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SYMPOSIUM

Analysis of Hemoglobin A1c from Dried Blood Spot Samples with the Tina-quant® II Immunoturbidimetric Method

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Abstract

Background:

Hemoglobin A1c (HbA1c) has been endorsed as a tool for the diagnosis of diabetes. This test requires instrumentation that may not be available in underdeveloped areas. Dried blood spot (DBS) samples collected by finger stick procedures offer a mechanism to transport samples to laboratories that do measure HbA1c.

Methods:

Whole blood (ethylenediaminetetraacetic acid) was applied to Ahlstrom 226 filter paper. These DBS samples were compared to whole blood samples using the Roche Tina-quant® II immunoturbidometric assay. Hemoglobin A1c stability on DBS was assessed at three temperatures—4, 25, and 40°C—for up to 9 days. A 44-day study was also done for DBS at 20–25°C.

Results:

The Tina-quant® II DBS method showed excellent agreement with whole blood HbA1c results (r^2 = 0.99) with a slight positive mean bias of 0.08 ± 0.04% HbA1c (95% confidence interval). The variation in HbA1c on DBS samples subjected to different temperatures and times did not exceed 5.6%.

Conclusions:

Dried blood spot samples represent an alternative to whole blood for HbA1c by measurement when transporting whole blood is not feasible.

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Introduction

Hemoglobin A1c (HbA1c) has been used for many years to assist in the monitoring and treatment of diabetes. Increased emphasis has been placed upon use of this marker as a diagnostic tool. In July 2009, an international expert committee (IEC) recommended the use of HbA1c to diagnose diabetes when HbA1c

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Abbreviations: (%CV) percentage coefficient of variance, (CI) confidence interval, (DBS) dried blood spot, (HbA1c) hemoglobin A1c, (HPLC) high-performance liquid chromatography, (IEC) international expert committee, (MDP) medical decision point, (NGSP) National Glycohemoglobin Standardization Program, (USPS) U.S. Postal Service

Keywords: diabetes, dried blood, glycohemoglobin, hemoglobin A1c

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levels are ≥6.5%.[1] The American Diabetes Association formally endorsed the recommendations of the IEC in January 2010.[2] The authors cited lack of availability of HbA1c testing as a possible limitation to its use in diagnosing diabetes, especially in developing parts of the world. Hemoglobin A1c testing from samples collected by finger stick onto filter paper may increase availability in areas where venipuncture is impractical. With this technique, self-collection is also possible and has been shown to improve patient satisfaction.[3]

A variety of methods for HbA1c analysis of dried blood spot (DBS) samples have been described in the literature, including affinity chromatography,[4] ion-exchange chromatography,[5] and immunoturbidimetric methods.[6,7] This article describes use of an immunoturbidimetric technique to measure HbA1c in samples collected and dried on filter paper.

Methods

Whole Blood Samples

All whole blood samples were obtained by venipuncture and collected in ethylenediaminetetraacetic acid-preserved collection tubes. Samples used for studies were selected from specimens submitted for routine testing in our laboratory.

Preparation of DBS Samples

Filter paper used for these studies was Ahlstrom 226 (ID Biological, Greenville, SC). The paper conforms to the Clinical and Laboratory Standards Institute LA4-A5 standard for use in neonatal screening programs[8] and has been approved by the Centers for Disease Control as an acceptable blood spot collection paper.

Dried blood spot samples were prepared by spotting 20 µl of whole blood onto the paper and drying at room temperature for at least 3 hours.

The National Glycohemoglobin Standardization Program (NGSP) used this method to prepare DBS samples for testing in our laboratory.[9]

Roche Tina-quant® Hemoglobin A1c II Method

The Roche Tina-quant® II assay was performed on the Hitachi Modular P analyzer. The method is based on the turbidimetric inhibition immunoassay. In the standard assay, whole blood is first hemolyzed with a hemolyzing reagent and the resulting hemolysate is added to the antibody reagent. The anti-HbA1c antibody reacts with a single binding site on HbA1c, forming a soluble complex. A second reagent containing a polyhapten is then added and binds with the remaining anti-HbA1c antibody to form an insoluble complex. The amount of complex formed is determined turbidimetrically, and total hemoglobin is determined spectrophotometrically on a second channel. The ratio of these two values gives percentage HbA1c. Whole blood samples were analyzed in accordance with the manufacturer's recommended procedures. The assay system has been calibrated to meet Diabetes Control and Complication Trial standards.[10]

Dried Blood Spot Sample Elution

Dried blood spot samples were prepared for analysis by cutting two, 1/8-inch-diameter punches from each DBS using a common paper hole punch. The punches were placed into a 12 × 75-mm test tube and eluted for 3 hours at room temperature (20–25°C) with 500 µl of Roche hemolyzing reagent. Samples were mixed gently without removing the disks, and analysis was performed directly from the test tube.

Correlation Studies

Two correlation studies were performed to evaluate the performance of DBS specimens compared to whole blood HbA1c. The first study used 73 whole blood samples with values from 3.8 to 15.3% HbA1c selected from samples submitted for routine HbA1c measurement in our laboratory. Whole blood and DBS samples were analyzed with the Tina-quant® assay system on the Hitachi Modular P analyzer. A second correlation study was performed using 40 samples with HbA1c values from 5.0 to 12.2% provided by the NGSP. Whole blood samples were tested by the NGSP using the Tosoh ion-exchange high-performance liquid chromatography (HPLC) method. DBS samples were prepared by the NGSP using the method described previously and were shipped to the laboratory for analysis by the Tina-quant® method.

To evaluate the relative performance of the DBS Tina-quant® method at critical HbA1c values, a medical decision point (MDP) analysis was performed. Comparisons were made at HbA1c values of 6.0, 6.5, and 7.0%. These values were chosen based on recommendations of the IEC[1] for individuals at risk for diabetes (≥6.0 to <6.5%), those with diabetes (>6.5%), and the commonly used target of ≤7.0% HbA1c for diabetic control. Estimates of the equivalent points on the test method were determined by application of the derived regression equation. Confidence intervals (95%) were calculated for each estimate.

National Glycohemoglobin Standardization Program criteria for level II certification were used as the measure of MDP analysis acceptability. For level II certification, NGSP requires that the 95% confidence interval (CI) of the difference between methods falls within ±0.75% HbA1c.

Precision Studies

Six DBS samples were prepared from whole blood with HbA1c values from 5.0 to 12.4% for within-run Tina-quant® precision studies. Five disks were punched from each DBS sample, eluted as described earlier, and tested in a single run.

Forty DBS samples, prepared by the NGSP, were used for between-run precision studies. Each sample was analyzed twice per run for 5 consecutive days. To better understand between-run precision as a function of HbA1c concentration, results were sorted by percentage HbA1c value, and the data set was segmented into four groups. Each group contained a total of 100 observations over five separate runs. Mean, standard deviation, and percentage coefficient of variance (%CV) were calculated for each group.

Stability Studies

Five whole blood samples were selected at random to study the effect of long-term storage on specimens prepared as DBS and analyzed with the Tina-quant® method. Samples stored at room temperature (20–25°C) were analyzed on day 0, 1, 2, 43, and 44.

To simulate temperature exposure and U.S. Postal Service (USPS) delivery times within the continental United States, a 9-day stability study was performed at 4, 25, and 40°C. Samples delivered to our laboratory by the USPS average 4.5 days from the date the sample was collected. Three samples were spotted onto filter paper as described previously. Samples were removed from storage on day 1, 3, 5, 7, and 9 and stored at –20°C until the end of the study. Five eluates from each sample were prepared and analyzed in a single run with the Tina-quant® method.

National Glycohemoglobin Standardization Program criteria for level II certification were used as the measure of acceptability for Tina-quant® stability studies. The 95% CI for measurements, over time, must fall within ±0.75% HbA1c of the original value on day 0.

Statistical Analysis

Statistical analysis was performed with JMP Statistical Discovery software. JMP is a product of SAS, Inc. (Cary, NC).

Results

Correlation Studies

Two correlation studies were performed to investigate the relationship between whole blood and DBS HbA1c results. The first study, shown in **Figure 1**, compared 73 matched whole blood and DBS samples, analyzed for HbA1c with the Tina-quant® assay. The relationship was highly correlated with $r^2 = 0.996$ ($y = 0.984x + 0.189$, $p < 0.0001$). The second study, shown in **Figure 2**, compared 40 whole blood and DBS samples provided by the NGSP. Whole blood samples were analyzed by the NGSP using their reference Tosoh ion-exchange HPLC method. Dried blood spot samples were analyzed using the Tina-quant® assay method. The two methods were highly correlated with $r^2 = 0.970$ ($y = 0.998x - 0.204$, $p < 0.0001$).

Bland–Altman plots of differences between DBS and whole blood HbA1c for both correlation studies are shown in **Figures 3** and **4**. A mean HbA1c value of 6.66% was observed in whole blood and 6.74% in DBS samples when analyzed by the Tina-quant® method (**Figure 3**). The mean difference between whole blood and DBS HbA1c was 0.08% HbA1c (±0.042, 95% CI).

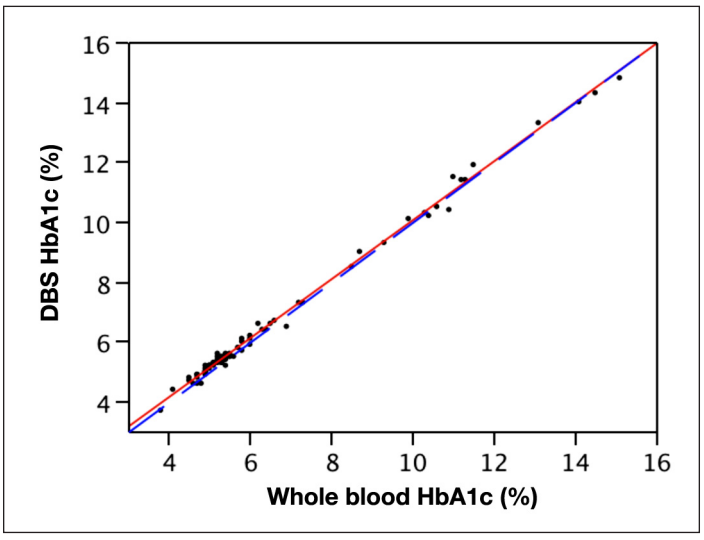

Figure 1. Comparison of whole blood HbA1c and DBS HbA1c analyzed by the Tina-quant® immunoturbidimetric method ($y = 0.984x + 0.189$, $r^2 = 0.996$).

This difference was statistically significant ($p = 0.0002$). For the correlation study performed with specimens provided by the NGSP, a mean value of 8.22% HbA1c for whole blood by the Tosoh method and 8.00% HbA1c by the Tina-quant® DBS method was observed. The mean difference between the two methods (**Figure 4**) was –0.22% HbA1c (±0.112, 95% CI). While the mean difference was small, it was statistically significant ($p = 0.0004$).

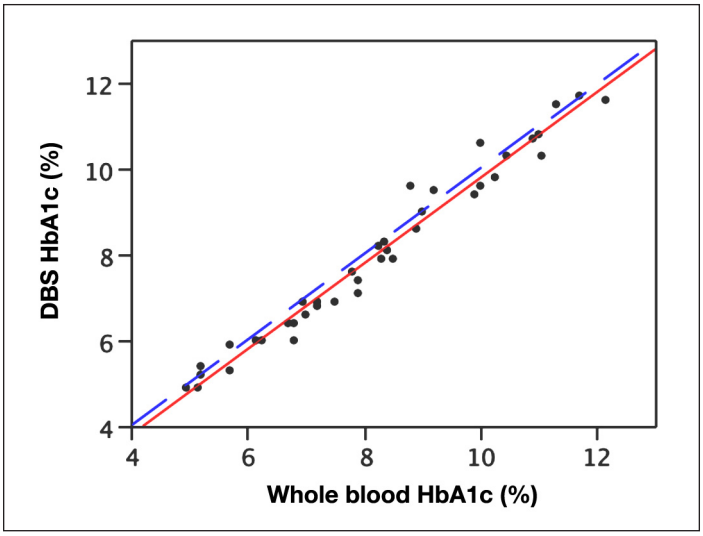

Figure 2. Comparison of whole blood HbA1c analyzed by Tosoh ion-exchange HPLC and DBS HbA1c analyzed by the Tina-quant® immunoturbidimetric method ($y = 0.998x - 0.204$, $r^2 = 0.970$).

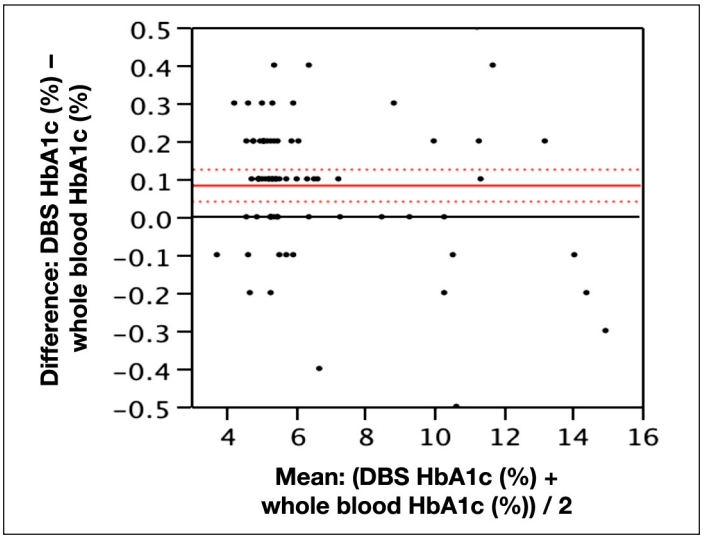

Figure 3. Bland–Altman plot of difference in whole blood HbA1c and DBS HbA1c analyzed by the Tina-quant® immunoturbidimetric method (the reference line at $x = 0$ represents the zero mean difference between the two methods; dashed lines represent the 95% CI of the observed mean difference).

To determine if the mean difference between the methods was clinically significant, MDP analysis, at the critical HbA1c values of 6.0, 6.5, and 7.0%, was performed. For the correlation study involving whole blood and DBS HbA1c by the Tina-quant® method, MDP values were calculated to be 6.09, 6.58, and 7.08%. For the correlation study using whole blood HbA1c by the Tosoh method (NGSP) and DBS HbA1c by the Tina-quant® method, MDP values were 5.79, 6.29, and 6.79%. All values and their 95% confidence limits were within the acceptability criterion of ±0.75% HbA1c.

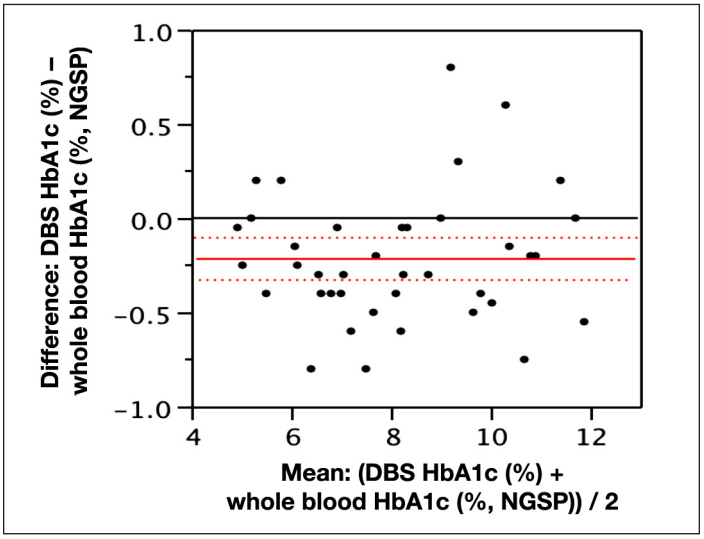

Figure 4. Bland–Altman plot of difference in whole blood HbA1c analyzed by Tosoh ion-exchange HPLC and DBS HbA1c analyzed by the Tina-quant® immunoturbidimetric method (the reference line at $x = 0$ represents the zero mean difference between the two methods; dashed lines represent the 95% CI of the observed mean difference).

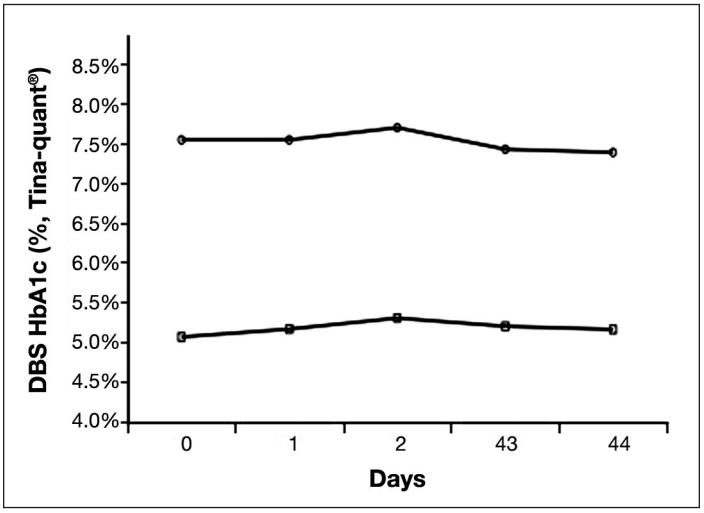

Figure 5. Long-term stability (44 day) study for two DBS samples stored at room temperature (20–25°C).

Precision Studies

Within-run precision studies were performed for DBS samples tested on the Tina-quant® system. The observed %CV was from 0.9 to 3.2%. This compares to the whole blood within-run %CV reported by Roche[11] of 1.8 to 2.3%.

Between-run precision for the Tina-quant® method was determined from DBS samples provided by the NGSP. Results (**Table 1**) show %CV values from 2.2 to 3.0% for DBS samples. This compares to between-run precision results for whole blood samples published by Roche[11] of 2.2 to 4.4%.

Table 1.
Between-Run Precision Results[a]

	Number of replicates	Mean (%HbA1c)	Standard deviation	Coefficient of variation (%)
Group 1 (n = 10)	10 @	5.6	0.16	3.0
Group 2 (n = 10)	10 @	6.9	0.19	2.8
Group 3 (n = 10)	10 @	8.6	0.25	2.9
Group 4 (n = 10)	10 @	10.7	0.23	2.2

[a] The NGSP supplied DBS samples analyzed with the Tina-quant® method.

Stability Studies

The effect of long-term storage at room temperature (~25°C) on specimens collected as DBS and analyzed with the Tina-quant® system is shown in **Figure 5**. The three samples not included in the graph had initial values of approximately 5% HbA1c and showed changes similar to graphed data. The observed change in percentage HbA1c over the 44 days of the study averaged 1.78% with a maximum change of 3.9%. All results were within the predefined acceptability criterion of ±0.75% HbA1c.

A 9-day storage study was performed at 4, 25, and 40°C with DBS samples tested with the Tina-quant® system. **Figure 6** shows the mean of five observations for each sample. The average change for all samples was 2.6% with the largest observed change of 5.6%. All results were within the predefined acceptability criterion of ±0.75% HbA1c.

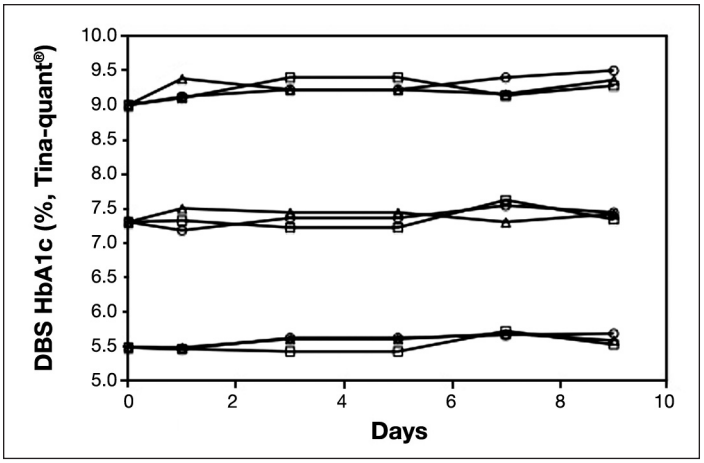

Figure 6. Nine-day stability study for three DBS samples at 4°C (□), 25°C (○), and 40°C (Δ).

Discussion

Correlation results reported show good agreement between whole blood and DBS HbA1c using the Tina-quant® immunoturbidimetric method. These findings are in agreement with previously published data showing good correlation between whole blood and dried blood using the Agappe Diagnostics immunoturbidimetric system.[6] This, however, is in contrast to an earlier study[7] that found a 12% high bias for DBS HbA1c compared to whole blood using the Tina-quant® II assay system.

A negative bias (0.22% HbA1c) was observed for comparisons between whole blood tested with the Tosoh ion-exchange HPLC method and matching DBS samples tested by the Tina-quant® method. A negative bias has also been reported for the Tina-quant® method compared to the Tosoh ion-exchange method.[12]

Stability studies for DBS samples tested with the Tina-quant® system show sufficient robustness to meet the requirements for time and temperature exposure anticipated within the continental United States. These studies are consistent with previously published results showing statistically insignificant changes over a 15-day period[6] and an extensive room temperature study over a 4-week period.[12] This is in contrast to stability studies that show significant temperature-independent increases in HbA1c values over time using an affinity chromatography method.[4]

The ability to provide reliable HbA1c results from samples collected on filter paper makes testing for HbA1c more viable in developing parts of the world or in other situations where venipuncture is not an option. Correlation studies show that DBS samples tested on the Tina-quant® systems are capable of producing reliable results consistent with results obtained by conventional whole blood testing methods.

These studies suggest that use of DBS collection and testing for HbA1c is an alternative to venipuncture sampling. One concern is the ability of the individual to self-collect an adequate specimen for testing. Our initial experience with self-collected DBS HbA1c specimens showed that sample insufficiency rates were about 15%, even though individuals with diabetes, who were experienced in finger stick collection methods, collected the samples. While the HbA1c assay requires a very little sample (~20 μl), the volume required is still considerably greater than what is needed for home glucose monitoring (typically 0.35 to 3 μl). A change in collection instructions to emphasize the required amount and quality of specimen needed along with the availability of a training video has reduced sample insufficiency rates to about 3.5%.

Conclusion

The Tina-quant® II immunoturbidimetric method for the analysis of HbA1c shows good correlation (r^2 = 0.996, $p < 0.001$) between whole blood and samples collected and dried onto filter paper. A slight high bias of 0.08% HbA1c (absolute difference) was observed for dried blood specimens compared to whole blood analyzed by the same method. This bias, while statistically significant, was not considered clinically significant. A negative bias of 0.22% HbA1c (absolute difference) was observed for dried blood specimens analyzed with the Tina-quant® method compared to whole blood analyzed with the Tosoh ion-exchange method.

Dried blood spot samples are stable at room temperature for up to 44 days and up to 9 days (the typical time required for transport through the mail) under a variety of conditions. These characteristics make this specimen collection and analysis system ideal for HbA1c measurement using remote and/or self-collected samples.

Disclosure:

All authors are employed by Heritage Labs International, LLC. Heritage Labs produces and tests the Appraise® and ReliOn® dried blood spot hemoglobin A1c self-collection kits.

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