ABSTRACT

Objective: To validate an ion exchange high-pressure liquid chromatography (HPLC) method for measuring glycated hemoglobin (HbA1c) in gingival crevicular blood (GCB) spotted on filter paper, for use in screening dental patients for diabetes.

Methods: We collected the GCB specimens for this study from the oral cavities of patients during dental visits, using rigorous strategies to obtain GCB that was as free of debris as possible. The analytical performance of the HPLC method was determined by measuring the precision, linearity, carryover, stability of HbA1c in GCB, and correlation of HbA1c results in GCB specimens with finger-stick blood (FSB) specimens spotted on filter paper.

Results: The coefficients of variation (CVs) for the inter- and intrarun precision of the method were less than 2.0%. Linearity ranged between 4.2% and 12.4%; carryover was less than 2.0%, and the stability of the specimen was 6 days at 4°C and as many as 14 days at -70°C. Linear regression analysis comparing the HbA1c results in GCB with FSB yielded a correlation coefficient of 0.993, a slope of 0.981, and an intercept of 0.13. The Bland-Altman plot showed no difference in the HbA1c results from the GCB and FSB specimens at normal, prediabetes, and diabetes HbA1c levels.

Conclusion: We validated an HPLC method for measuring HbA1c in GCB; this method can be used to screen dental patients for diabetes.

Keywords: HbA1c, gingival crevicular blood, dried blood spot, HbA1c stability, HPLC assay, screening for diabetes

According to the Centers for Disease Control and Prevention (CDC) National Diabetes Fact Sheet for the year 2011, diabetes affects 25.8 million individuals in the U.S.1 Of these, 7 million are unaware that they have diabetes. It is estimated that 79 million adults aged 20 years and older in the United States have prediabetes, a condition in which blood, plasma, or serum glucose and/or glycated hemoglobin (HbA1c) levels are higher than normal but not high enough to meet the criteria for diabetes. Progression from prediabetes to type 2 diabetes is reversible. With lifestyle changes, some people with prediabetes can bring their glucose levels back to normal and others can delay the progression to diabetes.

In 2014, the American Diabetes Association recommended monitoring of HbA1c levels to diagnose patients with diabetes and prediabetes, and to monitor glycemic control in patients with diabetes.2 The diabetes range was defined as an HbA1c level of 6.5% or greater. After treatment, the goal of most adult patients with diabetes is an HbA1c level of less than 7.0%. The prediabetes range was defined as an HbA1c level of between 5.7% and 6.4%.

Abbreviations:
CDC, Centers for Disease Control and Prevention; HbA1c, glycated hemoglobin; NGSP, National Glycohemoglobin Standardization Program; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; EDTA, ethylenediaminetetraacetic acid; DBS, dried blood spot; HPLC, high-pressure liquid chromatography; GCB, gingival crevicular blood; NYU, New York University; CUMC, Columbia University Medical Center; FSB, finger-stick blood; WBS, whole blood specimens; WB, whole blood; CVs, coefficients of variation; r², correlation coefficients; AUC, area under the curve; CI, confidence interval; ND, not determined; RT, room temperature

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In this article, HbA1c results will be reported in percentage of HbA1c units, as recommended by the National Glycohemoglobin Standardization Program (NGSP). HbA1c results can also be reported in mmol/mol, as recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). Conversion of HbA1c percentage to mmol/mol is by the following equation:

\[
\% \text{ HbA1c} = (0.0915 \text{ mmol/mol}) + 2.15
\]

Some advantages of using HbA1c levels in diabetes testing include the low preanalytical and biological variation of HbA1c and the fact that HbA1c results reflect overall glycemic exposure with no requirement that the patient be fasting before specimen collection. HbA1c is often measured in a hospital laboratory using blood collected via venipuncture into ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. Screening for diabetes outside a hospital laboratory (ie, in the office of a primary care provider) often involves collecting blood by finger-stick puncture and measuring HbA1c levels on site using a point-of-care analyzer or spotting the blood on filter paper and sending the dried blood spot (DBS) to a clinical laboratory for analysis. HbA1c levels have been measured in DBSs obtained by finger-stick puncture via ion exchange high-pressure liquid chromatography (HPLC), affinity chromatography, and turbidimetric immunochemical assays.

In this article, we present the results of our analyses, which support the use of another blood-specimen source, gingival crevicular blood (GCB), to screen dental patients for diabetes. The results of a pilot study that used HbA1c levels from GCB to screen dental patients for diabetes showed that prediabetes or diabetes could be detected via this method. However, the analytical data presented to validate the HPLC method were limited; also, 26% of the specimens could not be analyzed due to an interfering peak in the chromatogram. Therefore, herein, we provide detailed and optimized analytical data to validate an ion-exchange HPLC procedure for measuring HbA1c in GCB spotted on filter paper.

**Materials and Methods**

This study was approved by the institutional review boards of the New York University (NYU) Langone Medical Center and Columbia University Medical Center (CUMC). We collected GCB and finger-stick blood (FSB) from patients in the general practice clinics at the NYU College of Dentistry. FSB was collected by registered nurses or trained nursing students, and GCB was collected by dental providers or trained dental and dental hygiene students. HbA1c levels were measured at CUMC.

**Specimen Collection**

**GCB Specimens**

All dentists and dental hygienists were trained to collect the GCB specimen from patients while they were seated in the dental chair. After probing and selecting a site that exhibited erythema and/or edema, the dental provider isolated the area using cotton rolls to prevent saliva contamination, scaled the site, dried the area with gauze to eliminate contaminants from the tooth, and then reprobed the site, resulting in a steady flow of debris-free blood. The dental provider collected blood using a micropipette and transferred the blood to 1 or more discs on Whatman 903 filter paper (Whatman, Inc, Sanford, ME). The blood was allowed to dry at room temperature for 1 hour before refrigeration, in anticipation of transfer to the laboratory for HbA1c analysis.

**FSB Specimens**

FSB was also collected from patients while they were seated in the dental chair. After the side of the fingertip of the patient was cleaned with an alcohol prep pad, the alcohol was allowed to evaporate. After the skin was dry, the dental practitioner punctured the side of the fingertip with a sterile lancet. The first drop of blood was wiped away with a sterile gauze pad. With hand of the patient positioned palm down, the blood of the patient was collected using a micropipette and then transferred to 1 or more discs on Whatman 903 filter paper. As with the GCB specimen, the FSB was allowed to dry at room temperature for 1 hour before refrigeration, in anticipation of transfer to the laboratory for HbA1c analysis.

**Whole Blood Specimens (WBSs)**

Blood for HbA1c analysis was collected from hospital patients at CUMC by venipuncture in 5-mL BD Vacutainer tubes with EDTA (Becton, Dickinson, and Company. Franklin Lakes, NJ). Dried blood spots from these specimens were obtained by spotting enough blood to
completely cover 1 disc (a circle 0.5-in in diameter) of Whatman 903 filter paper.

**Instrumentation and Reagents**

We measured HbA1c levels using the D-10 ion exchange HPLC whole blood (WB) procedure from Bio-Rad Laboratories (Hercules, CA). Use of this method is traceable to the reference methods of the National Glycohemoglobin Standardization Program (NGSP) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The D-10 software program calculates the HbA1c area using an exponentially modified Gaussian algorithm that excludes the labile A1c and carbamylated peak areas from the HbA1c peak area.

We obtained reagents from Bio-Rad Laboratories, Inc. Those reagents consisted of bis-triphosphate elution buffers of pH 6.0 and pH 6.7, a wash-diluent solution of deionized water, a cation-exchange resin, and a WB primer.

**Procedure**

**For GCB, FSB, and WB Specimens Spotted on Filter Paper**

The accuracy of the HbA1c results depends on the collection method for blood specimens. When conducted properly, the method avoids introduction of contaminants during the specimen collection process and ensures collection of a sufficient volume of blood (spotted on the filter paper). There are 5 discs on each Whatman filter paper. If sufficient blood was collected to cover 1 entire disc, one 3/16 inch punch was used. If small amounts of blood are spotted in several discs, 2 to 4 punches are needed to obtain enough blood for analysis. We placed the punched disc(s) from each filter paper spot into a vial containing 1 mL of dilutent. After each disc stood at room temperature for 1 hour, it was removed from the vial, and the vial was inverted and placed on the analyzer.

**Preparing WB Specimens for the Analyzer**

We prepared each specimen by mixing 5 μL of blood with 1 mL of dilutent. All vials were mixed before placing them on the analyzer.

**HbA1c Assay**

The Bio-Rad D-10 extended program (Bio-Rad Laboratories, Inc., Hercules, CA) is a 6.5-minute procedure that we used to measure HbA1c levels. After placing the vials on the analyzer, the specimens are automatically injected onto a column containing a negatively charged cation-exchange resin that has an affinity for the positively charged hemoglobins. Buffers of increasing ionic strength pass through the column, and the hemoglobin fractions are separated based on their ionic interactions with the column. The separated hemoglobin fractions pass through the flow cell of the filter photometer, where the absorbances are measured at a wavelength of 415 nm. Background is subtracted by measuring the absorbance at a wavelength of 690 nm.

WB control materials containing high (9.1%) and normal (5.2%) levels of HbA1c, as supplied by Bio-Rad Laboratories, Inc., were measured before each run of specimens. At the beginning of the study, the control material was spotted onto Whatman 903 filter paper and its levels of HbA1c were measured. When HbA1c levels were measured directly from the vial (5 μL of control material with 1 mL of dilutent) and compared with the DBS control results, we observed no significant difference in HbA1c values. In this study, we analyzed the control material directly from the vial.

All specimens were stored at 4ºC or -70ºC and analyzed within 5 days of the blood being drawn. After analysis, leftover specimens were stored at -70ºC.

**Precision Studies**

We chose specimens that had HbA1c values in normal and diabetes ranges. Precision was determined using GCB, FSB, and WB specimens spotted on filter paper. For the intra-assay precision study, 5 dilutions from each specimen were prepared from 5 different blood spots, and each was measured in duplicate. For the inter-assay precision, the dilutions were prepared daily from the GCB, FSB, and WB spots, and HbA1c levels were measured via duplicate testing sessions for 5 days.

**Linearity**

Linearity was determined by measuring HbA1c in WB spotted on filter paper. A specimen with an HbA1c level of
12.4% was diluted with a specimen containing HbA1c at a concentration of 4.2% to obtain HbA1c levels of 10.4%, 8.3%, 6.3%, 5.3%, and 4.5%.

**Carryover Studies**

We determined carryover by performing 3 successive measurements of high-level HbA1c in GCB, FSB, and WB specimens spotted on filter paper. This was followed by 3 successive measurements of low-level HbA1c in GCB, FSB, and WB specimens.

**Readable Area Range**

We determined the readable area range of the assay by spotting WB specimens with normal, prediabetes, and diabetes HbA1c levels onto filter paper. Specimens were prepared using the following steps. First, we used the standard procedure: one 3/16 punch and 1 mL of diluent. Second, we increased the readable area by adding 1 mL of diluent to 2, 3, or 4 punches. Third, we decreased the readable area by using 1 punch, adding 2 mL of diluent, and making serial 1.5- and 2.0-fold dilutions. We measured HbA1c levels in these specimens in duplicate.

**HbA1c Stability Studies**

We determined the stability of HbA1c using different GCB and FSB specimens from dental patients with HbA1c values in the normal, prediabetes, and diabetes ranges. The WB specimen was collected from a hospital patient at CUMC. The GCB, FSB, and WB specimens were spotted on filter paper and stored at room temperature, 4°C, and -70°C. We measured the HbA1c levels of the specimens for as long as 28 days.

**Method Comparison**

We determined the HbA1c levels in 50 GCB and matched FSB specimens spotted on filter paper. Also, we determined these levels in 60 WB specimens and the corresponding WB specimens spotted on filter paper.

**Statistical Analysis**

Statistical calculations were performed using GraphPad Prism software, version 5.0 (GraphPad Software Inc, La Jolla, CA). We determined means, SDs, coefficients of variation (CVs), correlation coefficients ($r^2$), and SEs, as well as creating Bland-Altman charts.

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**Results**

**Precision Studies**

Table 1 shows the mean percentages, SDs, and coefficients of variation (CVs) for the inter- and intra-assay studies for the GCB, FSB, and WB specimens spotted on filter paper. The average CVs for the inter-assay precision for specimens with normal and elevated HbA1c levels were 1.5% and 1.0%, respectively. The average CVs for the intra-assay precision for specimens with normal and elevated HbA1c levels were 1.4% and 0.9%, respectively. There was no difference in the CVs when GCB, FSB, or WB specimens spotted on filter paper were used to measure HbA1c. The CVs that we obtained with this method met the interassay precision specifications of less than 3.0% recommended by Sacks et al.

**Carryover Studies**

Carryover was measured using GCB and FSB from dental patients and venous WB from hospital patients; all these specimens were spotted on filter paper. High and low HbA1c levels in the GCB specimens were 8.9% and 4.2%, respectively; in the FSB specimens, those levels were 9.1% and 4.3%. In the WB specimens, those levels were 13.1% and 4.2%, respectively. We did not perform carryover studies using GCB and FSB specimens with HbA1c levels of greater than 10% because of the lack of specimens with HbA1c at these levels. Three consecutive measurements of HbA1c in a specimen with a high HbA1c level were followed by 3 measurements of HbA1c in a specimen with a low HbA1c level. The percentage of carryover was calculated using the following formula:

$$\% \text{ Carryover} = \frac{[L1 - L3]/(H3 - L3)] \cdot 100}{H}$$

where H is the concentration of HbA1c in the specimen with a high HbA1c level and L is the concentration of HbA1c in the specimen with a low HbA1c level. The percentage of carryover in the GCB and FSB specimens was 2.0%; in the WB specimen, it was 1.1%. This meets the acceptance criteria for carryover of less than or equal to 2.0%.
Linearity

Linearity was determined by measuring HbA1c in duplicate in the WB specimens spotted on filter paper. Linear regression analysis of the expected and measured HbA1c results yielded a slope of 0.991, intercept of 0.01, and SE of 0.08. The percentage of recovery averaged 101% (range, 100% to 102%). The linearity of the method extends from 4.2% to at least 12.4%. We did not determine linearity using GCB and FSB because an insufficient amount of blood was collected to perform linearity studies.

Readable Area Range

The readable area range is the difference between the maximum and minimum area counts of the HbA1c peak in the chromatogram. If the total area falls within this range, the areas of each peak in the chromatogram are used in the calculation of HbA1c levels. Bio-Rad Laboratories, Inc. recommends that the readable area range for measuring HbA1c with the D-10 6.5-minute procedure be between 1 million and 4 million µvolt per second. The units in µvolt per second are obtained by measuring the absorbance at 415 nm and converting the absorbance units to voltage units (1 voltage unit = 1 absorbance unit).

Table 2 shows the readable area ranges and the HbA1c levels for specimens in the normal, prediabetes, and diabetes ranges. We obtained acceptable HbA1c results when the total readable area of analysis was between 0.7 million and 4.0 million µvolt per second. HbA1c levels were not reported when the range was less than 0.7 million or greater than 5.0 million µvolt per second.

In this study, the readable area range for most of the specimens was between 0.8 million and 2.0 million µvolt per second. HbA1c levels were not reported when the range was less than 0.7 million or greater than 5.0 million µvolt per second.

Method Comparison

Figure 1 shows the linear regression analysis for 50 matched GCB and FSB specimens. HbA1c concentrations ranged between 4.8% and 9.9%. A slope of 0.981 (95% confidence interval [CI], .95 to >.99), intercept of 0.13 (-.08 to .34), SE of 0.08, and a correlation coefficient of 0.993 were obtained. There were 17 specimens with HbA1c levels of between 4.8% and 5.6%, 23 specimens with HbA1c levels of between 5.7% and 6.4%, and 10 specimens with HbA1c levels between 6.5% and 9.9%. A relative difference of ±6% is used by NGSP to identify bias between HbA1c results. For the 50 matched GCB and FSB specimens, none of the relative differences in the HbA1c results exceeded the 6% limit. The mean difference (FSB-GCB) for the 50 specimens was -0.2% (range, -4.8% to 3.9%). Good agreement was obtained between the HbA1c results from the GCB and FSB specimens.
Figure 2 shows the Bland-Altman plot of the differences in HbA1c results obtained with the GCB and FSB specimens. Mean HbA1c values of 6.03% were obtained from the FSB specimens and 6.04% from the GCB specimens. The mean difference between the results was 0.01%.

We also performed linear regression analysis for the HbA1c results obtained from 60 matched WB and WB specimens spotted on filter paper. HbA1c levels ranged from 4.0% to 13.8%. There were 21 specimens with HbA1c levels between 3.7% and 5.4%, 14 specimens with levels between 5.9% and 6.6%, 12 specimens with levels between 7.0% and 9.5%, and 13 specimens with levels between 10.0% and 13.8%. A slope of 0.975 (95% CI, 0.96 to 0.99), an intercept of 0.09 (-0.04 to 0.23), SE of 0.16, and correlation coefficient of 0.997 were obtained. For the 60 matched WB and WB specimens spotted on filter paper, the mean difference (WB specimens spotted on filter paper - WB) for the 60 specimens was 1.1% (range, -5.1% to 6.1%). In 1 specimen, the difference in the HbA1c results was greater than 6%. Good agreement was obtained between the HbA1c results from WB and WB spotted on filter paper.

Specimen Stability

Table 3 shows the stability of HbA1c in GCB, FSB, and WB specimens spotted on filter paper and stored at room temperature, 4°C, and -70°C. At HbA1c concentrations of greater than 9.5%, HbA1c is stable for as many as 28 days in GCB, FSB, and WB specimens stored at room temperature, 4°C, and -70°C.

HbA1c levels are stable in GCB and FSB specimens with prediabetes HbA1c levels that are stored at 4°C or room temperature for as many as 6 days, after which there is an increase in the HbA1c level. For the GCB and FSB specimens stored at 4°C, the relative increase in HbA1c was greater than the 6% limit established by the NGSP after 21 days and for the GCB specimen stored at room temperature, the relative increase in HbA1c was greater than 6% after 14 days.

For the GCB and FSB specimens with normal HbA1c levels that were stored at room temperature, the relative increase in HbA1c was greater than 6% after 6 days. For the specimens that were stored at 4°C, the relative increase in HbA1c was greater than 6% after 14 days.

Specimen Stability

HbA1c is stable in GCB and FSB specimens with normal and prediabetes levels for at least 14 days if stored at -70°C. We did not test the stability of such specimens after 14 days in the GCB and FSB specimens due to insufficient specimen volume. Although not shown in Table 3, the stability of HbA1c in WB specimens with normal and prediabetes levels spotted on filter paper shows the same pattern that we observed in the normal and prediabetes GCB and FSB specimens.

Discussion

The ideal scenario to validate a method for measuring HbA1c in GCB would be to collect venous blood and GCB and to measure the HbA1c in both sets of specimens. Although dentists are familiar with using FSB for glucose measurements,12,13 they may not be proficient in collecting venous blood. In our study, we used GCB and FSB collected and spotted on filter paper in a dental setting and WB and WB spotted on filter paper from a hospital-based population to evaluate the HPLC assay. The close correlations between HbA1c levels of WB and WB spotted on filter paper and between the HbA1c levels of GCB and FSB spotted on filter paper indicate the GCB HbA1c results are accurate.
Among the 50 GCB specimens, 3 had hemoglobin AS trait and 2 had hemoglobin F concentrations between 5% and 7%. Examination of the chromatograms for these specimens revealed acceptable separation of the hemoglobin fractions. Studies have shown that with the D-10 assay, there is no interference from specimens with hemoglobin AS or from hemoglobin F at concentrations of as high as 12.5%.

For an accurate measurement of HbA1c, adequate GCB is required. Our protocol requires the laboratory technologist to examine the blood spot; if a small amount of blood is present, several punches are performed. In this study, only 1 specimen was rejected for insufficient quantity of blood. The AUC for this specimen was 0.4 million µvolt per second, which is less than the 0.7 million µvolt per second that is required for analysis.

Spotting of FSB on filter paper and sending the DBS to a laboratory for HbA1c analysis has been used for home screening and to screen patients in third-world countries for diabetes. The stability of HbA1c in the DBS plays an important role in these screening programs. In this study, we found that the rate of increase of HbA1c in GCB, FSB, or WB specimens spotted on filter paper depends on the level of HbA1c and the temperature at which the DBS is stored. The rate of increase is greatest when the specimen is stored at room temperature and when the HbA1c level is within the normal range. The reason for the increase in HbA1c could be continued glycation of hemoglobin. The stability profile of HbA1c in GCB, FSB, and WB spotted on filter paper is essentially identical and does not vary depending on the source of the specimen.

Table 3. Stability of the HbA1c Specimens Spotted on Filter Paper

<table>
<thead>
<tr>
<th>Specimens With HbA1c Level of &gt;9.5%</th>
<th>WBS</th>
<th>GCB</th>
<th>FSB</th>
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<td>RT</td>
<td>4°C</td>
<td>-70°C</td>
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<td>9.9</td>
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<td>9.9</td>
<td>9.9</td>
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<td>9.9</td>
<td>9.8</td>
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</table>

<table>
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<tr>
<th>Specimens With HbA1c Level of 5.7%-6.4%</th>
<th>GCB</th>
<th>FSB</th>
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<tr>
<td>Days, No.</td>
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</tr>
<tr>
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<table>
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<th>FSB</th>
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</tbody>
</table>

HbA1c, glycated hemoglobin; WBS, whole-blood specimens; GCB, gingival crevicular blood; FSB, finger-stick blood; RT, room temperature ND not determined due to insufficient specimen volume; *Exceeds the ±6 % relative differences in HbA1c results based on the NGSP criteria.
When the 6.5-minute instead of the 3.0-minute Variant II program was used, separation of the hemoglobin peaks improved. In an earlier study in which HbA1c was measured in GCB using the Variant II analyzer, HbA1c levels could not be reported in 26% of the specimens because of an unidentifiable peak in the chromatogram that coeluted with HbA1c. The reason for the poor separation of the hemoglobin fractions could be the use of the 3.0-minute program to measure HbA1c levels. We used the 6.5-minute program on the D-10 analyzer to measure HbA1c and found good separation between HbA1c and the other hemoglobin fractions for all 50 GCB specimens.

In a recent study to determine whether the HbA1c levels from GCB could be viable for screening dental patients for diabetes, GCB was collected and spotted on filter paper from 245 adults who were older than 45 years and from 96 patients with an age range between 18 years and 44 years. These patients are at high risk for diabetes but had never been told this. HbA1c was measured in the DBSs from this cohort using the D-10 HPLC procedure detailed herein. A total of 51% of the patients that were older than 45 years and 23% of the patients aged 18 years to 44 years had GCB HbA1c levels in the prediabetes or diabetes range. These results show that the HbA1c levels obtained from GCB could be used to screen for diabetes in dental patients who have not been diagnosed with the disease.

HbA1c testing during a dental visit offers an opportunity to screen a large segment of the U. S. population that may not know whether they have diabetes or prediabetes. A study has shown that most dental patients support diabetes testing in the dental office and that most dentists are willing to collect blood for diabetes screening. Many patients who had GCB and FSB collected for diabetes screening preferred GCB because the GCB collection felt like a routine dental cleaning, whereas the FSB collection could be painful. Many dental providers also preferred GCB to FSB because they often observe considerable bleeding related to gingival inflammation. Although controls and safeguards need to be taken to obtain GCB that is as free of debris as possible, dental professionals still viewed the collection of oral blood as being easier than collection of FSB.

In conclusion, this article validates the measurement of HbA1c in GCB spotted on filter paper. This ion exchange HPLC method is precise, yielding CVs of less than 3%, linearity of at least 12.4%, and no specimen carryover. Excellent correlation (r = 0.993) was obtained between the HbA1c levels in GCB and the corresponding FSB specimens spotted on filter paper. The HbA1c levels obtained from GCB can detect patients with prediabetes or diabetes; and as such, GCB represents another blood specimen source that can be used to screen dental patients for diabetes.

Acknowledgments

We thank Bio-Rad Laboratories, Inc., for providing the D-10 HPLC analyzer that we used in this study. This research was partly funded by a grant from the National Institute of Dental and Craniofacial Research of the National Institutes of Health (grant no. 1R15DE023201). LM

References


