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Original Article

Unlocking precision: evaluating the impact of K2 and K3 EDTA anticoagulants on HbA1c analysis

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Abstract

HbA1c plays a vital role in the diagnosis and monitoring of diabetes mellitus. The variety of blood collection tubes is a significant pre-analytical aspect that could affect test results. The influence of blood assortment devices on laboratory tests is often unnoticed. This study investigates whether K2-ethylenediaminetetraacetic acid (EDTA) and K3-EDTA vacuum tubes affect the analytical results of glycosylated haemoglobin, type A1C (HbA1c), measured via a Bio-Rad Analyser D10 HPLC analyser. This study was an analytical, observational, cross-sectional study, where we collected samples from 100 patients at Chettinad Hospital and Research Institute, Kelambakkam. Blood was taken simultaneously in both K2 RDTA and K3 EDTA tubes. The samples were analyzed immediately in a Bio-Rad analyzer D10 after collection. We employed the Wilcoxon test and passed the Bonferroni regression for comparing the two tubes. The information are represented as median and interquartile range. The standard deviations for K2 and K3 were 2.06 and 2.05, indicating the degree of variability in the data. Even though there is a very minor difference in the Wilcoxon test, there was no statistically significant p-value and passing the Bablok regression showed the Y=0.000+1.000x. This study showed very little noteworthy variance between K2 and K3 EDTA anticoagulant tubes. K2 and K3 tubes have a very slight difference, but it is not statistically significant and falls within the limit of agreement (LOA) range of 95%.

Keywords: HbA1c, K2-EDTA, K3-EDTA, anticoagulant comparison, pre-analytical variability

Introduction

The non-enzymatic binding of circulating blood glucose to hemoglobin results in glycated hemoglobin (HbA1c). HbA1c is crucial in assessing diabetes and glycemic status. The mainstream of laboratory medicine errors happens during the pre- and post-analytical phases of the analysis process [1–3]. This is the most critical stage, but preanalytical errors may remain undetected until post-analytical validation and interpretation, potentially affecting results [4, 5]. Thus, following standardization, measuring HbA1c is the most reliable laboratory method for assessing long-term glycemic control in diabetic patients [2, 6, 7].

In addition to the traceability of HbA1c results to the reference system, it is crucial to establish analytical goals to ensure the clinical reliability of HbA1c measurements. To improve the accuracy of HbA1c measurements, a test center must eliminate all potential sources of pre-analytical and analytical errors to achieve a diagnostic goal for imprecision of 2.0% and a maximum allowable bias of 2.8%. Anticoagulant use is an important pre-analytical factor in hematological tests. EDTA is a polyprotic acid with four carboxylic acid groups and two amine groups containing lone pairs of electrons that chelate calcium and several other metal ions while preserving cellular components and blood cell morphology [8, 9].

For HbA1c assays, laboratories require commercially available EDTA anticoagulated whole blood collection tubes [10]. EDTA salts such as di-potassium, tri-potassium, and sodium EDTA are available in blood

tubes. Because tri-potassium EDTA is dispensed as a liquid, the specimen is slightly diluted. Because of its less hyperosmolar effect on blood cells, the Global Council for Standardization of Hematology (ICSH) claims K2EDTA as an anticoagulant of choice for hematology testing [11, 12].

In this study, we compared the measurement outcomes of HbA1c using K2 EDTA and K3 EDTA as variations in EDTA use. For HbA1c testing, we use a Bio-Rad D-10 cation exchange high-performance liquid chromatography platform (Bio-Rad Laboratories) [13].

Material and methods

Study design and participants

A cross-sectional study included 100 apparently healthy participants (age ≥18 years) recruited from the outpatient department of Chettinad Hospital, India, between January and April 2023. The purpose was to compare analytical methods under typical conditions. Exclusion criteria included a known history of diabetes, recent blood transfusions (<3 months), or hemoglobinopathies.

Sample collection and processing

Between January 2023 and April 2023, specimens were collected in the outpatient clinical chemistry unit of the Department of Biochemistry at Chettinad Hospital and Research Institute, Kelambakkam, Chennai, Tamil Nadu, India. Outpatients over the age of 18 (n=100) who had fasted for at least 8 hours were used in the study. A well-trained phlebotomist was involved in sample collection. Two 3 mL plastic vacuum tubes were obtained for each patient. One is coated with K2-EDTA, while the other is coated with K3-EDTA. This study was conducted as an internal method comparison and quality assurance activity for laboratory procedures.

As such, in accordance with the policies of Chettinad Hospital and Research Institute at the time of the study, such internal quality control analyses utilizing leftover anonymized samples are exempt from formal review by the Institutional Ethics Committee. Blood samples were collected as part of a routine check-up, and the leftover anonymized portions were used for this comparative analysis.

Laboratory testing

After retrieving the specimen from the chute room, the technician mixed tube 8 to 10 times per the manual's recommendation before transporting it to the biochemistry laboratory for processing. If the sample is less than 2 mL or the tube is abnormal, we predilute it in a 1:300 ratio (i.e., 5 µL of sample in 1.5 mL of wash/diluent solution) before analysis. Appropriate blood collection and well-timed processing are precarious pre-analytical steps mandatory for laboratory outcomes to be precise. Within 30 minutes of our specimen retrieval, samples are processed and analyzed. We measured HbA1c concentrations on the Bio-Rad D10 HPLC analyzer, which separates Hb kinds based on charge modifications between HbA1c and other hemoglobin. Before processing the samples, we double-checked the daily maintenance pre-run checklist and the Quality indicator to ensure the accuracy of the results. Samples with total areas that fall outside the expected range should be diluted and rerun until they achieve values in the 1.0–5.0 million total area count range. HbA1c levels are expressed as a percentage.

Statistical analysis

To assess normality, we used the Kolmogorov-Smirnov test. Since the distribution was not regular, we presented our information as medians and interquartile ranges. The nonparametric Wilcoxon test for paired observations was used to investigate the differences between K2-EDTA and K3-EDTA measurements. Bablok regression analysis and Bland-Altman plotting were used to assess the comparability of outcomes obtained with two different additive tubes. According to the current recommendation for HbA1c measurement, the diagnostic goal for imprecision must be 2.0 percent, with a maximum allowable bias of 2.8 percent. A p-value of 0.05 is considered statistically significant.

Table 1: Descriptive statistics of HbA1c (%).

	K2	K3
Mean	7.1145	7.1054
Standard error	0.205572	0.205284

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Table 1: Continued.

	K2	K3
Median	6.4	6.4
Mode	5.6	5.6
Standard deviation	2.055718	2.052839
Sample variance	4.225974	4.214148
Kurtosis	0.48341	0.508623
Skewness	1.098238	1.090383
Range	9.4	9.7
Minimum	4.5	4.3
Maximum	13.9	14
Sum	711.45	710.54
Count	100	100
Confidence level (95.0%)	0.407899	0.407328

Results

Descriptive statistics

Median HbA1c values were identical (6.4%) for both anticoagulants (Table 1). Variability measures (SD: 2.05–2.06) and ranges (4.3–14.0%) aligned closely, with minimal differences in central tendency (mean: 7.11% for both) and dispersion (variance: ~4.22).

Table 1 shows that the mean value of central tendency for both k2 and k3 is approximately 7.11, indicating that both sets of values are close to the central point, with a median of 6.4 and a mode of 5.6. K2 and k3 standard deviations were 2.06 and 2.05, respectively, indicating the degree of variability in the data. The variance between the extreme and least values observed is 9.4 for K2 and 9.7 for K3.

Comparative analysis

Wilcoxon Signed-Rank Test: No significant difference between K2- and K3-EDTA results (Z=−0.973, *p*=0.330). Negative ranks (K3<K2: 29 cases) slightly outweighed positive ranks (K3>K2: 22 cases), but the median difference was negligible (Table 2). Passing-Bablok Regression: Demonstrated perfect agreement between methods (Y=0.000+1.000X), with a slope of 1.000 (95% CI: 0.98–1.02) and intercept of 0.000 (95% CI: −0.05–0.05) (Figure 1). Bland-Altman Analysis: Mean difference =−0.01% (95% limits of agreement: −0.15% to +0.17%), confirming no clinically significant bias (Figure 2).

In Table 2, the comparison of HbA1c values was measured in K3-EDTA and K2-EDTA anticoagulant tubes using the Wilcoxon Signed-Rank Test (n=100). No statistically significant difference was observed

Table 2: Wilcoxon Signed-Rank test results comparing K3-EDTA and K2-EDTA HbA1.

		N	Mean rank	Sum of ranks
k3-k2	**Negative ranks**	29[a]	26.24	761.00
	Positive ranks	22[b]	25.68	565.00
	Ties	49[c]		
	Total	100		
		Test statistics		
	Z		-.973[b]	
	Asymp. Sig. (2-tailed)		.330	

Note: [a] –k3<k2; [b] – k3>k2; [c] – k3=k2.

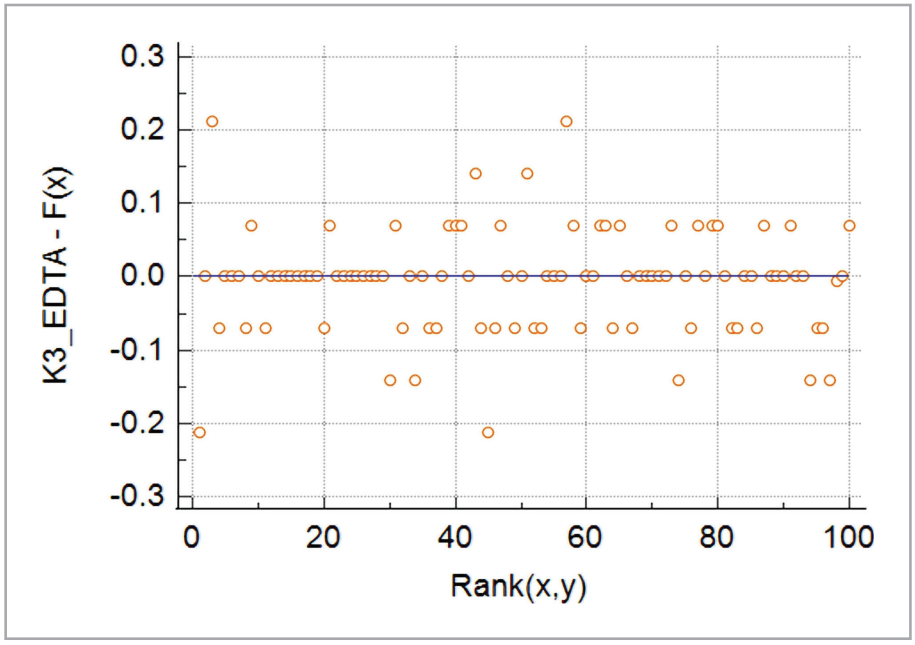

Figure 1: Passing-Bablok regression analysis.

(Z=−0.973, p=0.330). Negative ranks (K3<K2: 29 samples) and positive ranks (K3>K2: 22 samples) reflect directional differences, while ties (49 samples) indicate identical values. Mean and sum of ranks quantify the magnitude of differences between paired samples.

Figure 2 is a comparison of HbA1c values between K2-EDTA and K3-EDTA anticoagulant tubes using Wilcoxon signed-rank test. Boxplots show median values and interquartile ranges. No statistically significant difference was observed (p=0.330).

Figure 1 illustrates the scatter plot showing the Passing-Bablok regression line comparing two quantitative measurement methods. The plot includes the regression line, 95% confidence intervals, and identity line (y=x) for visual assessment of agreement and systematic bias.

Figure 3 explains the Residuals from the Passing-Bablok regression analysis plotted against the fitted values or the average of the two methods. The plot helps visualize deviations, assess linearity, and

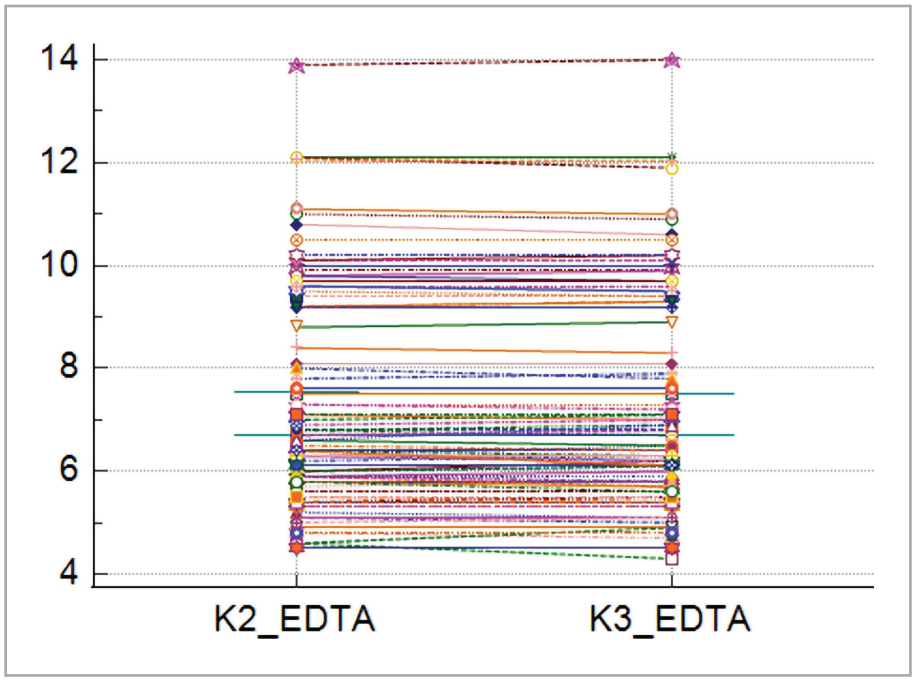

Figure 2: Wilcoxon test – multiple variable grap.

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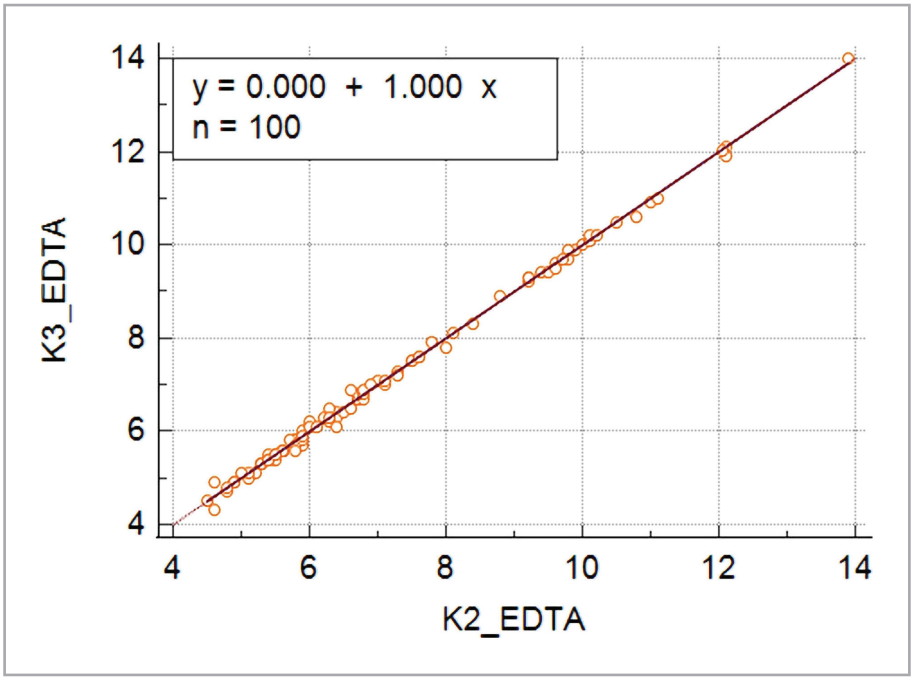

Figure 3: Residual plot of Passing-Bablok regression.

detect outliers or heteroscedasticity in the agreement between methods.

Discussion

Biochemical laboratories are critical components of patient care and diagnosis. Despite the fact that hematology and clinical pathology labs are highly automated, there are numerous variables that can impact the outcomes. Recent automated methods are designed to meet the needs of high-volume test centers. As a therapeutic and medicinal device, blood collection tubes must perform as expected under specific conditions of use [14, 15]. Even minor variations in the concentration of the anticoagulant in the blood collection tubes can cause significant differences in the test results. A total of 100 samples were obtained and processed for HbA1c measurement, yielding results. There have been few studies comparing HbA1c measurement using K2 and K3 anticoagulants. In our study, the lowest measured concentration of HbA1c was 4.3%, and the highest concentration was 14.0%.

To determine the difference between K3 and K2, the Wilcoxon signed rank test was used [16]. In this case, we obtained negative difference ranks that were higher than positive difference ranks. The negative difference has a rank sum of 761, while the positive difference has a rank sum of 565. The mean rank for the negative difference is 26.24, while it is 25.68 for the positive difference. Because the sum of ranks for the negative difference is greater, it implies that K3 is significantly smaller than K2, but the test statistics obtained were -0.973, and the asymptotic significance (p-value) obtained was o.330 [17]. We do not reject the null hypothesis because the p-value is greater than 0.05. This leads to the conclusion that there is insufficient evidence to conclude a significant difference between K2 and K3 EDTA tubes [18–21]. Using the Bablok regression analysis, we obtained Y=0.000+1.000x, indicating a proportional relationship between the two variables [22]. The cumulative sum test (p=0.88) revealed that the intercept A and slope B were 0.000 (95% confidence interval) and 1.000, respectively, with no significant deviation from linearity.

Furthermore, the random difference, as measured by the residual standard deviation RSD, was within acceptable limits [23]. These findings imply that the measurements from the two statistical methods have a proportional relationship with no systematic or random variation. The CUSUM (or cumulative sum control chart) confirmed the validity of the linear model, thereby bolstering the reliability of the regression results. One possible limitation of our study is that we did not check the occurrence of other haemoglobin alternatives that could impact HbA1c measurements [24, 25]. Interference may occur depending on the manufacturer. All possible interferences, such as HbF and Hbs, will be listed in production units. However, the purpose of our study is to compare the HbA1c

concentrations of two samples from the same patient. It is not uncommon to find K2 and K3 EDTA tubes in the same laboratory, particularly in recent years [26–29].

This study has several limitations that must be acknowledged. One key limitation is the exclusion of patients with hemoglobinopathies, such as sickle hemoglobin (HbS) and fetal hemoglobin (HbF). These abnormal haemoglobin variants can significantly interfere with the accuracy of HbA1c measurements, particularly when using high-performance liquid chromatography (HPLC) methods, and their exclusion may limit the generalizability of the findings to broader patient populations. Additionally, the study was conducted in a single-centre setting, which may introduce centre-specific biases and limit the external validity of the results. Consequently, larger, multi-center studies are necessary to validate the findings across diverse demographic and clinical settings.

Future research should focus on evaluating the long-term stability of HbA1c in blood samples collected using K2- and K3-EDTA anticoagulants under various storage conditions, including different temperatures and durations, to better inform pre-analytical handling protocols. Furthermore, it would be valuable to investigate the influence of different anticoagulants on HbA1c measurements across a variety of analytical platforms, such as immunoassays, capillary electrophoresis, and enzymatic assays. Such comparative analyses would help determine the consistency and reliability of HbA1c results regardless of the method or anticoagulant used, thereby enhancing clinical decision-making and laboratory standardization.

Conclusion

From the previously reported literature, both K2 and K3 have shown both negative and positive correlations, but not supported by absolute evidence stating the interference of the anticoagulant with the levels of glycosylated haemoglobin. Overall, our study concludes that there is an absence of any clinically and statistically noteworthy difference between K2 and K3 EDTA tubes. Since our study was conducted in a localized centre, future research may focus on covering the global population with a maximized sample size.

Conflict of interest

The authors declare no conflict of interest.

Ethics approval

This study was conducted as an internal method comparison and quality assurance activity for laboratory procedures. As such, in accordance with the policies of the Institute at the time of the study, such internal quality control analyses were exempt from formal review by the Institutional Ethics Committee. The procedures followed were in accordance with the ethical standards.

Consent to participate

Informed consent was waived for this study by the institutional policy, as it utilized leftover anonymized blood samples from routine clinical testing. All patient data were fully anonymized prior to analysis.

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