Precision of CAPILLARYS 2 for the Detection of Hemoglobin Variants Based on Their Migration Positions

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ABSTRACT

Objectives: *In this report, we evaluated utility of the capillary electrophoresis (CE) migration position of the CAPILLARYS 2 CE instrument.*

Methods: *The precision of this x-axis number was determined on a selection of common hemoglobin (Hb) variants (Hb S, Hb C, Hb D-Punjab, Hb E, Hb Hope), and the reproducibility of this number was evaluated by comparing the results obtained by two large reference laboratories on 81 Hb variants. Additionally, the CE migration position is given for a total of 409 Hb variants.*

Results: *The x-axis migration position showed excellent intra- and interassay precision. Comparison of Hb variants seen by both laboratories showed that 83% had a difference in migration position of 1 unit or less. Only three rare Hb variants showed a difference of more than 2 units.*

Conclusion: *In summary, the CE migration position is a reproducible value and can be used as an aid in the identification of Hb variants.*

Hemoglobinopathies are the most common autosomal recessive disorder worldwide, with 7% of the global population carrying an abnormal hemoglobin (Hb) gene.[1] Thus, the screening of hemoglobinopathies and thalassemias is of growing importance. The laboratory diagnosis of an Hb variant is typically done by a variety of methods using a protein analysis, such as alkaline acid Hb electrophoresis, isoelectric focusing (IEF), and high performance liquid chromatography (HPLC).[2] A more recent addition has been the application of capillary electrophoresis (CE) for the evaluation of Hb disorders.

Alkaline electrophoresis is simple, inexpensive, rapid, reproducible, and capable of separating common Hb variants. But Hb S and Hb D-Punjab are unresolved from each other, as are Hb C, Hb E, and Hb O-Arab. However, when combined with acid gel electrophoresis, the most common clinically significant Hb variants can be confirmed. Still, there are many other variants with electrophoretic mobilities identical or similar to these common variants.[3]

IEF is the most widely used method for investigating Hb disorders. It has a greater ability to resolve different variants due to differing isoelectric points, but it still has the same limitations as alkaline electrophoresis in that many rarer variants can show similar mobilities.[2,4]

More recently applied to the analysis of Hb disorders, cation-exchange HPLC is now usually considered as the gold standard technique, and there are several companies that manufacture these instruments.[5-11] HPLC analysis time is very rapid, and most instruments are semiautomated. The retention time of an Hb variant by HPLC is an objective and reproducible number. Many

variants also have a very characteristic shape to their elution peak. However, many Hb variants will have similar retention times.[12-14] A disadvantage is that Hb H and Hb Barts cannot be quantitated because of their very fast elution times. Additionally, the presence of Hb adducts, particularly glycosylated variants, can interfere with the quantitation of both Hb A_2 and some Hb variants.[15]

The accuracy of identification of Hb variants can be greatly improved by combining methods. The electrophoretic mobilities on alkaline/acid electrophoresis and IEF can be converted to a numeric value using published equations.[16] These numbers, called Schneider-Barwick ratios, can be used to develop a database that can be searched to more precisely identify rarer Hb variants, particularly when combined with the HPLC retention time. Both laboratories involved in this study utilized this approach. However, definitive identification of Hb variants can still only be achieved by either DNA sequencing of the affected globin gene or amino acid sequencing of the abnormal protein, typically by mass spectrometry.[17,18]

Developed recently, CE appears to be a reliable additional method to HPLC for the initial screening of quantitative and qualitative common Hb abnormalities providing high-resolution separation (Hb S, Hb C, Hb D-Punjab, and Hb E are separated) and accurate quantitation of the Hb fractions, with a user-friendly interpretive format. Multiple studies have documented the precision of the quantitation of Hb A_2 and Hb F,[19-33] and have compared CE with other methodologies, particularly HPLC.[33-37]

Using CE, the presumptive identification of Hb variants usually relies on their migration position on the electropherogram. While retention time is used in HPLC for the presumptive identification of the Hb variants, the numeric migration position on CE is not used and the presumptive identification of the Hb variants is inferred from their electrophoretic mobility in migration zones defined by the manufacturer. These migration zones are less specific than retention time, as these zones can sometimes be quite wide and include several Hb variants with close electrophoretic mobilities. The instrument does give an x-axis number, which represents a relative migration position. The units used to determine the migration position do not correspond to migration time; they are arbitrary units defined by the manufacturer that appear on the x-axis below the electropherogram, from 0 to 300. However, this x-axis number has not been specifically used by the manufacturer in the identification of Hb variants.

In this study, we wished to determine the utility of the x-axis migration position in a manner similar to the HPLC retention time to allow for greater discrimination between Hb variants of similar electrophoretic mobility and therefore higher specificity for the presumptive identification of Hb variants. We assessed the precision and reproducibility of the CE migration position on a selection of common Hb variants (Hb S, Hb C, Hb D-Punjab, Hb E, and Hb Hope). Interlaboratory precision was assessed by comparing the migration positions of a large number of rare variants obtained in two large laboratories.

Material and Methods

All evaluations were performed on either samples from the routine workflow (both institutions) or from samples stored at −80°C at Henri Mondor Hospital. However, the data collected on these samples for this study and shared between institutions were deidentified. Only the data and not the actual samples were shared. Thus, this study was determined to be exempt by the corresponding institutional review boards.

Capillary Electrophoresis

Hb CE was performed on a CAPILLARYS 2 instrument (Sebia, Lisses, France) using the CAPILLARYS HEMOGLOBIN(E) kit, following the manufacturer's instructions. Given the length of this study and the number of specimens involved, multiple lots of kits/reagents were used.

The CAPILLARYS 2 system is a fully automated system that uses the principle of liquid-flow CE in free solution. With this technique, charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH. Separation also occurs according to the electrolyte pH and electroosmotic flow. The separation of the different Hb fractions takes place in eight silica capillary tubes of 25 µm internal diameter, and the migration is performed at a high voltage of 9,800 volts under a tight temperature control of 34°C using a Peltier device. The Hbs are directly detected at a specific absorption wavelength of 415 nm at the cathodic end of the capillary. The optical detector consists of a deuterium lamp, optical grating, CMOS (complementary metal-oxide semi-conductor), diode array detector, and optical fibers. The system has a primary capacity of 104 tubes (ie, 13 sample racks), with uninterrupted throughput of 40 samples/h and continuous loading of the sample racks.

The separation of Hb fractions is based on their electric charge, and the CAPILLARYS 2 instrument records Hb migration on the x-axis from 0 to 300. The migration position for each Hb is normalized relative to the standardized position of Hb A (position 150) and Hb A_2 (position 243). If Hb A and/or Hb A_2 is present, the electrophoretic profile is divided in 15 migration zones, and presumptive identification of any abnormal

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fraction is obtained according to the migration zone in which the abnormal fraction migrates. This division in 15 zones allows discrimination between the most common Hb variants: Hb S, Hb C, Hb D-Punjab, and Hb E. This method also allows the measurement of Hb A_2 in the presence of Hb E. This has been previously impossible with other methods in which Hb E and Hb A_2 have consistently comigrated. Because of the electroosmotic flow that is part of the system, the migration of Hb fractions is the reverse of older electrophoretic methods, ie, Hb C and Hb A_2 are some of the first to be detected, whereas Hb H, Hb I, and Hb Barts are the last. Putative identification of the abnormal fraction is facilitated by placing the mouse pointer on the zone heading, thereby producing a drop-down library with numerous Hb variants potentially migrating in the zone. The profiles are color-coded to help the visualization of abnormal profiles.

We recorded the positions of the peak of the Hb variant in each sample **Figure 1**.

Statistics

Precision studies were performed at Henri Mondor Hospital on fresh samples of whole blood collected in EDTA and containing Hb variants. Cases containing Hb S, Hb C, Hb D-Punjab, Hb E, and Hb Hope were included.

The intra-assay precision of hemoglobin variants migration position was assessed by selecting seven fresh samples: one normal sample (homozygous Hb A), five samples heterozygous for a common Hb variant (one A/S, one A/C, one A/D-Punjab, one A/E, one A/Hope) and one normal control (Sebia, cat. No. 4774). For each sample, washed RBCs were distributed in 4 aliquots of 500 µL each and then stored at −80°C. Each aliquot was then analyzed twice during the same day on the eight capillaries simultaneously, once in the morning, once in the afternoon, using two different buffer lot numbers. Mean values, standard deviations, and coefficients of variation (CVs) for the migration position of all fractions were then calculated. The interassay precision of Hb variants migration position was assessed on the same seven samples after having repeated the analysis of each aliquote 12 times during 12 consecutive runs: six runs in the morning and six runs in the afternoon using two different buffer lot numbers. Mean values, standard deviations, and coefficients of variation for the migration position of all fractions were then calculated.

The interindividuals precision of Hb variants migration position was assessed by selecting 61 samples heterozygous for common Hb variants: 20 A/S, 10 A/C, 10 A/E, 10 A/D-Punjab, and 11 A/Hope. These samples were run randomly during several days. Mean values, standard deviations, and CVs for the migration position of each Hb variant were then calculated.

Interlaboratory Precision

To determine whether the x-axis number is reproducible between different laboratories and on different instruments, the migration positions of a large number of rarer Hb variants on the CAPILLARYS 2 were compiled and compared between the Genetic Biochemistry Department at Henri Mondor Hospital, and the Metabolic Hematology Laboratory at Mayo Clinic. Both

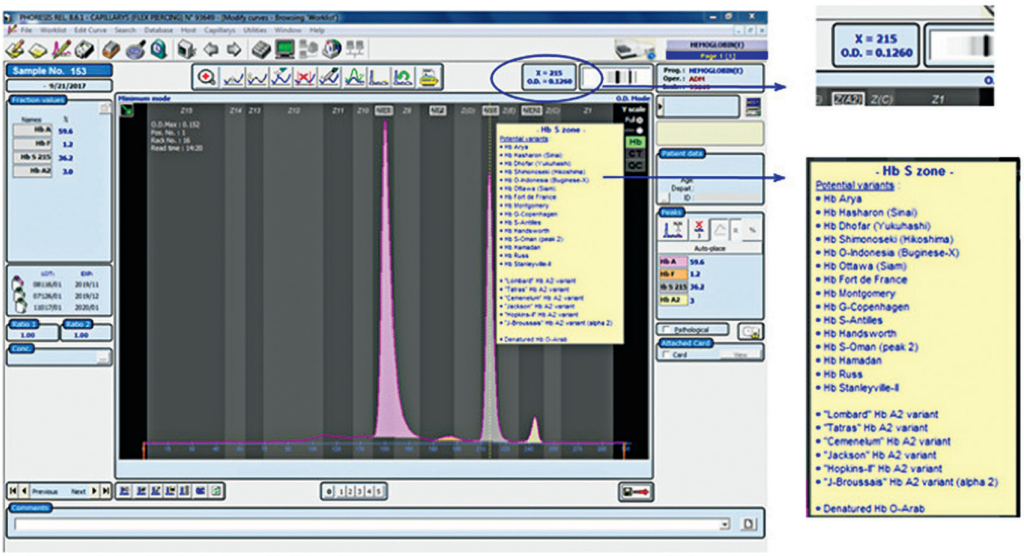

Figure 1 Screenshot of the Phoresis display (with Hb variant and cursor positioning x = 215). The instrument gives a pop-up screen with potential Hb variants at this position.

laboratories used the latest version (8.6.1) of the Phoresis software (Sebia) available at the time of this study, as supplied by the manufacturer.

In addition to samples used for intra- and interassay precision, 151 samples of rare Hb variants stored at −80°C at Henri Mondor Hospital were used. All variants were identified using a combination of CE, globin chain electrophoresis, IEF, HPLC, and reverse phase HPLC, and were confirmed by DNA sequencing, peptide structural study, or by mass spectrometry. Hb variants identified at Mayo Clinic were a part of the routine workflow, and identified by a combination of CE, HPLC, IEF, and mass spectrometry. All variants included in the study were confirmed by DNA (Sanger) sequencing. A total of 409 Hb variants were included in the study.

Results

Migration Position of Normal Hemoglobins

For both the intra- and interassay precision studies, the migration positions of Hb A and Hb A_2 were always consistent, Hb A always migrating in position 150 and HbA_2 always migrating in position 243. This perfect repeatability is obtained after the standardization of the migration positions for Hb A and Hb A_2. On each sample, when present, Hb A and Hb A_2 are normalized to migrate at position 150 and 243, respectively, based on a migration control previously run at the beginning of the series. If either Hb A or Hb A_2 is not present in a sample (case for a homozygous variant), the normalization is not possible and the assignment of the migration zones is not effective. Then, the CAPILLARYS 2 uses information from the most recent previous samples analyzed to estimate the position of Hb A in order to generate migration points. In the absence of Hb A and/or Hb A_2, the migration position is imprecise. Thus, the manufacturer recommends mixing the sample with a normal control in order to introduce Hb A and Hb A_2 in the sample and therefore get the normalization of the migration positions. Consequently, the assignment of the migration zones allows for the presumptive identification of the abnormal fraction, while the accurate quantitation of the fractions is obtained from the initial pattern before mixing with the normal control **Figure 2**.

Precision of the Migration Patterns of Common Hemoglobins

By CE, the migration position for each abnormal Hb is standardized relative to the position of Hb A and Hb A_2. The intra- and interassay precision of the migration positions for the common Hb variants tested, as well as the interindividuals precision of the migration positions of Hb variants are shown in **Table 1**.

Interlaboratory Precision

A total of 81 Hb variants were seen by both laboratories, and a total of 409 Hb variants were seen by either laboratory. To determine whether freezing at −80°C could affect the migration position of the Hb variant, the results obtained on fresh specimens at the Mayo Clinic were compared to results obtained on previously frozen specimens. Although there were readily identifiable degradation peaks (particularly in zones 11-12, x = 110-130), there was no difference in the migration position between fresh and frozen specimens (data not shown). With unstable Hb variants, the amount of the abnormal protein could be reduced with freezing.

The 40 most common variants seen by both laboratories, based on a previous study,[14] are shown in **Table 2**. A full listing of Hb variants seen and their migration positions are contained in supplemental material available at *American Journal of Clinical Pathology* online. Of the 81 Hb variants seen by both laboratories, 67 (83%) had a difference of migration position of 1 unit or less. Only three Hb variants had a difference of more than 2 units: Hb Aztec, Hb Bassett, and Hb Share Zedek. All three Hb variants are very rare and only one to three cases were available for comparison. Hb Aztec had a peak at position 113, which may represent degradation, but was seen in all Mayo Clinic cases and had a slightly higher percentage than what is typically seen with degradation peaks.

A list of those Hb variants found in the study that migrated similar to the three most common Hb variants (Hb S, Hb C, and Hb E) are shown in **Table 3**. This table illustrates two important points. First, these data illustrate why migration position is more useful than migration zones. For example, there were 22 Hb variants in the study that migrated in the same zone as Hb S (zone 5). However, there are fewer variants, 13, that migrate within +/− 2 units of Hb S. Secondly, the data show that there are very few Hb variants that can be confused with HB C or Hb E on CE, particularly if the migration position and the percentage of the Hb variant are taken into account. For Hb C, the only variant is the doubly substituted Hb variant, Hb C-Harlem. Lack of recognition of the presence of Hb C-Harlem could have potential clinical consequences. For example, an Hb C/C-Harlem compound heterozygote would act like Hb S/C disease, and not as homozygous Hb C, and Hb S/ Hb C-Harlem would have clinical features equivalent to homozygous Hb S and not

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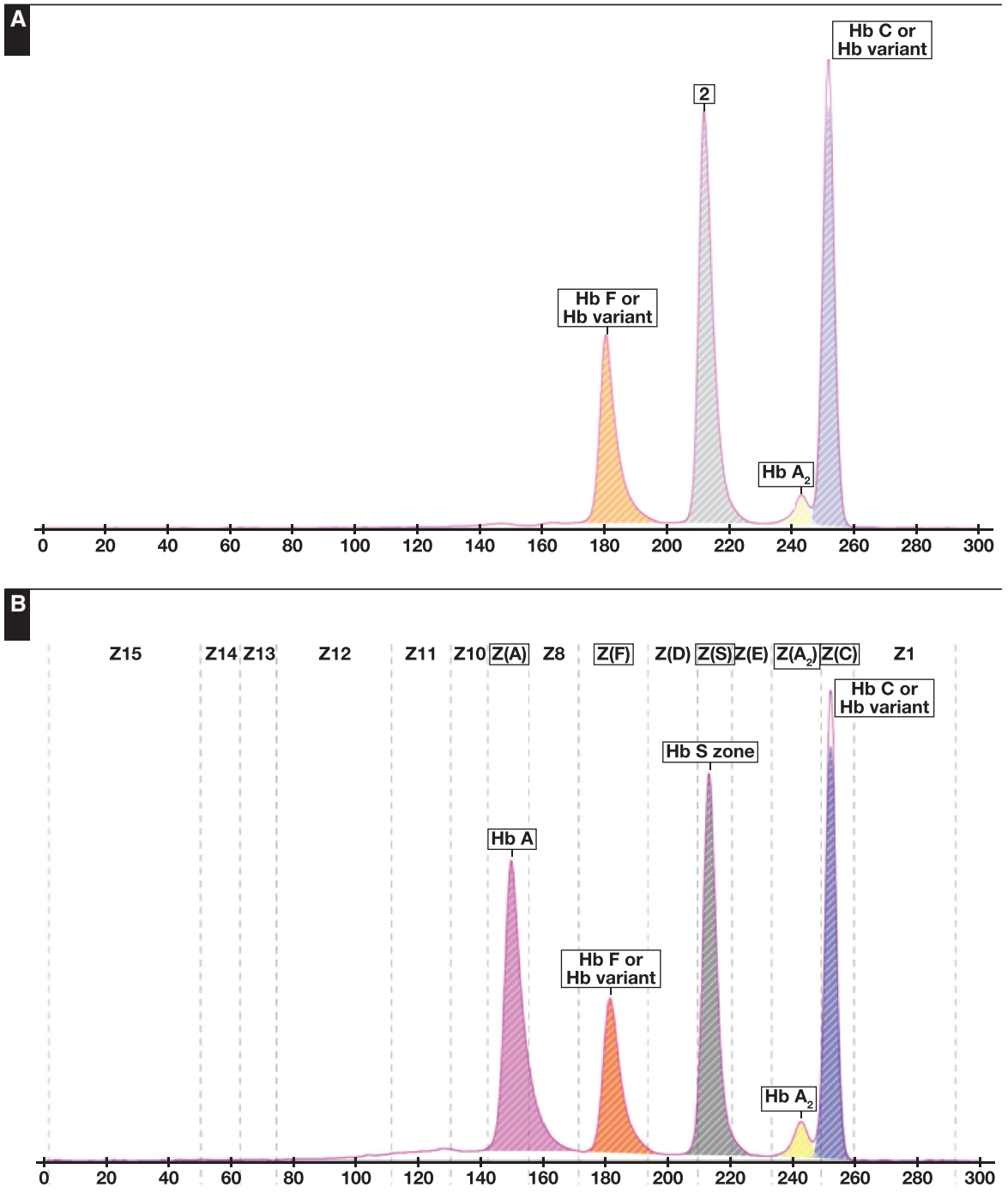

Figure 2 Compound heterozygous profile (Hb S/C) before (**A**) and after (**B**) mix with normal control.

Hb S/C disease. However, without associated thalassemia, the differentiation of Hb C and Hb C-Harlem can be done through their quantifications, as their expected levels of expression do not overlap (Table 3). The only variant that was found that could be misidentified as Hb E on CE was Hb Agenogi, a rare variant. Again, the difference of expression level between these two variants is enough to allow discrimination (Table 3).

Discussion

A high degree of reproducibility and accuracy is essential for the initial screening of Hb disorders.

HPLC is sometimes described as more specific than electrophoresis for the presumptive identification of Hb variants, since it provides retention times that are more precise than migration zones. It must be noted that

Table 1
Precision Studies Performed on CAPILLARYS 2

	Hb Hope	Hb D	Hb S	Hb E	Hb C
Intra-assay reproducibility of the variants migration positions (n = 16)					
Min	132	207	213	227	252
Max	134	208	214	228	253
Mean	132.8	207.1	213.5	227.7	252.2
SD	0.66	0.34	0.52	0.48	0.40
CV	0.49%	0.16%	0.24%	0.21%	0.16%
Interassay reproducibility of the variants migration positions (n = 12)					
Min	132	207	214	227	252
Max	134	209	214	228	253
Mean	133.1	207.9	214.0	227.8	252.5
SD	0.51	0.51	0.00	0.45	0.52
CV	0.39%	0.25%	0.00%	0.20%	0.21%
	Hb Hope (n = 11)	**Hb D (n= 10)**	**Hb S (n=20)**	**Hb E (n=10)**	**Hb C (n=10)**
Interindividual repeatability of the variants migration positions					
Min	132	207	212	227	251
Max	134	209	216	228	252
Mean	133,0	207.3	213,9	227.9	251,8
SD	0.63	0.67	1.33	0.32	0.42
CV	0.48%	0.33%	0.62%	0.14%	0.17%

Hb, hemoglobin.

definitive identification of Hb variants by HPLC alone is unachievable, since overlap of retention times of variants is still possible.[12-14] Moreover, it has been described that the retention time of some Hb variants can change windows with different lots of reagents.[13] Thus, HPLC should not be used as the sole means of identification of Hb variants, but its use, combined with another electrophoretic method, appears to be an effective combination for the first line screening of Hb disorders.

CE has been used for the screening of Hb disorders for decades.[19-33] It offers the advantage of a clear pattern with no separation of glycated fraction and other Hb adducts, accurate measurement of the Hb fractions (such as Hb A_2),[22,31,32] and high resolution at a high throughput. Developed in the past decade, the Sebia CAPILLARYS 2 has a superior throughput compared with HPLC systems, providing an easy pattern for interpretation, and does not have the need to account for glycated and breakdown products when measuring the most common Hb variants.[29,30,35,36]

In this study, we have investigated the possibility to use the migration position of the Hb variants on the Sebia CE pattern in order to refine the presumptive identification of the Hb variants by narrowing their search zone. We have selected common Hb variants showing different electrophoretic mobility, from anodic (Hb Hope) to very cathodic (Hb C) positions, in order to cover a wide range of migration positions and assess whether the precision of the migration positions is acceptable throughout the

Table 2
Top 40 Variants in Common Between Institutions and Difference in Mobility

Zone	Variant Name	Chain	Mondor Mean	Mayo Mean	Difference
2	C	β	252	252	0
2	C-Harlem	β	251	251	0
3	Chad	α	243	243	0
3	E-Saskatoon	β	241	241	0
3	O-Arab	β	241	241	0
4	Seal Rock	α	232	232	0
4	Koln	β	229	229	0
4	E	β	227	227	0
5	Arya	α	220	220	0
5	Dhofar	β	218	218	0
5	O-Indonesia	α	216	216	0
5	S	β	214	214	0
5	G-Copenhagen	β	214	213	1
5	Handsworth	α	214	213	−1
6	D-Punjab	β	208	208	0
6	Matsue-Oki	α	208	207	−1
6	Osu-Christiansborg	β	207	208	1
6	Q-Iran	α	207	207	0
6	G-Waimanalo	α	207	206	−1
6	G-Philadelphia	α	206	204	−2
6	Korle Bu	β	202	202	0
6	G-Pest	α	197	198	1
6	P-Galveston	β	197	196	−1
6	P-Nilotic	βδ	195	195	0
7	Bassett	α	185	181	−4
7	G-Georgia	α	182	182	0
7	Abruzzo	β	178	176	−2
9	Malmo	β	150	150	0
9	Olympia	β	150	150	0
9	Rhode Island	β	150	150	0
9	Old Dominion/ Burton-upon-Trent	β	145	144	−1
10	Hope	β	133	133	0
11	Andrew-Minneapolis	β	123	122	−1
11	New York	β	122	122	0
11	Fannin-Lubbock I	β	121	123	2
11	Jackson	α	121	123	2
12	Hopkins-II	α	106	106	0
12	J-Broussais	α	97	99	2
12	Hofu	β	93	94	1
12	Riyadh	β	89	88	−1

electrophoretic pattern. Comparison of the migration positions between two laboratories demonstrates excellent interlaboratory reproducibility.

Recently, Keren et al[38] have determined the precision of CE in the identification of relatively common and two closely migrating Hb variants. They found that the CVs of Hb S, Hb C, Hb D, and Hb G-Philadelphia fluctuate around 1% from the direct migration data. They suggested that the precision of several common Hb variants was improved by expressing the migration relative to that of Hb A_2 in the same specimen, and that expression of other variants relative to the migration of Hb A_2 may be helpful in creating a more precise library of variants for CE. With the latest Phoresis

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Table 3
Variants Similar to Hemoglobin (Hb) C, Hb E, and Hb S

Zone	Variant Name	Chain	Mondor Mean	Mayo Mean	Trait % by Sebia
2	C-Ziguinchor	β	259		
2	Constant Spring	α		253	0-5
2	Pakse	α		253	4
2	C	β	252	252	30-40
2	C-Harlem	β	251	251	27-30
2	Contaldo	α		251	0.2-0.5
4	Buenos Aires	β		232	1
4	Hornchurch	β		232	34-35 (not an accurate quantitation due to baseline)
4	Seal Rock	α	232	232	1-3
4	Koln	β	229	229	3-5 (additional peaks may be present at approximately 204 and 165)
4	Cleveland	β		227	31
4	E	β	227	227	15-30
4	Agenogi	β		226	34-39
4	O-Padova	α		225	16
4	Savaria	α		222	19-24
5	Arya	α	220	220	29
5	Sabine	β		220	10
5	Hasharon	α		219	19-20
5	Kenya	γβ	219	219	20
5	Shimonoseki	α		219	9 (only examples are in an infant or with a β-thalassemia)
5	Dhofar	β	218	218	14-16 (all examples in combination with β-thalassemia)
5	Machida	β	216		
5	O-Indonesia	α	216	216	12-13
5	Russ	α		216	18-19
5	Corbeil	β	215		
5	Hamadan	β		215	46
5	Fort de France	α	214		
5	Ottawa	α		214	25
5	S	β	214	214	34-40
5	S-Antilles	β		214	35
5	G-Copenhagen	β	214	213	39-43
5	Handsworth	α	214	213	16
5	Lavagna	β		213	39
5	Poissy	β	213		
5	Stanleyville-II	α		212	25-33
5	Cocody	β	210		
5	Evanston	α		210	0.0-8.0
5	Reims	α	210		

software development, Sebia has followed this recommendation and the migration positions of all abnormal fractions are now normalized based on the standardized Hb A andHb A_2 migration positions, while this standardization was previously done on the Hb A only.

In this study, we used this last software release, and we found that the CVs of the migration positions for Hb S, Hb C, Hb D-Punjab, Hb E, and Hb Hope were below 0.49% and below 0.39% for the intra- and interassay precision study, respectively. For the interindividuals precision study, CVs were found to be lower than 0.62%. Therefore, the migration positions of Hb variants obtained by CE proved to be highly reproducible. It can be expected that these migration positions visualized on the x-axis can be used, rather than migration zones, to refine the presumptive identification of Hb variants and increase their detection specificity. Based on these results, we have observed that the variation of the migration position on the CE pattern for the majority Hb variants tested is very low, comprised between +/− 1 unit on the x-axis, which appears to be more precise than the ranges of retention times usually offered by HPLC manufacturers.[12]

Despite this very low variation, we noticed a higher variation of the migration positions during the interindividuals precision study on samples presenting unusual levels of Hb S.

The detailed analysis of these data, in particular the electrophoresis patterns, indicated the presence of transfused patients in this study, with either very low (around 10%) or very high (around 60%) Hb S levels, leading to a shift of the migration position of +/−2 units on the x-axis, (Table 1). In other words, a lower than normal percentage of Hb S results in a higher migration position (215-217),

whereas a higher than normal percentage of Hb S results in a lower migration position (212-213). A similar situation can occur in neonates with low percentages of Hb S, and in patients with Hb S/β + thalassemia, where percentages greater than 50% would be expected. If we exclude from these data transfused patients, the migration position of Hb S remains centered on 214, with a variation comprised between +/− 1 unit on the x-axis, and a CV lower than 0.2%. A similar observation was seen in other variants, such as Hb C, Hb E, and Hb D-Punjab. This also explains the slightly wider mobility range observed with these more commonly seen variants where wider Hb variant percentages would be expected. These data strengthen the need to interpret profiles in combination with the patient status and the clinical data.

Furthermore, Keren et al[38] reported that it may be that variants with a more anodal migration may require a different internal standard to achieve similar results. Indeed, since on CE pattern the migration position for all Hbs is normalized based on the fixed position of Hb A and Hb A_2 fractions, it might be believed that all Hb variants that migrate outside of the Hb A-Hb A_2 curve area will show imprecise migration positions. In our study, we also assessed the reproducibility of the migration position for Hb Hope, which migrates in zone 10. We found the CVs to be 0.49%, 0.39%, and 0.48% for the intra-assay, interassay, and interindividuals precision studies, respectively. Although there were no variants in zones 13 to 15 that were seen by both laboratories, those seen in zones 10 to 12 by both laboratories showed excellent agreement (see supplemental materials). Therefore, our study demonstrates that when using Hb A and Hb A_2 for the normalization of the CE pattern, the migration position of anodic fractions, such as Hb Hope, shows excellent precision. This leads us to the conclusion that Hb A and Hb A_2 are effective internal standard fractions.

Conclusion

After having demonstrated the excellent intra- and interlaboratory reproducibility of the migration positions, both for anodic and cathodic Hb variants, we have combined our data on Hb variants (409 total) to create a more precise library of variants on CAPILLARYS 2 than the one provided by the manufacturer through the migration zones. Based on the electrophoretic mobility of each Hb variant, this library might be useful for refined presumptive identification of Hb variants.

Nevertheless, due to the very large number of abnormal Hbs reported so far, no separation method is currently able to separate all known Hb variants, and therefore no definitive identification can be obtained from a single method nor from two or three methods.

Neither HPLC nor CE allows the identification of Hb variants, if they are not coupled to complementary tests like we described above. Even though CE offers high-resolution separation with clear patterns, comigration of some Hb variants is unavoidable, and definitive identification will only be obtained by DNA analysis or aminoacid sequencing.

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