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Role of HbA1c in the Diagnosis of Hemoglobinopathies

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ABSTRACT

Introduction: Hemoglobinopathies are common genetic disorders resulting from qualitative (hemoglobinopathies) or quantitative (thalassemias) abnormalities affecting hemoglobin. The Hb S variant is of particular concern, as it causes severe sickle cell syndromes. Screening for these conditions is essential, especially in regions such as Morocco, where consanguinity increases the prevalence of genetic disorders.

Patients and Methods: This study included 17 patients, aged between 23 and 70 years, who were followed at the Cheikh Zaid University Hospital in Rabat from 2018 to 2022. Among them, six were from Morocco, and eleven were from sub-Saharan Africa. All patients underwent comprehensive hemoglobin testing and HbA1c measurement.. Two primary methods were used for analysis. The first was alkaline agarose gel electrophoresis with the Hydrasys® analyzer, which identifies normal hemoglobins (A, A2) and abnormal variants (S, D, C, E). The second was high-performance liquid chromatography (HPLC) using the D-10® and G7® analyzers. Although primarily designed for HbA1c measurement, these analyzers can also detect hemoglobin variants. The D-10® identifies the Hb S variant but may occasionally confuse it with Hb D.

Results : Electrophoresis identified Hb S as the only abnormal variant. One patient had homozygous sickle cell disease (S/S), and ten were heterozygous carriers (A/S). The remaining six patients showed no hemoglobin abnormalities. The D-10® analyzer detected the Hb S variant in several patients but misidentified one case as Hb D. Additionally, the G7® analyzer detected some variants, although it lacked clear differentiation. The correlation between HPLC and electrophoresis for HbA and HbS measurements was generally acceptable. However, the correlation for HbA was weaker, likely due to differences in the detection of specific hemoglobin fractions.

Discussion : This study highlights that while HPLC is primarily used for diabetes management, it also serves as a valuable complementary tool for the incidental detection of hemoglobin variants, such as Hb S. This approach is particularly useful in asymptomatic patients, enabling the early identification of carriers and sickle cell syndromes. Integrating HPLC with electrophoresis could improve patient care and reduce diagnostic delays, especially in high-prevalence regions such as Morocco. However, it is important to account for HPLC's limitations, including its inability to distinguish certain variants accurately.

Conclusion : In conclusion, although HPLC is not specifically intended for the diagnosis of hemoglobinopathies, it represents a useful complementary tool. It facilitates the detection of variants such as Hb S, guiding further diagnostic investigations. A combined strategy using both HPLC and electrophoresis could enhance patient care and minimize diagnostic delays, particularly in regions with high prevalence. Special attention should be given to HPLC's limitations, notably its difficulty in distinguishing between certain hemoglobin variants.

Keywords: Hemoglobinopathies, sickle cell disease, screening, high-performance liquid chromatography (HPLC), agarose gel electrophoresis.

INTRODUCTION

Hemoglobinopathies, common genetic disorders typically inherited in a recessive manner, result from abnormalities in hemoglobin. They are divided into qualitative abnormalities (hemoglobinopathies) and quantitative abnormalities (thalassemias). Over 1,500 different variants have been identified, with the Hb S variant being the most significant, as it leads to severe sickle cell syndromes in its homozygous form or through various compound heterozygous states. Diagnosing these disorders is essential not only when symptoms are present but also for identifying heterozygous carriers, allowing for potential genetic counseling in cases of family planning [1].

The World Health Organization (WHO) estimates that more than 70% of all hemoglobin disorders occur in Africa [2], with a high prevalence in Morocco, partly due to consanguinity [3]. Although precise epidemiological data are lacking, the WHO suggests that approximately 6.5% of the population carries a pathological allele, translating to around 30,000 major cases of thalassemia and sickle cell disease.

In 2009, HbA1c was adopted as a diagnostic criterion for diabetes, requiring highly precise measurement methods. High-performance liquid chromatography (HPLC), based on ion exchange, allows the separation and quantification of various hemoglobin fractions, including variants, offering a significant advantage in their detection.

The combined use of ion-exchange high-performance liquid chromatography (HPLC) and hemoglobin electrophoresis enables the detection and identification of key pathological variants.

Objective:

This study aims to assess the reliability of results obtained by HPLC in detecting hemoglobin variants, even though this method is not typically used for diagnosing hemoglobinopathies. By comparing HPLC results with those from agarose gel electrophoresis, a reference technique, the objective is to evaluate to what extent HPLC could serve as a complementary or screening tool. The study also focuses on the incidental discovery of hemoglobin variants, often in heterozygous states, during chromatographic analyses conducted for glycosylated hemoglobin (HbA1c) monitoring.

PATIENTS AND METHODS

Patients

This retrospective study included 17 patients for whom clinicians prescribed both a hemoglobin study and glycated hemoglobin (HbA1c) measurement. These analyses were performed at the International Cheikh Zaid University Hospital in Rabat between 2018 and 2022. The patients ranged in age from 23 to 70 years (10 men and 7 women). Six patients were of Moroccan origin, while the remaining 11 were from sub-Saharan Africa.

Methods

This study employed both alkaline gel electrophoresis and liquid chromatography, which is also used for measuring HbA1c, crucial for diabetic monitoring.

The analyses were performed on venous blood samples collected in EDTA tubes, with a minimum volume of 5 mL. Hemoglobin electrophoresis was conducted using the HYDRAGEL HEMOGLOBIN kit and the Hydrasys® analyzer. This kit enables the separation of normal hemoglobins (A and A2) and the detection of major abnormal hemoglobins (S, D, C, E) through agarose gel electrophoresis. The hemoglobins are separated using various buffers and stained for qualitative analysis, and densitometry is used for precise quantification of the different bands.

HbA1c analysis was performed using two analyzers, D-10® and G7®, both featuring short cycles. Hemoglobin variants such as HbS, HbC, and HbD have retention times distinct from HbA and do not interfere with HbA1c measurements. However, the G7® analyzer does not clearly identify these variants, while the D-10® identifies them based on retention time, although HbS and HbD can be confused.

The D-10® analyzer's HbA1c program is based on high-performance liquid chromatography (HPLC) using ion exchange. Samples are automatically diluted, injected into an analysis column, and hemoglobins are separated based on their ionic interactions. Absorbance variations are measured at 415 nm to produce the results.

The Tosoh G7® analyzer also operates on the principle of HPLC. It dilutes the sample, injects it into an analytical column, and separates the different hemoglobin fractions using ionic interactions. Components are eluted in stages using different elution buffers and measured at 415 nm to calculate the relative percentages of each fraction. This analysis is rapid, taking only 2.2 minutes.

In summary, these analyses employ HPLC- and electrophoresis-based techniques to separate and quantify different forms of hemoglobin in blood samples.

This study utilized the paired Student's t-test to compare the mean hemoglobin values (HbA, HbS, and HbF) obtained by agarose gel electrophoresis and HPLC. The t-test evaluates whether the differences between the two methods are statistically significant by testing the null hypothesis that there is no difference between the means. Results include the observed t-value, p-value, and degrees of freedom, which help determine whether the null hypothesis can be rejected. Additionally, Pearson's correlation coefficient was calculated to assess the strength and direction of the linear relationship between the hemoglobin values measured by the two methods, providing an indication of their concordance.

Thus, this study relies on the analysis of HbA1c chromatograms to explore potential hemoglobin variants without aiming for diagnostic purposes.

RESULTS

In this study, the S variant was the only variant detected through hemoglobin electrophoresis, and all retention times on both the G7® and D-10® analyzers occurred after the elution of HbA0 (Table 1). The D-10® analyzer mainly identified the S variant, while one specific case revealed the simultaneous presence of both S and D variants, without the ability to distinguish between HbS and HbD (Figure 1). In contrast, the G7® analyzer detected variants but did not provide precise identification.

In general, a patient with a heterozygous S variant shows an expression level between 35% and 40%. A patient whose hemoglobin is primarily composed of HbS, without HbA, is most likely affected by a major sickle cell syndrome, with HbF present at variable levels. An HbS level above 40%, combined with the presence of HbA and HbF, could suggest a recent blood transfusion (< 3 months) in a patient with this syndrome [4]. In the absence of transfusion, this might indicate a composite βS/β+-thalassemia heterozygote [5] or an alpha triplication [6].

It should be noted that no clinical information, particularly regarding a recent transfusion, was provided during hemoglobin electrophoresis or glycated hemoglobin measurements. Therefore, the study based its hypothesis on the hemoglobin values obtained through electrophoretic methods to determine heterozygous or homozygous status.

Among the 17 patients analyzed, one Moroccan patient presents with a major sickle cell syndrome (Figure 2), while 10 patients exhibit a probable heterozygous S variant (Table 2, Figure 3,4). The remaining 6 patients show no visible hemoglobin abnormalities nor any variant detected by the two methods of analysis (Table 3, Figure 5).

Table 1. Retention times of hemoglobins (min)

Automate	Type of hemoglobin	Retention time
D-10®	Hb A0	1,46 à 1,49
	Hb S	1,65 à 1,66
G7®	Hb A0	1,01 à 1,04
	Hb S	1,39 à 1,44

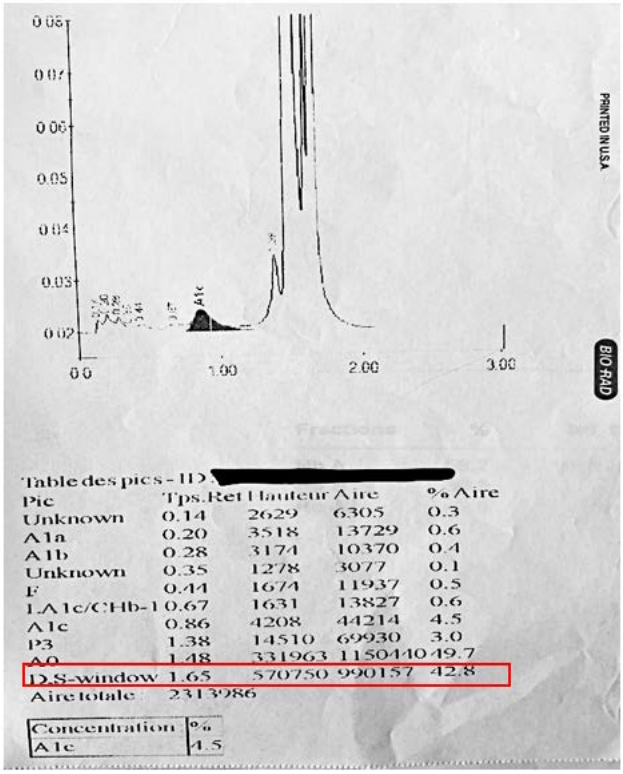

Figure 1. Chromatogram of HbA1c measurement in an A/S patient (Bio-Rad D-10®).

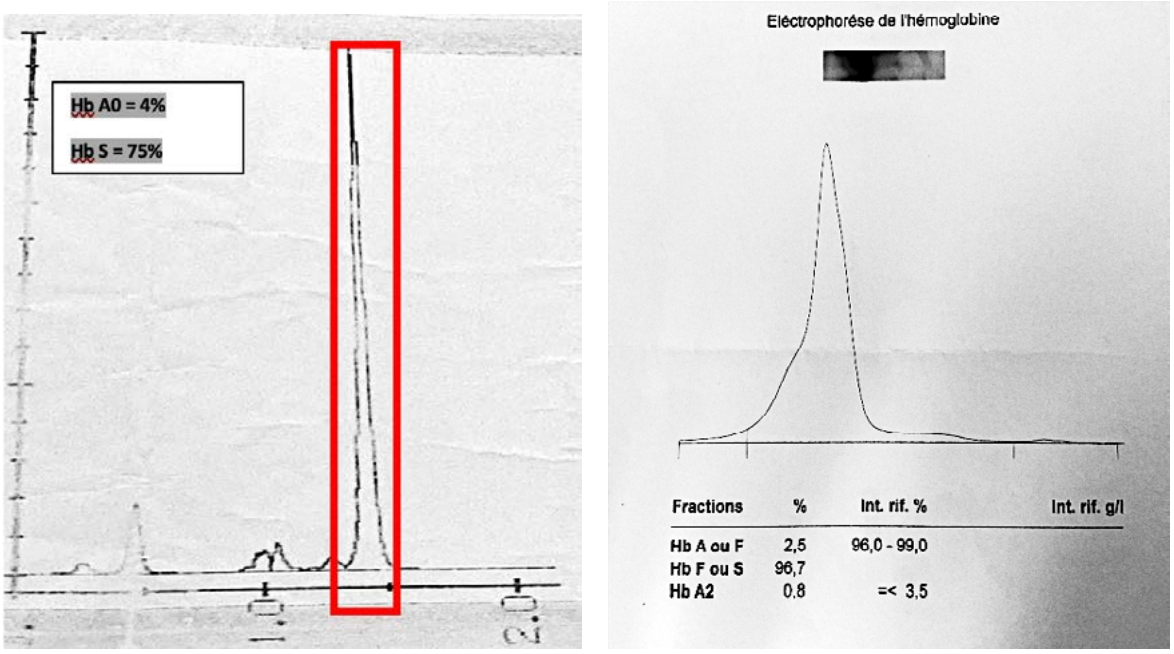

Figure 2. Profil électrophorétique et Chromatogramme de la mesure de l'HbA1c chez la patiente S/S (automate Tosoh G7®)

Table 2. Comparison of values obtained by electrophoresis (E) and HPLC

PATIENT A/S	E(HbA%)	E(HbS%)	E (HbF%)	HPLC (HbA0%)	HPLC (HbS%)	HPLC (HbF%)
A	62.6	35	0	56.9 (TOSOH)	34.8 (TOSOH)	1.3 (TOSOH)
B	61.9	36.1	0	57 (TOSOH)	36 (TOSOH)	0.5 (TOSOH)
C	72.9	24.3	0	67.5 (TOSOH)	24.3 (TOSOH)	0.9 (TOSOH)
D	58.4	37.6	0	55 (D-10)	35.7 (D-10)	0.5 (D-10)
E	62.2	35	0	54.8 (D-10)	32.2 (D-10)	0 (D-10)
F	60.8	36.5	0	46.9 (D-10)	31.6 (D-10)	2.7 (D-10)
G	35.9	31.3	29,4	57.7 (TOSOH)	22.4 (TOSOH)	12 (TOSOH)
H	65.2	33.1	0	58.9 (TOSOH)	32.8 (TOSOH)	0.8 (TOSOH)
I	58.1	40.6	0	56.5 (TOSOH)	37.6 (TOSOH)	0.5 (TOSOH)
J	58.2	38.6	0	49.7 (D-10)	42.8 (D-10)	0.5 (D-10)

Table 3. Comparison of values obtained by electrophoresis (E) and HPLC in patients without hemoglobin abnormalities

PATIENT A/A	E(HbA%)	E (HbF%)	HPLC (HbA0%)	HPLC (HbF%)
J	97.3	0	89.5 (G7)	1.1 (G7)

K	96.7	0	91.4 (G7)	0.5 (G7)
L	97.1	0	82.2 (D-10)	0.3 (D-10)
M	97.8	0	77.7 (D-10)	0.5 (D-10)
N	95.7	0	85.3 (D-10)	0.8 (D-10)
O	97	0	84 (D-10)	0 (D-10)

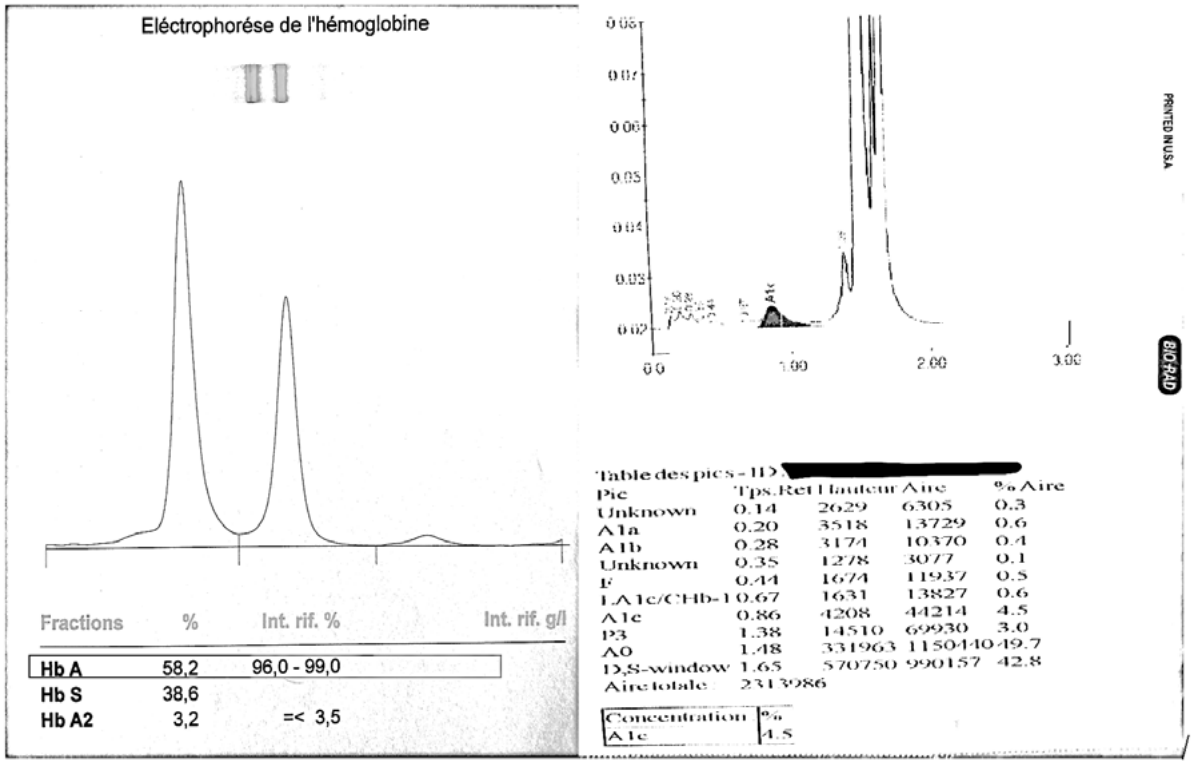

Figure 3. Electrophoretic profile of an A/S patient and chromatogram of HbA1c measurement in an A/S patient (Bio-Rad D-10®).

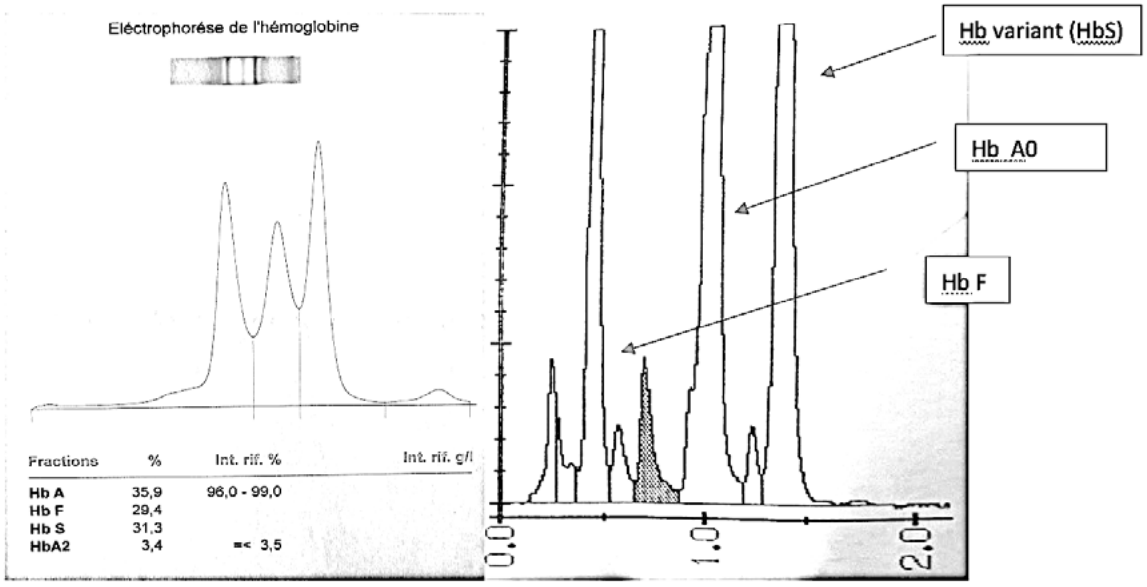

Figure 4. Electrophoretic profile and chromatogram of an A/S patient with elevated HbF (Tosoh G7® analyzer).

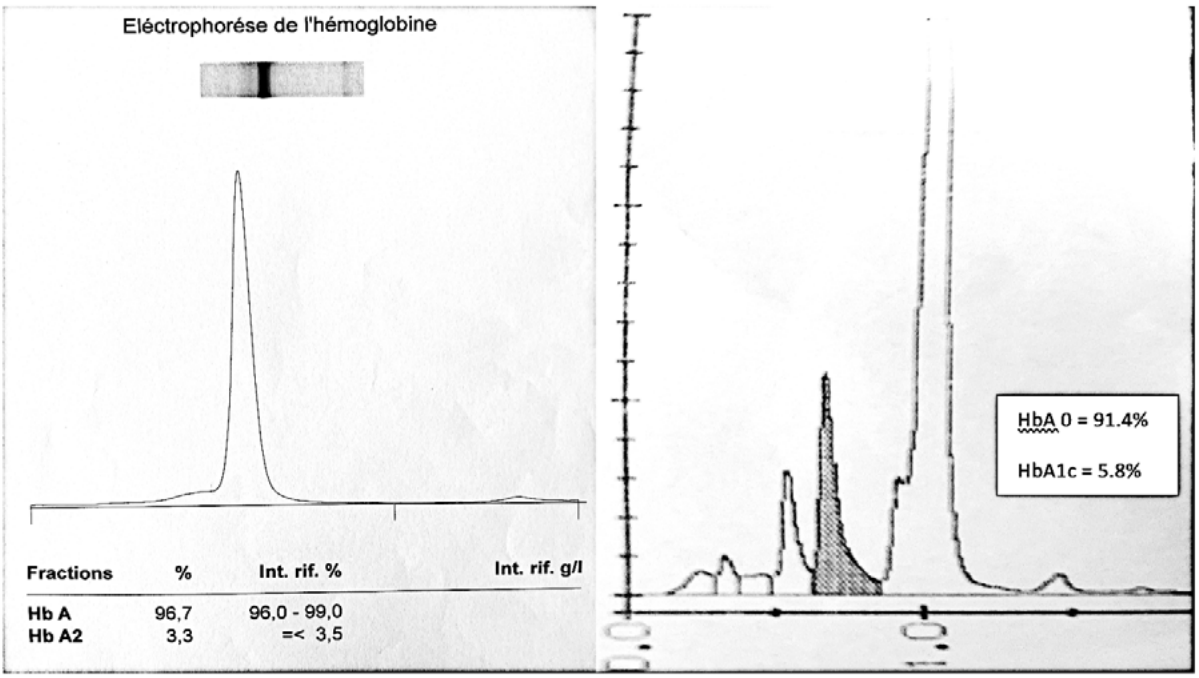

Figure 5. Electrophoretic profile and chromatogram of an A/A patient (Tosoh G7® analyzer).

STATISTICAL ANALYSIS: COMPARISON OF MEASUREMENT METHODS

In this study, we compared two commonly used techniques for the diagnosis and monitoring of hemoglobinopathies: agarose gel electrophoresis and high-performance liquid chromatography (HPLC). The comparison focuses on the results obtained for three main types of hemoglobin: HbA, HbS, and HbF.

We first compared the HbA values obtained by agarose gel electrophoresis and HPLC using the paired Student's t-test. The confidence interval (CI) around the mean difference was [-3.408; 10.068], with a mean difference of 3.330. The observed t-value was 1.118, while the critical t-value was 2.262, with 9 degrees of freedom (DF). The two-tailed p-value was 0.293, greater than the significance level alpha of 0.05. Therefore, we could not reject the null hypothesis (H0), which states that there is no difference between the mean HbA values from the two methods.

For HbS values, we also used the paired Student's t-test. The CI around the mean difference was [-0.704; 4.284], with a mean difference of 1.790. The observed t-value was 1.623, and the critical t-value remained 2.262 with 9 DF. The two-tailed p-value was 0.139, also greater than alpha of 0.05. This indicates that we could not reject H0, suggesting no difference between the mean HbS values obtained by the two methods.

Finally, for HbF values, the same test was applied. The CI around the mean difference was [-3.193; 5.133], with a mean difference of 0.970. The observed t-value was 0.527, with a critical value of 2.262 and 9 DF. The two-tailed p-value was 0.611, greater than alpha of 0.05, leading us not to reject H0, indicating no difference between the mean HbF values from the two methods.

Regarding Pearson correlation coefficients, we found an r-value of 0.278 for HbA, with a coefficient of determination r^2 of 0.077, indicating a weak correlation. For HbS, the r-value was 0.819 with an r^2 of 0.670, showing good correlation. Finally, for HbF, the r-value was 0.979 with an r^2 of 0.959, indicating a very strong correlation. These results suggest that the measurements obtained by the two methods are generally interchangeable, with some nuances in the correlation of results.

Thus, the analyses show that both methods are generally interchangeable for all types of hemoglobin studied. However, a significant difference was noted in the correlation of results for HbA. This weakness may be explained by methodological differences: HPLC primarily measures HbA0 without including certain fractions, such as HbA1c or the P3 fraction, which may be detected by other methods like electrophoresis. These nuances could affect the accuracy of the results, especially for diabetic patients, where HbA1c is an essential component.

Table 4. Comparison of Hemoglobin Measurement Methods: Statistical Analysis of Agarose Gel Electrophoresis and HPLC Results

Type of Hemoglobin	Mean Difference	95% CI	t (p)	Correlation (r)	Conclusion
HbA	3.33	[-3.408; 10.068]	1.118 (p = 0.293)	0.278 (r^2 = 0.077)	Weak correlation, but methods remain interchangeable with caution.
HbF	0.97	[-3.193; 5.133]	0.527 (p = 0.611)	0.979 (r^2 = 0.959)	Very strong correlation, methods are interchangeable.
HbS	1.79	[-0.704; 4.284]	1.623 (p = 0.139)	0.819 (r^2 = 0.670)	Good correlation, methods are interchangeable.

DISCUSSION

Sickle cell disease is a major public health issue, especially on the African continent, where approximately 66% of the 120 million people affected by the disease reside. Every day, one thousand new cases of children with sickle cell disease are reported, with more than half of them dying before the age of five, mainly due to severe infections or episodes of anemia. Morocco is not exempt from this reality, with around 5,600 severe cases being monitored. However, the absence of national systematic screening programs often leads to delayed diagnoses and preventable deaths.

Early diagnosis is therefore crucial to reducing infant mortality associated with sickle cell disease. In this context, the measurement of glycated hemoglobin (HbA1c) is emerging as a promising tool. Although traditionally used for monitoring diabetes, it can also provide valuable insights into detecting hemoglobin variants. Indeed, some variants—particularly in heterozygous (A/S) or homozygous (S/S) individuals—can be identified through high-performance liquid chromatography (HPLC). While this method is not exhaustive for all types of variants, it allows for faster and more precise clinical interventions.

Illustrative Case Study

This case, which was handled at our laboratory, perfectly illustrates the importance of HbA1c measurement in screening for hemoglobinopathies. It demonstrates how this analysis can assist in diagnosing serious conditions, such as sickle cell syndromes, even when they are not initially suspected.

A patient was referred by her clinician for a blood test due to symptoms of anemia. The results revealed:

- **Red blood cells:** 2.56 x 10^6/L
- **Hemoglobin:** 7.96 g/dL
- **Hematocrit:** 24.1%
- **Mean Corpuscular Volume (MCV):** 94.3 fL
- **Mean Corpuscular Hemoglobin (MCH):** 31.1 pg
- **Red Cell Distribution Width (RDW):** 13.5%

A blood smear analysis highlighted the presence of numerous sickle cells (Fig. 6), prompting further investigation into a sickle cell disorder. At the same time, the HbA1c measurement (Fig. 7) revealed a fortuitous hemoglobin variant, potentially homozygous (S/β^0). However, precise characterization of this variant could not be achieved using the Tosoh G7® analyzer. Hemoglobin electrophoresis ultimately confirmed the presence of a major sickle cell syndrome in the patient.

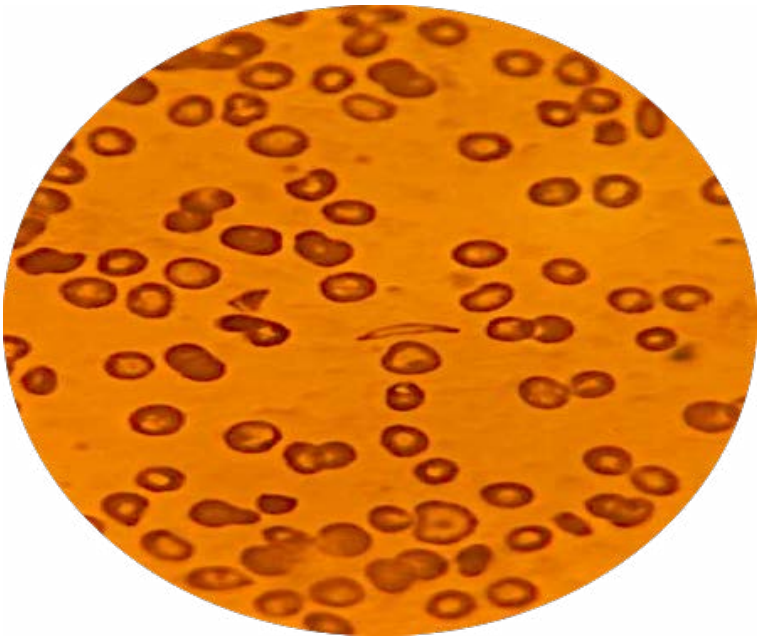

Figure 6. Presence of numerous sickle cells in the blood smear.

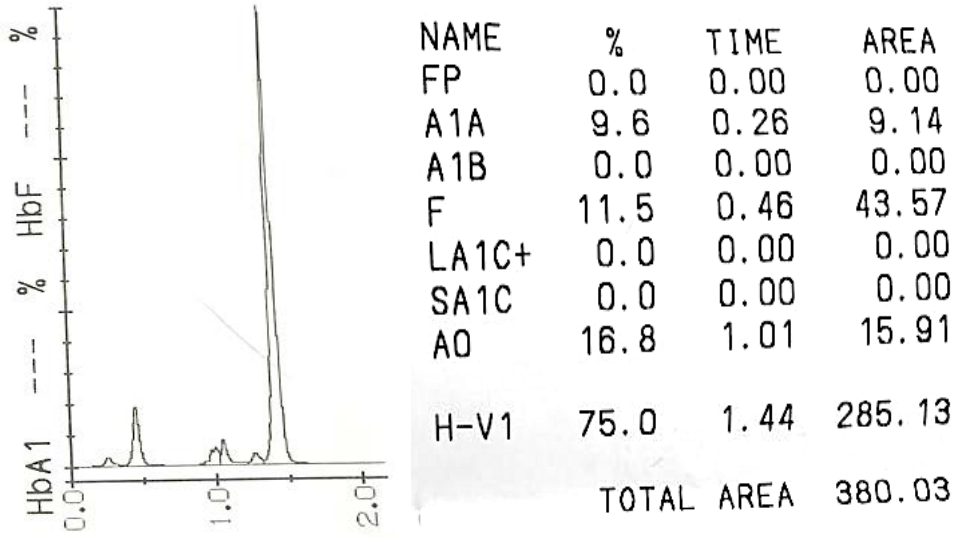

Figure 7. Detection of a hemoglobin variant represented at 75% (TOSOH G7).

This clinical case illustrates the importance of integrating HbA1c measurement in the screening of hemoglobinopathies. Although this approach does not allow for the detection of all variants, it proves useful for identifying the most common heterozygous and homozygous profiles. By adopting a broader diagnostic strategy, including HPLC chromatography and electrophoresis, it becomes possible to better target at-risk patients and improve their management. In contexts where diagnosis is often delayed, such as in Morocco, such an approach could significantly contribute to reducing mortality associated with hemoglobinopathies.

Precautions and Limitations When Measuring Hba1c in Homozygous Patients or Carriers of a Variant

HPLC methods indicate the presence of a hemoglobin variant but lack the resolution to differentiate silent variants, which may lead to additional peaks in chromatograms [7]. Some hemoglobins can be eluted in the same fraction as HbA0 or HbA1c, resulting in false increases or decreases in HbA1c [8]. Over a thousand hemoglobin variants have been identified, but their impact on HbA1c measurement is not always known [9]. Many are clinically silent and detected incidentally. The identification of variants is crucial for interpreting results for several reasons: they can alter red blood cell survival [10], making clinically misleading results even if they are analytically accurate. Moreover, unresolved peaks can contribute to falsely elevated or decreased results: overlapping peaks hinder the correct integration of relevant peaks , thereby compromising area calculations and affecting the reported percentage of HbA1c. Finally, variants can also influence hemoglobin glycation [11].

A study at the University of Virginia showed that measuring glycated hemoglobin by capillary electrophoresis can identify variants not detected by HPLC, although this is not systematic; thus, these methods are complementary. Other variants have been detected by capillary electrophoresis but not by agarose gel electrophoresis [12]. Each method provides distinct information, making it essential to understand their limitations to optimize result interpretation.

Comparison of Hb measurement by HPLC on G7® (TOSOH bioscience) and D-10® (BIO-RAD) [13]

This study compares two analyzers for glycated hemoglobin to evaluate the reliability of their results. A French study conducted in Roanne revealed that for within-series precision tests (repeatability), the coefficients of variation (CV) ranged from 0.51% for G7® to 1.17% for D10®, deemed excellent. For between-series precision (reproducibility), the CVs were 0.3% for G7® and 1.46% for D10®. The G7® also achieved 75 strictly identical pairs, compared to 42 for D10®, and showed no deviation greater than 0.4% among 105 pairs, while D10® displayed a CV of 1.7%, acceptable for HbA1c measurement. The correlation coefficients between the two analyzers were close to 1, with considerable commutability of results (mean ratio equal to 1.001). It is noteworthy that a patient who is heterozygous A/E or A/D will have an HbA1c level decreased by 25% if analyzed with G7® [14].

Both methods meet the precision and accuracy criteria set by AFSSAPS, but operators prefer G7® for its accuracy and specificity [13]. Another study on the D10® analyzer showed that it detects all hemoglobin variants (HbS, HbC, HbE, HbD) as peaks following HbA0, identifying HbS and C on the chromatogram (S and C window) [15].

Comparison of Results with Other Studies

A study published in the Journal of Clinical Pathology examined the incidental discovery of hemoglobin variants during HbA1c measurement in 800 patients. HPLC analyses were performed using the Tosoh G8® analyzer, and any detected variant was confirmed with the Bio Rad Variant II analyzer. The results were compared to a previous study using the Tosoh G7®, revealing that retention times for the variants were different, but the order remained the same: HbD, HbS, Hb Setif, and HbC [16]. This study concluded that the detection of variants by the Tosoh G7® and G8® analyzers is similar to that obtained with the Bio-Rad Variant II.

Another study at the Military Hospital of Rabat, involving 79,066 patients, analyzed the presence of variants in diabetic patients using the ADAMS® A1c HA-8081V Arkray analyzer, detecting 264 carriers of variants, primarily S and C, confirmed by capillary electrophoresis. The prevalence of sickle cell disease in this study was 0.28% [17].

These studies corroborate the results obtained with the Tosoh G7® and Bio Rad D-10® analyzers, emphasizing that chromatogram analysis during HbA1c measurement can provide crucial information regarding the presence of pathological variants.

CONCLUSION

The measurement of HbA1c by HPLC remains a routine test indicated for the monitoring of diabetic patients. Results are obtained fairly quickly with the various analyzers available on the market. As demonstrated, this test provides complementary information beyond the simple measurement of glycated hemoglobin. It would be beneficial to systematically examine HPLC chromatograms during glycated hemoglobin measurement. This examination would collect useful information for the patient on one hand and epidemiological data concerning the number of carriers or patients with hemoglobinopathies in Morocco on the other. Some laboratories take time to report the results of hemoglobin electrophoresis, whereas glycated hemoglobin can be obtained in a very short time. Therefore, it would be prudent to raise awareness among clinicians, especially hematologists, about prescribing glycated hemoglobin, whether it is performed by capillary electrophoresis or chromatographic methods, to quickly guide them regarding the presence or exclusion of a potential pathogenic hemoglobin variant. Indeed, the most common variants, namely S and C, are easily identifiable during this analysis. This could have beneficial consequences for the diagnosis, management, and therapeutic or transfusion follow-up of patients.

ABBREVIATIONS

AFSSAPS: Agence Française de Sécurité Sanitaire des Produits de Santé (French Health Products Safety Agency),

CV: Coefficient of Variation, D-10®: Bio-Rad D-10 Analyzer,
EDTA: Ethylenediaminetetraacetic Acid,
G7®: Tosoh G7 Analyzer,
HbA: Hemoglobin A, HbA1c: Glycated Hemoglobin,
HbA2: Hemoglobin A2,
HbC: Hemoglobin C,
HbD: Hemoglobin D,
HbF: Fetal Hemoglobin,
HbS: Hemoglobin S,
HPLC: High-Performance Liquid Chromatography,
WHO: World Health Organization.

Authors' Contributions

I.H.Z and B.A conceptualized the study and collected relevant data, which B.A designed and supervised. W.A.M, and H.I did the literature review and produced the initial draft of the manuscript. All authors contributed to interpreting the data, structuring, and writing the article. All authors approved the final version and take full responsibility for all its parts.

Declaration of Conflict of Interest

The authors declare that the research has no commercial or financial relationships that could be construed as a potential conflict of interest.

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