

## Glycated hemoglobin measurements from dried blood spots: reliability and relation to results obtained from whole blood samples

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### Abstract

**Background:** Main objective was to measure glycated hemoglobin (HbA1c) in dried blood spots on paper filter and in whole blood samples in diabetic patients to evaluate relationship between two methods and their respective reliability.

**Methods:** The 20×10 µl of venous blood samples of 33 diabetics were blotted onto the filter paper allowed to dry at room temperature and then stored at 25°C and 4°C. HbA1c was measured via the Turbidimetric Inhibition Immunoassay Technique. The relation was evaluated with correlation and linear regression tests using STATA software and SPSS. Agreement between the results obtained from the dried blood spots and others was evaluated using the Bland and Altman. The pitman's permutation test was also employed to compare the difference in variance.

**Results:** A high positive correlation was detected between whole blood samples and dried blood spots stored at 4°C ( $r^2 = 0.90$ ) and at 25°C ( $r^2 = 0.95$ ). The Bland and Altman graphs, as well as the Pitman tests, showed statistically significant differences in variability between the values obtained from whole blood samples and those derived from dried spots stored at 4°C ( $p=0.05$ ) or 25°C ( $p=0.004$ ).

**Conclusion:** HbA1c measurements from dried blood spots on the filter paper yielded reliable results. That the Hitachi autoanalyzer is available in most countries renders this assay less costly than the High Performance Liquid Chromatography Method (HPLC). In addition, the filter paper method for Immuno-turbidimetric estimations of HbA1c at different temperatures is reliable and may be particularly useful in outpatient diabetes clinic.

**Keywords:** Glycated hemoglobin, Paper filter, Diabetes, Dried blood spot.

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## Introduction

Diabetes mellitus (DM) is a major cause of morbidity and mortality worldwide, and in Iran it afflicts approximately 4 million adults' aged  $\geq 20$  (1). One study conducted in 2001 estimated that the burden of DM and its complications in Iran equaled 306,440 years in terms of disability adjusted life years (DALYs) (2).

To achieve good control of DM and to regulate anti-diabetes drugs such as insulin, diabetic patients need to regularly measure their blood glucose (3). Random measurements of glucose cannot be a reliable indicator of glycemic control in the long term. Indeed, the long term indicator of glucose control is glycated hemoglobin (HbA1c), which shows an average level of blood glucose in the previous 2-3 months and reflects the long-term risk of DM complications (4).

Most diabetic patients should be referred to clinical laboratories for HbA1c testing via at least 1mL of venous blood. Nevertheless, apart from creating inconvenience for the patient, HbA1c measurement is limited due to its sample collection and storage. Given the paucity of health resources in Iran in the face of increasing DM prevalence, there is urgent need to facilitate the diagnostic modalities of this disease so as to reduce cost and burden inflicted on the country's health care system.

Recent years have witnessed the development of at home test kits as a filter paper assay for HbA1c measurement (5-8), our DM patients, however, had no experience with this method and we employed the Turbidimetric Inhibition Immunoassay Technique to measure HbA1c in dried blood spots on the filter paper at different temperatures and in whole blood samples and subsequently evaluated the relation between the two methods and their respective reliability.

## Methods

### Study Population

Forty-two patients with type 2 DM were selected from our outpatient diabetes clinic. Those with renal failure sever or moderate anemia and pregnancy were excluded from the study. Overall, 33 diabetic patients, who provided informed written consent for participation in the study were recruited. The study was approved by the Ethics Committee of Tehran University of Medical Sciences.

### Laboratory methods

The process of laboratory testing was performed in three steps for each subject. Venous blood samples were collected in EDTA containing vacutainers. Thereafter,  $20 \times 10 \mu\text{l}$  of the blood samples were blotted onto the filter paper (Whatman number 1) and allowed to dry at room temperature. These dried blood spots were then placed in sealed polythene covers and stored at two different temperatures; room ( $25^\circ\text{C}$ ) and  $4^\circ\text{C}$ , separately. Also, the whole blood samples were stored at  $4^\circ\text{C}$ . HbA1c was measured for all of above samples via Turbidimetric Inhibition Immunoassay Technique with a Hitachi Autoanalyzer.

Our study was carried out in two phases:

#### 1- The measurement phase

Day 0 was considered the day of whole blood sample collection. The HbA1c tests were conducted for all the blood samples of our 33 patients, 10 times on the first day.

#### 2- The comparison phase

The HbA1c values of the dried blood spots were compared with those obtained from the whole blood samples. Comparisons of these HbA1c values reflected the relationship between the two methods and the reliability of HbA1c measurement on dried blood spots.

### Statistical analysis

Statistical analysis was performed with the STATA software, version 10 and SPSS version 16. The relation between the different methods was assessed using correlation and Linear regression analysis (after assessing normality and linearity assumption in the study variables), and the agreement between the results obtained from the dried blood spots and those derived from the whole blood samples was evaluated using the Bland and Altman (9). The pitman's permutation test was used to compare the difference in variance for the paired data (10, 11). P-value  $\leq 0.05$  was considered as statistically significant.

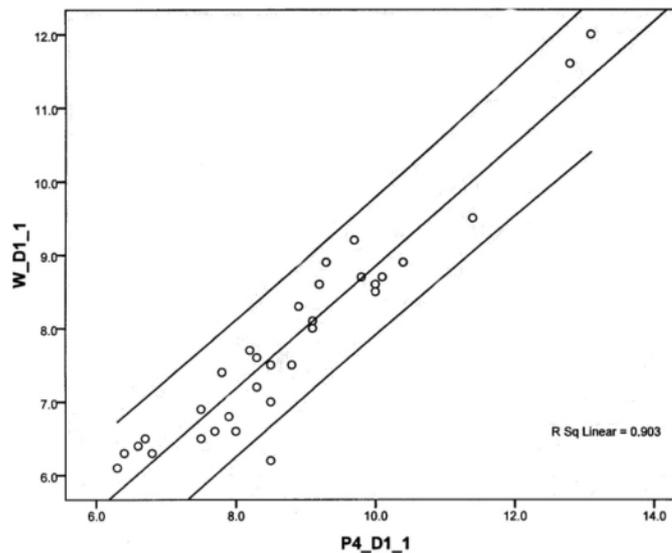
## Results

In our 33 diabetic patients, 57.6% (19 subjects) were female. The range of age in our patients was 33-80 years ( $58.7 \pm 11.1$ ), and the range of type 2 DM duration was 2-25 years ( $9.8 \pm 5.8$ ). The mean  $\pm$  SD of the HbA1c values in the whole blood samples and the dried blood spots stored at  $25^\circ\text{C}$  or  $4^\circ\text{C}$  was  $7.9 \pm 1.4$  (assay range 5.90-

11.97%),  $8.9 \pm 1.7$  (assay range 6.66-13.61%), and  $8.8 \pm 1.6\%$  (assay range 6.48-13.24%), respectively. Both of HbA1c values in dried blood spots stored at 25°C or 4°C had statistically significant differences with those derived from the whole blood samples ( $p < 0.001$ ). The relationship between the first whole blood samples and the first dried blood spots at 4°C is shown as below formula and also in Figure 1. HbA1c whole blood (y) =  $0.528 + 0.832$  HbA1c dried spot at 4°C (x). The  $r^2$  was 0.903 with a statistically significant difference ( $P < 0.001$ ) which indicates a reliable relationship between these two methods. This formula for dried blood spots at 25°C was as following with  $r^2 = 0.948$  and  $p < 0.001$  (Fig.2). HbA1c whole blood (y) =  $0.805 + 0.798$  HbA1c dried spot at 25°C (x).

Bland and Altman graphs, as well as the Pitman's tests, showed statistically significant differences in variability between the values

derived from the whole blood samples and those obtained from the dried blood spots stored at 4°C (Figure 3). Bland-Altman limits of agreement (Reference Range for difference) for them were -1.993 to 0.084 with a mean difference -0.955 (95% CI -1.139 to -0.770) and range; 6.200 to 12.550. The Pitman's Test of difference in variance for 33 samples had an  $r$  (-0.396), and a significant  $p$  value (0.049). The above results for HbA1c values derived from the whole blood samples and those obtained from the dried blood spots stored at 25°C is shown in Figure 4. Bland-Altman limits of agreement (Reference Range for difference) for them were -1.938 to -0.026 with a mean difference -0.982 (95% CI -1.151 to -0.812) and range; 6.300 to 12.700. The Pitman's Test of difference in variance for 33 samples had an  $r$  (-0.660), and a significant  $p$  value (0.004).



**Figure 1. Relationship between the first whole blood samples (W\_D1\_1) and the first dried blood spots stored at 4°C (P4\_D1\_1).**

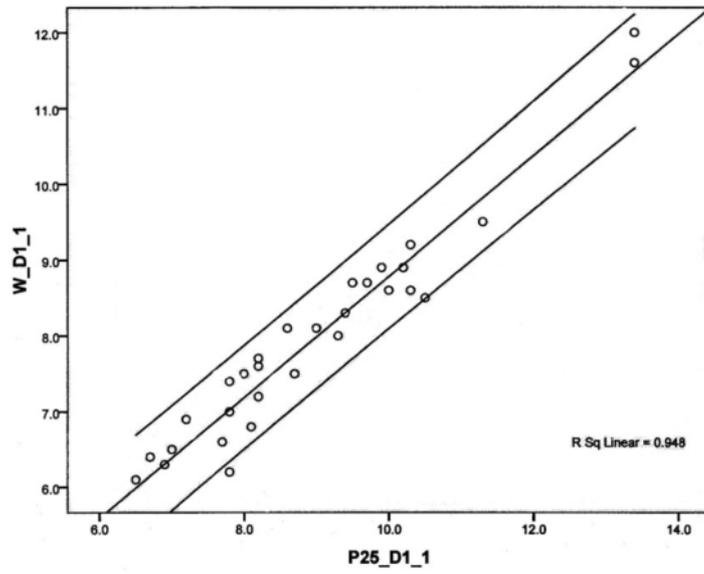


Figure 2. Relationship between the first whole blood samples (W\_D1\_1) and the first dried blood spots stored at 25°C (P25\_D1\_1).

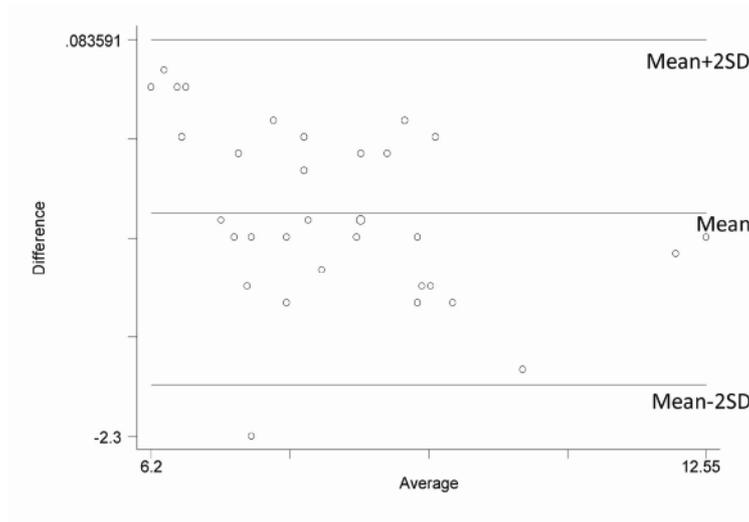


Figure 3. Bland-Altman plot of the comparison between the first whole blood samples and the first dried blood spots at 4°C.

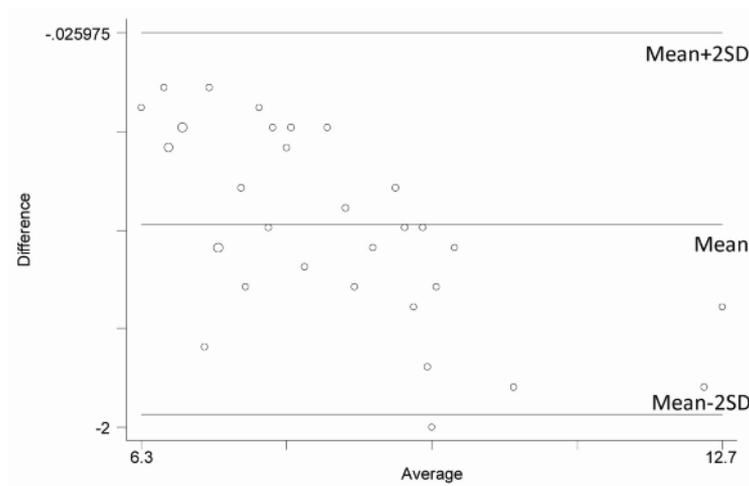


Figure 4. Bland-Altman plot of the comparison between the first whole blood samples and the first dried blood spots at 25°C.

## Discussion

We found a similarity between HbA1c values in dried blood spots stored at different temperatures. Also, there was a reliable relationship between the first whole blood samples and the first dried blood spots at 25°C or 4°C. The Bland and Altman graphs, and the Pitman's tests showed requirement to appropriate coefficients for providing equivalency between the values obtained from the dried blood spots stored at 4°C or 25°C and those derived from the whole blood samples.

There are factors that are of particular significance in the measurement of HbA1c, as a routine diabetic patients care modality, such as the convenience of blood sample collection and the handling and effects of sample storage on measured HbA1c (12). Amongst the different measurement techniques, the filter paper method is discussed in considerable length in the existing literature (7, 12-16). Not only is this method convenient for patients and health care providers but it also technically easier and faster than- for example- is the High Performance Liquid Chromatography method (HPLC).

In our study, the HbA1c levels obtained from the dried blood spots stored at 4°C or 25°C were similar together, with statistically significant differences with those values derived from the whole blood samples. Overall, the mean measured HbA1c of our dried blood spots on the untreated paper was 0.96% (for 4°C) or 0.97% (for 25°C), which was higher than the HbA1c values of our whole blood samples. Little and colleagues (14,17) showed that the drying and storage of whole blood samples on the filter paper could increase the final measured HbA1c value of the whole blood. This increase was directly related to both the duration of storage and the concentration of free glucose in the blood spots. Their research, therefore, reflected the rapid *in vitro* glycation of hemoglobin and plasma proteins during drying.

A comparison of the dried blood spots HbA1c values and those of the Turbidimetric Inhibition Immunoassay Technique (Figs. 1 and 2) revealed a significant correlation ( $r^2 = 0.90$  and  $r^2 = 0.95$ , respectively) and a statistically significant difference with the measured HbA1c values of the whole blood samples. The interpretation of the results obtained from the HbA1c values of the dried blood spots required the use of an appropriate regression equation that

would make the prediction of the whole blood possible. Our regression equation at 25°C had some differences with the Anjali et al. study (13) which described a regression equation as follows: capillary HbA1c on the filter paper at room temperature  $= 0.95$  (venous HbA1c)  $+ 1.4$ . The observed difference in the above regression equation may be attributed to the comparison time of the samples. Whereas we compared the first HbA1c values of the dried blood spots with the HbA1c values of the whole blood samples on the very same day, Anjali et al. compared the HbA1c values obtained on days 4, 7, and 10 with the baseline values. This difference should render our results more reliable. What our study and the Anjali et al. study have in common, however is the significant correlation in the HbA1c values of the dried blood spots and the statistically significant differences between them and the HbA1c values of the whole blood samples ( $r = 0.889$  in the study of Anjali and  $r = 0.974$  in our study).

Although the accuracy and reliability of HbA1c values on the filter paper have been borne out by previous investigations (18) we made use of the Bland and Altman method as well as the Pitman's test to assess the variability between the values derived from the dried blood spots at different temperatures and those obtained from the whole blood samples. These analytic tests constitute some other differences between our study and that of Anjali et al. The Bland and Altman methods, as well as the Pitman's test, revealed that there were statistically significant differences between the variability of the values obtained from the dried blood spots stored at 4°C or 25°C and those derived from the whole blood samples in our study. In other words, appropriate coefficients were required to provide equivalency between the HbA1c levels obtained from the dried blood spots stored at 4°C or 25°C and the values derived from the whole blood samples. It is worthy of note that our regression equations may be useful in assay ranges of 5.9 - 12% of whole blood sample HbA1c levels.

The present study had some limitations, first and foremost amongst which was the non-use of the HPLC assay as a reference for baselines HbA1c values. That the blood spots were not immersed in alcohol may also have increased the HbA1c values on the filter paper. We would suggest that further studies with larger sample sizes be

conducted and comparisons be made between the filter papers and the HPLC assay.

In our study, HbA1c measured in the dried blood spots on the filter paper stored at 4°C or 25°C yielded reliable results. Given the availability of the Hitachi Autoanalyzer in most countries, this assay is less expensive than is the HPLC assay. Furthermore, the paper filter method for the Immuno-turbidimetric estimation of HbA1c at

different temperatures is reliable and may be particularly useful in outpatient diabetes clinic.

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