ORIGINAL ARTICLE

The Effect of Storage at -20°C and -80°C on HbA1c Measurements in Whole Blood Samples Analysed Using Bio-Rad D-10

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ABSTRACT

Introduction: Glycated haemoglobin (HbA1c) is a test commonly measured in many clinical laboratories to monitor and diagnose diabetes mellitus (DM). The laboratory in Hospital Tengku Ampuan Afzan (HTAA) receives HbA1c samples internally and from several other hospitals and clinics in the state of Pahang, at which, before transportation and analysis, the samples are often stored. This study determined the stability of HbA1c samples following different storage temperatures and duration for up to 30 days. **Method:** Whole blood samples for HbA1c analysis were collected from 222 healthy blood donors and type 2 DM (T2DM) patients. Each sample was prepared into four aliquots, which were then stored at temperatures -20°C and -80°C. HbA1c analyses were performed at baseline, days 15 and 30 using ion-exchange high-performance liquid chromatography (HPLC) assay technique on Bio-Rad D-10 analyser. HbA1c levels following sample storage were compared to the levels at baseline. **Results:** The baseline HbA1c (mean±SD) was 6.6±2.2%. At -20°C of storage, the HbA1c levels decreased with a mean difference of 0.1% and 0.3% on days 15 and 30, respectively. Storage at -80°C resulted in a mean difference of 0.1% on day 15 whilst no change was noted on day 30. The number of samples that showed clinically significant HbA1c change (defined as a change of >0.5%) was less for the samples stored at -80°C (4.5%) as compared to -20°C (23%). **Conclusion:** HbA1c concentration is affected by temperature and duration of sample storage. In instances where prolonged storage of HbA1c samples is expected, storage at -80°C is preferable.
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Keywords: Diabetes mellitus, HbA1c, High-performance liquid chromatography, Stability, Storage

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INTRODUCTION

Glycated haemoglobin (HbA1c) is a biomarker widely used for monitoring glycaemic control and for the diagnosis of diabetes mellitus (DM). Compared to other biomarkers, HbA1c offers the advantage of sample stability, low intra-individual variability and eliminates the necessity for a fasting sample (1). It reflects the average blood glucose level for the past eight to 12 weeks and the level correlates well with the development of diabetic complications (1). Measurement of HbA1c in Malaysia's public healthcare system is often performed centrally as batch-test analysis in identified biochemistry laboratories nationwide. The laboratory of Hospital Tengku Ampuan Afzan (HTAA) is one of the designated laboratory for HbA1c analysis. Generally, the laboratory receives HbA1c samples internally as well as from several healthcare centres in the state of Pahang, Malaysia. In HTAA, the analysis is performed bi-weekly in batches using an ion-exchange high-performance liquid chromatography (HPLC) method on Bio-Rad D-10 (Bio-Rad Laboratories). Normally, the blood samples are stored at 4-8°C before transportation or analysis. In some situations, the storage intervals were prolonged, exceeding the manufacturer's recommendation of seven days at 2-8°C or three days at 15-30°C (2). The reasons for the prolonged storage include delayed transportation of samples from peripheral collection centres, instrument malfunction and the need for re-testing (3). The presence of high degradation products from prolonged storage and improper temperature conditions may compromise the integrity and the reproducibility of HbA1c measurements (4).

Several studies had looked at the effect of sample storage on HbA1c measurements. The studies vary in terms of duration and conditions of sample storage, characteristics of subjects included (only diabetics, only non-diabetics or both) and methods for HbA1c measurement. The studies reported that, for prolonged storage, the samples were best stored at temperatures of -70°C or lower, with reported stability between 12 weeks to up to one year of storage (5, 6). On the other

hand, studies looking at HbA1c sample storage at -20°C are limited, despite it being more readily available as compared to freezers with temperatures of -70°C or lower. Thus, this study aimed to evaluate the effects of HbA1c sample storage at -20°C and -80°C up to 30 days of storage in both diabetic and non-diabetic subjects, which would allow a wide range of HbA1c levels to be assessed. The result of this study will aid our laboratory to optimise the storage temperature and duration for HbA1c samples from various centres, especially in cases where prolonged sample storage is expected.

MATERIALS AND METHODS

Sample size and subject recruitment

Using universal sampling, 222 subjects were recruited either i) among blood donors of HTAA during their donation day or ii) among T2DM patients during their routine clinic follow-up between August 2018 – December 2018. The inclusion criteria were consented Malaysian citizens aged 18-year-old and above. Insufficient sample volume and individuals with condition(s) known to affect HbA1c analysis were excluded. A questionnaire consisting of questions on their medical history and demographics was given to the subjects with all subjects giving informed written consent.

Sample preparation

Non-blood donor subjects had a blood sample taken from the antecubital fossa whereas, for the blood donors, the sample collection was from the donor diversion pouch bag. The samples were collected into EDTA tubes and were immediately brought to the laboratory and processed. Each sample was aliquoted into four aliquots (minimum of 1.5 ml each), labelled and stored accordingly, as shown in Table I, within two hours of sample collection. The baseline HbA1c results were obtained from the analysis of fresh samples before storage.

Sample analysis

The HbA1c analysis was performed on a single ion-exchange HPLC analyser (Bio-Rad D-10), which is certified by the National Glycohemoglobin Standardization Program (NGSP) and International Federation of Clinical Chemistry (IFCC) according to the laboratory's standard operating procedure. Internal quality control was performed using the Biorad Lypocheck Diabetes Control before each run as per the lab practice.

Table I: Sample storage duration and temperature

Description	Record code
Baseline sample	A0
Stored at -20°C and measured after 15 days	A1
Stored at -20°C and measured after 30 days	A2
Stored at -80°C and measured after 15 days	B1
Stored at -80°C and measured after 30 days	B2

Data analysis

Statistical calculations were performed using the standard statistical software package, IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp. A paired-sample t-test was performed to compare the HbA1c levels on day 0 and following storage at various storage conditions whilst linear regression analysis was performed to determine their correlations. Bland-Altman plots were constructed to determine the agreement between HbA1c levels on day 0 and following sample storage. The y-axis is the difference between HbA1c levels on day 0 and after storage [HbA1c levels on storage – HbA1c levels on day 0], whilst the x-axis is the average HbA1c = [HbA1c levels on day 0 + HbA1c levels on storage (%)]/2. 95% limits of agreement were obtained as mean ± (1.96 x SD). The mean bias for HbA1c between the fresh and stored samples was calculated using the following formula (6).

$$\text{Mean Bias (\%)} = \frac{\text{(Mean HbA1c on fresh sample – Mean HbA1c after storage)}}{\text{Mean HbA1c baseline}} \times 100\ \%$$

Ethics

Ethical approval was obtained from the Medical Research Ethical Committee (MREC) Ministry of Health.

RESULTS

Out of 222 samples, 145 (65.3%) were obtained from those with known T2DM diagnoses. The HbA1c mean±SD at baseline was 6.6±2.2%. The lowest and highest HbA1c levels were 4.0% and 14.0%, respectively. Table II shows the mean±SD and means differences in HbA1c levels on sample storage. At -20°C of storage, the HbA1c level significantly decreases with increasing storage time, with a mean of 6.6±2.0% and 6.4±2.0% on days 15 and 30, respectively ($p<0.001$). At -80°C, the mean HbA1c on day 15 was significantly higher, with a mean of 6.7 ±2.1% ($p<0.001$) whilst on day 30 the mean was 6.6 ±2.1%. The mean difference was largest when the samples were stored at -20°C for 30 days.

Fig. 1 shows the linear regression analysis plot of the HbA1c levels on day 0 and following storage at various storage conditions. The correlations between the HbA1c measurements were excellent. At -80°C, r=0.992 and r=0.994 for day 0 vs day 15 and day 0 vs day 30, respectively. For sample storage at -20°C, r=0.989 and r=0.986 for day 0 vs day 15 and day 0 vs day 30, respectively. Fig. 2 shows the Bland-Altman plots for the measurements of HbA1c in samples stored at -20°C and -80°C compared with day 0. All the figures revealed a wider distribution of the HbA1c measurements with increasing HbA1c concentrations.

Table II: Mean and mean difference of HbA1c value at different storage temperatures and duration

Storage Temperature	Day	HbA1c (%) Mean ±SD	Mean difference	95% Confidence Interval for difference	t	p-value
Baseline	0	6.6 ±2.2	-	-		
-20°C	15	6.6 ±2.0	-0.1	-0.13 – -0.56	-5.116	<0.001
	30	6.4 ±2.0	-0.3	-0.31 – -0.23	-12.130	<0.001
-80°C	15	6.7 ±2.1	0.1	0.03 – 0.09	4.399	<0.001
	30	6.6 ±2.1	0	-0.07 – -0.02	-3.503	0.001

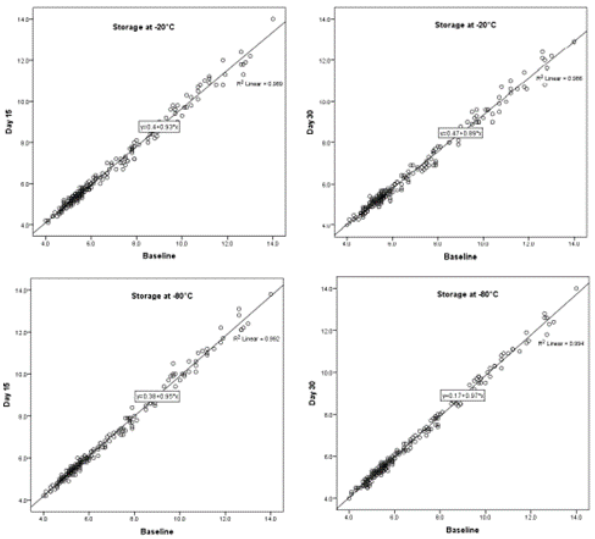

Figure 1: Scatterplot comparison between HbA1c levels on day 0 and days 15 and 30

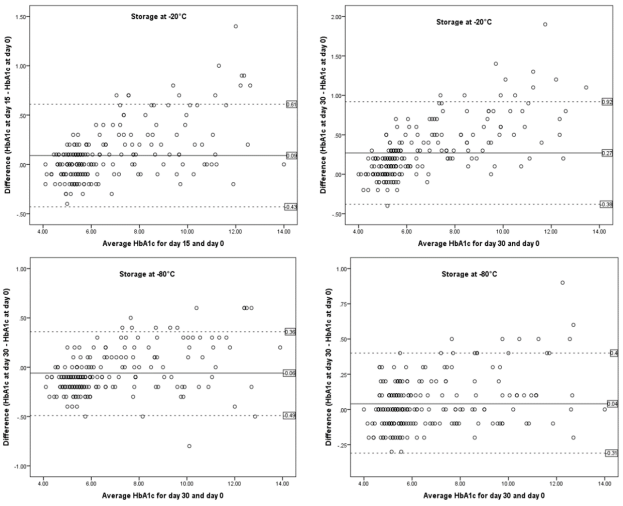

Figure 2: Bland-Altman plots for the measurements of HbA1c in samples stored at -20°C and -80°C compared with day 0. The horizontal line in the middle is the mean difference and the dotted lines above and below it are the mean ± 1.96 SD

The mean bias was highest in the stored HbA1c samples at -20°C on day 30, followed by samples on day 15 of the same temperature (Table III). However, the mean biases were lower in the samples stored at -80°C for both storage duration.

An absolute difference of HbA1c level of ≥0.5% was considered clinically significant (6). Fig. 3 shows the number of samples with and without clinically significant change in HbA1c. The majority demonstrated no clinically significant change in HbA1c levels on storage. However, storage at -80°C had a lower percentage of samples with a clinically significant change in HbA1c level (n=10, 4.5% at day 15 and n=7, 3.2%, at day 30) as compared to those stored at -20°C (n=23, 10.4%, at day 15 and n=52, 23.4%, at day 30).

Table III: Calculated bias for each storage condition

Temperature	Time	Mean Bias (%)
-20°C	Day 15	1.39
	Day 30	4.09
-80°C	Day 15	0.95
	Day 30	0.66

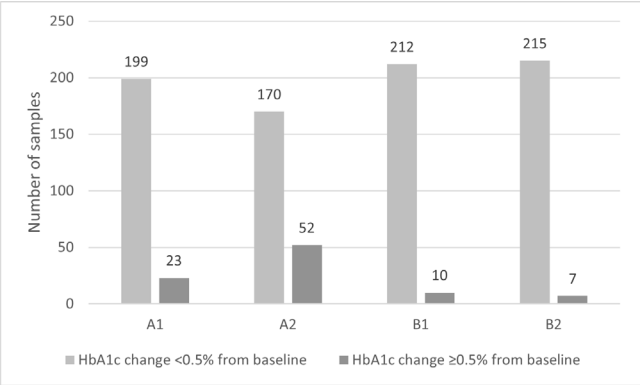

Figure 3: Samples with and without clinically significant change in HbA1c on storage. A difference in HbA1c level compared to the baseline value of 0.5% or more was considered a clinically significant change. A1= storage at -20°C for 15 days, A2= storage at -20°C for 30 days; B1= storage at -80°C for 15 days, B2= storage at-80°C for 30 days.

DISCUSSION

This study observed that samples stored at -80°C demonstrated the least change in HbA1c. This was comparable with a previous study which demonstrated that samples taken from patients with and without DM and stored at -80°C were stable up to 12 weeks (6). An even longer study period (up to 1.5 years) was demonstrated by Liotta et al 2013, with HbA1c measurement performed using HPLC ion-exchange chromatography, but on the ADAMS A1C HA8160 platform (7). In their study, the mean HbA1c±SD at baseline and after one year of storage was 6.9±1.2% and 6.6±1.1%, respectively. A further 6 month-refrozen

storage resulted in a mean HbA1c of 6.4±1.0 % (7).

On the other hand, studies looking at the effect of sample storage at -20°C are limited with a study demonstrating stability only for up to two weeks (4). Storage beyond two weeks resulted in large variability in HbA1c level, as compared to pre-storage level, with discrepancies between HbA1c levels on storage compared to baseline increased with the increasing length of storage (4). The reported mean differences between day 0 and day three, seven, 15, and 30 were 0.0, 0.2, 0.3, and 0.5%, respectively (4). In our study, we obtained a much lower mean difference on day 15 (0.1%) whilst a mean difference of 0.3 was obtained on day 30. In contrast, they demonstrated a mean difference of 0.2 as early as day seven. The higher HbA1c levels in their samples (median HbA1c 7.6% (range 4.8 – 10.5%) could contribute to the earlier changes seen in sample storage. This was also demonstrated by Liotta et al. 2013 where the storage of samples with higher HbA1c levels tends to result in a statistically significant greater error suggesting a higher rate of degradation of these samples and hence underestimation of the HbA1c on storage (7). The exact mechanisms for these changes are nevertheless unclear.

In terms of bias, samples stored at -20°C had a higher bias (1.39% on day 15 and 4.09% on day 30) compared to those stored at -80°C (0.95 % on day 15 and 0.66% on day 30). The Guideline-Driven Medical Decision Limits states that a bias of less than 1% is recommended for a clinically useful HbA1c result (8). It was observed that the false positive number tends to double for +2% bias (8). Hence, the bias obtained in this study indicates that HbA1c samples that require prolonged storage should be stored at -80°C than -20°C for the results to be clinically useful.

When interpreted by clinical significance, the majority had no significant change i.e. HbA1c change of ≥0.5% (6). This is critical as HbA1c concentration is being used widely as part of the diagnosis and also monitoring of DM. Therefore, the difference in HbA1c level of more than 0.5% may lead to the misclassification of a patient's diagnosis. The difference may also influence the decision on treatment modifications since glycaemic control typically depends on the HbA1c levels. Considering the importance of accurate HbA1c levels in diabetic management, it is crucial to ensure proper sample storage is practised in every laboratory running the test.

Sample stability may also be affected by the HbA1c assay methods (9). The ion-exchange assay methods tend to be more sensitive to storage conditions compared to boronate affinity or immunoassay methods (9). A study that compared several HbA1c assay methods reported that for the ion-exchange methods [Tosoh G7 and G8 (Tosoh Bioscience) and Bio-Rad Variant II (Bio-Rad Laboratories)], the 4°C storage is preferable (14–21 days) compared to storage at -20°C (4–10 days) (9). On the other hand, for the immunoassay method [Siemens DCA 2000+ (Siemens Healthcare Diagnostics)], the best stability was at -20°C, whereas for the boronate-affinity high-performance liquid chromatography (Trinity Biotech ultra2), the best sample stability was at 4°C. Nevertheless, whenever long-term stability is required, the samples should be stored at -70°C or lower.

CONCLUSION

Sample storage duration and temperature affect HbA1c concentration. Thus, in cases where prolonged storage of HbA1c samples is required, it is recommended that the samples are stored at -80°C as compared to -20°C.

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