International Journal of Innovative Research and Advanced Studies (IJIRAS)
Volume 1 Issue1, December 2014

High Performance Liquid Chromatography (HPLC) In Diagnosis of Sickle Cell Disorders

Sonam M. Dixit
Student, M.E, Dept. of CSE,
SMIT, India

Abstract: ***More than 900 structural changes in the α,β,γ,δ-globin chains have been reported to cause abnormal hemoglobins. Four abnormal hemoglobins (HbS, HbC, HbE, HbD Punjab) are relatively frequent recessive traits. Carriers of the common variants are healthy individuals. But their children may be born with severe sickle cell disease (SCD) when both the parents are carriers. The laboratory diagnosis of hemoglobinopathies and thalassemias, both of which are common may be required (a) to confirm a provisional diagnosis, (b) to explain a hematologic abnormality, (c) to identify an abnormality in the presymptomatic phase, as in neonatal screening, (d) to predict serious disorders of globin chain synthesis in the fetus and offer the option of termination of pregnancy, (e) to permit genetic counselling of prospective parents, and (f) to allow preoperative screening.***

Keywords: Hemoglobins (Hbs), SCD, HPLC, electrophoresis, AD test, Retention time, Hb S carrier, Hb S Homozygous

I. OBJECTIVE

Alkaline electrophoresis is capable of separating Hemoglobin A (HbA), HbF, HbS and HbC but HbS, HbD, HbG, Hb Lepore are unresolved, as are HbC, HbA_2, HbO-Arab and HbE. In addition, there are other variants with electrophoretic mobilities identical or similar to those of HbS and HbC and acid electrophoresis is needed for identification. Hemoglobin fraction analysis by cation exchange HPLC has the advantage of quantifying HbF and HbA_2 along with hemoglobin variant screening making it an excellent technology to screen for hemoglobin variant and hemoglobinopathies along with thalassemias. The simplicity of the automated system with internal sample preparation, superior resolution, rapid assay time, and accurate quantification of hemoglobin fraction makes this an ideal methodology for the routine clinical laboratory.

II. MATERIAL AND METHODS

NORMAL AND ABNORMAL HEMOGLOBINS

Hemoglobins (Hbs) are polypeptide tetramers consisting of two pairs of unlike globin chains (α,β,δ,γ), to each of which is bound a heme group. Hb in normal adult human blood is >96% Hb A ($\alpha_2\beta_2$), ~2-3% Hb A2 ($\alpha_2\delta_2$), and < 1% Hb F ($\alpha_2\gamma_2$). Normal newborn blood contain Hb F as the major constituent (60-80%) and the rest is Hb A[1].

More than 900 structural changes in the α,β,γ,δ-globin chains have been reported to cause abnormal hemoglobins.(http://globin.cse.psu.edu/globin/hbvar). Most of these abnormal hemoglobins are rare and either recessive or associated with clinical conditions such as hemolysis (unstable hemoglobins), thalassemia minor phenotype (unstable or low expression mutants), erythrocytosis (high O_2 affinity mutants), or cyanosis (low O_2 affinity mutants).

Some are semi dominant and result in intermediate to severe pathological phenotypes in the carriers that generally, due to the presence of cellular inclusions, are classified as Heinz body hemolytic anemia. Only a few abnormal hemoglobins are very common and population specific.

Four abnormal hemoglobins (HbS, HbC, HbE, HbD Punjab) are relatively frequent recessive traits. The first three have been strongly selected by malaria and are very common in specific populations. Carriers of the common variants are healthy individuals. But their children may be born with severe sickle cell disease(SCD)or β-thalassemia major when both the parents are carriers.

Six mutation events, which occurred on a preexisting HbS allele, have been described. These are HbS –Antilles, HbC-Ziquinchor, HbC-Harlem, HbS-Providence, HbS-Oman, and HbS-Travis. Although rare, these mutations should be kept in mind as they may cause SCD in combination with HbS, β-

thalassemia, HbC, HbE, HbD Punjab, HbO-Arab and Hb-Lepore.

Due to population specificity, SCD is likely to be caused by different genotypes combinations depending upon the geographical area. Also in immigration countries, the genotype depends upon the ethnic background of the immigrant populations and upon the degree of intermixture among them[2].

THE LABORATORY DIAGNOSIS OF HEMOGLOBINOPATHIES AND THALASSEMIAS

The laboratory diagnosis of hemoglobinopathies and thalassemias, both of which are common may be required (a) to confirm a provisional diagnosis, such as significant sickling disorders or β-Thalassemia major , (b) to explain a hematologic abnormality such as anemia, microcytosis, or polycythemia, (c) to identify an abnormality in the presymptomatic phase, as in neonatal screening, (d) to predict serious disorders of globin chain synthesis in the fetus and offer the option of termination of pregnancy, (e) to permit genetic counselling of prospective parents, and (f) to allow preoperative screening for the presence of sickle cell hemoglobin[3].

The identity of hemoglobin variants is generally inferred from electrophoretic mobility, its quantity, and the patient's ethnic background. Family studies can be of considerable importance in elucidating the nature of disorders of hemoglobin synthesis, but the definite identification can be achieved only by DNA analysis or amino acid sequencing [3-5].

Alkaline and acid hemoglobin electrophoresis are the most widely used methods for investigating hemoglobin variants and hemoglobinopathy. Alkaline electrophoresis is rapid, reproducible, and capable of separating common hemoglobin variants such as hemoglobin A (HbA), HbF, HbS and HbC but HbS, HbD, HbG, Hb Lepore are unresolved from each other, as are HbC, HbA_2, HbO-Arab and HbE. In addition, there are other variants with electrophoretic mobilities identical or similar to those of HbS and HbC. Consequently acid electrophoresis is needed for identification for the aforementioned variants. Nevertheless electrophoretic methods are still not able, in most cases, to separate HbD from HbG and Hb Lepore and in some cases, HbE from HbO-Arab[4].

Hemoglobin fraction analysis by cation exchange HPLC has the advantage of quantifying HbF and HbA_2 along with hemoglobin variant screening in a single, highly reproducible system, making it an excellent technology to screen for hemoglobin variant and hemoglobinopathies along with thalassemias[3,6-9]. The simplicity of the automated system with internal sample preparation, superior resolution, rapid assay time, and accurate quantification of hemoglobin fraction makes this an ideal methodology for the routine clinical laboratory[7,8].

III. HPLC IN DIAGNOSIS OF SICKLE CELL DISORDERS

We discuss HPLC method for diagnosis of Sickle Cell Disorders using Bio-Rad Variant Hemoglobin Testing System employing β-Thalassemia Short Program.

PRINCIPLES OF THE OPERATION

The VARIANT provides an integrated method for the separation and determination of the relative percent of specific hemoglobins in whole blood samples. It uses the principle of high performance liquid chromatography.

Two dual-piston pumps deliver a consistent flow of buffer to the analytical cartridge and the detector. Whole blood samples drawn in tubes containing Dipotassium EDTA are mixed and diluted with specific hemolysing/wash buffer reagent and are introduced to the analytical flow path using automatic injection. Between sample injections, the automated sampler is rinsed with wash solution, in order to minimize the possibility of sample carry-over.

The sample is carried by the buffer through the analytical cartridge, where the sample is separated into its individual components. The separated components then pass through the dual-wavelength detector, where absorbance of the sample components is measured at 415 nm; background noise is reduced with the use of a secondary wavelength at 690 nm. The absorbance data is transmitted from the central processing unit and displayed as a real-time chromatogram (graph of time vs. absorbance) within the right panel of the liquid crystal display [10]. The integrated peaks are assigned to manufacturer-defined windows derived from retention time, i.e., the time in minutes from sample injection to the maximum point of the elution peak, of normal hemoglobin fractions and common variants (Table 1). If a peak elutes at a retention time not predefined, it is labelled as an unknown[11].

The processed data is incorporated into a printed report, which contains (1) a complete summary of the sample's detected components (i.e., peak identification, retention time, relative percent, area); (2) the sample's chromatogram; (3) date and time of analysis; (4) vial and sample number[10].

There are several programs available for this system. One program is the Sickle Cell Short Program, which is a rapid 3-min assay and uses either filter paper blood spots or whole blood samples. This program is specifically designed to provide a qualitative result for hemoglobins (Hbs) A, F, S, C, D and E in the **neonate**.

Another program is the β Thalassemia Short Program, which is a 6.5-min assay designed to quantify Hbs A_2 and F. The performance of this program for the quantification of these Hbs and the identification of other Hbs, including A, S, C, D and E in **adults**[12].

HPLC analysis should be undertaken after evaluation of detail clinical history with emphasis on previous blood transfusion, family history, ethnic background, complete blood count with peripheral smear examination.

HbS confirmation to be done by use of the sodium metabisulfite reduction test[13]. All non-A non-S variants to be confirmed by alkaline and acid electrophoresis. The presence of HbH to be confirmed by use of the brilliant cresyl blue test

for inclusion bodies[14]. The presence of Hb F to be confirmed by use of AD test and Kleihauer test to see the distribution of Hb F within the cell. And any other complimentary test to establish specific diagnosis of hemolytic anaemia.

Those haemoglobins commonly seen in India are discussed.

Evaluation of the Retention Times[11]

HEMOGLOBIN VARIANTS WITH RETENTION TIMES <0.63 MIN

The tetramers Hb Barts (γ4) and HbH (β4) and HbF_1, the acetylated form of HbF all elute before chromatogram integration; they therefore are not indicated on the chromatogram report. The elution peaks are detected only by visual analysis of the chromatogram. HbF_1 was seen mostly in newborns and was ~10-15% of the total HbF present. Hb Bart's is seen in newborns at risk for at least two gene deletions of the α globin chain.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE P1 WINDOW (0.63-0.85 MIN)

No hemoglobin variants were detected in this window.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE F WINDOW (0.98-1.20 MIN)

At least seven hemoglobin variants (four β and three α-variants) expected to elute in this window, all in quantities >10%.

Note: All laboratories must establish their own range for Hb F.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE P2 WINDOW (1.24-1.40 MIN)

HbA_1c eluted in the P2 window. When the elution peak was >7% of the total hemoglobin, the patient's records were indication of diabetes and HbA_1c quantification. The only hemoglobin variant found to elute in this window was Hb Hope, which had a mean (SD) %Hb [45.9(2.2%)] much greater than would be expected for HbA_1c.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE P3 WINDOW (1.40-1.90 MIN)

Nine hemoglobin variants (four α and five β- variants) had elution peaks in the P3 window. Elecrophoretic mobility, Retention time and percentage of Hb will differentiate them from each other. Hb J-Meerut has the Retention time of 1.88 and Hb percentage around 25%. It is predicted that Hb J-Meerut can be differentiated and identified based solely on its retention time (d=0.12 min from Hb J-Mexico).

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE A_0 WINDOW (1.90-3.10 MIN)

Six hemoglobin variants (two α and four β- variants) have elution peaks in the A_0 window.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE A_2 WINDOW (3.30-3.90 MIN)

Five hemoglobin variants (one δβ hybrid, one α and three β- variants) had elution peaks in the A_2 window. Hb Lepore could be differentiated and identified based solely on its retention time. The %Hb and the characteristic hump on the downward slope of the elution peak were additional distinguishing features of Hb Lepore. The retention time for Hb D-Iran appear to be significantly different from those of Hb Lepore (d=0.12 min) and HbA_2 (d=0.14 min). In addition, the %Hb of Hb D-Iran (47.7%) was significantly greater than either of these variants [Hb Lepore, 12.1 (1.5)%; HbA_2, 3.63 (0.04)%]. The retention time and %Hb for HbA_2 and HbE were significantly different (P=0.001 for both)[11].

The Hb A2 percentage for the identification of the various abnormalities:

- ✓ Thalassemia minor : 4-8 %
  - To be differentiated from associated Iron deficiency anemia (IDA) as Hb A_2 is low in IDA with raised RDW. Repeat HPLC after treatment of IDA.
  - Falsely low Hb A2 in Hb D^{punjab} . Electrophoretic mobility, characteristic chromatogram helps in diagnosis.
- ✓ Hb Lepore : 10-14 % with characteristic hump on downward slope of chromatogram.
- ✓ Hb E carrier: 14-33% associated thalassemic element with microcytic hypochromic red cells and target cells.
- ✓ Homozygous E : 85-95%
- ✓ Hb D Iran: 45-48% has electrophoretic mobility similar to Hb S on alkaline pH and has a characteristic chromatogram.
- ✓ Raised Hb A_2 value can be seen with Megaloblastic Anemia.

Note: All laboratories must establish their own range for Hb A_2.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE D WINDOW (3.90-4.30 MIN)

Three hemoglobin variants have elution peaks in the D window, all of which are β variants. The mean Hb A_2 values for the HbD-Punjab trait [1.4 (0.4)%] and HbG-Philadelphia trait [1.30 (0.4)%] were significantly lower (P<0.001, respectively) than the range for Hb A_2 in the normal specimens. Whereas HbD-Punjab and HbD-Iran had identical electrophoretic mobility, the retention times were quite different (d=0.69 min).

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE S WINDOW (4.30-4.70 MIN)

Six hemoglobin variants (three α, two β and one δ variant) had elution peaks in the S window. HbE-Saskatoon and HbS appeared to have significantly different retention times (d=0.18 min). Although Hb Manitoba, Hb Montgomery and HbA_2, all appeared to have identical retention times, their retention times and %Hb were statistically different from those of HbS (P<0.001 for all).

Although Hb S is the most common variant, confirmation using the simple sickling test is imperative.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE UNKNOWN WINDOW (4.70-4.90 MIN)

The elution peak Hb-Hasharon, an α-variant, fell in the time interval for unknowns.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE C WINDOW (4.90-5.30 MIN)

Three hemoglobin variants (three β-variants) had elution peaks in the C window. HbO-Arab and HbC had statistically different retention times (P<0.001).

The common Hb Variants in neonates[16]

β- thalassemia major, HbS, HbC, HbE, HbD are detectable at birth on both the variant and variant II using a fresh cord or peripheral blood. However, the routine cord blood analysis is feasible only in case of hospital delivery and when an agreement is reached between the neonatology department and local laboratory.

Screening on the fresh cord blood is feasible at the local level but not very efficient in a centralized national screening programme. For this purpose dry blood extracted from Guthrie cards is the current solution.

PERFORMANCE CHARACTERISTICS

- ✓ HPLC is sensitive, fast, fully automated test for identification & quantitation of normal & abnormal hemoglobins in adult & newborn samples in a single test.
- ✓ No Interference with Lipemia- Triglyceride levels up to 4600mg/dl , EDTA concentration, Conjugated bilirubin up to 20mg/dl, Hemolysed sample .
- ✓ No carry over of specimens and the detection limit between 0.5 to 0.75%
- ✓ Minimal effect of ageing of the dried blood spots on HPLC in neonatal screening
- ✓ Sample preparation for HPLC method are simple and lead to very low technical repeats
- ✓ Stat option on Bio-Rad variant allows urgent queries to be met

IV. RESULTS

Tables and graphs:

Table 1. Manufacturer assigned windows Bio-Rad variant HPLC System[11]

Peak name	Retention time(min)
P1 window	0.63-0.85
F window	0.98-1.20
P2 window	1.24-1.40
P3 window	1.40-1.90
A_0 window	1.90-3.10
A_2 window	3.30-3.90
D window	3.90-4.30
S window	4.30-4.70
C window	4.90-5.30

Table 2: percentage of Hb S and MCV values in Hb AS individuals with or without α+- thalassemias[15]

	αα/αα	-α/αα	-α/-α
HbS (%)	35 - 39	29 - 34	24 - 28
MCV (fL)	80 - 90	75 - 85	70 - 75

Graph 1: Chromatogram of normal adult

V. CONCLUSION

HPLC is an excellent powerful diagnostic tool for direct identification of sickle cell disorders and thalassemias however use of other complimentary techniques may help in arriving at the final conclusions in certain situations. One cannot underestimate the importance of family screening and ethnic background. Chromatogram obtained with HPLC are less subjective as results are obtained in preset windows. α variants have a mean % Hb values < 30%, β variants have a mean % Hb value > 30-34% , except for the Hb E which is < 30%. HPLC can avoid misidentification of Hb D^{Punjab} and Hb D Iran having interaction with Hb S.

REFERENCES

[1] Steinberg HM, Benz EJ, Hemoglobin synthesis, structure and function. In: Hoffman R. ed. Hematology, basic principles and practice, New York: Churchill Livingstone, 1991:291-302.1

[2] Giordino PC In: Diagnostics and Prevention of Hemoglobinopathies using High Performance Liquid Chromatography 2006:17-20

[3] Working Party of the General Haematology Task Force of the British Committee for Standards in Haematology. Guideline: the laboratory diagnosis of Haemoglobinopathies. Br J Haematol 1998;101:783-92.

[4] Bain BJ. Haemoglobinopathy diagnosis ,oxford,England, Blackwell science Ltd.[2001:260PP]

[5] Clarke G, Higgins TN. Laboratory investigation of Haemoglobinopathies and Thalassemia, review and update. Clin Chem 2000,46;128490.

[6] OU C-N, Rogerud CL. Rapid analysis of hemoglobin variants by cation-exchange HPLC. Clin Chem. 1993;39:820-4

[7] Wild BJ, Stephens AD. The use of automated HPLC to detect and quantitative haemoglobins. Clin Lab haematol 1997;19:171-6.

[8] OU C-N, Rogerud CL. Diagnosis of Haemoglobinopathies: Electrophoresis vs. HPLC. Clin Chim Acta 2001, 313:187-94.

[9] Lufferty JD, Mc Farlane AG, Chui DHK, Evaluation of dual hemoglobin A2/A1c quantification, Kit on the Bio-Rad Variant 11 automated hemoglobin analyzer. Arch Pathol. Lab. Med 2002, 126:1494-9.

[10] Operation manual: VARIANT™ Hemoglobin Testing System march 1996:700:114-2

[11] JouTvsk A,H adzi-Nesic J, Nardi M A HPLC Retention Time as a Diagnostic tool for hemoglobin variants and haemoglobinopathies : A study of 60,000 samples in a clinical Diagnostic laboratory Clinical chemistry 2004; 50:10:1736-1747.

[12] Campbell M, Henthorn JS, Davies SC. Evaluation of cation exchange HPLC campared with isoelectric focusing for neonatal Haemoglobinopathy screening. Clinical chemistry 999; 45:7;969-975.

[13] Doland GA, Castle WB. A simple and rapid method for demonstrating sickling of the red blood cells: the use of reducing agents. J Lab Clin Med 1948;33:1082-88

[14] US Department of health and human services/public health service. Brilliant cresyl blue test for inclusion bodies. In:laboratory methods for detecting hemoglobinopathies, Atlanta: centre for disease control, 1984:129-31

[15] Renzo G. Screening and diagnosis of Hemoglobin disorders In: Prevention of Thalassemias and other Hemoglobin disorders volume 1, 2003:58

[16] Giordino PC In: Diagnostics and Prevention of Hemoglobinopathies using High Performance Liquid Chromatography 2006:58