

# Evaluation and Validation of Utility of BD Glucose Vacutainer for Glycosylated Hemoglobin Assay for Timely Therapeutic Management of Diabetes Mellitus

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## Abstract

Blood glucose and glycosylated haemoglobin are the two vital biochemical tests widely prescribed for the therapeutic management of diabetes and require different anticoagulants during specimen collection. If both tests can be performed using single vacutainer, it reduces the laboratory costs at a larger scale and facilitates timely intervention. The anticoagulants NaF/Na<sub>2</sub> EDTA and K2 EDTA are used conventionally for blood glucose and HbA1c estimations respectively for specimen collection. In the current study we have investigated whether NaF/Na<sub>2</sub> EDTA can be used for HbA1c assay without the need of separate vacutainer i.e.K2EDTA. A total of 125 subjects (25 non-diabetic and 100 diabetic) were enrolled for this study. Parallel samples were collected in NaF/Na<sub>2</sub> EDTA and K2 EDTA to compare HbA1c levels in both the anticoagulants. The stability of HbA1c was elucidated in both anticoagulants till 72hrs of collection. The HbA1c levels in both anticoagulants showed excellent correlation ( $R^2$ : 0.998). Bland and Altman analysis revealed that the bias between methods is -0.22 to +0.22%. The changes in HbA1c levels were unaltered till 72 hrs of collection in NaF/Na<sub>2</sub>EDTA and K2EDTA (mean SD: 0.066 vs. 0.058; %CV: 0.94 vs. 0.81). These results conclude that the same vacutainer used for glucose estimation can be used even for HbA1c till 72 hr of collection thus preventing the need of second sampling specifically in monitoring diabetic control. This study emphasized that a single vacutainer of NaF/Na<sub>2</sub>EDTA can be used safely for both glucose and HbA1c assays.

**Keywords:** NaF/Na<sub>2</sub> EDTA; K2 EDTA; Glucose vacutainer; HbA1c assay; Diabetes.

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## INTRODUCTION

The normal adult haemoglobin HbA consists of 2  $\alpha$  and 2  $\beta$  chains ( $\alpha_2$  &  $\beta_2$ ) and make upto 97% of normal adult haemoglobin. By the process of post translational modification, HbA is modified into minor types- HbA1a, HbA1b, HbA1c, of which HbA1c is the abundant form. Hb A1c is formed by a non enzymatic glycation process and occurs in two steps i.e reversible binding as an aldimine/schiff base and irreversible binding through amadori rearrangement to form a ketoamine linkage [1]. The process of post-translational modification of HbA to HbA1c occurs at a slow rate throughout the lifespan of RBC. In 1976, HbA1c assay is proposed for the first time as a biomarker for monitoring the levels of glucose among diabetic patients [2].

In recent years, globally India has emerged as one of the epicentres of diabetic mellitus pandemic with more than 69.2 million diabetic individuals in a total population of 1339.2 million accounting for an incidence of 5.2% [3]. Glycosylated haemoglobin (HbA1c) and fasting blood glucose are the two standard biochemical tests that are used for the diagnosis and therapeutic management of diabetes. Physicians are tailoring the diabetes therapy based on HbA1c levels in type 2 diabetes with HbA1c levels between 7.0-7.4% for monotherapy (metformin preferably), HbA1c between 8.0-8.4% for dual therapy and HbA1c between 9.0-9.4% for triple therapy [4]. Poor glycemic control was reported to increase incidence of severe hypoglycaemia, diabetic ketoacidosis, and increase risk for microvascular complications such as nephropathy and neuropathy in type 1 diabetes [5]. Fasting blood glucose >172mg/dl and HbA1C>9.0% were shown to

be associated with microalbuminuria [6]. In view of the pivotal role of these two biochemical tests as surrogate markers for diabetic related complications, frequent monitoring is mandatory.

Sodium fluoride and potassium oxalate was the conventional anticoagulants for glucose vacutainer where in sodium fluoride acts as inhibitor of glycolytic enzymes. Recently, potassium oxalate is replaced by Na<sub>2</sub> EDTA as it precipitates as calcium oxalate, which is toxic. A small pilot study on three diabetic and three non-diabetic samples evaluated the stability of HbA1c in EDTA, heparin, citrate and fluoride and found no significant changes when sample was stored at -4°C for seven days [7]. Another study found no significant difference in HbA1c or its stability between parallel post-mortem samples collected in EDTA and sodium fluoride vacutainers and stored at 4°C [8].

As there are a very few studies that substantiate the use of glucose vacutainer for HbA1c and all the available studies have limitations such as sample size, lack of validation studies across broad coverage of HbA1c, and have not evaluated stability of HbA1c at the ambient temperature.

The current study was aimed to substantiate the utility of glucose vacutainer for HbA1c assay by addressing these lacunae in a systematic manner: i) By incorporating both diabetic and non-diabetic subjects, ii) By covering different ranges of HbA1c i.e. (<6.0%, 6.0-8.0%, 8.1-10.0%, 10.1-12.0% and 12.1-16.0%); iii) By evaluating the reproducibility and repeatability of HbA1c, iv) By studying the stability of HbA1c at ambient temperature till 72 hrs of sample collection.

## MATERIALS AND METHODS

### Recruitment of Subjects

A total of 125 subjects, which includes 25 (15 men and 10 women) non-diabetic controls and 100 (60 men and 40 women) diabetic patients were enrolled for this study at the out-patient department of Nizam's Institute of Medical Sciences, Hyderabad, India. The mean ages of non-diabetic and diabetic subjects were 55.8±11.9 yrs and 59.2±12.9 yrs, respectively. The Institutional Ethical Committee of Nizam's Institute of Medical Sciences, Hyderabad, India approved the study protocol. Informed consent was obtained from all the study participants.

### Specimen collection and Biochemical testing

Whole blood samples were collected in two Becton Dickinson (BD) vacutainers i.e. NaF/Na<sub>2</sub> EDTA (3mg/6 mg) and K2 EDTA (5.4 mg) from each participant. In these parallel samples, HbA1c levels were measured by ion exchange high performance liquid chromatography (HPLC) using Bio-Rad D-10™ Dual program. The samples are automatically diluted on the D-10 and injected into the analytical cartridge. The D-10 delivers a programmed buffer gradient of

increasing ionic strength to the cartridge, where the hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell of the filter photometer, where changes in the absorbance 415 nm are measured. The HbA1c area is calculated using an exponentially modified Gaussian (EMG) algorithm that excludes the labile A1c and carbamylated peak area from the A1c peak area. In five representative samples stored at ambient temperature, HbA1c is repeated at 24 hr, 48 hr and 72 hr after collection. In two representative samples, assay was performed in 20 replicates to evaluate repeatability and reproducibility.

## STATISTICAL ANALYSIS

Spearman rank correlation coefficient (r) was used to compare HbA1c levels in both vacutainers. Paired t-test was used to assess whether there is any statistically significant difference between HbA1c levels in both vacutainers. Bland and Altman analysis was performed by plotting average vs. difference plots that indicate the bias between parallel samples. Coefficient of variation (CV%) was used to calculate the precision in HbA1c measurement. Paired t-test was used to study stability of HbA1c at ambient temperature up to 72 hrs of blood collection.

## RESULTS

### Comparison of HbA1C in NaF/Na<sub>2</sub> EDTA and K2 EDTA

In non-diabetic subjects, we observed HbA1c levels in the range of 4.8-6.0%. The HbA1c levels in this group showed a correlation coefficient of 0.96. In all the diabetic subgroups, the correlation coefficient value is consistent i.e. 0.99. Cumulatively, across the broad range of 4.8-16.0% HbA1c the correlation was 0.99 (Figure-1). The regression equation showed a slope value of 0.998 and intercept value of -0.023.

Paired t-test showed that mean difference between both methods is 0.041 (95% CI: 0.021-0.061). The HbA1c levels in K2 EDTA and NaF/Na<sub>2</sub> EDTA were 9.08±2.92% vs. 9.04±2.92% (p<0.0001), respectively.

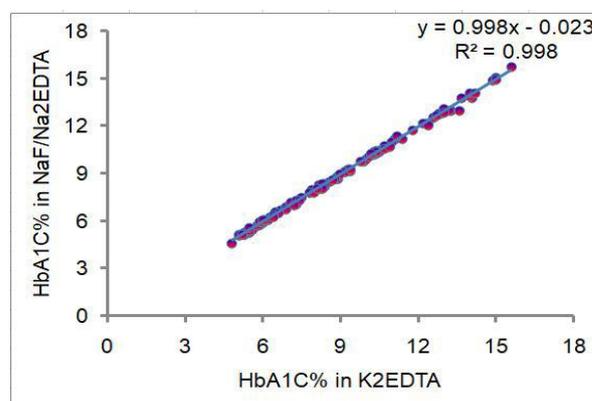
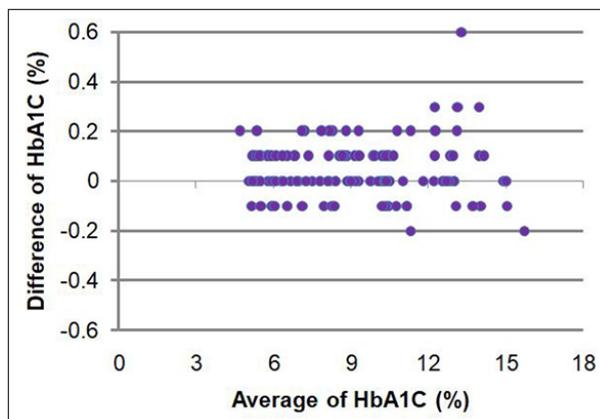


Fig-1: Correlation between NaF/Na<sub>2</sub> EDTA and K2 EDTA anticoagulants

This illustrates correlation plot of HbA1c levels in NaF/Na<sub>2</sub> EDTA and K<sub>2</sub> EDTA. The correlation coefficient is 0.99. The regression equation depicted a slope value of 0.998 and intercept value of -0.023.

**Bland and Altman analysis**

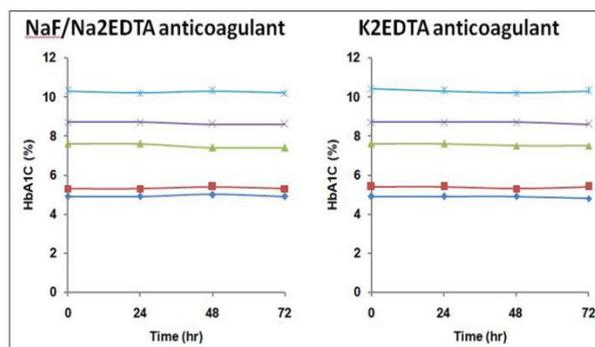


**Fig-2: Bland and Altman analysis of HbA1c levels in NaF/Na<sub>2</sub> EDTA and K<sub>2</sub> EDTA anticoagulants**

Bland and Altman analysis was performed by plotting average vs. difference between HbA1c levels in NaF/Na<sub>2</sub> EDTA and K<sub>2</sub> EDTA. The bias between the methods was ±0.02.

As shown in Figure-2, average vs. difference plot was computed to assess the bias in measuring HbA1c levels in both the anticoagulants. The bias between methods is -0.22 to +0.22%.

**Stability of HbA1c at ambient temperature**



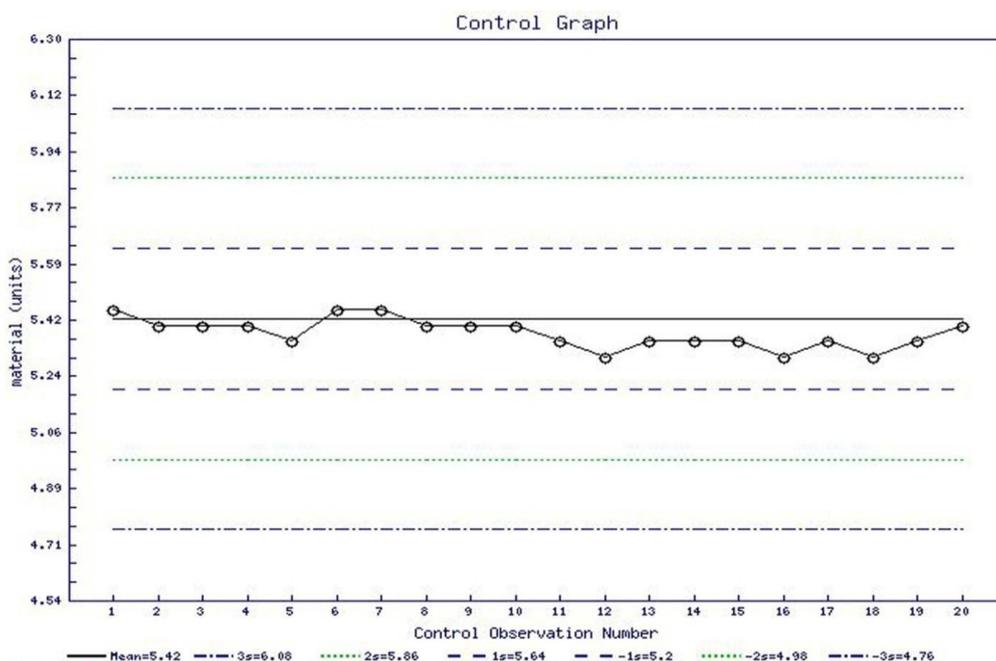
**Fig-3: Stability of HbA1c at ambient temperature NaF/Na<sub>2</sub> EDTA and K<sub>2</sub> EDTA anticoagulants**

In five representative subjects, parallel samples of NaF/Na<sub>2</sub> EDTA and K<sub>2</sub> EDTA were used to measure HbA1c at different time intervals till 72 hrs of sample collection. No significant difference was obtained from the time of collection to till 72 hrs.

As shown in Figure 3, five representative samples whose HbA1c levels are in the range 4.9-10.4% were tested for stability at room temperature at 24 hr, 48 hr and 72 hr after sample collection. In K<sub>2</sub> EDTA, the CV ranged from 0.58-1.02% while in Na<sub>2</sub> EDTA the CV ranged from 0.55-1.53%.

**Repeatability and reproducibility**

Two representative samples were processed 20 times to check their repeatability and reproducibility. The coefficient of variation was 0.71% and 1.56% in these two samples. The measurement of uncertainty was 0.076 and 0.16 in these samples. Levy Jenning plot of the mean of both samples in 20 different runs showed values within 1 SD (Figure-4).



**Fig-4: Levy Jenning Plot showing inter-assay precision**

The mean values of two representative samples processed in 20 runs were depicted in the Levy Jenning plot. The values are within 1SD.

## DISCUSSION

This is the first systematic investigation in the clinical utility of glucose vacutainer for HbA1c measurement. This study substantiates that same vacutainer can be used for glucose and HbA1c measurements with high precision across a broad range of HbA1c (4.8-16%). The HbA1c levels were stable till 72 hr of collection at ambient temperature. Although, mean difference between both the methods i.e. 0.04 is statistically significant, it will not have practical implications on its clinical utility as the difference is small. The slope value of 0.998 and intercept value of -0.023 in the regression equation substantiate this statement further. This is consistent with findings of Chakraborty *et al.*, who demonstrated slope value of 1.00 and intercept value of 0.14 in the regression model of HbA1c in EDTA vs. Fluoride vacutainers [9]. In addition to the three studies mentioned earlier, two more studies compared HbA1c levels in sodium fluoride and K2 EDTA in limited number of subjects, which are in agreement with our findings [10, 11].

Vrtaric A and his colleagues have observed that there is no clinically and statistically significant bias between K2-EDTA and K3-EDTA HbA1c measurements [12]. Also, Sarmah D *et al.*, have demonstrated that HbA1c values in fresh and stored whole blood sample does not change when analysed in K3-EDTA, Na-citrate, lithium-heparin and Na-fluoride/Na<sub>2</sub> EDTA anticoagulant vials [13]. Recently, Kumawat R and her colleagues have also observed that there was no significant difference in the HbA1c values in samples collected in sodium fluoride/potassium oxalate and EDTA tubes at the time of initial estimation and after 7 days of collection under proper storage [14].

Our study demonstrated that there was no significant difference in the HbA1c values when it was measured in NaF/Na<sub>2</sub> EDTA. In view of increasing incidence of diabetes mellitus in India, there is an increase in the numbers of HbA1c test requests from the treating physicians. It is known that HbA1c test is costlier than blood glucose test; use of a separate K2 EDTA vacutainer for HbA1c testing increases the cost further. If both plasma glucose and HbA1c can be performed from the same NaF/Na<sub>2</sub> EDTA vacutainer, there is no need of additional blood sample which in turn reduces the cost associated with HbA1c testing.

The major strengths of the current study are: i) Inclusion of non-diabetic and diabetic subjects, ii) Equal representation of subjects across HbA1c measurable range of 4.8-16%, iii) Comparison of HbA1c levels between both vacutainers using different statistical approaches such as correlation coefficient, regression equation, paired t-test, etc, iv) Demonstration

of repeatability and reproducibility in HbA1c, and v) Stability assessment at ambient temperature up to 72 hrs of blood sample collection.

## CONCLUSION

The current study along with the previous pilot studies emphasized that single BD glucose vacutainer is sufficient and can be safely used for HbA1c measurement from whole blood and glucose measurement from plasma after centrifugation of same sample, which is cost-effective in terms of laboratory perspective, particularly medium and large-sized laboratories, and facilitates timely therapeutic intervention in terms of clinical perspective. Future studies are warranted to examine the utility of glucose vacutainers in non-HPLC based HbA1c assays.

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