

HPLC determination of hemoglobins to establish reference values with the aid of statistics and informatics

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ABSTRACT. The purpose of the present study was to establish reference values for hemoglobins (Hb) using HPLC, in samples containing normal Hb (AA), sickle cell trait without alpha-thalassemia (AS), sickle cell trait with alpha-thalassemia (ASH), sickle cell anemia (SS), and Hb SC disease (SC). The blood samples were analyzed by electrophoresis, HPLC and molecular procedures. The Hb A2 mean was $4.30 \pm 0.44\%$ in AS, $4.18 \pm 0.42\%$ in ASH, $3.90 \pm 1.14\%$ in SS, and $4.39 \pm 0.35\%$ in SC. They were similar, but above the normal range. Between the AS and ASH groups, only the amount of Hb S was higher in the AS group. The Hb S mean in the AS group was $38.54 \pm 3.01\%$ and in the ASH it was $36.54 \pm 3.76\%$. In the qualitative analysis, using FastMap, distinct groups were seen: AA and SS located at opposite extremes, AS and ASH with overlapping values and in-

intermediate distribution, SC between heterozygotes and the SS group. Hb S was confirmed by allele-specific polymerase chain reaction. The Hb values established will be available for use as a reference for the Brazilian population, drawing attention to the increased levels of Hb A₂, which should be considered with caution to prevent incorrect diagnoses.

Key words: Reference values, High-performance liquid chromatography, Hemoglobins

INTRODUCTION

Electrophoresis on cellulose acetate at alkaline pH is a widely used method in the diagnosis of hemoglobinopathies, since analyses can be done quickly and at a low cost. It should, however, be used only as an initial screening test for the detection of hemoglobin (Hb) variants. Electrophoresis at acidic pH can help confirm the presence of some hemoglobins such as Hb A, Hb F, Hb S, and Hb C, but it does not allow the distinction between Hb D and Hb G and Hb O and Hb E. Even though these methods are easily reproducible, they do not provide a precise identification of the variant, due to the co-migration of resembling forms. Therefore, complementary methods have to be used to characterize these hemoglobins (Clarke and Higgins, 2000; Zamaro et al., 2002).

High-performance liquid chromatography (HPLC), established by the automated system VARIANT (Bio-Rad), is a method that allows the detection of abnormal Hb quickly and precisely, using a small sample amount (Ou and Rognerud, 2001; Old, 2003). Ion exchange HPLC allows the quantification of Hb A₂, Hb F, Hb A, Hb S, and Hb C and screening for Hb variants, thereby being an important method for the investigation of hemoglobinopathies in routine laboratories (Fisher et al., 1997; Clarke and Higgins, 2000; Joutovsky et al., 2004). The determination of the percentage of an Hb variant and its retention time often allow a more precise identification of the mutant than do electrophoresis methods (Joutovsky et al., 2004). Thus, information about the reference values for the different Hb fractions obtained by HPLC is necessary for the correct identification of these Hb variants (Roa et al., 1995).

Based on this information and in view of the phenotypic diversity of the Brazilian population, we aimed to establish reference values for hemoglobins in normal adults and in adults carrying Hb S in Brazil, comparing the different phenotypes found with the aid of statistics and informatics.

MATERIAL AND METHODS

After informed consent, 751 peripheral blood samples were analyzed, which had been collected with EDTA as anticoagulant from subjects older than one year, regardless of gender and ethnicity, coming from different states of Brazil.

The samples were analyzed by hemoglobin electrophoresis on cellulose acetate at pH 8.6 (Marengo-Rowe, 1965), by agar gel electrophoresis at pH 6.2 (Vella, 1968), and by HPLC in an automated VARIANT system (Bio-Rad) using the "Beta Thalassemia Short Program" for the detection of hemoglobin variants and the quantification of Hb A₂ and Hb F.

With HPLC, the Hb fractions are separated based on their ionic interactions with the cationic column under high pressure and by elution with two phosphate buffers differing in pH and ionic strength. The result is a chromatogram with the percentage and retention time of each Hb fraction (Instruction Manual of BIO-RAD, 2003).

For the values of Hb A0 obtained by HPLC, the glycosylated Hb A and acetylated Hb A subfractions were included, designated P2 and P3, respectively, using the software of the equipment. For the control group, the Hb A2 values considered normal ranged from 2.0 to 3.5%, and the Hb F values up to 1.3%, which were provided by the manufacturer and previously compared with other analytical systems for the laboratory conditions. Using the limits established by Shokrani et al. (2000), the Hb A2 values considered for the definition of the Hb S sample group were up to 5.2% for the heterozygous groups and up to 5.9% for the groups with Hb SS and Hb SC.

The Hb S mutation was confirmed by allele-specific polymerase chain reaction. The leftward primer pair consisted of: upstream primer B5a (5'-GGC TGT CAT CAC TTA GAC CTC-3') and downstream primer B5b (5'-AGA AGG GGA AAG AAA ACA TCA-3') as internal control of the reaction, which produce a 660-bp fragment. The downstream primer BA (5'-CAG TAA CGG CAG ACT TCT CCT C-3') was used for amplification of allele normal beta and primer BS (5'-CAG TAA CGG CAG ACT TCT CCA-3') for amplification of allele beta mutant, which produce a 210-bp fragment. Two test tubes were used containing the primer for normal sequence and the primer for mutant sequence (Fischel-Ghodsian et al., 1990).

The diagnosis of alpha-thalassemia was determined by electrophoretic analysis at pH 7.0 (Dacie and Lewis, 1985) and microscopic visualization of Hb H inclusions in unfixed cells with Brilliant cresyl blue which is an oxidative dye (Papayannopoulou and Stamatoyannopoulos, 1974).

After the analyses, the subjects were separated into five groups, according to their phenotype: with normal Hb (AA), with sickle cell trait without alpha-thalassemia (AS), with sickle cell trait and alpha-thalassemia (ASH), with sickle cell anemia (SS), and with Hb SC disease (SC).

Descriptive statistics of the groups was performed using a 3-dimensional (3-D) visualization data mining tool (FastMap) to detect the behavior patterns of the different phenotypes. FastMap is a tool that maps conventional, multidimensional databases, with attribute domains of the numerical, temporal and textual type. Two-dimensional or 3-D images are generated, attributing dots of different shapes and colors to the stored data. These dots are grouped or distant from each other, identifying the behavior of the base. FastMap allows the reduction of dimensionalities and performs quick data mapping in spaces of large dimensions to spaces of smaller dimensions, preserving as much as possible the distances between the objects (Keim, 2001).

To compare the Hb values among the groups, the Kruskal-Wallis non-parametric test was used, while the Mann-Whitney non-parametric test was used to compare the ratio between Hb A and Hb S. The criterion for significance was $P < 0.05$ (Zar, 1999).

RESULTS

Of all the samples, 482 were found with phenotype AA, 136 AS, 106 ASH, 18 SS, and 9 SC, and the group of normal individuals was used as control for the establishment of normal values. All samples with Hb S showed elution of the Hb variant within the Hb S window, established for the instrument, with a mean retention time of 4.57 ± 0.028 min, varying from 4.51

to 4.63. The mean values of the Hb fractions for the different phenotypes, including minimums and maximums, are presented in Table 1.

Table 1. Percentages of Hb fractions obtained by HPLC in the control group and in the groups with Hb S.

Phenotypes	AA (N = 482)	AS (N = 136)	ASH (N = 106)	SS (N = 18)	SC (N = 9)
Hb A	96.47 ± 0.77 (93.8-98.2)	56.57 ± 2.73 (51.7-67.7)	58.81 ± 3.75 (52.1-68.0)	– –	– –
Hb A2	2.81 ± 0.31 (2.0-3.5)	4.30 ± 0.44 (2.9-5.2)	4.18 ± 0.42 (2.8-5.2)	3.90 ± 1.14 (0.8-5.6)	4.39 ± 0.35 (3.8-5.7)
Hb F	0.37 ± 0.35 (0.0-1.3)	0.50 ± 0.34 (0.0-1.3)	0.53 ± 0.36 (0.0-1.3)	15.75 ± 5.27 (7.2-23.1)	1.70 ± 1.72 (0.4-5.5)
Hb S	– –	38.54 ± 3.01 (27.2-43.4)	36.54 ± 3.76 (26.6-43.0)	75.13 ± 4.73 (69.3-84.8)	45.65 ± 1.05 (43.8-47.1)
Hb C	– –	– –	– –	– –	46.71 ± 1.35 (44.01-49.41)

Data are reported as mean ± SD with minimum-maximum values in parentheses.

The exploratory evaluation of the reference values using FastMap showed four distinct groups: with Hb AA, with Hb S in heterozygosis (AS and ASH), with Hb S in homozygosis, and with Hb S associated with Hb C, being diametrically opposed on a 2-D plane. In Figure 1, these groups can be observed by their representation in different colors, according to each phenotype. The Hb values, which were the attributes used in the analysis, were previously stored in a database.



Figure 1. Image obtained by FastMap for the AA (red), AS (blue), ASH (green), SS (purple), and SC (black) groups, including all percentages of Hb.

Homozygotes AA and SS were located at the opposite extremes, while the heterozygotes, AS and ASH, showed an intermediate distribution, between the two homozygote positions and with overlapping values, demonstrating a similarity between them. The SC group showed an intermediate disposition between heterozygotes AS and ASH and the SS group. The analysis parameters for this procedure included fractions of Hb A, Hb A₂, Hb F, and Hb S.

Statistical analyses demonstrated that all groups with Hb S present showed a significant difference in the mean values of Hb A, Hb A₂ and Hb F ($P < 0.05$) as compared to the AA group, besides the fraction corresponding to Hb S, the phenotype-determining variant.

Between the AS and ASH groups, no statistically significant difference was found in the comparison of the amounts of Hb A, Hb A₂ and Hb F, thus justifying the overlap of the groups in the exploratory analysis. In this comparison, only the amount of Hb S showed a statistically significant difference, as it was higher in the AS group.

The ratios between Hb A and Hb S were also calculated, in order to draw the profile of the means of those Hb. In the AS group, the mean ratio found was 1.48 ± 0.20 , varying from 1.2 to 2.4, which is similar to the ratio 1.4 ± 0.2 found by Roa et al. (1995) in an adult African-American population with Hb S. In the ASH group, the ratio of 1.64 ± 0.30 , varying from 1.2 to 2.6, was statistically different ($P < 0.01$) from that of the AS group.

Analysis by FastMap using only the Hb A values showed three linearly arranged groups, with AA at one extremity, with values which are statistically different from the others, an intermediate group with AS and ASH arranged in parallel, and a group with SS and SC at the opposite extremity of the AA group. The values of Hb A in the AS and ASH groups did not show statistically significant differences.

The analysis of the Hb S values showed an arrangement that resembles that of the Hb A values, but the SC group was arranged in parallel to the AS and ASH groups. In the AS, ASH and SC groups, the Hb S values were statistically different, as it was higher in the SC group, even though they occupied the same position in space. It was, therefore, not possible to precisely infer the similarities among the groups by the behavior pattern of the data generated by FastMap alone.

The analysis of the Hb A₂ values using FastMap showed three groups, of which AA was the most externally located, AS and ASH had a close and intermediate distribution, and the group with SS and SC had values which were distant from the others. Nevertheless, by rotating the image, the groups with Hb S appeared in a parallel arrangement, and AA continued as a group located externally to the central plane. It was found that in the groups with Hb S, there was no statistically significant difference for the Hb A₂ values, which were higher than expected for the normal range and different from the group used as control, explaining the spatial arrangement of the groups.

Regarding the Hb F values, the AA, AS, ASH, and SC groups were found to be in a parallel arrangement, with SS located more externally on the plane, due mainly to the higher values of Hb F. The Hb F values of all groups with Hb S showed statistically significant differences when compared to those of group AA, and mean values higher than the normal mean. The values of the SS group were higher than those of all other groups, and those of the AS, ASH and SC groups showed no differences between each other.

DISCUSSION

Routine electrophoresis methods were used to screen normal and variant Hb, and HPLC allowed the verification of the Hb observed with electrophoresis and precise quantification of their proportion. As observed by Fisher and co-workers (1997), in this study there was concordance between the electrophoretic and chromatographic findings for the samples examined.

In 2004, Joutovsky et al. demonstrated that HPLC is an important analytical tool for the identification of Hb variants, mainly if the information regarding their retention time is used. In this study, the mean percentages of Hb obtained by HPLC were shown to be also very useful, especially for the identification of Hb variants and demonstrating associations between different hemoglobinopathies, by comparing them with normal values, thus permitting the determination of phenotypes.

The exploratory analyses carried out with FastMap using all Hb values showed spatial overlap of the AS and ASH groups, for they only differed in the amount of Hb S. It was also found that the groups considered heterozygous, AS and ASH, showed an intermediate distribution between the AA and SS groups, since they have both normal and variant Hb. The double heterozygote also showed an intermediate distribution, but between the AS/ASH groups and the SS group, since it had Hb S in heterozygosis, associated with Hb C, enhancing the interfaces of inheritance between genotypes and phenotypic expression.

By analyzing the Hb values separately, it was possible to obtain a 3-D spatial view of them, corroborating the findings for normal and abnormal homozygotes, heterozygotes and double heterozygotes. However, the statistical analyses were fundamental for testing those differences, showing that exploratory analysis alone did not allow inferring precisely the differences which exist among the groups studied.

The reference values for Hb A, Hb A2 and Hb F observed in the AA group can be used as parameters when the presence of an abnormal Hb is suspected and when the percentages obtained are not compatible, corroborating the findings of Roa et al. (1995) in an African-American population.

In the ASH group, the amount of Hb S was smaller than in the AS group, and the difference was statistically significant. This decrease is due to the association with alpha-thalassemia that results from a deficiency in the synthesis of the alpha chain, which in turn causes a decrease in the formation of Hb S (Serjeant, 1997; Shokrani et al., 2000). This difference was confirmed in the evaluated group by calculating the ratios between Hb A and Hb S, which showed a higher ratio in the ASH group than in the AS group. Similar values were observed by Roa et al. (1995) in an adult African-American population with Hb S. The alteration of these ratios may be indicative of an association with another hemoglobinopathy (Roa et al., 1995). It was thus possible to differentiate the AS and ASH groups by comparing the mean percentages of Hb S and the ratios between Hb A and Hb S. The higher values for Hb S in the SC group are due to the similar affinity of beta S and beta C for alpha-globin chain with a ratio of approximately 50:50.

The groups with Hb S were also found to have Hb A2 values higher than normal, a finding that should be considered with caution. Shokrani et al. (2000) proposed that in such situations, the association between the alpha-globin chain and the variant beta-globin chain is compromised,

which promotes the association of the alpha-globin chain with the delta-globin chain, increasing the Hb A2 levels, in addition to the co-elution of glycosylated Hb S and other subfractions in the Hb A2 window. More recent studies using mass spectrometry techniques have proposed that the increase in the Hb A2 values of subjects with Hb S may be due to the co-elution of a minor component of Hb formed by an alpha-globin chain that was post-translationally modified by carbamylation associated with the beta S chain (Zurbriggen et al., 2005).

Shokrani et al. (2000) determined the Hb A2 values in samples containing Hb S, and proposed that values up to 5.2% in heterozygotes and up to 5.9% in individuals with Hb SS and Hb SC should be considered normal, provided there is no other sign of beta-thalassemia. These values were used here to define the cutoff values of the sample groups. In the Brazilian population, the following values can be considered normal: Hb A2 from 2.9 to 5.2% for Hb AS; 2.8 to 5.2% for Hb ASH; 0.8 to 5.6% for Hb SS, and 3.8 to 5.7% for Hb SC. This was the diagnostic reference by HPLC in the VARIANT system.

The Hb F values found in the subjects with Hb S in heterozygosis, although within the range of normality, were statistically higher than those of the control group, due to the presence of the Hb variant and of its possible haplotypes. In the SS group, all samples showed values higher than normal, which may have resulted from the use of medications, hereditary persistence of Hb F or a characteristic of the haplotype. In the SC group, the Hb F values were not considered in the global evaluation in view of the small number of samples, which could harm this analysis.

CONCLUSION

In the present study, it was possible to establish Hb values that will be available for use as a reference in population studies by HPLC, and to identify the differences in Hb values between carriers of Hb S, for the Brazilian population. Hb A values lower than those of the normal group, increased Hb A2 and Hb F, and the presence of a variant fraction eluted in the Hb S window are phenotypic characteristics suggestive of Hb S. These variations in Hb values should be examined separately, to determine possible associations with other hemoglobinopathies. The increased Hb A2 levels should also be regarded with caution, to prevent incorrect diagnoses and to confirm the suspicions of Hb S, considering the absence of these adducts in other hemoglobinopathies.

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