Performances of Capillary Electrophoresis and HPLC Methods in HbA_{1c} Determination: Diagnostic Accuracy in HbS and HbD-Iran Variants' Presence

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Background: Glycated hemoglobin (HbA_{1c}) provides a useful estimate of mean glycemia in patients with diabetes and is directly related to risks for diabetes complications. The aim of this study is to compare a capillary electrophoresis method and two high-performance liquid chromatography (HPLC) cation-exchange analyzers (Variant II (Bio-Rad Laboratories, Inc., Hercules, CA) and G8 (Tosoh Biosciences, San Francisco, CA)) to identify the most reliable method in Hb variants' presence. **Methods:** Measurements of HbA_{1c} were carried out in blood samples from 200 Tor Vergata Hospital patients, using G8 Tosoh, and from 107 San Filippo Neri Hospital patients, using Variant II Bio-Rad methods. All samples were analyzed by Capillarys 2 Flex Piercing (FP; Sebia, Lisses, France). **Results:** There

was a good concordance between the results of capillary electrophoresis and HPLC methods ($R^2 = 0.99$, $P < 0.0001$ for G8 HPLC; $R^2 = 0.99$, $P < 0.0001$ for Variant II HPLC). During the study, we observed that some Hb variants, HbS and HbD-Iran, can alter the HbA_{1c} level. **Conclusions:** Since the HbA_{1c} test is now recommended for diagnosing diabetes, and minimal variation of the concentration affects the clinical therapy, it is very important that the results are reliable and interference-free. Capillarys 2-FP analyzer is suitable for this purpose and sometimes it showed some advantages with respect to the HPLC analyzers tested, especially when Hb variants are present. J. Clin. Lab. Anal. 29:57–60, 2015. © 2014

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INTRODUCTION

Glycated hemoglobin (HbA_{1c}) is the stable glucose adduct to the N-terminal group of the beta chain of HbA₀. It is considered the gold standard of diabetic survey, since its evaluation provides retrospective information on the glycemic balance of patients for the past 6–8 weeks. In addition, this marker is correlated with the risk of long-term complications in both type 1 and type 2 diabetes (1, 2), and its use has been suggested for screening and diagnosis of diabetes (3, 4).

Therefore, accurate and precise measurement of HbA_{1c} is extremely important. The routine determination of HbA_{1c} can be achieved by methods based on different principles: immune-turbidimetric, boronate affinity chromatography, ion-exchange high-performance liquid chromatography (HPLC), and enzymatic assay (5–7); among

these, cation HPLC is one of the methods generally used for the HbA_{1c} determination. Several studies have reported a significant bias among analytical methods to measure HbA_{1c} levels (8). Therefore, standardization and comparability of HbA_{1c} results with different methods appear to be an important issue.

Recently, a new HbA_{1c} assay based on the separation of Hb fractions by capillary electrophoresis has been

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developed on a Capillarys 2 Flex Piercing (FP) analyzer by Sebia (Lisses, France) (9, 10), making this technology available for HbA_{1c} quantification in routine practice.

The aim of our study is to compare Capillarys 2-FP HbA_{1c} with G8 (Tosoh Biosciences, San Francisco, CA) and Variant II (Bio-Rad Laboratories, Inc.) HPLC cation-exchange analyzer, in order to evaluate the HbA_{1c} concentration and to see pathological Hb possible interference on HbA_{1c} levels.

MATERIALS AND METHODS

The study protocol complied with the Declaration of Helsinki (1964) and was approved by the Ethical Committee of the University of Rome "Tor Vergata" and "San Filippo Neri" Hospital.

In the present study blood samples were obtained from the subjects for routine/glycated testing in San Filippo Neri Hospital (107 patients) and Tor Vergata University Hospital (200 patients).

The first group was evaluated by Variant II A_{1c} Dual and the second group by G8 (HLC-723G8; HbA_{1c} variant mode) HPLC cation-exchange analyzers; all the samples were then analyzed by Capillarys 2-FP. Whole blood samples were drawn by venipuncture and collected in K₂ EDTA (Ethylenediaminetetraacetic acid) tubes. The samples were processed within 4 hr and stored at 4°C if evaluated within 24 hr. The analyzers as well as the reagents used for this evaluation were used according to the manufacturer's instructions.

Pearson correlation analysis and Bland–Altman plots were performed from method comparison data using SPSS 17.1 statistical software. All results are expressed as means $\pm$ standard deviation (SD).

RESULTS

Figure 1 shows correlation and Bland–Altman plots between G8 HPLC and Sebia Capillarys 2-FP. The graphic (Fig. 1A) highlights a good correlation (G8,

Fig. 1. Comparison between HbA_{1c} values obtained with Capillarys 2-FP, G8, and Variant II analyzers. (A) Correlation plot with linear regression line ($y = 0.9636x + 0.227$, $R^2 = 0.9939$ for G8 HPLC; 200 blood samples). (B) Bland–Altman difference plot comparing HbA_{1c} results of Capillarys 2-FP and G8 analyzer. (C) Correlation plot with linear regression line of HbA_{1c} values obtained with Capillarys 2-FP and Variant II analyzer for 107 blood samples ($y = 0.9662x + 0.279$, $R^2 = 0.9958$ for Variant II HPLC). (D) Bland–Altman difference plot comparing HbA_{1c} results of Capillarys 2-FP and Variant II analyzer. Dotted lines represent ± 2 SD.

TABLE 1. Quantification of HbA_{1c} in Millimoles per Mole and in Percentages of Six Heterozygote HbAS Patients

Tosoh G8 (mmol/mol)	(%)	Capillarys 2 FP (mmol/mol)	(%)	Variant II (mmol/mol)	(%)	HbS/HbAS (%)
42	6.0	45	6.4	46	6.5	31.8
33	4.7	40	5.7	39	5.6	36.1
34	4.8	41	5.8	42	6.0	35.2
27	3.8	30	4.3	32	4.6	33.3
39	5.6	42	6.0	41	5.8	31.3
112	16.0	126	18.0	128	18.3	31.3

HbS/HbAS shows percentages of HbS in each HbAS sample.

47.21 ± 16.08 mmol/mol, 6.5 ± 1.5%; Capillarys 2-FP, 47.08 ± 15.54 mmol/mol, 6.4 ± 1.4%; $R^2 = 0.99$, $P < 0.0001$; Fig. 1A) and a minimal dispersion respect to media, the Bland–Altman plot shows good agreement (95% C.I.; Fig. 1B). During the evaluation, we ran six samples from known patients bearing an HbS variant; the HbA_{1c} levels were underestimated when analyzed by G8, whereas the values detected by Variant II analyzer are similar to that obtained by Capillarys 2-FP analyzer (Table 1).

The same statistical analyses were performed between Variant II and Capillarys 2-FP (Variant II, 48.68 ± 14.85 mmol/mol, 6.6 ± 1.3%; Capillarys 2-FP, 49.29 ± 14.37 mmol/mol, 6.7 ± 1.3%; Fig. 1C). The correlation value is significant ($R^2 = 0.99$, $P < 0.0001$) and the dispersion shown in the Bland–Altman plot is good (95% C.I.; Fig. 1D).

Anyway, both for Capillarys 2-FP vs. G8 and both vs. Variant II, the differences shown in HbA_{1c} measurement are not related to the level of HbA_{1c} (Bland–Altman plot).

One case drew our attention. A San Filippo Neri Hospital patient showed very high HbA_{1c} level (681 mmol/mol, 97.3%) and abnormal elution curve (Fig. 2A). To confirm this result, the sample was analyzed again by the VII beta thal dual program (Bio-Rad; Variant II), dedicated to screening for hemoglobinopathies. This system uses the same reagent kit, but differs for calibrators, controls, running time, and analytical software. With the latter method, the HbA_{1c} value decreases substantially (76 mmol/mol, 10.8%). We believe that the program time for HbA_{1c} (3.5 min; VII A1c dual) is too short to discriminate the presence of the variant HbD Iran, which instead highlights with longer hemoglobinopathies program (6.5 min; VII beta thal dual).

The same sample was analyzed with G8 (HbA_{1c} variant mode) and Capillarys 2-FP: G8 underestimated the HbA_{1c} value (48 mmol/mol, 6.8%; Fig. 2B), whereas Capillarys 2-FP showed the Hb variant and detected carefully the HbA_{1c} level (75 mmol/mol, 10.7%; Fig. 2C). Using the program for identification of Hb fractions and Hb variants in Capillarys 2-FP, we identified in this sample

Fig. 2. Chromatograms of the same whole blood sample (HbD Iran) analyzed by Variant II and G8 HPLC methods (A and B). Separation profile of HbD Iran sample using Capillarys 2-FP (C); number 4 indicates Hb variant.

an HbD variant, than confirmed by genetic typing (HbD Iran; beta 22 Glu-Gln).

DISCUSSION

The importance of HbA_{1c} levels in the management of diabetes mellitus requires efficient and reliable methods for the measurement of glycated Hb. There was a good concordance between the results of Capillarys 2-FP and HPLC methods.

Many methods have been shown to be affected by the Hb variants such as Hb C, D, E, and S (11; <http://www.ngsp.org/interf.asp>). During this study, we found an Hb variant that produced an extra peak on the Variant II chromatogram. The same sample analyzed with G8 showed an underestimated HbA_{1c} level. Only Capillarys 2-FP did not show any interference with the variant trait. Our data show an uncorrected value of HbA_{1c} in presence of HbD Iran variant using the two HPLC analyzers (G8 and Variant II). A recent article of Bhat et al. (12) shows that HbD Iran, found mainly in Iranian and Pakistani families, migrates with HbA₂ and HbE using HPLC methods. For this reason, care must be taken and efforts must be made to get reliable results; many physicians have suggested that 0.5% HbA_{1c} is a clinically significant change. Treatment guidelines and algorithms from ADA/EASD and National Institute for Clinical Excellence in the United Kingdom recommend evaluating new treatment regimens in terms of whether HbA_{1c} is lowered by 0.5% points or more. Therefore, it is important to be sure that a change of this magnitude is statistically significant and not due to analytical variation (13, 14).

Further, when we run samples from six heterozygote HbAS patients, Capillarys 2-FP, Variant II, and G8 measured a different HbA_{1c} concentration, with HbA_{1c} underestimated by G8. This agrees with the results obtained by Lin et al. (11). According to these authors, Capillarys 2-FP shows higher accuracy than G8 in the HbA_{1c} measure when HbS variant is present.

Since the HbA_{1c} test is now recommended for diagnosing diabetes, and minimal variation of the concentration affects the clinical therapy, one must become more stringent that any unacceptable results are detected, not reported and each method is evaluated for Hb variant interference (11; <http://www.ngsp.org/interf.asp>). Laboratories must be aware of the interferences and limitations of their assay method and should consider the prevalence of hemoglobinopathies in their respective patients and populations, considering the ever-increasing migration flows.

Therefore, it is very important that the results are reliable and interference-free. It is also essential for the economy of the laboratory, that the method be accurate, precise and does not require verification.

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