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Stability and reliability of glycated haemoglobin measurements in blood samples stored at −20 °C

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ABSTRACT

Aim: To validate the stability of glycated haemoglobin (HbA1c) measurements in blood samples stored at −20 °C for up to one month.
Methods: The study group comprised 142 type 2 diabetic subjects visiting a tertiary centre for diabetes at Chennai city in south India. The HbA1c assay was done on a fasting blood sample using the Bio-Rad Variant machine on Day 0 (day of blood sample collection). Several aliquots were stored at −20 °C and the assay was repeated on the 3rd, 7th, 15th, and 30th day after the sample collection. Bland–Altman plots were constructed and variation in the HbA1c levels on the different days was compared with the day 0 level.
Results: The median differences between HbA1c levels measured on Day 0 and the 3rd, 7th, 15th, and 30th day after blood collection were 0.0%, 0.2%, 0.3% and 0.5% respectively. Bland–Altman plot analysis showed that the differences between the day '0' and the different time points tend to get larger with time, but these were not clinically significant.
Conclusions: HbA1c levels are relatively stable up to 2 weeks, if blood samples are stored at −20 °C.

1. Introduction

Glycated haemoglobin (HbA1c) is the most widely used test to assess long term diabetes control (International Expert Committee, 2009; Sacks et al., 2002; WHO Consultation, 2011). HbA1c has also been recently approved as a diagnostic and screening test for diabetes (American Diabetes Association, 2005; Goldstein et al., 2004). Elevated HbA1c levels are strongly associated with microvascular complications in both type 1 diabetes (T1DM) as well as type 2 diabetes (T2DM) (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998)

Current clinical chemistry guidelines suggest that whole blood samples remain stable for at least 1 year when stored at −70 °C. However, HbA1c is usually measured on fresh blood samples (Jones et al., 2004). Moreover, −70 °C deep freezers are not available at most centres, especially in developing countries with limited resources whereas −20 °C freezers, being less expensive, are more widely available. In large population based studies, it is often difficult to measure HbA1c on the day of blood sample collection, necessitating the use of stored samples (Blake et al., 2004; Meyer et al., 2003; Schulze, Rimm, Shai, Rifai, & Hu, 2004). In the ongoing national Indian Council of Medical Research India Diabetes (ICMR-INDIAB) study, samples from all parts of the country are sent to a centralised lab at Chennai, South India and usually a week or two elapses before the HbA1c assay is performed (Anjana et al., 2011a, 2011b). However, for many assays used to measure HbA1c, significant amounts of degradation products from long term storage may compromise the integrity of the assay and the reproducibility of the measurements. This could have serious implications if HbA1c is used as a diagnostic test in epidemiological and clinical studies. Finally, conditions, temperature, humidity and storage conditions vary in developing countries. To the best of our knowledge, some studies have not been published from developing countries like India where 80% of people with diabetes live. We therefore undertook this study to assess the reproducibility and accuracy of HbA1c measurements from whole blood samples that had been stored at −20 °C for periods up to one month.

Conflict of interest: None declared.

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Table 1
Clinical characteristics of type 2 diabetic subjects (n = 142).

Variables	mean ± SD
Males, n (%) *	87 (61.2%)
Age (years)	57 ± 11
Duration of diabetes (years)	12 ± 8
Body mass index (kg/m^2)	26.9 ± 3.8
Waist circumference (cm)	94 ± 10
Systolic blood pressure (mm Hg)	134 ± 18
Diastolic blood pressure (mm Hg)	80 ± 8
Fasting plasma glucose (mg/dL)	132 ± 37
Glycated haemoglobin (%)	7.7 ± 1.2
Total serum cholesterol (mg/dL)	149 ± 38
Serum triglycerides (mg/dL)	141 ± 88
Serum HDL cholesterol (mg/dL)	40 ± 9
Serum LDL cholesterol (mg/dL)	80 ± 29
Serum urea	26 ± 11
Serum creatinine	0.8 ± 0.3
Treatment	
Oral hypoglycaemic agents, n(%) *	91 (64.1%)
Insulin and oral hypoglycaemic agents, n(%) *	51 (35.9%)

2. Materials and methods

One hundred and forty two consecutive T2DM subjects above 20 years of age attending a tertiary diabetes care centre at Chennai in southern India were enrolled into the study. Along with the blood sample, clinical details including age, sex and duration of diabetes were recorded. A venous sample was drawn in EDTA-containing tubes in all subjects for assessment of HbA1c. After measurement of HbA1c on the day of blood collection (Day 0), all blood samples were centrifuged within 1 h of collection, and the aliquots were stored in a deep freezer at −20 °C for a period of one month. The HbA1c assay was repeated on the 3rd, 7th, 15th and 30th day after the blood collection. At the time of analysis, 0.5 μl of whole blood samples was taken and reconstituted with 1 ml of deiodinised water. HbA1c was estimated by high-pressure liquid chromatography (HPLC) using the Variant II Turbo machine (Bio-Rad, Hercules, CA). All the assays were carried out by the same team of laboratory technicians using the same method throughout the study period. Our centre participates in the Unity Program of Bio-Rad HBA1C standardisation and is also certified by the College of American Pathologist (CAP) and National Academy of Biological Lab (NABL) by the Govt of India. The CV for HbA1c assay in house quality control was <2.5% indicating good reproducibility.

3. Statistical analysis

The reproducibility of the HbA1c values across each follow up time point was assessed using the day 0 HbA1c value as the gold standard. Scatter plots were drawn to find the correlation between HbA1c values at different time periods. Intra-Class Correlation Coefficient (ICC) was computed to test reliability. Bland–Altman plot analysis was used to find the agreement between HbA1c in fresh and stored blood at each time point. 95% limits of agreement are obtained as the mean ± 1.96 SD. Regression equations were derived for day 0 HbA1c using the HbA1c values at different time points. All statistical analyses were done using SPSS (version 15.0).

4. Results

The mean age of the study subjects (n = 142) was 57 ± 12 years (range: 23–91 years) and 61.2% were males. The clinical characteristics of the study subjects are presented in Table 1. The mean duration of diabetes was 12 ± 8 years. The mean HbA1c of the study subjects was 7.7% ± 1.2% (day 0), and the mean fasting plasma glucose was 132 ± 37 mg/dl. Ninety-one patients (64.1%) were on treatment with oral hypoglycaemic agents (OHA), and the remainder were on a combination of OHA and insulin.

The median differences in HbA1c levels between day 0 and the 3rd, 7th, 15th, and 30th days were 0.0, 0.2, 0.3 and 0.5 respectively as shown in Table 2.

Scatter plot comparison of the HbA1c levels showed that there was no significant underestimation or overestimation of HbA1c levels measured on 3rd, 7th, 15th and 30th days (Fig. 1). The correlations between measurements on different time points were high (day 0 vs day 3, r = 0.99; day 0 vs day 7, r = 0.99; day 0 vs day 15, r = 0.99 and day 0 vs day 30, r = 0.94; $p < 0.0001$), as shown in Table 3. The intra class correlation (ICC) of all five measured time points was 0.94 (95% CI: 0.87–0.97), $p < 0.001$, and the pairwise comparisons showed that the ICC was over 0.95 for comparisons between day 0 and the 3rd, 7th and 15th day, whereas it decreased to 0.85 at the 30th day.

Fig. 2 shows the Bland–Altman plots displaying the differences in HbA1c values at different time periods compared to day 0, to show the magnitude of disagreement. The mean differences between day 0 and 3rd, 7th, 15th and 30th day were 0.11, 0.17, 0.25 and 0.51 respectively. These plots reveal a slightly wider distribution of the HbA1c measurements with increasing time periods particularly by day 30 but up to day 15, the variations are quite small.

Fig. 3 shows the regression slope for HbA1c in the difference over time, which helps to estimate the Day 0 value from the stored specimens. The slope is, Day 0 HbA1c value = (0.016 × stored HbA1c value) + 0.019.

5. Discussion

Our data demonstrate that reliable HbA1c measurements can be obtained from whole blood samples stored at −20 °C for up to two weeks. The median difference observed was 0.5% after a storage period of 30 days. The College of American Pathologists (CAP) has graded the accuracy for future reduction of HbA1c based on the NGSP 2011–2012 survey and the acceptable limit reported for error was ± 7% of the target value (National Glycohemoglobin Standardization Program, 2015). Previous studies have shown that exposure of whole blood samples to improper temperature conditions can cause erroneous HbA1c results and that, in general, ion-exchange assay methods tend to be more sensitive to storage issues than boronate affinity or immunoassay methods (Little, England, Wiedmeyer, & Goldstein, 1983; Little, England, Wiedmeyer, & Goldstein, 1986; Little,

Table 2
Descriptive statistics for median differences in HbA1c levels (n = 142).

Time	Median value (Range)	Bootstrap Confidence Interval	Median Difference[a]	Interquartile Range of differences	
				25th Percentile	75th Percentile
Day 0	7.6 (4.8–10.5)	7.4–7.8	–	–	–
Day 3	7.6 (5.0–10.4)	7.3–7.9	0.0	0.0	0.3
Day 7	7.4 (4.8–10.3)	7.2–7.6	0.2	0.1	0.3
Day 15	7.3 (4.8–10.1)	7.1–7.5	0.3	0.2	0.4
Day 30	7.1 (4.9–9.7)	6.8–7.4	0.5	0.2	0.8

[a] The reported percentage changes are absolute.