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ORIGINAL ARTICLES

Measurement of Glycated Hemoglobin A1c from Dried Blood by Turbidimetric Immunoassay

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Abstract

Background:

Glycated hemoglobin A1c (A1C) is an important marker in the diagnosis and treatment of diabetes. Dried blood measurement of A1C is useful in large scale epidemiological evaluation of A1C, especially to assess the impact of intervention programs. The possibility of using dried blood for measurement of A1C by the immunoturbidimetric method was explored in the present study.

Method:

Blood was collected from 30 patients, and blood spots were prepared and dried. The dried blood spot samples were kept for different lengths of time at 4 °C to assess stability. Glycated hemoglobin was measured in whole blood and dried blood on the day of collection as well as on days 10 and 15 by immunoturbidimetric method.

Results:

The A1C values of 30 samples analyzed for comparison between whole blood estimation and dried blood ranged from 4.6% to 9.9%. The mean A1C on the day of sample collection was 6.01% ± 1.58% in fresh whole blood samples and 5.94% ± 1.58 % in dried blood spots. A linear and highly correlated relationship was observed between dried blood A1C values and those in whole blood (r = 0.986 and intraclass correlation value = 0.993). Glycated hemoglobin values on day 10 and day 15 were comparable with the values on day 1 with a shift in mean of just 1% on day 10 and 3.04% on day 15.

Conclusion:

In conclusion, dried blood can be used for measurement of A1C by immunoturbidimetric method, and further stability of A1C measurement from dried blood for up to 15 days at 4 °C makes it an ideal matrix for transportation in developing countries like India.

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Abbreviations: (A1C) glycated hemoglobin A1c, (EDTA) ethylenediaminetetraacetic acid, (HPLC) high-pressure liquid chromatography, (IgG) immunoglobulin G

Keywords: dried blood, glycated hemoglobin, immunoturbidimetric assay, stability

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Introduction

Measurement of glycated hemoglobin A1c (A1C) in human blood is an indicator for long-term control of glycemic state in diabetes patients and is an important marker in the diagnosis and treatment of diabetes. The significance of A1C has been emphasized by the report of the Diabetes Control and Complications Trial.[1] There are currently four principal A1C assay techniques (ion exchange chromatography, electrophoresis, affinity chromatography, and immunoassay) that measure different glycated products and report different units. For large-scale epidemiological evaluation of A1C, especially to assess the impact of intervention programs, storage and transportation of samples at -20 °C or lower temperatures poses a challenge in developing countries like India. A method that circumvents the need to store and ship the samples at a low temperature would be highly desirable, and assays using dried blood appear to be a viable alternative. Several community-based studies have shown dried blood to be convenient and reliable for evaluation of a number of analytes.[2] Measurement of A1C in dried blood by colorimetry,[3] affinity chromatography,[4] and ion exchange chromatography[5] have been reported and found to be useful. The immunoturbidimetric inhibition method for measurement of A1C is a relatively recent technique.[6] We report herewith the measurement of A1C from dried blood by the immunoturbidimetric method. We also evaluated the stability of dried blood stored at 4 °C for 15 days for A1C measurement.

Methods

Thirty patients visiting the outpatient department of the All India Institute of Medical Sciences, New Delhi, India, for routine A1C evaluation were selected at random. Blood was collected by venipuncture into tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. All procedures were in accordance with the ethics standards of our institution. Blood spots were prepared by pipetting 10 µl blood on to the Whatman filter paper (No. 3)[7] kept on a nonadsorbent thermacol surface and were kept at room temperature for drying. After drying, the filter discs were kept in sealed plastic bags to protect from dust and moisture and stored at 4 °C. One aliquot of blood from the 30 individuals was directly analyzed on the day of collection for comparison. The dried blood spots were analyzed on the same day, on the 10th day, and on the 15th day. A punch of 6 mm diameter was used to cut the blood spots. Glycated hemoglobin estimation was by immunoturbidimetric latex method using kits from Agappe Diagnostics (India). The method utilizes the interaction of antigen and antibody to directly determine A1C in whole blood. As per the method, when mouse antihuman A1C monoclonal antibodies are added to whole blood, latex A1C antihuman A1C antibody complex is formed. Agglutination occurs when goat antimouse immunoglobulin G (IgG) polyclonal antibody interacts with the monoclonal antibody. The amount of agglutination that is measured as absorbance would be proportional to the amount of A1C absorbed onto the surface of latex particles. Multipoint calibration was used for preparing calibration curves. One dried blood spot corresponding to 8 µl of blood was punched out and dispensed in 400 µl of hemolysis reagent, mixed well, and kept at room temperature for 30 min for complete lysis. The hemolysate was then incubated with latex reagent followed by the addition of antibody reagent containing mouse antihuman A1C antibody and goat antimouse IgG polyclonal antibody in glycine buffer and further incubation followed by measurement of absorbance at 600 nm. The measurement was carried out in a Beckman Autoanalyzer (CX9). For whole blood estimation, hemolysate was prepared by incubating 10 µl with 500 µl of hemolysis reagent for 10 min. The rest of the procedure was the same as described for dried blood. The immunoturbidimetric latex method was compared with the widely used high-pressure liquid chromatography (HPLC) method on Biorad variant in 15 samples.

For assessing the stability of dried blood stored at 4 °C for A1C measurement, the dried-out spots were punched out from 25 samples at the end of 10 and 15 days, and A1C was measured. Values obtained at days 10 and 15 were compared with day 1 values using repeated measures analysis of variance.

Results

The relationship between samples analyzed by immuno-turbidimetric latex method and HPLC was linear and highly correlated, with an r value of 0.987 (**Figure 1**). The intra-assay and interassay coefficient of variation of the modified method for dried blood was 4.05% and 5.85%, respectively. The A1C values of 30 samples analyzed for comparison between whole blood estimation and dried blood ranged from 4.6% to 9.9%. Twenty-two

samples had an A1C value of less than or equal to 6.5% (upper limit of reference range) and eight samples had an A1C value of more than 6.5%. The mean A1C on the day of sample collection was 6.01% ± 1.58% in fresh whole blood samples and 5.94% ± 1.58% in dried blood spots. The values were compared by paired *t*-test, and the difference was not statistically significant (p = .087). Mean recovery from dried spots were 98.9% (91.0–109.2%). **Figure 2** depicts the relationship between dried blood A1C values and those in whole blood. The relationship was linear and highly correlated, with an r value of 0.986 and an intraclass correlation value of 0.993. The Bland-Altman plot of difference in A1C values in fresh blood and dried blood (**Figure 3**) showed a good agreement between the two methods, with 25 out of 30 samples falling within the one standard deviation limit and none outside the two standard deviation limits.

To assess storage stability of dried blood for A1C estimation, 25 samples were reanalyzed at the end of 10 days of storage of filter disc at 4 °C. Glycated hemoglobin values were comparable with a shift in mean of just 1% (mean at day 0 was 5.92% ± 2.66%, and at day 10, it was 5.86% ± 2.81%) on day 10. At the end of 15 days, the mean value was 6.10% ± 3.77% and the values were 3.04% higher than those at day 0 (**Figure 4**). Analysis by repeated measures analysis of variance gave a p value of 0.686 (F = 0.380), suggesting no significant difference in the values at days 0, 10, and 15.

Discussion

We report a good correlation between A1C measured by latex immunoturbidimetric method in dried blood and fresh blood. The analyte was stable until 15 days in dried blood. Previous studies have reported measurement of A1C in dried blood by thiobarbituric acid colorimetry, affinity chromatography, and HPLC. Little and colleagues,[4] who measured A1C by affinity chromatography in dried blood, showed an increase in A1C

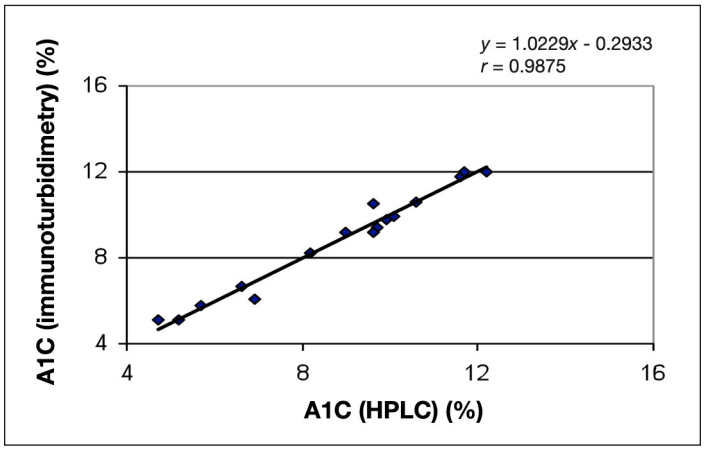

Figure 1. Comparison of A1C measurement by HPLC and the immunoinhibition method.

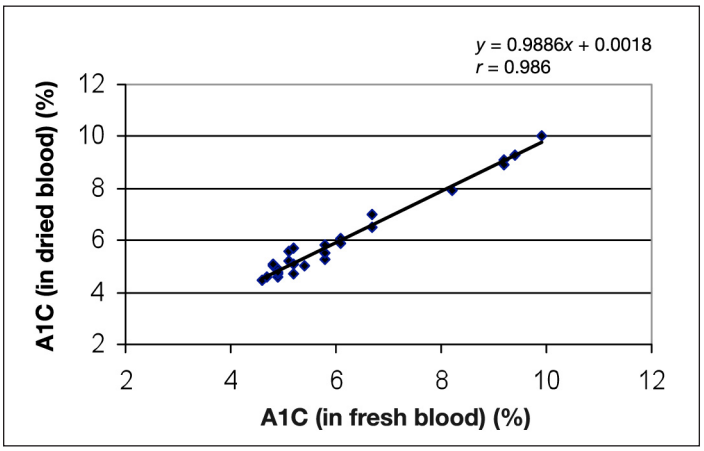

Figure 2. Comparison of A1C values obtained in fresh and dried blood.

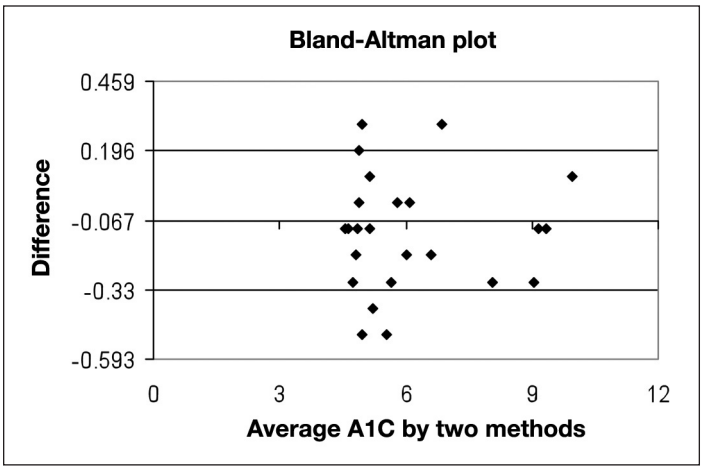

Figure 3. Bland-Altman plot of difference in A1C values obtained from fresh blood and dried blood on the day of sample collection.

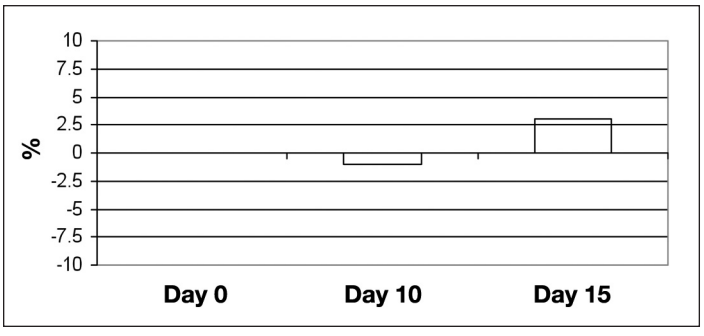

Figure 4. Shift in mean A1C expressed as a percentage of day 0 values.

from day 1 to 14 when stored at higher temperatures but a relatively consistent result when dried blood spots were stored at 4 °C. The authors used glucose oxidase impregnated paper. In another study, a special paper that quickly dries the blood was used for A1C analysis by HPLC, and stability was found good for 2 weeks.[8] Several authors have reported an increase in A1C due to *in vitro* glycation of hemoglobin in stored samples and recommend pretreatment with ethanol/isopropanol,[9] glucose oxidase,[4] boric acid,[3] ethylene glycol[10] phosphate buffered saline with EDTA and p-hydroxy methyl ester[11] to prevent the same. We did not pretreat the Whatman filter paper, but at 4 °C, we found A1C to be remarkably stable until 15 days in dried blood stored in Ziploc bags to prevent moisture. Anjali and coworkers[12] reported stability of A1C in untreated filter paper for 10 days using the turbidimetric immunoinhibition method for estimation. Methodology for A1C measurement reported by different authors differs in the glycated products measured (hemoglobin A1 or hemoglobin A1c). The turbidimetric immunoassay described here measures A1C programmable on autoanalyzer and is easy to carry out. The stability of dried blood up to 15 days makes sample transportation to a distant laboratory with an adequate facility easier and has great applicability in developing countries with considerable rural populations who have limited accessibility to diagnostic laboratories performing the investigations. In India, dry ice and liquid nitrogen is not easily available, and transportation at ultra low temperatures is a problem. Demonstration of stability at 4–8 °C would mean that ice packs could be used.

Conclusion

A good correlation between A1C values measured by latex immunoturbidimetry in fresh and dried blood supports the validity of the assay and the stability of dried blood stored at 4 °C for up to 15 days, making it an ideal matrix for transportation and amenable for widespread usage.

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