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A comprehensive analysis of hemoglobin variants by high-performance liquid chromatography (HPLC)

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SUMMARY

Introduction: High-performance liquid chromatography (HPLC) is a method commonly used for the detection of hemoglobin (Hb) variants. In addition to providing precise quantitation of Hb A_2 and Hb F, the reported retention time and peak shape of a high number of hemoglobin (Hb) variants are very helpful for presumptive identification. However, there is a scarcity of summarized data in the literature of the mobility of Hb variants on this method.
Methods: A total of 383 Hb variants were studied on the Bio-Rad Variant ^{TM} Classic HPLC instrument. Hb variant identification used a number of methods, including confirmation by DNA sequencing in at least one case for all alpha and beta chain Hb variants.
Results: Retention time data and the number of occurrences of each Hb variant were obtained. This showed that rare Hb variants can have similar retention times to the five most common alpha or beta chain Hb variants.
Conclusion: HPLC is a very powerful tool in the evaluation of Hb variants, particularly when combined with other methods. However, it should not be used as a stand-alone method for definitive identification of Hb variants.

INTRODUCTION

There are many methods currently available for the evaluation of hemoglobin (Hb) variants, including alkaline and acid electrophoresis, capillary electrophoresis (CE), globin chain electrophoresis, isoelectric focusing (IEF), mass spectrometry (MS), and HPLC. These methods are all typically successful in detecting the common Hb variants. However, a limitation of many methods is the lack of detailed data regarding the migration or mobility of Hb variants that are less frequently encountered.

Since its adaptation to hemoglobinopathy diagnosis in 1983 [1], HPLC has become a common method utilized by many laboratories. Several manufacturers have produced similar systems utilizing a weak cation exchange column. An attractive feature of HPLC is that in contrast to gel-based methods, it provides precise quantification of Hb A_2 and Hb F. Additionally, the Hb variant mobilities expressed as a retention time are fairly constant and reproducible. Several studies have documented the precision, linearity, and reproducibility of HPLC systems [1–5]; however, most studies have been limited in the number of Hb variants

studied [6–11]. A number of validated, dedicated automatic devices for the detection of common and uncommon abnormal hemoglobins and beta thalassemia have been evaluated [12]. This study presents our experience in a clinical laboratory using the Bio-Rad Variant™ Classic HPLC system over an extended period of time in the evaluation of 383 Hb variants.

METHODS

This study encompasses 298 276 samples analyzed over a 16-year period. The samples were received from a wide geographic distribution, predominantly the Midwestern United States, but also many other areas of the United States and from several international clients. Many ethnic groups are represented, particularly those that have clinically significant Hb disorders, such as those of African or Southeast Asian heritage. All samples in this study were analyzed with the Bio-Rad Variant ™ Classic HPLC instrument (Bio-Rad Laboratories, Hercules, CA, USA) utilizing the β-thalassemia short program, according to the instructions of the manufacturer. The Bio-Rad Variant ™ uses two buffers with increasing ionic strengths to elute hemoglobin molecules from a weak cation exchange column. Elution of the fractions is monitored by a dual wavelength photometer (415 and 690 nm); a chromatogram is produced, which shows the percentage of each hemoglobin fraction present, the retention time of each hemoglobin fraction, and any distinguishing peak characteristics (widening and shape variations). The instrument identifies a series of 'windows' that aid in interpretation. Each window has a retention time range, and each reagent lot includes a ROM (read only memory) card. The ROM card allows the user to download all assay parameters, which specifies the ranges for each window. In addition to the A_0, A_2, and F windows, there are also S, D, and C windows, and a series of 'fast' windows (Hb F, P1, P2, and P3). If a Hb variant falls between these windows, or there are multiple peaks in the same window, it is labeled as unknown. Data that were collected for each Hb variant includes the number of cases seen (including the number of cases molecularly sequenced), retention time range, midpoint of the retention time range, and the approximate percentage of each Hb variant. Table 1 lists the approximate HPLC peaks and retention time windows that the Bio-Rad Variant ™ identifies. Windows can shift slightly with different reagent lots and ROM card versions, so retention time ranges listed in Table 1 are approximate ranges observed in our laboratory over the course of this study.

In addition to HPLC, all samples were analyzed with alkaline and acid electrophoresis (SPIFE 3000® electrophoresis system, Helena Laboratories, Beaumont, TX, USA). This combination was sufficient to identify the common Hb variants of S, C, and E. Other, less common Hb variants were then further analyzed using IEF (Perkin Elmer Resolve® Hemoglobin Kit, Perkin Elmer, Waltham, MA, USA) and globin chain electrophoresis, using methods as previously described [13]. In addition to the HPLC retention times, all mobilities for the above-listed methods are maintained in a comprehensive database for every Hb variant. Because multiple methods are used, the database can successfully identify Hb variants based on the collective mobility characteristics for most specimens. However, if there was a question of the identity of the Hb variant or was the first time seen in our laboratory, DNA sequencing was performed. Thus, although each Hb variant in this study has been confirmed by DNA sequencing on at least one occasion, (except for fusion proteins Hb Lepore, P-Congo, P-Nilotic and delta variants Hb A_2', Hb A_2-Babinga, and Hb A_2-Flatbush), in actuality, many Hb variants have been confirmed by DNA sequencing on multiple occasions.

DNA sequencing was performed using standard automated-sequencing instruments (ABI Prism ™ 3130, Applied Biosystems Life Technologies, Carlsbad, CA, USA). More recent specimens in the study have also been analyzed utilizing intact globin protein mass spectrometry using an electrospray ionization quadruple-time-of-flight mass spectrometer (Q-ToF Premier™ Waters Corp., Milford, MA, USA) and results were analyzed using Waters BioPharmalynx software. The approach utilized is similar to one previously published [14].

The types of Hb Lepore were not routinely further subclassified as they were assumed to be Hb Lepore-Boston (confirmed in several cases by mass spectrometry). However, a subset of cases was definitively identified as Hb Lepore-Hollandia or Hb Lepore-Baltimore and is included in the study. Hb H and Hb Bart's were excluded from this study as they are not assigned a specific retention time by the instrument

Table 1. Analyte windows and the most common Hb variants in each window

Analyte Window	RT on Bio-Rad Variant (min)	# of Hb Variants in Window	Most Common Hb Variants in each Window (Number of cases seen)
P1	0.63–0.85	1	Providence (1)
Between P1 and F	0.85–0.92	0	
F	0.92–1.25	9	Wayne (62), J-Sardegna (5), South Florida (5), J-Iran (4), Marseille (3)
P2	1.24–1.46	10	Hope (164), Raleigh (35), K-Woolwich (17), Camperdown (8), Norton (7)
Between P2 and P3	1.38–1.50	4	I (132), Osler (8), Wood (5), Pisa (1)
P3	1.47–1.90	63	J-Baltimore (263), N-Baltimore (133), Fannin-Lubbock (103), J-Oxford (60), Camden (48)
Between P3 and A	1.87–2.30	30	Athens-GA (38), J-Bangkok (19), N-Seattle (18), Hofu (17), J-Meerut (16)
A_0	1.87–2.84	83	New York (55), A_2 Babinga (53), Twin Peaks (53), P-Nilotic (40), Tacoma (28)
Between A and A_2	2.50–3.38	25	Fontainebleau (34), P-Galveston (26), Silver Springs (20), Loves Park (9), Port Phillip (7)
A_2	3.38–3.90	35	E (5796), Lepore* (523), G-Coushatta (108), Osu-Christiansborg (75), D-Iran (50)
D	3.80–4.30	43	D-Punjab (1240), G-Philadelphia (729), Korle-Bu (148), Tarrant (71), Inkster (44)
S	4.11–4.69	33	S (38038), A_2 Prime (1812), Q-Thailand (46), Stanleyville-II (42), Russ (40)
Between S and C	4.47–4.92	36	Hasharon (225), G-San Jose (125), Montgomery (75), Koln (72), Q-India (54)
C	4.87–5.18	11	C (9062), Constant Spring (512), O-Arab (267), O-Indonesia (31), Agenogi (11)

*Assumed to be Hb Lepore-Boston.

because of their extremely fast elution from the ion-exchange column. Gamma chain variants and delta chain variants other than Hb A_2′, Hb A_2-Babinga, and Hb A_2-Flatbush were also not included as a part of the study. This study was reviewed by the Mayo Clinic Rochester Institutional Review Board. Because of the blinded nature of this study (no patient identifiers were used), it was given exempt status.

RESULTS

Over the 16 years that our laboratory has used HPLC, we have definitively identified 383 different Hb variants (159 α-, 215 β-, 3 δ-, 3 δ/β fusion-, 2 β/δ fusion-, and 1 γ/β fusion- variants) and observed 62,604 occurrences of a Hb variant. Because of the fact that one sample can have more than one Hb variant; Hb variants were counted by occurrence rather than number of samples. For example, a patient with Hb S and Hb C would be two occurrences. Table 1 shows the number of Hb variants observed in each HPLC window and the five most common Hb variants in that window. A complete listing of all Hb variants identified and their retention times and ranges are given in Table S1. This also includes the number of times each Hb variant has been sequenced. The five most common Hb variants (Hb S, Hb C, Hb E, Hb A_2′, and Hb D-Punjab) accounted for 55,948 (89%) of the total number of occurrences. The next 10 most common Hb variants (G-Philadelphia, Hb Lepore, Hb Constant Spring, Hb O-Arab, Hb J-Baltimore, Hb Hasharon, Hb Hope, Hb Korle-Bu, Hb N-Baltimore, and Hb I) accounted for 3096 (5%) of all occurrences. Thus, the 15 most common Hb variants accounted for approximately 94% of all occurrences, indicating that most Hb variants are extremely rare. However, within certain ethnic groups, a particular Hb variant may be seen in higher frequencies.

General observations

Before outlining information about specific windows, there are several general observations that can be made from the analyzed data:

- In general, the elution of a Hb variant from the ion-exchange column has some analogy with that Hb variant's mobility on alkaline electrophoresis and IEF. Hb variants that migrate ahead (anodal) of Hb A on these methods will generally elute from the HPLC column before Hb A and *vice versa* (Hb F being the primary exception).
- The presence of a double Hb A_2 peak (because of the alpha variant/normal delta chain hybrid) and the percentage of the major Hb variant peak are helpful features in recognizing the presence of an alpha chain variant. However, the hybrid is best seen with Hb variants that elute slower than Hb A. It is usually difficult to identify for alpha chain variants that elute faster than Hb A. Additionally, the percentage of alpha chain variants can be modified by the presence of coexistent alpha thalassemia.
- The shape of the peak can be a very helpful feature in recognizing a particular Hb variant, as the abnormal shape is typically present in all examples of that Hb variant and acts as a 'fingerprint'. Some Hb variants, although they do not separate distinctly from Hb A, will display an abnormal shoulder on the upslope or down slope. Figure 1 gives examples of distinctive chromatograms.
- Many Hb variants will have similar retention times. Furthermore, there is a range of retention times for most Hb variants. This range is variable; it is generally tighter for more common Hb variants but is wider with Hb variants that are infrequently seen.
- When the Hb variant elutes very close to Hb A, the instrument may 'flip' the window assignments, that is, mislabel the Hb variant as Hb A_0 and Hb A as 'Unknown'. This implies that the unknown peak is the Hb variant and will result in an erroneous retention time for the Hb variant. This can occur on both sides of the Hb A_0 peak, from the end of the P3 window into the unknown area between the P3 and A_0 windows and also the unknown area between the A_0 and the A_2 windows. This does not happen with all Hb variants and is not even consistent with a single Hb variant. The reason for this is if the two peaks are very close to the retention time range of Hb A, the instrument assigns the peak with the higher percentage as Hb A. Careful inspection of the chromatogram in these situations and knowledge of the retention time range for Hb A (typically 2.30–2.50 min) are necessary to ensure the correct retention time has been assigned to the Hb variant. Figure 2 presents two examples in which this situation has occurred.
- Hb variants will generally run in a certain window but occasionally can move to an adjacent window. This is because of the fact that Hb variants can have slightly shifting retention times. This shift can happen because of a reagent lot change, column change, instrument re-calibration, integration mode, or Hb concentration of the loaded sample [15]. Review of our data shows that we have seen this shift occur with 68 Hb variants that have retention times near the border of two adjacent windows.
- Several Hb variants can interfere with accurate Hb A_2 quantitation. This can happen even if they do not directly co-elute with Hb A_2, often because of the abnormal shape of the peak. Table 2 shows Hb variants that can interfere with Hb A_2 quantitation.
- It is important to recognize three fractions that could possibly be confused with a Hb variant, particularly if the percentage is over 10%. These are (i) Hb A_{1C} which elutes in the P2 window, (ii) degraded Hb which elutes in the P3 window and is because of the age of the specimen or storage conditions of the sample, and (iii) sulfhemoglobin which elutes in the unknown area between the S and C windows (approximate retention time of 4.65 min).

Fast windows (P1, F, P2, P3, 2 unknown areas)

Hb variants in these fast windows correspond to Hb variants that migrate ahead (anodal) of Hb A on alkaline Hb electrophoresis or IEF. Although there are a significant number of Hb variants that elute in these areas (117 total Hb variants, 53 α-, 64 β- variants), there are none that are particularly prominent. It is important to mention again that both Hb H and Hb Bart's elute very quickly from the column, before the time that the instrument begins to quantitate. The nine Hb variants that elute in the F window prevent accurate Hb F quantitation. All of the Hb

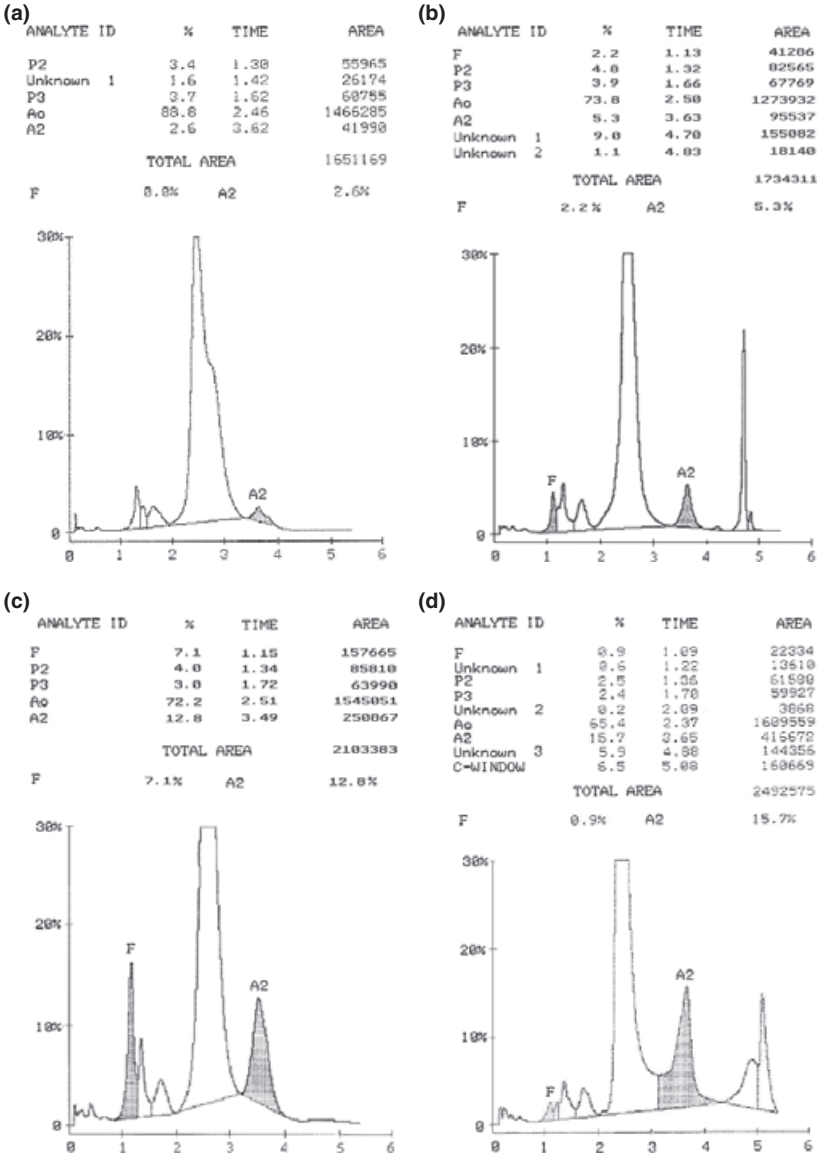

Figure 1. Examples of characteristic chromatograms. (a) Hb Twin Peaks trait, which does not separate completely from Hb A but produces a shoulder on the downslope of the Hb A peak. (b) Hb Q-India trait, which has a very narrow elution peak. This is typically seen with all Q-Hbs. (c) Hb Lepore trait, showing an increase in the Hb A_2 peak in the range of 10-15%. This elevation occurs regardless of the Hb Lepore subtype. (d) Hb M-Saskatoon, showing a very unique chromatogram. In addition to peak eluting in the C window, there is an elevation in the A_2 peak as well.

variants in both the P2 and P3 windows could potentially interfere with the measurement of hemoglobin A_{1C} if ion-exchange HPLC methods are used.

A_0Window

There were 83 different Hb variants observed in the A_0 window (29 α-, 50 β-, 2 δ-, and 2 β/δ fusion- variants). This was the window with the largest number of different Hb variants; however, there were none that predominated. Importantly, 51 of the 83 (61%) Hb variants observed in the A_0 window showed no sign of their presence with a completely normal chromatogram. There were 8 Hb variants that inconsistently split from Hb A. The remaining Hb variants typically displayed a shoulder on the up- or downslope, but in some cases could also have a normal chromatogram. Although the chromatogram may appear normal, the presence of a Hb variant in some situations was suspected if the retention times for Hb A fell outside the typical range (Figure 2c). Significantly, of the 83 Hb variants in the A_0 window, 38 had functional abnormalities (altered O_2 affinity, abnormal Hb stability, and microcytosis). Of the 19 Hb

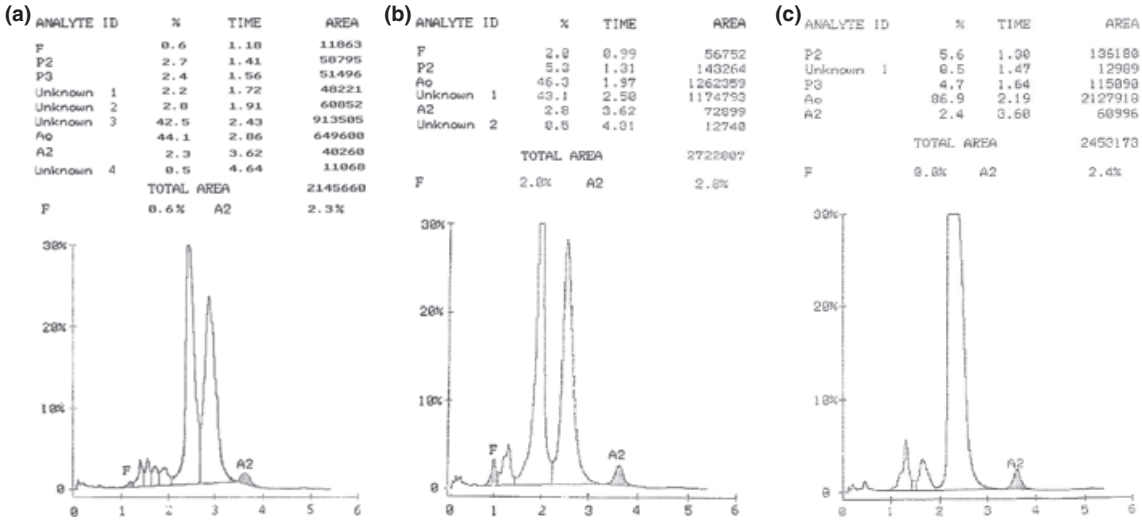

Figure 2. Incorrect assessment of Hb A retention time. (a) Hb P-Galveston trait, which elutes after Hb A. The instrument has labeled the true Hb A peak as 'unknown 3' (2.43 min) and labeled Hb P-Galveston as 'Ao' (2.86 min). (b) Hb Old Dominion trait, which elutes before the Hb A peak. The instrument has labeled the Hb Old Dominion peak (1.97 min) as Ao and mislabeled Hb A as 'unknown 1' (2.5 min). (c) Hb San Diego elutes only slightly faster than Hb A but the instrument does not separate the peaks. Instead, the single Hb A peak has a retention time of 2.19 min, which is highly unusual.

variants with increased O_2 affinity, 13 had clinical manifestations of erythrocytosis. Of the 19 Hb variants which were unstable, 11 had clinical manifestations of hemolytic anemia.

A_2Window

There were 35 Hb variants identified, which eluted in the A_2 window (7 α-, 24 β-, 3 δ/β-fusion, 1 γ/β fusion- variants), with Hb E accounting for 85% of all Hb variant occurrences. All Hb variants in this window will be labeled and shaded as Hb A_2 by the instrument and will interfere with Hb A_2 measurement (Table 2). This misidentification as Hb A_2 will cause confusion only with those Hb variants that are present in smaller percentages, such as Hb Lepore (Figure 1c).

D window

There were 43 Hb variants seen in the D window (20 α- and 23 β- variants), with two Hb variants, Hb D-Punjab (1240 occurrences) and Hb G-Philadelphia (729 occurrences) accounting for 80% of all occurrences. Notably, 24 of the 49 Hb variants that interfere with Hb A_2 measurement (Table 2) elute in the D window.

S window

There were 33 Hb variants seen in the S window (19 α-, 13 β-, and 1 δ- variant). Hb S is overwhelmingly the most common Hb variant in this window and the most common Hb variant overall, accounting for almost 95% of all S window occurrences and 61% of all total Hb variant occurrences. The next most common Hb variant was Hb A_2', which accounted for approximately 5% of all S window occurrences. Hb A_2' has a midpoint retention time very close to Hb S; thus, this Hb variant will not be seen if Hb S is present. This combination (Hb S/Hb A_2') is not a rare occurrence, as both of these Hb variants are most commonly seen in those of African descent. The percentage of Hb A_2 can give a clue to the presence of Hb A_2'. In cases of Hb S trait alone, the Hb A_2 level is typically mildly elevated on HPLC, because of the presence of Hb S adducts [16, 17]. However, when Hb A_2' is present with Hb S, Hb A_2 is in the range of 2.0-2.5%.

Table 2. Hb variants that co-elute with Hb A_2 or interfere with Hb A_2 measurement

Interferes with (increases or decreases) A_2 measurement (# cases in seq. examples)	Co-elutes with Hb A_2 (# of cases in seq. examples)	Sometimes interferes and sometimes co-elutes (# interfering and # co-eluting)	Sometimes interferes and sometimes does not interfere (# interfering and # not interfering)
Abington (1)	Abruzzo (5)	Bethesda (2,4)	Coimbra (3,2)
Agenogi (4)	Akron (2)	D-Iran (1,23)	Creteil (1,1)
Alabama (2)	Boras (1)	Denver (5,1)	Hekinan (7,5)
Alberta (1)	Chandigarh (1)	Ethiopia (1,3)	Louisville (3,4)
Atlanta (1)	Deer Lodge (4)	Jeddah (1,5)	M-Boston (1,2)
Bellevue (1)	E (62)	Korle-Bu (24,5)	M-Iwate (7,5)
Beth Israel (2)	Fort Worth (4)	Loves Park (4,3 (2 cases no interference))	P-Galveston (4,2)
Bunbury (1)	G-Copenhagen (10)	M-Saskatoon (3,10)	Port Phillip (2,1)
Caen (1)	G-Coushatta (22)	Osu-Christiansborg (1,29)	Sogn (1,2)
Caribbean (2)	G-Ferrara (20)	San Bruno (3,2)	Sunshine Seth (5,2)
Chelmsford (1)	G-Galveston (7)	Santa Juana (1,2 (2 cases no interference))	Twin Peaks (24,6)
Chiapas (4)	G-Honolulu (4)	Willamette (2,2)	
Connecticut (2)	G-Taipei (5)		
D-Ibadan (9)	Hamadan (4)		
D-Punjab (85)	Hoshida (1)		
Dhofar (7)	Kenya (1)		
Fontainebleau (22)	Lake Tapawingo (1)		
G-Philadelphia (49)	Lepore (10)		
G-San Jose (21)	Muravera (1)		
G-Siriraj (3)	Ocho Rios (2)		
G-Szuhu (1)	Rocky Mountain (2)		
Gerland (1)	Sheffield (1)		
Hammersmith (1)	Spanish Town (1)		
Inkster (15)	Toulon (3)		
Karlskoga (1)	Tubingen (1)		
Kenitra (1)	Zurich (4)		
Khartoum (3)			
M-Milwaukee I (1)			
Matsue-Oki (5)			
Miyagi (1)			
Mobile (3)			
O-Padova (2)			
Oleander (1)			
Park Ridge (5)			
Presbyterian (3)			
Q-India (11)			
Ravenscourt Park (1)			
Rockaway (1)			
Roseau-Pointe-a-Pitre (1)			
Roubaix (1)			
S			
San Antonio (5)			
Shelby (7)			
Swan River (3)			
Tak (2)			
Tamano (1)			
Taradale (2)			
Ube-4 (2)			
Vaasa (1)			

C window

Only 11 different Hb variants were observed in the C window (2 α- and 9 β- variants). Most prominent was Hb C (9062 occurrences) which accounted for 92% of all cases in the C window. Although the second most common variant in this window, Hb Constant Spring, has a very similar retention time to Hb C, these variants are very rarely confused because of the very low percentage of Hb Constant Spring. A notable rare Hb variant is Hb M–Saskatoon, which results in a peak in the Hb C window but also usually a peak in the A_2 window as well. This is a Hb variant which has a very characteristic chromatogram and is easily recognizable (Figure 1d.).

As mentioned previously, the retention time ranges for the different windows provided by the instrument have changed slightly over time. More than seven different versions of ROM cards were used over the course of this study and slight shifts in analyte windows were noted. For example, early in the study, the retention time range of the S window was 4.33–4.65 min but has shifted to 4.15–4.47 min in more recent versions. For the D window, the retention time range has changed from 3.91 to 4.33 min in earlier versions to 3.82–4.14 min in later ROM cards. Similar, but smaller shifts have occurred in all other windows with the exception of the F window. Because of this change, Hb variants which have retention time ranges which lie on the border of two windows may elute in different windows over time

Thus, the window in which a Hb variant elutes is a helpful feature, but is not precise enough to aid in the distinction between two Hb variants. The midpoint or mean retention time of a Hb variant is more helpful; however, the retention time is not absolute because there is typically a range of the retention times seen with each Hb variant or with different reagent lots. The retention time for a Hb variant with the same reagent lot varies very little, but there may be greater variation over an extended period. This variation is not a problem for the commonly seen Hb variants, but may be a concern for Hb variants that are rare.

We examined the five most common alpha or beta chain variants (Hb A_2′ was excluded as it is a delta chain variant) and used the data only from sequenced examples of these Hb variants to determine the mean retention time and standard deviation of the mean retention time for each Hb variant. This demonstrated that the mean retention time and the midpoint retention time as listed in the online supplement were virtually identical for these 5 Hb variants. We then looked at the number of Hb variants whose midpoint retention time would fall within a range of 0.05, 0.10, and 0.15 min on either side of the mean retention time of the five most common Hb variants. This showed than for these 5 Hb variants, 0.10 min is close to the retention time range. Table 3 is a summary of this data.

Table 3 illustrates that retention times are not unique to Hb variants and cannot be used to identify Hb variants by this single method. As an example, Hb E (Figure 3a.) cannot easily be distinguished from Hb Osu-Christiansborg (Figure 3b.), because their retention times are within 0.09 min. Furthermore, it is important to note that if two Hb variants with similar retention times are present together, it may not be apparent by HPLC alone that there are two Hb variants. Figure 3c shows an example of Hb S with Hb

Table 3. Five most common alpha or beta Hb variants and the number of Hb variants with similar midpoint retention times

Most Common Hb Variants	Mean RT (min)	RT Range (min)	SD (min)	# of Hb variants within 0.05min of mean*	# of Hb variants within 0.10 min of mean*	# of Hb variants within 0.15 min of mean*	# of Hb variants within RT Range
S	4.42	4.31–4.52	0.04	9	18	26	18
C	5.14	5.10–5.17	0.02	0	1	1	0
E	3.67	3.59–3.74	0.04	7	13	20	10
D-Punjab	4.07	3.98–4.18	0.04	18	27	33	26
G-Phil	4.09	4.02–4.24	0.05	17	26	32	27

*On either side of the mean retention time.

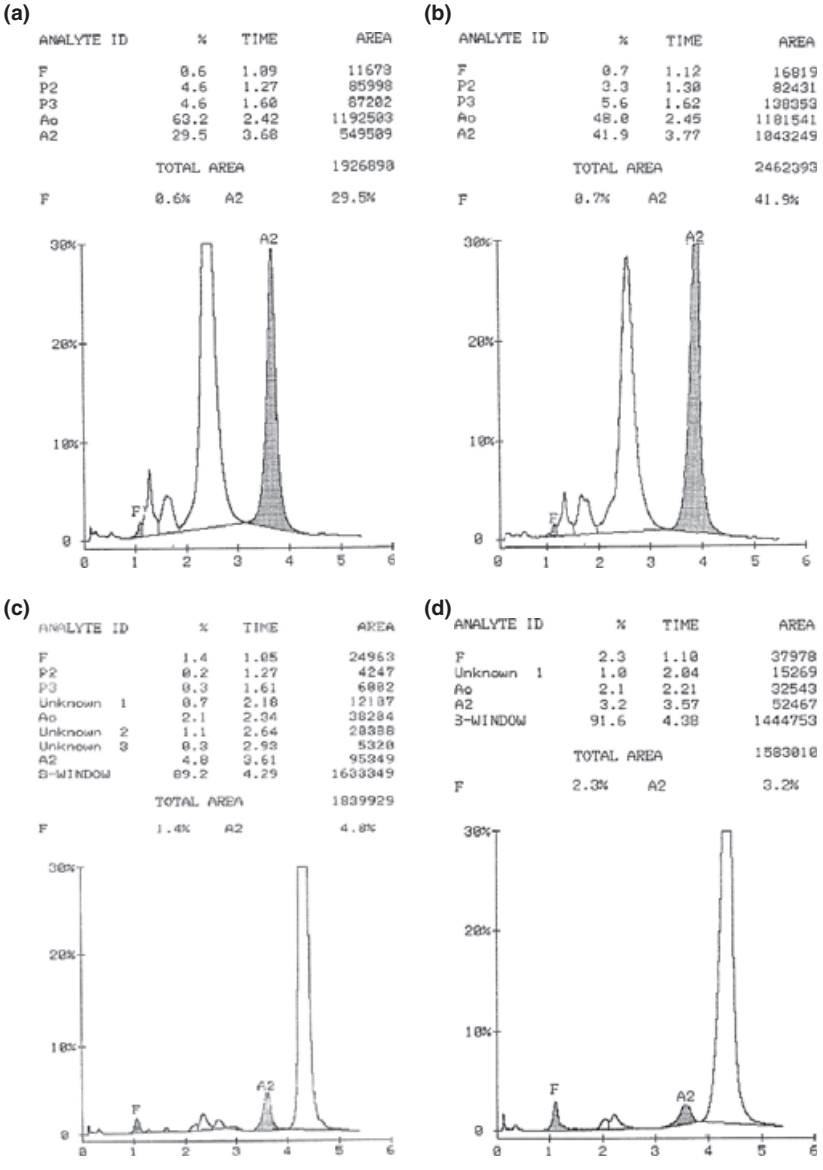

Figure 3. -Examples of Hb variants with virtually identical retention times and chromatograms (a) Hb E which has a similar retention time as (b) Hb Osu-Christiansborg. The 2 Hb variants cannot be distinguished on this method. (c) An example of Hb S in combination with Hb Richmond. The 2 Hb variants have such similar retention times that they elute as a single peak. The chromatogram looks very similar to (d) homozygous Hb S.

Richmond. Because the mean retention times of Hb S and Hb Richmond differ by only 0.03 min, they appear as a single peak, thus leading to a possible erroneous diagnosis of homozygous Hb S (Figure 3d.) if HPLC is used as a sole method. These two Hb variants, however, do clearly separate on other methods such as alkaline electrophoresis, acid electrophoresis, capillary electrophoresis, IEF, or mass spectrometry.

DISCUSSION

This study represents the largest data set for the retention times of Hb variants using the HPLC method. Although this study was conducted on the Bio-Rad Variant ™ Classic, the results should be similar on other HPLC platforms as well [7, 12]. All HPLC instruments utilize a weak cation exchange column and differ only in the overall run lengths and graphic display of data. Thus, although the exact retention times may be different on different instruments, the relative positions of Hb variants, such as those which migrate very close to each other, should be analogous regardless of the system. For example, these data should be very similar on the Bio-Rad Variant II ™ system, which uses identical chemistry.

Differences in HPLC retention time can be used to differentiate Hb variants that may co-migrate or co-elute on other methods. A very important example of this is the distinction between Hb D-Punjab and Hb Korle-Bu. These two Hb variants have almost identical mobilities on alkaline and acid Hb electrophoresis and IEF but have significantly different HPLC retention times. This distinction is important when Hb S is present, as HB S/Hb D-Punjab is a sickling syndrome, whereas Hb S/Hb Korle-Bu is not. A second example is the distinction of Hb S/C from Hb S/O-Arab. Both have identical patterns on alkaline electrophoresis; however, there is distinct separation on HPLC [18], with a midpoint retention time difference of 0.26 min. Although both disorders are sickling disorders, Hb S/O-Arab is a clinically more severe disorder [19]. Finally, both homozygous Hb C and Hb C/C-Harlem show identical patterns on capillary electrophoresis, as both Hb C and Hb C-Harlem have identical migration patterns. However, these two Hb variants separate distinctly on HPLC. Hb C-Harlem is a rare doubly substituted Hb variant in which one of the mutations is the Hb S mutation. Thus, the combination of Hb C with C-Harlem is equivalent to Hb S/C disease and not homozygous C.

This study shows that HPLC is an excellent tool to aid in the identification of Hb variants. Many Hb variants have a very characteristic elution peak, which can be a helpful indicator when differentiating Hb variants. Furthermore, the retention time of a Hb variant is an even more influential piece of data to use when trying to classify a Hb variant. However, because of the short run length of the HPLC program (approximately 6 min), it is intuitive that many Hb variants must have a similar retention time. Also, the retention time of a particular Hb variant will have some variability over time, such as with differing reagent lots. Thus, HPLC cannot be used as a single, stand-alone method for the identification of Hb variants, particularly rare Hb variants, as has been advocated previously [8, 18, 20]. Although the combination of HPLC with other hemoglobin analysis methods, such as IEF or CE, can add to the putative identification of common and less common variants [6, 9, 21, 22], the ultimate rapid identification method, essential in risk assessment and prognosis, remains DNA analysis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Haemoglobin Variants.