05$), with greater bias observed below the diagnostic threshold of 6.5%. Lithium heparin demonstrated acceptable stability compared to K3-EDTA but introduced variability at higher HbA1c levels. Bias analysis indicated minimal clinical relevance when anticoagulants were used appropriately, but underfilled samples produced erroneous classifications in up to 10% of cases.

Conclusions: Preanalytical variables, particularly fill volume, must be tightly controlled in HbA1c testing to ensure diagnostic reliability. While alternative anticoagulants like lithium heparin may provide flexibility, their adoption requires thorough validation under specific conditions. Laboratories should implement strict quality control protocols to mitigate preanalytical errors.

1. Introduction

Glycated haemoglobin (HbA1c) is a cornerstone biomarker for diabetes mellitus (DM) diagnosis and management due to its ability to reflect average blood glucose levels over 120 days. The American Diabetes Association recommends HbA1c testing as a primary diagnostic and monitoring tool for Diabetes Mellitus

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[1]. However, its accuracy is susceptible to preanalytical variables such as anticoagulant type, sample volume, and storage conditions [2].

EDTA, either in its K2 or K3 form, is the standard anticoagulant for HbA1c testing due to its stability and compatibility with automated analysers [3]. However, clinical situations, such as poor venous access or paediatric patients, often lead to underfilled tubes or the use of alternative anticoagulants like lithium heparin. Accordingly, inadequate sampling may be possible in patients who had HbA1c test orders, due to the vascular changes in DM. These preanalytical deviations can compromise HbA1c results and lead to misclassification of diabetic status [4][5].

2. Objectives

This study was planned to investigate the impact of underfilled EDTA tubes and the use of alternative anticoagulants on HbA1c accuracy and diagnostic validity.

3. Methods

Study Design

This prospective study was conducted in the Clinical Biochemistry Laboratory of Pacific Medical College and Hospital, Udaipur, Rajasthan, India. Written informed consent was obtained from all participants, and ethical approval was secured (Approval No. PMU/PMCH/IEC/2024/271, Dated-29/07/2024).

Sample Collection

Venous blood samples were collected from 150 adult patients requiring routine HbA1c testing. Samples were drawn using K3-EDTA, K2-EDTA, and lithium heparin tubes. Each patient provided three sets of anticoagulant tubes with three sets of fill volumes, as mentioned below, for a total of 9 samples to assess the impact of the anticoagulants and fill volumes on HbA1c values. Tubes were classified based on fill volume as:

1. <25% (<0.5 mL)
2. 25–50% (0.5–1.0 mL)
3. 50% (1.0–2.0 mL).

All blood collection tubes from each patient were taken at the same time, and all samples were sent for routine HbA1c measurement as per standard manufacturer protocol, as per established lab guidelines. Transportation of samples from phlebotomy to the laboratory in terms of temperature and transport conditions was also kept similar for all samples of a patient set, and analysis of all samples from a single patient was also performed in the same batch at the same time.

HbA1c Measurement

HbA1c was measured using the immunoturbidimetric Tina-quant HbA1c Gen. 3 assay on the Cobas c 6000 analyser (Roche Diagnostics, Germany). The assay is certified by the NGSP and standardized by the IFCC [6]. Quality control was ensured using two levels of control samples, with coefficients of variation (CVs) maintained below 2%. The assay used in this study is accredited by the National Glycohemoglobin Standardization Program (NGSP) and aligned with the standards set by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). All measurements were conducted per the manufacturer's guidelines, employing the same batches of reagents, calibrators, and quality control materials throughout the study. Quality assurance was maintained using two levels of control samples, with all internal quality control data falling within the acceptable limits during the evaluation period.

To confirm the analytical precision of the Cobas HbA1c assay, we followed the CLSI EP5-A2 protocol (8). The within-run coefficients of variation (CVs) were found to be 1.1% for the normal control and 0.5% for the high-level control. The between-run CVs were 1.1% and 1.7%, respectively. HbA1c concentrations were reported in NGSP units as percentages (%) and calculated using the formula derived from the A1c-to-total haemoglobin ratio: HbA1c (%) = (A1c/Hb) × 91.5 + 2.15.

Statistical Analysis

The distribution of the data was evaluated using the Shapiro-Wilk test for normality. As the data did not follow a normal distribution, results were reported using the median, interquartile range, and minimum-to-maximum values. To compare HbA1c levels between underfilled and properly filled blood collection tubes, the Wilcoxon matched-pairs signed-rank test was employed. A p-value of less than 0.05 was considered statistically significant for all analyses. Bias analysis evaluated the

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differences between sample conditions, and clinical implications were assessed against the diagnostic cutoff of HbA1c ≥6.5%. Bias in % HbA1c was determined by calculating the difference between underfilled and filled sample results (% HbA1c underfilled − % HbA1c filled), and the corresponding 95% confidence intervals (CIs) were computed using SPSS version 23.

4. Results

Impact of Fill Volume

The underfilled blood samples were divided into three groups based on their anticoagulants: Group 1 with K2 EDTA, Group 2 with K3 EDTA, and Group 3 with Lithium Heparin. Each group was further split into three subgroups based on volume: 0.5 mL or less (<25%), between 0.5 and 1.0 mL (25–50%), and between 1.0 and 2.0 mL (>50%). In total, the three subgroups combined included 150 patients, with 450 samples in each group. A comparison of HbA1c levels between these underfilled groups and properly filled tubes is shown in Table 1. Notably, a statistically significant difference in HbA1c percentages was observed for all three groups among samples filled to less than 25% (p-value < 0.05*).

To assess the impact of sample volume on HbA1c concentration more precisely, we stratified the results based on the diagnostic threshold of 6.5% for HbA1c. Among the 150 total patient samples, 77 (51%) had HbA1c values below 6.5%, while 73 (49%) exceeded this threshold.

Comparison of Anticoagulants

Lithium heparin and K2-EDTA showed comparable performance to K3-EDTA at >50% fill volume (P > 0.05). However, variability increased with lithium heparin at HbA1c levels exceeding 7.5%, likely due to altered reagent-anticoagulant interactions.

Bias Analysis

To determine the significance of differences between paired observations, we conducted a bias analysis. As shown in Table 2, greater bias was evident in samples from patients with HbA1c values below 6.5%, particularly when the tube fill volume was 50% or less.

Underfilled EDTA tubes (<50%) demonstrated significant deviations in HbA1c measurements compared to appropriately filled tubes (P < 0.05). Bias was particularly pronounced in samples below the diagnostic cutoff of 6.5%, resulting in a potential misclassification of up to 8% of cases.

Bias analysis revealed mean deviations of +1.2% for underfilled EDTA tubes and ±0.3% for alternative anticoagulants. The clinical impact was negligible when anticoagulants were used correctly, but critical when combined with low fill volumes.

Table 1- Comparison of HbA1c concentrations in underfilled and appropriately filled tubes among different anticoagulants

HbA1c%	Group 1			Group 2			Group 3		
	<0.5mL	**0.5-1.0mL**	**1.0-2.0mL**	**<0.5mL**	**0.5-1.0mL**	**1.0-2.0mL**	**<0.5mL**	**0.5-1.0mL**	**1.0-2.0mL**
Median (IQR)	6.2 (5.5-8.6)	6.5 (5.8-7.4)	6.6 (6.0-8.2)	6.1 (5.4-8.5)	6.4 (5.7-7.5)	6.5 (6.1-8.2)	6.1 (5.2-9.5)	6.4 (5.4-8.5)	6.5 (6.3-9.2)
Min-Max	5.0 - 12.0	4.9 - 10.9	5.2 - 10.1	4.9 - 11.7	4.9 - 11.1	5.3 - 10.0	4.9 - 11.7	4.9 - 11.1	5.3 - 10.0
P-Value	0.030*			0.018*			0.027*		

Group 1 – Samples with Lithium Heparin. Group 2 - Samples with K2-EDTA. Group 3 - Samples with K3-EDTA. IQR- interquartile range. Min - lowest value. Max - highest value. Statistical significance was set at P < 0.05.

Table 2- Biases obtained in tubes with underfilling ratios compared to standard volume according to the cut-off value for HbA1c

HbA1c (%)	**Filling ratio of tubes**		
	< 25%	**25-50%**	**> 50%**
Number of samples with HbA1c < 6.5%	25	17	14

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Mean bias (95% Cl)	0.1 (- 0.2 to 0.3)	0.1 (- 0.2 to 0.3)	0 (- 0.2 to 0.3)
Mean bias (%)	1.3	1.1	-0.5
Number of samples with HbA1c ≥ 6.5%	19	19	15
Mean bias (95% Cl)	0 (- 0.5 to 0.3)	0 (- 0.5 to 0.2)	0 (- 0.6 to 0.2)
Mean bias (%)	0	0	0.2

CI - confidence interval.

5. Discussion

This study underscores the importance of preanalytical variables in HbA1c testing, highlighting the susceptibility of EDTA tubes to fill volume effects. Consistent with prior reports [7–10], underfilled tubes (<50%) led to significant errors in HbA1c measurement.

While lithium heparin offers a viable alternative, its use should be restricted to specific scenarios where EDTA is unavailable, and only after thorough validation [11-14]. Laboratories must adopt stringent preanalytical protocols to ensure accuracy and mitigate bias.

Our findings indicate that underfilling EDTA blood collection tubes to only 25% can result in statistically significant deviations in HbA1c levels. Bias analysis revealed that in samples with HbA1c values below 6.5%, tubes filled to 50% or less showed a consistent positive bias. This suggests that when HbA1c is used for diagnostic purposes, especially in samples with less than 50% fill, there is a risk of misclassification from "normal" to "diabetic." Given the clinical sensitivity of HbA1c, even minor inaccuracies can substantially influence medical decisions, underscoring the importance of minimizing analytical bias.

These discrepancies may also stem from the inherent variability in the HbA1c assay. We assessed the assay's performance by evaluating its precision, and the coefficient of variation (CV) values fell within the manufacturer's recommended and acceptable limits. Furthermore, since unequal effects were not observed across all tube fill levels, analytical variability alone does not fully account for the observed differences.

There is limited research addressing the impact of underfilled anticoagulant-containing tubes on analyte measurement [15-18]. It is generally accepted that underfilling up to 75% in such tubes rarely introduces clinically relevant bias. Although few studies have examined the specific impact of underfilled K2-EDTA or K3-EDTA tubes on HbA1c levels, existing evidence—based on limited sample sizes—suggests negligible effects [19-21]. However, due to the lack of comprehensive studies evaluating the relationship between EDTA tube fill volume and HbA1c measurement, our results may offer valuable guidance. We recommend a minimum fill volume of 50% for accurate HbA1c analysis, especially in laboratories dealing with insufficient sample volumes.

The primary limitation of our study is the unequal sample distribution across the test groups. Additionally, our analysis was restricted to a single instrument—the Cobas 6000—and its specific reagents.

In summary, to minimize potential clinical misinterpretations, we advise that EDTA blood collection tubes intended for HbA1c measurement be filled to at least 50%.

6. Conclusion

Preanalytical variables, particularly sample volume and anticoagulant type, play a critical role in HbA1c testing reliability. Laboratories should prioritize quality control measures, including:

1. Ensuring EDTA tubes are filled to at least 50% of their capacity.

2. Validating alternative anticoagulants, such as lithium heparin, under controlled conditions.

Future studies should focus on multicentre validations and explore innovative anticoagulant technologies for HbA1c testing.

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