Accuracy of Filter Paper Method for Measuring Glycated Hemoglobin

Anjali*, FS Geethanjali+, R Selva Kumar+, MS Seshadri*

Abstract

Background and Objectives: Glycated hemoglobin (HbA1c) provides an accurate and reliable method to assess the glycemic control in patients with Diabetes. Its measurement is limited by the inconvenience of sample collection that requires venipuncture, sample handling and storage factors. The aim of this study was to assess the feasibility of using a dried capillary blood spot on a filter paper to estimate HbA1c, to check its stability at room temperature and to compare these values with the venous sample HbA1c by Turbidimetric Inhibition Immunoassay (TINA, Tina-quant HbA1c II).

Methods: Venous blood samples of seventy eight patients with Type 1 or type 2 diabetes, were collected in EDTA containing vacutainers. Stability of HbA1c was studied in capillary blood samples blotted on to Whatman number1 filter paper and stored at room temperature, for the first 20 patients enrolled in the study. After establishing the stability over a ten day period, HbA1c values obtained on the capillary blood spots were compared with those obtained from the venous blood samples of the remaining 58 patients.

Results: Glycated hemoglobin is found to be stable in dried capillary blood spots on filter paper till the 10th day, stored at room temperature. It however, shows an inherent variability of ±15%, which falls with in the permissible variability (18%) of the quality control material. Seventy nine percent of the capillary HbA1c values were found to fall within this range. With linear regression, we derived the relationship between filter paper and venous HbA1c values. The regression equation was as follows: Cap.HbA1c = 0.95 (Ven.HbA1c) + 1.4. The filter paper results were highly correlated with the venous sample values (r = 0.889, p < 0.01).

Conclusion: Measurement of glycated hemoglobin in dried blood spots on filter paper gives reliable and reproducible results. In our study, the mean capillary sample HbA1c value was 12% higher compared to the venous sample HbA1c values. Therefore a higher normal range may have to be used for interpreting the dried blood spot capillary blood HbA1c values. ©

INTRODUCTION

Measurement of glycated hemoglobin in patients with Diabetes is accepted as a standard method for assessment of recent glycemic control and is a critical element in clinical practice. In our country, patients do not have ready access to reliable HbA1c measurements and visits to the hospitals become time consuming and expensive. The recent development of mail-in-filter paper method for capillary blood samples may ameliorate this problem.

Previous studies have compared HbA1c estimation on dried spots of capillary blood on filter paper versus venous samples using high performance liquid chromatography, Ion exchange chromatography, and Affinity chromatography. However, there is no published literature on comparison of glycated hemoglobin estimation in capillary versus venous samples assayed by Turbidimetric Inhibition Immunoassay. The present study was undertaken to estimate HbA1c in dried capillary blood spots on a filter paper, to determine its stability over a period of ten days when stored at room temperature and to compare these results with values determined on the same venous sample by TINA.

MATERIALS AND METHODS

Subjects

Seventy eight patients with type 1 or type 2 diabetes coming to our outpatient clinic were included in this study. Those with renal failure (serum creatinine > 1.5 mg/dl) and pregnant patients were excluded from the study. The study was approved by the institutional research committee and ethical clearance was also obtained.

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Sample collection

Venous blood samples were collected in EDTA containing vacutainers. Capillary blood samples were obtained by finger prick with a sterile lancet, after obtaining informed consent. 4 x 30 μl of capillary blood samples were blotted on to the filter paper (Whatman number1) and allowed to dry at room temperature. These were then placed in sealed polythene covers and stored at room temperature.

Method of HbA1c estimation in our biochemistry lab

Turbidimetric Inhibition Immunoassay technique is used in our laboratory for HbA1c estimation in hemolysed whole blood using Hitachi autoanayser. This method uses tetradecyltrimethylammonium bromide (TTAB) as the detergent in the hemolyzing reagent to eliminate interference from leukocytes (not lysed by TTAB). Sample pretreatment to remove labile HbA1c is not necessary. All hemoglobin variants that are glycated at the β-chain terminus and which have antibody recognizable regions identical to that of HbA1c are determined by this assay.

Step 1: HbA1c in the sample is made to react with anti HbA1c antibody (ovine serum) to form soluble antigen antibody complexes.

Step 2: The next step consists of adding a buffer that reacts with excess anti HbA1c antibodies to form an insoluble antibody – polyhapten complex that can be determined by turbidimetry. The buffer solution used contained MES (2-morpholinoethane sulfonic acid) and TRIS (tris hydroxymethylamino methane). The higher the turbidity, the lower would be the HbA1c levels.

Validation of turbidimetric inhibition immunoassay (Roche TinaQuant II assay) versus high performance liquid chromatography:

We compared our laboratory HbA1c values estimated by TINA (20 samples) with those estimated by high performance liquid chromatography (HPLC) (Bio-Rad variant) at the Department of Endocrinology, Lucknow. The intra class correlation coefficient between the two set of values were then computed.

Calculation of coefficient of variation for HbA1c estimation by TINA:

Both intra-assay and inter-assay coefficients of variation were calculated for high and low ranges of glycated hemoglobin in venous samples tested by TINA.

The study was carried out in three phases –

a) The Stability Study:

For the first twenty patients enrolled in the study, 4 x 30 μl of capillary blood samples were collected on the filter paper, dried and subsequently stored in sealed polythene covers at room temperature. A fixed diameter office punch was used to cut out the dried blood spots. These were then eluted with the buffer and the eluate used for estimating HbA1c. Elution was done on day 1, 4, 7 and day 10 (day 0 being the day of venous sample collection). The HbA1c values thus obtained, were then compared with the values obtained on the venous blood samples (Table 1). Comparison of the HbA1c values obtained on day 4, 7 and 10 with the baseline values reflected the stability of HbA1c on a dried blood spot.

The stability study was done in two batches. After the first batch of ten samples, slight modification was made in the methodology. Elution time was reduced to fifteen minutes as against 45 minutes for the first ten samples. This was similar to the time taken to hemolyse the venous sample. The punch was washed and dried before using for the next sample to avoid carry over contamination.

b) Comparison of HbA1c values obtained on capillary blood samples dried on filter paper versus those on venous blood samples –

After proving the stability of HbA1c on a dried blood spot, the second phase of the study was initiated. Capillary blood samples were collected from patients within twelve hours of collection of the venous samples for HbA1c estimation. HbA1c was then estimated in the eluate on the seventh day.

c) Estimation of HbA1c in capillary blood samples collected and mailed by the patients:

The last phase of the study was to provide filter papers, lancets, polythene covers and stamped envelopes to the patients who had venous sample HbA1c done as outpatients. The patients collected their own capillary blood samples at home the next day, placed them in sealed polythene covers, recorded the date of sample collection and mailed them to us. The envelopes were stored at room temperature, opened on day 7 of sample collection and HbA1c estimated on the eluate from these samples. Day 7 was arbitrarily chosen, as most of the mails even from distant parts of India would reach Vellore by this time.

Sample size calculation:

The published variance for HbA1c measurements in different studies varies from 3.17 - 5.48. In order to evaluate the filter paper method such that the sample variance will be ± 1 of the published population variance with 95% (P< 0.05) confidence and 80% power, the sample size was calculated to be 75 subjects [SPSS 9.0 software package].

Statistical analysis:

Linear regression and Intra class correlation coefficients (ICC) were computed using SPSS 9.0 software. Both intra and inter-assay coefficients of variation (CV) were calculated for the HbA1c values from venous blood samples and the dried blood spots on the filter-paper.
RESULTS

TINA was validated against HPLC technique for HbA1c estimation. Their controls ran as follows: Control 1 (mean 5.5, range 4.8-6.2) was 5.1% and Control 2 (mean 9.6, range 8.8-10.4) was 9.2%. Control 1 shows that the reading was (0.4/5.5 = 7.27%) and Control 2 shows that the reading is (0.4/9.6 = 4%) lower than expected. The HPLC values were corrected by 5%. ICC between the two sets of values was found to be 0.945 (95% confidence interval - 0.87 – 0.98).

Intraclass Correlation Coefficients for the second batch of 10 patients:

Day 1 (filter paper) Vs venous sample HbA1c – 0.99 (95%CI 0.97 - 0.99)

Day 4 (filter paper) Vs venous sample HbA1c – 0.98 (95%CI 0.93 – 0.99)

Day 7 (filter paper) Vs venous sample HbA1c – 0.91 (95%CI 0.69 - 0.98)

Day 10 (filter paper) Vs venous sample HbA1c – 0.99 (95%CI 0.95 – 0.94)

There is an excellent correlation between venous blood sample HbA1c on day zero and the dried blood spot HbA1c on days 1, 4, 7 and 10 with intra-class correlation coefficients varying from 0.91 – 0.99. The variability of HbA1c in dried filter paper samples is ±15% (Fig. 1). There was no significant difference between HbA1c values obtained on day 4 and day 1 (P = 0.46) and those between day 7 and day 1 (P = 0.54). Similarly there was no significant difference between HbA1c values obtained on day 10 when compared with day 4 HbA1c results (P = 0.29). This confirms that glycated hemoglobin is stable on a dried blood spot on filter paper for up to 10 days at room temperature.

Performance characteristics of the immunoturbidimetry method for HbA1c:

The interassay CV calculated from controls run in 11 assays over a period of 3 weeks was 4.57% for the lower quality control and 4.25% for the higher quality control. Two patient samples in high and low ranges were run ten times within the same batch and the intra-assay CV was calculated to be 3.27% for the lower range and 2.31% for the higher range. The mean CV of HbA1c estimation on capillary spots on filter paper is 4.72% (Table 1).

Precinorm HbA1c and Precipath HbA1c quality controls were used in HbA1c measurements. The company assigned value for Precinorm -

Control 1 - Mean 5.9, range 4.8 – 7 [permissible variation of 18.6% (5.9 ± 1.1)]

Control 2 - Mean 11.7, range 9.6 -13.8 [permissible variation of 17.9% (11.7 ± 2.1)].

The allowed variability for both the lower range and the higher values of HbA1c quality control material is approximately 18%. Out of the 58 samples that we tested prospectively, the HbA1c values in the dried blood spot in 46 samples (79.3%) fall within ±18% of concurrent venous sample HbA1c values.

Comparison of HbA1c values obtained on capillary blood samples dried on filter paper versus venous samples:

ICC between the capillary HbA1c and the venous sample HbA1c values is 0.887 (95% CI – 0.82 – 0.93) (Fig. 2). The mean value and range for venous blood and capillary blood samples were 8.46% (5.5 - 13%) and

Table 1: Coefficient of variation of the HbA1c values in capillary blood samples tested by the filter paper method:

<table>
<thead>
<tr>
<th>Hosp no.</th>
<th>Day 1 HbA1c (%)</th>
<th>Day 4 HbA1c (%)</th>
<th>Day 7 HbA1c (%)</th>
<th>Day10 HbA1c (%)</th>
<th>Mean HbA1c (%)</th>
<th>SD</th>
<th>CV(%)</th>
</tr>
</thead>
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<tr>
<td>304633C</td>
<td>6.1</td>
<td>6.7</td>
<td>6.1</td>
<td>6.8</td>
<td>6.4</td>
<td>0.33</td>
<td>5.15</td>
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<tr>
<td>026921B</td>
<td>9.0</td>
<td>9.4</td>
<td>8.8</td>
<td>9.1</td>
<td>9.1</td>
<td>0.22</td>
<td>2.41</td>
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<tr>
<td>304540C</td>
<td>8.2</td>
<td>8.3</td>
<td>7.4</td>
<td>8.3</td>
<td>8.1</td>
<td>0.58</td>
<td>4.69</td>
</tr>
<tr>
<td>001827O</td>
<td>7.6</td>
<td>8.5</td>
<td>7.9</td>
<td>8.0</td>
<td>8.0</td>
<td>0.32</td>
<td>4.0</td>
</tr>
<tr>
<td>304596C</td>
<td>10.5</td>
<td>10.9</td>
<td>10.9</td>
<td>11.6</td>
<td>10.9</td>
<td>0.40</td>
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<tr>
<td>302683C</td>
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<td>6.5</td>
<td>6.6</td>
<td>6.4</td>
<td>0.31</td>
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<tr>
<td>726671O</td>
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<td>0.22</td>
<td>3.49</td>
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<td>282843C</td>
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<td>8.4</td>
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<td>8.7</td>
<td>8.5</td>
<td>0.21</td>
<td>2.47</td>
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<tr>
<td>544387B</td>
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<td>13.8</td>
<td>11.6</td>
<td>13.9</td>
<td>13.1</td>
<td>0.92</td>
<td>7.02</td>
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<tr>
<td>304244C</td>
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<td>9.7</td>
<td>12.7</td>
<td>11.4</td>
<td>1.08</td>
<td>9.47</td>
</tr>
</tbody>
</table>

Mean CV – 4.72%
9.44% (6.4-13.3%) respectively, i.e., the mean capillary sample HbA1c value was 12% higher compared to the venous sample HbA1c values. With linear regression, we derived the relationship between filter paper and venous HbA1c values. The regression equation was Cap. HbA1c = 0.95 (Ven. HbA1c) + 1.4. The filter paper results were well correlated with the venous sample values (r = 0.889, p < 0.01), the standard error of estimate being 0.8602. In order to see if the capillary HbA1c values can be accepted for routine clinical use, we interpreted the values as acceptable if they fall within the permissible variability (μ 18%) of the quality control material (Fig. 3).

There was no significant difference between the two by paired ‘t’ test.

Paired samples statistics -

Venous HbA1c (mean – 8.46%, SD- 1.74, SE- 0.23)
Capillary HbA1c (mean- 9.45%, SD – 1.86, SE - .25), P value > 0.1

**DISCUSSION**

Measurement of glycated hemoglobin in dried blood spots on filter paper give reliable and reproducible results. TINA quant assay technique for HbA1c estimation when compared with the gold standard High Performance Liquid Chromatography shows a good correlation (r = 0.95). In the present study, HbA1c was found to be stable in the dried capillary blood spots till the 10th day, stored at room temperature. However, it shows an inherent variability of ±15% that falls within the permissible variability (±18%) of the quality control material employed in the HbA1c estimations. The coefficient of variation for HbA1c estimation in capillary blood samples by the filter paper method was found to be 4.72% and this compares favorably with the CV for venous blood HbA1c measurements.

Stability of HbA1c in the dried blood spots and in vitro glycation of glucose in the filter paper have remained a matter of concern and can cause an increase in capillary HbA1c values.10 Earlier studies by Jan Jeppson et al9 had established the stability of HbA1c on filter paper for 5-7 days at 20-21°C (room temperature), for 10 days at 4-6°C and several months at ~70°C. To prevent in vitro glycation, filter papers have been pretreated with ethanol/isopropanol,9 glucose oxidase containing Beckman glucose reagent11 and phosphate buffered sodium chloride 154 mmol/l with EDTA and p-hydroxybenzoic acid methyl ester in the earlier studies.14 In our study, though we had not pretreated the filter paper, majority (80%) of the results were within the allowed variability of ±18%. We find that the capillary blood HbA1c values estimated on day 7 after collection are on an average 12% higher than the concurrent venous sample HbA1c presumably due to in vitro glycation. Therefore it may be necessary to use a different normal range for capillary sample HbA1c estimations. The HbA1c values determined on the dried capillary blood samples showed a good intra-

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**Regression line**

\[
\begin{align*}
\text{Model} & \quad R & \quad R\text{ Square} \\
1 & \quad 0.889 & \quad 0.790 \\
2 & \quad 0.787 & \quad 0.602 \\
\end{align*}
\]

a Predictors: (Constant), VENHBA1C
b Dependent Variable: CAPHBA1C

*Fig 2 : Correlation between venous and Day 7 capillary HbA1c*

With linear regression, we derived the relationship between filter paper and venous HbA1c values. The regression equation was Cap. HbA1c = 0.95 (Ven. HbA1c) + 1.4. The filter paper results were highly correlated with the venous sample values (r = 0.889, p < 0.01), the standard error of estimate being 0.8602. In order to see if the capillary HbA1c values can be accepted for routine clinical use, we interpreted the values as acceptable if they fall within the permissible variability (±18%) of the quality control material (Fig. 3).

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**Fig. 3 : Capillary HbA1c values in the acceptable range**

Accepted variability: 82 – 118%

If the venous HbA1c value is taken as 100%, the accepted variability according to the quality control (QC) is ±18%(82-118%). If so, 11/58 (19%) of the capillary HbA1c values fall out of range, the scatter being from 119-146%. The mean Capillary HbA1c value was 12% higher compared to the venous samples.
class correlation of 0.89 (95% CI – 0.82 – 0.93) with those obtained from the venous samples, as has been shown in the previous studies.

Reliable HbA1c measurements are not available to the majority of patients who come from remote places. It is clear from our study that HbA1c measurements from dried capillary blood spots on filter paper are reliable. The stability of HbA1c in dried blood spots will permit sample collection at home and patients can mail the samples to the lab and the results can be communicated to the patients. This would reduce the number of hospital visits required by stable diabetics and allow physicians to spend more time with patients whose control is suboptimal. As long as the inherent variability in the measurements is known, one can interpret these results and make suitable alterations in the treatment plan. As Hitachi autoanalysers are available in a number of centers, validation of this filter paper method for immunoturbidimetric estimation of HbA1c will have wide applicability within the country. Further, this automated method for HbA1c testing is less expensive than HPLC.

We conclude that capillary blood collected as dried blood spots by patients and mailed to referral centers can be used for estimating HbA1c reliably but a higher normal range may have to be used for interpretation. The results can be communicated to them by post and appropriate changes made in the treatment plan.

REFERENCES


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