Abstract

The CAPILLARYS HbA1c kit used on the CAPILLARYS 2 Flex Piercing system is designed for separating and quantifying the HbA1c fraction of hemoglobin while detecting hemoglobin variants and HbA2. The aim of this study was to assess the performance of this new capillary electrophoresis kit for routine HbA1c quantification for patients with diabetes. The reliability of the system for HbA2 measurement was also assessed. Precision, correlation, and linearity studies were performed. The effect of hemoglobin concentration and the presence of coexistent interfering substances were evaluated. The results were found to be accurate and linear in the clinically significant analytic range with excellent precision (mean interassay coefficient of variation of 0.7%). HbA1c measurement is independent of the presence of common hemoglobin interferences for HbA1c quantification and the total concentration of hemoglobin. Moreover, the technique provides a rapid and reliable separation of HbA2. The measurement is reproducible and the results in β-thalassemia carriers were statistically different from those in noncarriers. Thus, this technique might be used for β-thalassemia detection. Given the short time of the analysis and the high throughput, this is a suitable system for the control of diabetes and the detection of hemoglobinopathies in laboratories with high workflow.

Hemoglobin (Hb) A1c, the major form of glycated hemoglobin, is the result of the nonenzymatic glycation of the N-terminal valine residues of the β chain of HbA. HbA1c has been shown to be a key biomarker whose measurement provides the most important medium to long-term time-averaged glycemic status. Its relationship with the clinical outcome and complications of diabetes mellitus has been convincingly demonstrated for both type I and type II subjects in 2 major clinical trials.1,2 The recently implemented consensus statement on worldwide standardization3 and the International Federation of Clinical Chemistry (IFCC) reference method for HbA1c measurement4 have contributed substantially to improving the quality of HbA1c measurements. Expanding the role of this hemoglobin fraction from monitoring to screening and diagnosis of diabetes5 leads to a greater use for this test.

Various methods have been used for the measurement of glycohemoglobins.6 Because of their automation and precision, the high-performance liquid chromatography (HPLC) methods have been favored, but recently capillary electrophoresis has been developed and adapted to the analysis of HbA1c. The CAPILLARYS HbA1c kit (Sebia, Lisses, France) is designed for the separation and quantification of the HbA1c fraction of hemoglobin in human blood by means of capillary electrophoresis in an alkaline buffer with the CAPILLARYS 2 Flex Piercing instrument (Sebia). By using alkaline pH buffer, normal and abnormal (or variants) levels of hemoglobin are detected in the following order, from cathode to anode: A2/C, E, S, D, F, A0, other hemoglobins (including minor HbA1) and then A1c.

Hemoglobinopathies and thalassemias are among the most common genetic disorders worldwide.7 Thus, hemoglobin variants can be a serious issue for the accuracy of HbA1c
quantification in the course of routine control of diabetes. Thalassemia is prevalent in the Mediterranean basin and in Southeast Asia, where it represents a major public health problem. However, nonendemic countries are also involved in thalassemia-related problems as a result of the migration of groups with a high frequency of thalassemic mutations.\textsuperscript{8} HbA\textsubscript{2} quantification plays a key role in screening programs for thalassemia: its decrease might reveal α-thalassemia and its increase β-thalassemia.\textsuperscript{9}

The analytic performance of the CAPILLARYS 2 Flex Piercing instrument was evaluated to verify the quality of HbA\textsubscript{1c} quantification, based on the criteria established in the recently published documents of consensus on HbA\textsubscript{1c}. Special attention was paid to the influence of the most frequent analytic interferences: labile HbA\textsubscript{1c} (LA\textsubscript{1c}), carbamylated hemoglobin (cHb), acetylated hemoglobin (AcHb), and fetal hemoglobin (HbF); the ability of the system to quantify HbA\textsubscript{1c} in the presence of the most frequent hemoglobin variants was also evaluated. The quantification of HbA\textsubscript{2} using both the CAPILLARYS hemoglobin program and the CAPILLARYS HbA\textsubscript{1c} program was compared to assess the ability of the HbA\textsubscript{1c} program to report a reliable HbA\textsubscript{2} value for the detection of β-thalassemia.

Materials and Methods

The CAPILLARYS 2 Flex Piercing analyzer as well as the reagents (HbA\textsubscript{1c} kit, reference no. 2015) used for this evaluation were provided by Sebia and used according to the manufacturer’s instructions. HbA\textsubscript{1c} values are reported in percentages; a master equation can be used to calculate values in millimoles per mole.\textsuperscript{3}

CAPILLARYS 2 Flex Piercing Analyzer

The CAPILLARYS 2 Flex Piercing is a multiassay fully automated analyzer that allows the analysis of whole blood samples directly from primary capped tubes. The CAPILLARYS 2 Flex Piercing instrument has 8 silica capillaries functioning in parallel, allowing 8 simultaneous analyses for HbA\textsubscript{1c} quantification from whole blood samples. The CAPILLARYS HbA\textsubscript{1c} kit (migration buffer, hemolysing solution, wash solution, and dilution segments, reference no. 2015), controls (levels 1 and 2, reference no. 4774), and calibrators (levels 1 and 2, reference no. 4755) were used. The assay was performed on the hemolysate of whole blood samples collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. A sample dilution with hemolysing solution was automatically prepared and injected by aspiration at the anodic end of the capillary. A high-voltage protein separation was then performed and hemoglobin directly detected at the cathodic end of the capillary at 415 nm, which is the specific absorbance wavelength for hemoglobin. Direct detection provides accurate relative quantification of all hemoglobin fractions and notably the HbA\textsubscript{1c} fraction. After completion of the electrophoretic separation, the Phoresis software (version 8.50) detected normal and abnormal peaks on the profiles (eg, the presence of hemoglobin variants), then integrated HbA\textsubscript{1c} and HbA\textsubscript{0} peaks. The formula used in this system was based on IFCC recommendations: HbA\textsubscript{1c} = HbA\textsubscript{1c}/(HbA\textsubscript{1c} + HbA\textsubscript{0}).

The CAPILLARYS 2 Flex Piercing HbA\textsubscript{1c} assay is certified by the IFCC and the National Glycohemoglobin Standardization Program (NGSP). The CAPILLARYS 2 Flex Piercing strictly follows the international consensus recommendations of 2010. The calibration is obtained by means of Sebia calibrators traceable to the IFCC measurement procedure, giving results in millimoles per mole. For the controls and samples analyzed, the software applies the master equation to move results from IFCC units (millimoles per mole) to NGSP units (percentage). It is an important difference from other systems in which the calibrators, also traceable to IFCC, are changed to NGSP percentages using the master equation. The master equation is then reused to obtain IFCC results (millimoles per mole) for the controls and samples.

Samples and Controls

Blood specimens were obtained from patients whose diabetic control or hemoglobinopathy were being assessed routinely and were used in this study according to the hospital’s ethics guidelines. Whole blood samples were obtained in the course of routine analysis and collected in EDTA anticoagulant tubes (Vacutainer Becton-Dickinson, Rutherford, NJ). Quality control materials used throughout the evaluations were provided by Sebia (levels 1 and 2, reference no. 4774).

Linearity Study

The linearity of the method was evaluated by measuring HbA\textsubscript{1c} in serial dilutions of 2 blood samples with high (14.6%) and normal (5.1%) values, containing the same total hemoglobin concentration.

Precision Study

Intra-assay precision was determined by 5 quantifications from the same sample for 3 levels of HbA\textsubscript{1c} (5.2%, 7.2%, and 10.9%) within the same series. Interassay precision was determined by 5 quantifications of 8 samples with different levels of HbA\textsubscript{1c}.

Correlation Study

HbA\textsubscript{1c} results obtained with the CAPILLARYS 2 Flex Piercing analyzer were compared with the results obtained with the ion-exchange HPLC system used routinely in the laboratory for HbA\textsubscript{1c} assay (Menarini/Arkay ADAMS A1c HA-8160, Menarini Diagnostics, Firenze, Italy). The study
was performed according to guideline EP9 of the Clinical Laboratory Standards Institute; 107 samples without hemoglobin variants were analyzed in duplicate.

Interferences

The interference of cHb was performed by incubating a pooled blood sample (HbA1c 5.0%) with increasing concentrations of a sodium cyanide solution (≤10 mmol/L). The samples were incubated for 2 hours at 37°C, and then analyzed.

The interference of LA1c was performed by incubating a pooled blood sample (HbA1c 5.2%) with increasing concentrations of a glucose solution (≤1,000 mg/dL [≤55.5 mmol/L]). The samples were incubated for 2 hours at 37°C, and then analyzed.

The interference of AcHb was performed by incubating a pooled blood sample (HbA1c 5.0%) with increasing concentrations of an acetaldehyde solution (2-10 mol/L). The samples were incubated for 2 hours at 37°C, and then analyzed.

The effect of HbF concentration was investigated by mixing blood samples (with known HbA1c levels) with umbilical cord blood to obtain HbF levels up to 15% (HbF values were determined using the CAPILLARYS 2 Flex Piercing hemoglobin method).

The potential interference of lipemia was investigated by overloading pooled blood samples (HbA1c 5.0%) by adding increasing amounts of pooled blood with high concentration of triglycerides (1,592 mg/dL [18 mmol/L]). The effect of bilirubin was evaluated similarly by adding pooled blood samples with high concentrations of bilirubin (16.5 mg/dL [282 µmol/L]).

Effect of Hemoglobin Concentration

The effect of varying hemoglobin concentrations on electrophoretic quantification was tested by mixing RBCs of a patient with a known HbA1c (7.1%) with their own plasma at different ratios. The range of hemoglobin concentrations studied was between 2 g/dL (20 g/L) and 19.5 g/dL (195 g/L).

HbA1c Quantification With the HbA1c Program

The HbA1c concentration was tested with the HbA1c program on CAPILLARYS 2 Flex Piercing for 279 normal individuals and 64 β-thalassemia carriers. An independent sample t test was performed to detect statistical differences between both groups. A P value less than .05 was considered statistically significant. Receiver operating curve (ROC) analysis was applied to calculate the best cutoff to discriminate noncarriers vs carriers of the β-thalassemia trait.

The HbA2 level was quantified in parallel using the hemoglobin program on CAPILLARYS 2 Flex Piercing for 60 normal individuals and 64 β-thalassemia carriers. Both results were plotted and the correlation between the 2 programs was studied with the Passing-Bablok regression. SPSS version 19.0 for Windows statistical software package (SPSS, Chicago, IL) was used for statistical analysis of the results.

Results

Analytic Performance of the HbA1c Quantification on CAPILLARYS 2 Flex Piercing

A typical electrophoretogram exhibits 3 peaks that correspond to HbA1c, HbA2, and HbA0, and an additional fraction identified as “other HbA” Figure 1A. All peaks are well separated, with a good return to baseline between them. Any modification of this profile, usually because of the presence of a hemoglobin variant, is indicated by the software as “atypical profile” with a special color code, thus allowing the rapid and easy identification of samples with hemoglobin variants.

The linearity of the method in the range of HbA1c values tested (5.1%-14.6%) was excellent, with a correlation coefficient of 0.9974 Figure 1B.

The intraassay coefficients of variation (CVs) were calculated for 8 HbA1c samples and remained less than 1.4% (1.4% for HbA1c of 5.2%; 0.9% for HbA1c of 7.2%, and 0.9% for HbA1c of 10.9%), when calculated from values expressed in NGSP units (%) (data not shown). The interassay CVs were calculated for 8 HbA1c samples and remained less than 1.4% with a mean CV of 0.7% when calculated from values expressed in NGSP units (%) (data not shown).

As shown in Figure 1C, HbA1c values obtained with CAPILLARYS 2 Flex Piercing and Menarini/Arkray HA-8160 analyzers were very well correlated; 107 samples (range, 3.4%-13.7%) extracted from patients with no hemoglobinopathies were analyzed with both systems. The coefficient of correlation obtained was 0.9970 (P < .0001); linear regression, y = 0.2000 + 1.0000x (95% confidence interval intercept, 0.2-0.31; slope, 0.985-1.000).

The Bland-Altman plot highlighted the fact that some values obtained with the Menarini analyzer were higher in the range of 12.0% to 16.0%, compared with CAPILLARYS 2 Flex Piercing Figure 1D. This representation also showed that 7.5% of values were discordant (differences outside the range, mean ± 2 SD).

Effect of Common Interferents on HbA1c Quantification With CAPILLARYS 2 Flex Piercing

The quantification of HbA1c with the CAPILLARYS 2 Flex Piercing analyzer was not influenced by the presence of LA1c or cHb in the range tested (≤5.1% of LA1c and 6.7% for cHb, as quantified with HPLC using the Menarini/Arkray Adams A1c HA-8160) Figure 2A and Figure 2B. With both interferences, the pattern was unchanged, suggesting that the LA1c and cHb comigrate with the HbA0 peak (data not shown).
The quantification of HbA1c with the CAPILLARYS 2 Flex Piercing analyzer was not influenced by the presence of AcHb in the range tested (≤6.5% of AcHb, as quantified with HPLC using the Menarini/Arkray ADAMS A1c HA-8160)

The influence of HbF on HbA1c quantification was assessed by mixing umbilical cord blood with 2 samples with different HbA1c values. HbA1c results were not modified for HbF percentages tested (≤15%) (data not shown).

No analytic interference of bilirubin and triglycerides was noticed for concentrations reaching 16.5 mg/dL (282 µmol/L) and 1,592 mg/dL (18 mmol/L), respectively (data not shown).
The influence of the most frequent hemoglobinopathies HbS, HbC, HbD, HbE, HbLepore, or β-thalassemia also was evaluated. These profiles were labeled “atypical” because of the presence of additional peaks. The hemoglobin variants were well separated from HbA1c and HbA0 peaks, and did not interfere with HbA1c peak quantification. HbA1c measurement was not affected by the variation of total hemoglobin concentration in the range of 2.1 g/dL (21 g/L) to 19.5 g/dL (195 g/L).

HbA2 Quantification With the HbA1c Program

The percentage of HbA2 was analyzed with the HbA1c program using CAPILLARYS 2 Flex Piercing for a non-β-thalassemia population (n = 279). The frequency of HbA2 (in percentage) is shown Figure 3A with minimum and maximum values of 1.4% and 2.8%, respectively. Sixty-four samples of β-thalassemia carriers were analyzed for HbA2 by using the HbA1c program Figure 3B. The box-and-whisker plot Figure 3C as well as the receiver operating...
The percentage of HbA2 was analyzed with the HbA1c program on CAPILLARYS 2 Flex Piercing for a non–β-thalassemic population (279 samples). A, The frequency of HbA2 (in percentage) is represented with a minimum and maximum HbA2 of 1.4% and 2.8% respectively. Samples of 64 β-thalassemia carriers were analyzed for HbA2 using the same program. B, The frequency of HbA2 is represented (in percentage), in which the lowest value obtained was 3.1% and the highest 6.3%. C, The box-and-whisker plot shows the frequency of HbA2 in patients with thalassemia and healthy subjects. D, The receiver operating characteristics curve shows the sensitivity and specificity, with an area under the curve of 1.00 and cutoff value higher than 2.8%.

Characteristics (ROC) curve Figure 3D show a complete separation of the HbA2 quantification with the HbA1c program between noncarriers and carriers of the β-thalassemia trait.

The results in β-thalassemia carriers (3.1%-6.3%) were statistically different from the noncarriers (1.4%-2.8%) (P < .001). ROC analysis rendered an area under curve of 1.0 (95% confidence interval, 0.988-1.00), with a cutoff value higher than 2.8% and sensitivity and specificity of 100%.

**Correlation Between HbA2 Quantification With the HbA1c Program and the Hemoglobin Program**

Finally, the HbA2 percentages obtained with both programs were plotted for 30 noncarriers and 64 carriers of the β-thalassemia trait with a range of 1.8% to 6.3% Figure 4D. The results clearly showed a very good correlation between both techniques, with a coefficient of correlation of 0.9766 (P < .001). These data suggest that, in addition to an accurate
interference-free measurement of HbA1c compared with the hemoglobin program, the HbA1c program on CAPILLARYS 2 Flex Piercing is suitable for detecting β-thalassemia with a reliable measurement of the HbA2.

Discussion

HbA1c is a highly requested test in the clinical laboratory, which requires efficiency in its performance. Complete automation, high result turnaround times, instrument robustness, and low costs are prerequisites for selecting a suitable analyzer. Efforts of manufacturers contributed to improvements in analyzers, creating systems with high throughput.

Capillary electrophoresis is widely used in the clinical laboratory for the separation and quantification of protein fractions, serum proteins, hemoglobin fractions for the diagnosis of hemoglobinopathies, and HbA2/HbF quantification. Technological development has allowed Sebia to adapt its CAPILLARYS 2 Flex Piercing instrument for the analysis of HbA1c. On the other hand, therapeutic strategies rely on reproducible and unbiased methods. These analytical and clinical requirements become even more important now, when HbA1c is on the threshold of becoming applicable for screening and diagnosis of diabetes.5

A high level of reproducibility of HbA1c measurement is essential for providing laboratory support for diabetes monitoring. Changes in results obtained between patient visits to the physician must reflect the pathology of the disease and its response to treatment rather than an analytic uncertainty. Biological variation generally has dictated the desirable targets for the performance of laboratory analysis.10

However, it has been shown that the situation in persons with diabetes is more complex, being affected by both clinical control and sampling intervals; a practical working CV of 2% has been proposed for analytic reproducibility in long-term monitoring.11

In the current study, the CAPILLARYS 2 Flex Piercing analyzer achieved this target and had excellent reproducibility (CVs <1.4%), far below the stringent requirement of 2%.12

Hemoglobin concentration had no significant effect on HbA1c. The results were reliable within a wide range of total hemoglobin values (2.1-19.5 g/dL [21-195 g/L]). This feature shows the robustness of the system, because the patient’s values could be controlled with confidence in different clinical situations.

The concentration of LA1c varies with blood glucose concentrations at the time of blood collection, and is an intermediate molecule in the production of corresponding stable HbA1c values. The isoelectric point of the LA1c fraction closely approximates that of its stable counterpart, which leads to little or no separation of the 2, giving falsely increased results in some methods that rely on charge separation.13,14 This is not the case with CAPILLARYS 2 Flex Piercing system; LA1c fraction comigrates with the HbA0 fraction without any effect on electrophoresis profile or HbA1c quantification. Most of the LA1c fraction will release glucose rendering HbA0. For this reason, it has to be included in the assessment of HbA1c fraction.

The current experiments show that HbA1c levels had no significant increase after incubation with glucose, suggesting that the tested method is specific for the stable HbA1c fraction. In the same samples, after incubation with glucose, the LA1c fraction had a fourfold increase over the basal level (1.4% to 5.3% in the sample with a glucose level of 1,000 mg/dL [55.5 mmol/L]) while HbA1c quantification remained stable at 5.1%.

Technological advances have enabled modern analyzers with high-resolution capabilities to handle these potentially interfering components. The presence of uremia in patients whose long-term glycemic status is being assessed is not an uncommon finding in patients whose renal function has deteriorated. Yet another potential interference with some, though not all, HbA1c methods, is that cHb elutes next to the HbA1c fraction.15-17 In vitro experiments using sodium cyanate showed that the cHb fraction was increased up to 6.7%, while the HbA1c fractions were stable within ± 0.3% of the initial sample, so the HbA1c results remain reliable.

In the same way, this study showed the absence of interference of AcHb on HbA1c measurement with CAPILLARYS Flex Piercing. This common modified hemoglobin found in pregnant women, alcoholic patients, or even patients being
treated with aspirin is not an analytic issue because \( \text{HbA}_{1c} \) quantifications show no variation in the presence of high concentrations of AcHb.

An additional benefit of the enhanced resolution is the ability to detect the presence of hemoglobinopathies: the presence of hemoglobin variants that may interfere with \( \text{HbA}_{1c} \) measurement and their influence is well studied in various systems currently available.\(^{18,19}\) In this study, a number of samples containing common variants were analyzed for \( \text{HbA}_{1c} \). The results indicate that this technique can be confidently used to measure \( \text{HbA}_{1c} \) in the presence of common hemoglobin variants.

The presence of HbS, HbC, HbD, HbE, and HbLepore variants produces an additional peak on the cathodic side of \( \text{HbA}_0 \), with no interference by \( \text{HbA}_{1c} \) fraction. Glycated forms of the variants also migrate far from \( \text{HbA}_{1c} \), thereby allowing precise \( \text{HbA}_{1c} \) quantification. Although an expert observer could presumably identify the common variants, a more precise presumptive identification can be done with the same instrument by using CAPILLARYS hemoglobin buffer.\(^{20}\) Moreover, the presence of atypical profiles (including variants, HbF, or any additional peak) is automatically detected with the software which uses a color-coded system to classify profiles.

It is not uncommon to detect the presence of hemoglobin variant in the course of routine diabetes control. This casual finding highlights the need to inspect electrophoretic profiles to detect abnormalities.\(^{21}\) The presence of any abnormality is readily detected by the analyst for further consideration of its implications by the physician, and is relevant for interpreting the \( \text{HbA}_{1c} \) results and for genetic counseling. But in these patients with reduced erythrocyte life spans, a non–hemoglobin-based method would be recommended for assessing glycemic control, because the \( \text{HbA}_{1c} \) level does not accurately reflect long-term glycemic control.\(^{22}\)

The results produced by the CAPILLARYS HbA1c Kit were found to correlate very well with those obtained with the Menarini/ARKRAY ADAMS Alc HA-8160 for the same samples. However, there was a constant 0.2% bias throughout the analytic range. Both instruments use the IFCC calibrator values and have been certified by the IFCC and NGSP. Although a bias of 0.2% could be judged statistically significant in the case of a subject with good metabolic control, from a practical point of view, it should be noted that the clinically significant change for physicians is 0.5%. Besides, it is assumed that the patients are analyzed in the same laboratory, generating data with the same technology over time. It also must be taken into account that a difference of ±0.75% \( \text{HbA}_{1c} \) between data using a laboratory method and an NGSP traceable value is considered acceptable by the NGSP.

\( \text{HbA}_2 \) is also well separated on the cathodic side and is quantified by the instrument with the \( \text{HbA}_{1c} \) program, while most other techniques are not able to separate \( \text{HbA}_2 \) from \( \text{HbA}_0 \). The \( \text{HbA}_2 \) is quantified using the Phoresis software, but to my knowledge, no study has ever shown the reliability of \( \text{HbA}_2 \) quantification with the \( \text{HbA}_{1c} \) program for diagnosis of \( \beta \)-thalassemia. The results in \( \beta \)-thalassemia carriers (3.1%-6.3%) were statistically different from the noncarriers (1.4%-2.8%) when \( \text{HbA}_2 \) was quantified using the \( \text{HbA}_{1c} \) program. In addition, after testing \( \text{HbA}_2 \) with both the \( \text{HbA}_{1c} \) and hemoglobin programs for 60 normal individuals and 64 \( \beta \)-thalassemia carriers, a good correlation was found between both results. However, the results obtained with the \( \text{HbA}_{1c} \) program were systematically lower than those obtained with the hemoglobin program, with an average bias of 0.29%.

These results suggest that it is possible to detect \( \beta \)-thalassemia using the \( \text{HbA}_{1c} \) Program on CAPILLARYS Flex Piercing, but the value obtained with the \( \text{HbA}_{1c} \) program needs to be corrected to obtain the real \( \text{HbA}_2 \) value for the patient.

Although an \( \text{HbA}_2 \) fraction higher than 2.8% strongly suggests the presence of the \( \beta \)-thalassemia trait, further studies need to be conducted to prove its precision,\(^{23}\) according to the recently published recommendations on \( \text{HbA}_2 \).\(^{24}\)

In summary, given the short time for analysis and the high throughput, this is a suitable system for diabetes control in patients and for detecting hemoglobinopathies in laboratories with high workflow.

**References**


