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Detection Of Abnormal Haemoglobin Variants By HPLC Method

Nilanjan Bhattacharya

Institute of Hematology and Transfusion Medicine (IHTM),
Kolkata, West Bengal

Abstract: ***Hereditary hemoglobinopathies currently accounts for the most frequent genetic disorders in nthe world, affecting approximately 4.5% of the world population. Every year about 300.000 affected homozygotes are born, equally divided between Hemoglobinopathies disorders and thalassemia syndromes (WHO Scientific Group, 1996). Diagnosis of these diseases requires the use of methods which allow for precise and accurate quantification of different hemoglobin fractions. At the molecular level the β thalassemias are very heterogeneous with more than 200 point mutations and deletions of different severity. The degree of severity generally correspond to the magnitude of residual output by the defective β globine gene, accordingly the β thalassaemia mutations are classified into severe, mild and silent.***

In several countries of the world there are screening programs with the aim of identifying carriers of hemoglobin disorders in order to assess the risk of a couple having a severely affected child and to provide information on the options available to avoid such an eventuality. Two methods are generally used to quantitate hemoglobin fractions that may be used in the diagnosis of thalassemia. These methods were hemoglobin electrophoresis and HPLC (High Performance Liquid Chromatography). In our study HPLC studies and haematological profile were utilized for the analytical prediction of the expanse of β-thalassemic carriers and affected individuals.

I. INTRODUCTION

Hemoglobinopathy is a kind of genetic defect that results in abnormal structure of one of the globin chains of the hemoglobin molecule. Hemoglobinopathies are inherited single-gene disorders; in most cases, they are inherited as autosomal co-dominant traits. The hemoglobinopathies encompass all genetic diseases of hemoglobin. They fall into two main groups: thalassemia syndromes and structural hemoglobin variants (abnormal hemoglobins). α- and β-thalassemia are the main types of thalassemia; the some of the main structural hemoglobin variants are HbS, HbE and HbH. Thalassemia is an autosomal recessive inherited group of disorders of hemoglobin synthesis characterized by the absence or reduction of one or more of the globin chains of hemoglobin. The α-thalassemias involve the genes HBA1 and HBA2, inherited in a Mendelian recessive fashion. Two gene loci and so four alleles exist. It is also connected to the deletion of the 16p chromosome. Alpha Thalassemias result in decreased alpha-globin production, therefore fewer alpha-globin chains are produced, resulting in an excess of β chains in adults and excess γ chains in newborns. Beta thalassemias are due to mutations in the HBB gene on chromosome 11, also inherited in an autosomal, recessive fashion. The structural variants result from substitution of one or more amino acids in the globin chains of the hemoglobin molecule.

Plethora of hemoglobin variants is prevalent in India owing to ethnic diversity of its population with minimal tomajor clinical significance. Being recessively inherited from the parents, the thalassemia and thalassemic hemoglobinopathies pose serious health problem leading to severe morbidity and mortality in Indian population. Detection of asymptomatic carriers by reliable laboratory methods is the cornerstone of prevention of this serious health problem. The preliminary selection of individuals at risk of being heterozygous for a form of thalassemia is based on the determination of MCV (Mean Corpuscular Volume) and MCH (Mean Corpuscular Haemoglobin). Thalassemic individuals have reduced MCV and MCH, an MCV of 72 fL or less and MCH of less than 27pg are suggestive of a presumptive diagnosis of thalassemia5. All β-thalassemias are characterized by an increase in HbA2 concentration. Cation exchange high performance liquid chromatography (HPLC) has become the preferred technique suitable in Indian

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scenario, as it can detect most of the clinically significant variants. The simplicity of the automated system with internal sample preparation, superior resolution, rapid assay time, and accurate quantification of haemoglobin fractions makes this an ideal methodology for the routine clinical laboratory. With increasing global awareness and mass screening programs undertaken at various levels by health care system, the responsibility for laboratory personnel has greatly enhanced in detection and prevention of this problem. Awareness about the diagnostic problems as well as their solutions is very important so that one does not miss a single case.

Many studies have been published from India on thalassemic hemoglobinopathies mostly putting emphasis on epidemiology and screening. However very few studies are available on the extensiveness of β-thalassemic carriers in the eastern part of India especially West Bengal determined from HPLC reports. The aim of this study is to quantify the magnitude of the β-thalassemia in the aforementioned region by analysing haematological profile and HPLC studies.

GENERAL THALASSEMIA SCREENING ALGORITHM

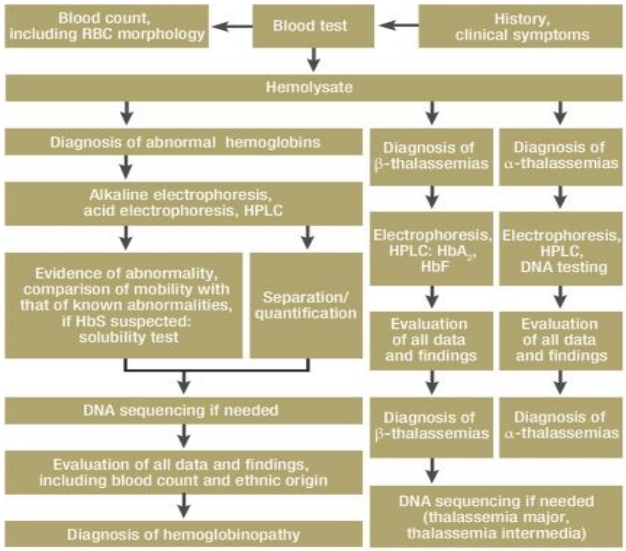

Figure 1

II. APPLICATION OF HPLC IN THE SCREENING OF BETA-THALASSEMIA

A. PRINCIPLE

An automated HPLC (VARIANT TM, Bio-Rad) system with the Beta-thalassemia Short Program was used. The Beta-thalassemia Short Program is applicable to the diagnosis of α-thalassemia and β-thalassemia disorders including HbH and HbBart's disease in adults, newborns and fetuses. This program has been designed to separate and quantify the hemoglobins HbA, HbA2, HbF, HbS, HbC from other hemoglobins in a 6.5 min run. The system cannot quantify accurately HbH and Hb Bart's.

The Beta-thalassemia Short Program utilizes the principles of cation-exchange high pressure liquid chromatography in which a mixture of molecules with a net positive charge is separated into their components by absorption onto a negatively charged stationary phase in a chromatography column, followed by their elution on theVariant II Sampling Station(VSS) with a mobile phase. Specifically the Beta –thalassemia Short program uses a 3cm×0.46cm cartridge packed with a 5µm silica-based weak cation exchange material. Samples of whole blood (5µl in 1 ml of buffer) are hemolyzed before injection. The analytes are eluted at a flow rate of 2ml/min using a step gradient of two phosphate buffers with different pH and ionic strengths at the Variant II Chromatographic Station (VCS). As the ionic strength of the mixture increases, HbA2/F are separated based on their ionic interaction with the analytical cartridge. A dual wavelength filter photometer (415nm and 690 nm) monitors the hemoglobin elution from the cartridge, detecting absorbance changes at 415 nm. The 690 nm secondary filter corrects the baseline for effects caused by mixing buffers with different ionic strengths. Changes in absorbance are monitored and displayed as a chromatogram of absorbance versus time. There are established windows with specific retention times for the most frequently occurring hemoglobins8.

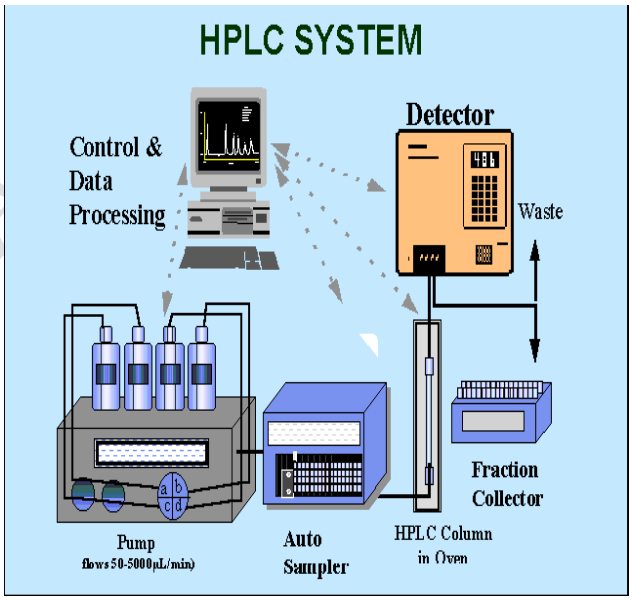

Figure 2

At the beginning of each run the HbA2/HbF Calibrator was analyzed to provide calibration factors for both hemoglobin A2 and F. These factors are used in the calculation of area percentages for HbA2 and F in all subsequent analyses in the run8. The Lyphocheck Hemoglobin A2 Control (Levels 1 and 2 from Bio-Rad) was also analyzed establishing that the concentration values of HbA2 and HbF were within acceptable limits. The Variant II CDM (CDM) Software performs reduction of raw data collected from each analysis. To aid in the interpretation of results, windows have been established for the most frequently occurring hemoglobins based on the characteristic retention time.For each sample a sample report and a chromatogram are generated by CDM showing all hemoglobin fractions eluted, their retention times, the area of the peaks, and values of the fractions. The age, ethnic origin, family history of the patient plus laboratory data including serum iron and red cell morphology was included. The erythrocyte indices MCV

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(mean corpuscular volume), MCH (mean corpuscular hemoglobin), RBC (red blood cell count), RDW (red cell distribution width)5 and hemoglobin were determined using the Sysmex NE1500 automatic cell counter.

This study was undertaken during a 1 month period at the Institute of Hematology and Transfusion Medicine (IHTM), Kolkata. A total of 100 adult and child blood samples were examined for routine thalassemia screening. The patients were guided to our department with the presumptive diagnosis of thalassemia.

B. REAGENTS REQUIRED

- ✓ Elution buffers (1,2): sodium phosphate buffer.
- ✓ Whole blood primer: lyophilized human red blood cell hemolysate with gentamicin, tobramycin, and EDTA as preservative.
- ✓ HbA2/F calibrator/diluent set: lyophilized human red blood cell hemolysate with gentamicin, tobramycin, and EDTA as preservative analytical cartridge. Diluent contains deionized water.
- ✓ Wash/diluent solution: deionized water.
- ✓ HB A2 Control: normal (HbF 1-2%, HbA2 1.8–3.2%) and abnormal (HbF 5–10%, HbA2 4–6%) controls.

C. SAMPLE COLLECTION

At least 5 μL of venous blood are required for this test. Whole blood samples should be collected in a vacuum blood collection tube containing EDTA as an anti-coagulent. The whole blood samples mixed with EDTA remain stable when stored at 2°–8°C for maximum 7 days if processing is delayed. No preparation was required unless the sample was in a tube other than the recommended tube or there was less than 500 μL of sample in the tube. In such case, sample was manually prediluted. Predilution was carried out by mixing 1.0mL wash/diluents with 5 μL of whole blood sample. HbA2/F calibrators and normal and abnormal controls were analyzed at the beginning of each run.

D. SAMPLE PREPARATION

- ✓ 5μL of whole blood sample from each patient sample was pipette into separate 1.5mL sample vials.
- ✓ 1.0mL Haemolysis Reagent was added to each sample vial.
- ✓ Each sample vial was covered with Parafilm and was mixed by inversion.
- ✓ The sample vials were placed into the VARIANT. Patient haemolysates are stable for 24 hours when stored at 2°–8°C.

E. INTERPRETATION OF REPORTS

Reports and chromatograms generated were studied and interpreted by observing HbA2 and F concentration for beta thalassemia and retention time and area percentage of other peaks and windows for structural variants. Each chromatogram shows peaks of Hb A0, A2, and Hb F alongwith C window, D window, S window, and two minor peaks, P2 and P3. Several haemoglobin variants elute same window; they were provisionally diagnosed by retention time and area percentage keeping in mind the ethnicity of the patients. Other relevant tests were done, for example, sickling test as supporting evidence of Hb S. Family study was carried out whenever possible and correlation with findings of Hb electrophoresis result was done in few cases.

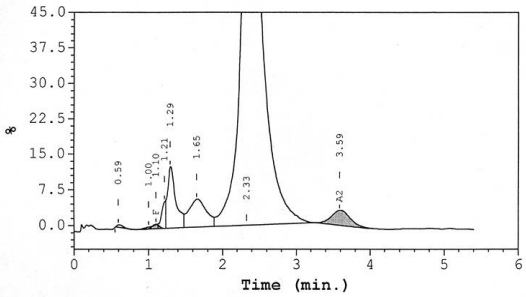
HPLC ANALYSIS - NORMAL ADULT

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.59	3217
Unknown	---	0.1	1.00	3321
F	0.3	---	1.10	7753
Unknown	---	1.1	1.21	29205
P2	---	4.8	1.29	125635
P3	---	4.7	1.65	121998
Ao	---	86.0	2.33	2232443
A2	3.1	---	3.59	70883

Total Area: 2594456

F Concentration = 0.3 %
A2 Concentration = 3.1 %

Analysis comments:



BETA THALASSAEMIA TRAIT

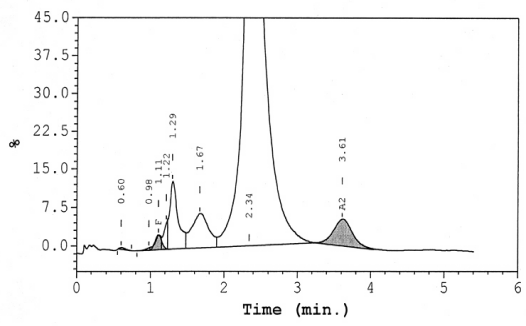
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.60	2439
Unknown	---	0.1	0.98	2463
F	1.0	---	1.11	23374
Unknown	---	1.0	1.22	26601
P2	---	5.0	1.29	128590
P3	---	5.2	1.67	133229
Ao	---	83.0	2.34	2115341
A2	5.2*	---	3.61	115816

Total Area: 2547853

F Concentration = 1.0 %
A2 Concentration = 5.2* %

*Values outside of expected ranges

Analysis comments:



HB-S THALASSAEMIA

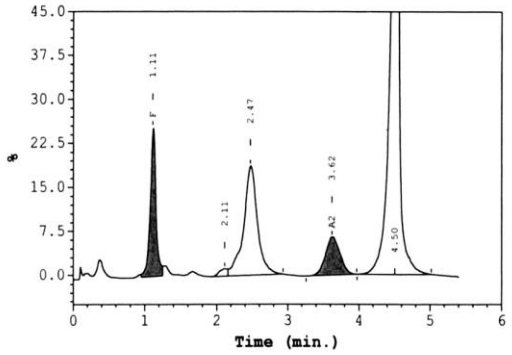
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	9.0*	---	1.11	153554
Unknown	---	0.7	2.11	11443
Ao	---	16.2	2.47	273375
A2	6.5*	---	3.62	99298
S-window	---	68.1	4.50	1149258

Total Area: 1686929

F Concentration = 9.0* %
A2 Concentration = 6.5* %

*Values outside of expected ranges

Analysis comments:



III. EXPERIMENTAL OBSERVATIONS

✓ Screening Data.

IV. RESULT AND DISCUSSION

From HPLC analysis report, It was observed that 24 out of 97 individuals had HbA2 values in and around the range for Beta thalassemia trait (typically between 4.0% and 6.0%, and is rarely outside the range of 3.5%–7.0% of the total haemoglobin), 18 out of 97 individuals had HbF values in and around the range for Beta thalassemia trait (typically between 1% to 5%) and only 4 individuals were found to have HbA0 values in the Beta thalassemia range. Thus 11% of the 97 individuals examined for Beta traits are reported to be carriers for Beta Thalassemia and 14% individuals are reportedly Beta Thalassemia patients.

Comparing the obtained results with the works of prior researchers give us an insight on the validity of our work as well as variations that might have occurred on the basis of geographic differences or other factors.

The work of Barbara Wild on 'Diagnosis of the Thalassemia Syndromes: Measurement of Haemoglobin A2' under the 'UK National External Quality Assessment Scheme, London' reported that of the 100 patients examined, 22 cases were just outside of the 'average' A2 and MCH groups (above2.2-3.3%), 35 patients with Hb A2 values >3.5% gave normal β gene sequencing results and 25 patients had a normal β-globin gene sequence. Rest of the subjects were found to have increased Hb A2 values e.i. in the range 3.5 - 7.0% or above. Thus 22% individuals were found to be Beta carriers and 18% were reportedly Beta Thalassemia patients.

The work done by Leela Pant, Dipti Kalita, Sompal Singh, Madhur Kudesia, Sumanlata Mendiratta, Meenakshi Mittal, and Alka Mathur on 'Detection of Abnormal Haemoglobin Variants by HPLC Method' at the 'Hindu Rao Hospital, Delhi 110007, India' stated that among 4800 cases screened, 290 (6.04%) cases were detected with abnormal haemoglobin in this study. Presumptive identification of haemoglobin variants was made primarily by retention time (RT) windows and area percent. The results were as follows:

Hemoglobin variant	Number (%)
Beta thalassemia trait (BTT)	216 (74.48)
Beta thalassemia intermedia/major (BTI/M)	9 (3.10)
Other thalassemia traits	65(22.39)
Total	290

Table 1

V. CONCLUSION

To conclude, RBC indices, HPLC finding, and family study are sufficient to detect and manage most of the haemoglobin variants prevalent in this country. However, one has to be aware of the limitations and problems associated with the diagnostic methods to avoid false negative diagnosis in day to day practice. Genetic studies are indicated to confirm borderline cases and to detect silent carriers of beta thalassemia, alpha thalassemia, and rare and novel variants in routine practice. The present study conducted using HPLC reflects the magnitude of thalassemia and haemoglobinopathies in a small hospital based population which may be in fact the tip of an iceberg, but this type of study can definitely help to increase awareness among both health care givers and general population.

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In June 2015 we were given the opportunity to work under the guidance Dr. Shila Chakraborty and observe the functionality of a Bio-RadVARIANT and obtain HPLC reports of 97 selected individuals, at the Institute of Haematology and Transfusion Medicine (IHTM) (Kolkata, West Bengal), which enabled us to make HPLC screening determinations.

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