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Incidence of hemoglobinopathies and hemoglobin variants using HPLC as a diagnostic tool in 6500 anemic patients in a tertiary care center in Western India

KEYWORDS: Hemoglobinopathies, Hemoglobin variants, Cation-exchange high performance liquid chromatography

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ABSTRACT *To determine the incidence of various hemoglobinopathies among anemic patients in Western India, a six year prospective study was conducted on 6,500 anemic patients. All the samples were analyzed on the Bio-Rad Variant II HPLC system.*

Out of 6500 patients, 740 (11.38%) cases showed abnormal Hb fractions. The major abnormality observed was of beta thalassemia trait (BTT) with a total of 522 cases (8.03%) followed by 42 (0.65%) cases of beta thalassemia major. Other hemoglobinopathies which were also identified are in the following proportions: HbE (67 (1.03%) cases), HbS (85 (1.31%) cases), HbD Punjab 12 (0.18%) cases, HbQ (2 (0.03%) cases), Double heterozygous HbE-BTT (3 cases), Double heterozygous HbS-BTT (6 cases) and one case of HPFH.

The reliability of HPLC for the detection of beta thalassemias is of great advantage in a country like India, where the prevalence of hemoglobinopathies and hemoglobin variants is high. An early and precise diagnosis is very important for their clinical management and providing genetic counselling to prevent more serious hemoglobinopathies.

INTRODUCTION

Among the various inheritable disorders in man, abnormalities in hemoglobin (Hb) synthesis constitute probably the commonest disorders. These disorders can be classified broadly into quantitative (e.g. the thalassemia syndromes) and qualitative (e.g. the variant Hbs) disorders, each having their own distinct etiopathogenesis.[1] The thalassemia syndromes (beta thalassemia major and few alpha thalassemias) forms the most important group, accounting for the most serious and lethal manifestations.[2]

India constitutes 10% of thalassemia in the world with 10,000 children being born with thalassemia major every year. [3] The frequency of beta thalassemia in India amongst general population ranges from 3.5 to 15%.[4] The treatment options for these children are monthly blood transfusion and regular iron chelation therapy.

Diagnosis of hemoglobinopathies in most centers in India relies upon conventional methods like, clinical and family history, complete blood counts (CBC), red cell indices, HbA_2, HbF estimation, sickling test, and Hb electrophoresis. Various limitations of these methods have been felt in recent years. One of the most important is the difficulty in the identification of Hb variants with same electrophoretic mobility, such as in A_2/E/C/O-Arab and S/D/G/Q/Lepore. Another issue comes up while diagnosing certain compound heterozygous states such as, HbD + HbE, HbS + β thalassemia, HbS + HbD, HbE + β thalassemia, HbD + β thalassemia.[5]

Therefore, an early and accurate diagnosis of hemoglobinopathies is required. One such reliable tool for diagnosis and early detection is cation exchange high performance liquid chromatography (CE-HPLC). [6] With the incorporation of CE-HPLC in the diagnosis of various types of abnormal hemoglobins, the prevalence in various parts of the world along with the changing trends, can be accurately determined.

The exact data pertaining to the spectrum of hemoglobinopathies in this region is scarce, hence this study attempted to find out the extent of burden of hemoglobinopathies in the Western part of India.

MATERIAL AND METHODS

This was a prospective study conducted on 6,500 consecutive anemic patients (i.e. Hb < 11 g/dl), who were referred by physicians as a part of anemia work-up. HPLC was run on samples of all patients along with their relevant family members for confirmation of clinically suspected thalassemias or hemoglobinopathies. Family history and history of blood transfusion were taken in all cases. The geographical distribution included states of Western India (predominantly from Maharashtra, Gujarat, Rajasthan and also some parts of Madhya Pradesh).

Vacutainer tubes containing dipotassium EDTA (Becton Dickinson) were used to draw specimens. The instrument, BIO-RAD 'VARIANT II' (beta thalassemia short program) (by BIO-RAD laboratories, USA), which utilizes the principle of CE-HPLC, was employed for performing the tests.

Sample preparation was performed by diluting 5 µl of EDTA anticoagulated blood in 1 ml of hemolysis solution which was provided with the kit. Once the samples were loaded in the instrument, the sample is diluted with specific buffer solutions, and then injected this into an assay-specific analytic cartridge, where the Hb fractions get separated out due to ionic interactions.

The separated hemoglobin fractions subsequently pass through a flow cell, which measures absorbance at 415 nm & 690 nm. A printed report is delivered by the software showing the chromatogram, and all the eluted hemoglobin fractions. Before every run, the instrument was calibrated

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with the help of Hb A_2/F calibrators, and two levels of controls (BIO-RAD).[7]

For every abnormal HPLC result, a further work up of peripheral blood smear (PBS) examination was done, along with the estimation of hemoglobin levels and red cell indices; performed using 3 part automated hematology cell counter (Sysmex KX-21 hematology analyzer, Sysmex Corporation, Japan). Clinical correlations as well as family studies were done for the results obtained by various tests. For all patients in whom a variant was detected in S-window, a sickling test was done using sodium meta-bisulphite. However, secondary confirmatory methods like Hb electrophoresis and molecular studies were not performed in this study.

Statistical analysis was done on Microsoft Excel 10. Continuous variables were expressed as range, median and mean ± SD. Categorical variables were expressed as percentages. The study was approved by the institutional ethical committee.

RESULTS

Of the total 6,500 samples tested, 740 (11.36%) cases showed abnormal Hb fractions. Among them, 414 (56%) were male and 326 (44%) female. The age of the patients ranged from 1 to 84 years (median 25.7 years). Table 1 shows manufacturer assigned windows for BIO-RAD variant HPLC system. Distribution of various hemoglobinopathies according to types and gender (n=740) are shown in Table 2 and Figure 1.

Normal adult chromatogram shows primarily HbA, a small percentage of HbA_2 (< 3.9%), and traces of HbF (< 1%) (Figure 2). In this study, the major abnormality observed was of high HbA_2. A total of 522 (8.03%) cases of BTT were diagnosed (Figure 3) with HbA_2 ranging from 4.2% to 6.6% (mean 5.4%). The RT for HbA_2 was between 3.30 and 3.90 min. There were 42 (0.65%) patients detected to have beta thalassemia homozygous pattern on HPLC (Figure 4). Cases diagnosed with thalassemia major presented within their first 2.5 years of life (range 1-14 years). Their Hb ranged from 2.5 g/dl to 9.5 g/dl, with PBS showing anisopoikilocytosis, marked microcytic hypochromic (MCHC) anemia, nucleated RBCs, and a raised RDW (range 20.2-35.6, mean 27.9). All the cases had HbF levels > 85%.

HbS eluted in a separate S-Window, which had a RT of 4.30-4.70 min. There were 85 (1.3%) cases of HbS with homozygous (SS) and heterozygous (AS) forms constituting 34 (0.34%) and 51 (0.78%) cases, respectively. Six (0.08%) cases were diagnosed as double heterozygous for HbS-BTT. Sickling test was positive in all these cases. The median age of incidence of HbAS was found to be 17.4 years (range 5-48 years) as compared to 11 years (range 1.4-37 years), in case of HbSS. The age at presentation of HbS-β thalassemia trait (BTT) patients ranged from 6 years to 45 years with a peak incidence at 10 years.

Mean Hb HbAS was 8.5 g/dl (range 4.6-12.5 g/dl) with mean HbS and HbA levels of 35.3% (range 14.5-45.7%) and 62.4% (range 34.5-70%), respectively (Figure 5). On PBS, out of 51 HbAS cases, 26 showed features suggestive of MCHC anemia, 14 of macrocytic hypochromic anemia and 11 of dimorphic anemia. In HbSS, mean Hb was 5.5 g/dl (range 2.8-8.5 g/dl) with mean HbS, HbA and HbF of 78.3% (range 64.5-89.5%), 3.5% (range 1.6-5.3%), and 16.5% (range 13.4-20.3%), respectively. All 34 HbSS cases, on PBS showed features suggestive of microcytic hypochromic anemia with anisopoikilocytosis and polychromasia.

Mean Hb in double heterozygotes of HbS and beta-thalassemia was found to be 6.3 g/dl (range 3.6-8 g/dl) with PBS showing severe degree of anisopoikilocytosis and target cells. Mean HbS, HbA, HbA_2 and HbF values were 65.6% (range 60.5-71.0%), 5.5% (range 2.7-8.4%), 6.5% (range 5.3-6.9%), and 15.4% (range 13.2-19.4%), respectively.

HbE variant included HbE heterozygous (26 (0.4%) cases), HbE homozygous (41 (0.63%) cases), and HbE-BTT double heterozygous (3 (0.04%) cases). HbE eluted in the A_2 window with RT ranging from 3.30-3.90 min.

Mean Hb in HbE trait (AE) patients was found to be 8.5 g/dl (range 4.5-13 g/dl) with PBS suggestive of MCHC anemia. HbE fraction ranged from 15.5% to 45.3% with a mean of 34.7%, HbA ranged from 53.6% to 75.4% with mean of 61.4% and HbF ranged from 1.3% to 1.7% with mean of 1.5%. Similarly, in HbE homozygous (EE) patients, the mean Hb was found to be 8.5 g/dl (range 4.5-13 g/dl) with PBS showing anisopoikilocytosis, few target cells and features suggestive of MCHC anemia. The HbE fraction ranged from 58.5% to 91.3% with a mean of 84.6%, HbA ranged from 2.4% to 6.6% with mean of 4.4% and HbF ranged from 1.7% to 8.7% with mean of 5.8%.

The HbE-BTT compound heterozygous patients had low mean Hb of 6.3g% (range 5.2-7.2%), high HbE fraction ranging from 60.4% to 55.0% (mean of 58.5%), low mean HbA of 4.6% (range 3.2-5.3%) and a high mean HbF level of 35.7% (range 30.2-42.0%). The median age of presentation was 16 years (range 4-28 years). Parental study was undertaken in all 3 cases to confirm beta thalassemia gene in either of the parents. PBS revealed a MCHC picture in all the 3 patients.

HbD-Punjab heterozygous constituted 12 (0.18%) cases. HPLC displayed a D Window with RT of 3.90-4.30 min. HbD ranged from 25.4% to 48.6% (mean of 38.4%). Hb levels and PBS were essentially within normal limits.

HbQ-India heterozygous constituted two (0.03%) cases. The characteristic findings included an unknown peak at 13% and 23% on HPLC with a typical RT of 4.77±0.01 min. Hematological parameters were essentially within normal limits.

Raised HbF (>1.5%) levels along with adult HbA was seen in 79 patients. 78 of these patients were pregnant, which was considered to be normal (not shown Table 2). The HbF levels were always below 5% in the above patients and all had normal blood counts with a normocytic normochromic picture on PBS. A possibility of heterozygous hereditary persistence of fetal Hb (HPFH) was raised in one case with HbF of 18% with a normal PBS and red cell counts. Molecular studies have been recommended for its confirmation.

DICSUSSION

Hemoglobinopathies and thalassemia both are common disorders and exert significant burden on various developed and developing countries of world including India. Hence, adequate measures and screening procedures are required for its confirmation.[8] HPLC has emerged to be a rapid, sensitive, specific and reproducible alternative to conventional hemoglobin electrophoresis, for screening and detection of various hemoglobinopathies. Incorporat-

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ing the basic hematological parameter testing, such as Hb and RBC indices along with HPLC, a laboratory is able to identify around 75% of the common variants encountered, without the need for confirmatory studies such as alkaline and acid electrophoresis.[9]

In our study, out of 6,500 patients, 740 (11.36%) patients had Hb variants with occurrence among males being slightly more as compared to female patients (56% vs 44%). This was in consensus from other studies[10,11] conducted in similar geographical areas. This may be due to the prevalent socio-cultural factors in our society, that more male patients seek medical attention.

BTT formed the predominant subgroup of abnormal Hb with 522 (8.03%) patients as compared to 42 (0.65%) cases of thalassemia major. Colah et al[12] also had similar findings in the states of Maharashtra and Gujarat in 2010. However, few studies[13] estimated a higher prevalence of BTT in this part of the country. In spite of these variations, the high incidence of traits highlights the need for antenatal screening for prevention of thalassemia major in offspring.

HbS gene was detected in 91 (1.23%) patients, which was lower than the study conducted by Urade[14] (i.e. 5.78%) around Pune area in 2012, in which a population within a restricted geographical area was considered for the study. Of the above, 34 (42.3%) patients were detected as HbS homozygous (SS), (with abnormal Hb ranging from 65 to 90%) when compared to 51 (56%) HbS heterozygous (AS) cases (with HbS ranging from 14 to 46%), which represented 0.78% of the total abnormal Hb. Sickling test was positive in all the above cases. As the study was a laboratory based study, the incidence of HbSS was found to be higher as compared to HbAS, as more of symptomatic HbSS patients were evaluated and diagnosed than HbAS patients. Double heterozygous HbS + BTT constituted 6 cases with formed 0.82% of total abnormal Hbs detected. Mean HbF level in case of HbSS patients is slightly higher in Indian subcontinent, than the other parts of the globe (which was also noted in our study i.e. 16.5%). The reason for this is that the haplotype of HbS gene, which is prevalent in India, is the Saudi Arabia/Indian haplotype, which reduces the clinical severity of the disease.[15]

HbE is the most frequent variant Hb in Asia, with a significant prevalence in the south-easterly direction from North East India and Bangladesh to as far as Indonesia. It is a beta chain variant resulting from substitution of a lysine residue for a glutamic acid residue at position 26 of the beta globin chain (β26). It tends to elute in A_2 window on HPLC. HbE homozygous presents with HbE values between 70 and 90% and heterozygous with HbE values < 40%. Compound heterozygous HbE-BTTs are important, as the clinical effects are more severe when they are co-inherited. Individuals who are $\beta^E\beta^{thalassemia}$ often have symptoms which resemble those of thalassemia major.[16] In our study, the overall incidence of HbE gene was found out to be 1.07% which is very low as compared to north-eastern part of our country (where it may be as high as 80%).[17-19]

The gene frequency of HbD (also known as HbD-Los Angeles) is relatively low with highest prevalence in north-western India (1% in Gujaratis, 2-3% in Sikhs). In HbD patients, there is a substitution of a glutamine residue for a glutamic acid residue at position 121 of the beta globin chain (β121).[16] As patients from Gujarat were also included in our study, the distribution of HbD gene was slightly more than normal population (i.e. 1.6% of total abnormal Hbs). On CE-HPLC, it gets eluted in the D-window, which is separate from HbS peak. On alkaline electrophoresis, it migrates in the S/D/G region. Both homozygous and heterozygous individuals are asymptomatic and are detected incidentally.[20]

HPLC can be highly useful to distinguish two Hb variants of HbD family; HbD-Iran and HbD-Punjab. HbD-Iran is a beta chain variant resulting from substitution of a glutamine residue for a glutamic acid residue at position 22 of the beta globin chain (β22) and is mostly concentrated in the north-western region of India. Both have similar electrophoretic motilities' but on HPLC, HbD-Iran gets eluted in A_2 window and HbD-Punjab in the D window. Both the situations are clinically important because HbD-Punjab produces a significant sickling disorder in compound double heterozygous HbD-HbS form but with HbD-Iran, the combination is clinically benign.[21]

HbQ-India heterozygous is a rare alpha1-chain structural variant caused due to a substitution of histidine residue with aspartic acid residue. Hb electrophoresis at acid and alkaline pH reveals migration of abnormal Hb S/D/G position and between A and S positions in acid electrophoresis.[22]

Conditions with borderline HbA_2 should be carefully interpreted. Iron deficiency may lead to a low HbA_2 and hence may mask a thalassemia trait, whereas B_{12}/folate deficiency may lead to slightly raised HbA_2 leading to a false diagnosis of a trait. Serum iron studies along with regular evaluation should be taken up in such cases.[23-26]

From this prospective study, we can conclude that HPLC can be an excellent and powerful diagnostic tool for direct identification of Hb variants. The simplicity and rapidity of sample preparation, accurate quantification of Hb concentration combined with complete automation, makes HPLC an ideal methodology for the routine diagnosis of Hb disorders. This is very important in India, where the incidence of beta thalassemia trait is quite high. But, it should be reinforced that, the final diagnosis based on HPLC findings, has to be individualized, and whenever required, must be supplemented by red cell counts with indices, Hb electrophoresis, family studies and even molecular studies.[27-30]

Table 1: Manufacturer assigned windows for Bio-Rad variant HPLC system

Peak name	Window (min)	Retention time (min)
F window	1.00-1.30	1.15
P2 window*	1.30-1.60	1.45
P3 window*	1.60-1.90	1.75
A0 window	1.90-3.30	2.60
A2 window	3.30-3.90	3.60
D window	3.90-4.30	4.10
S window	4.30-4.70	4.50
C window	4.90-5.30	5.10

*Shows P2 and P3 are minor peaks associated with Hb A.

Table 2:Incidence and gender distribution of various hemoglobinopathies (n=740)

Hemoglobinopathies	Male (%)	Female (%)	Cases (%)
Beta thalassemia trait (BTT)	287	235	522 (8.03)

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Beta thalassemia major (BTM)	18	24	42 (0.65)
HbS- Heterozygous (AS)	23	28	51 (0.78)
HbS- Homozygous (SS)	25	9	34 (0.52)
HbE- Homozygous	28	13	41 (0.63)
HbE- Heterozygous	23	3	26 (0.4)
HbD Punjab Heterozygous	6	6	12 (0.18)
HbE + beta thalassemia trait	1	2	3 (0.04)
HbS + beta thalassemia trait	2	4	6 (0.08)
HbQ Heterozygous	1	1	2 (0.03)
HPFH-Heterozygous	0	1	1 (0.02)
Total	414 (6.37)	326 (5.02)	740 (11.36)

Figure 1: Distribution of hemoglobinopathies and hemoglobin variants

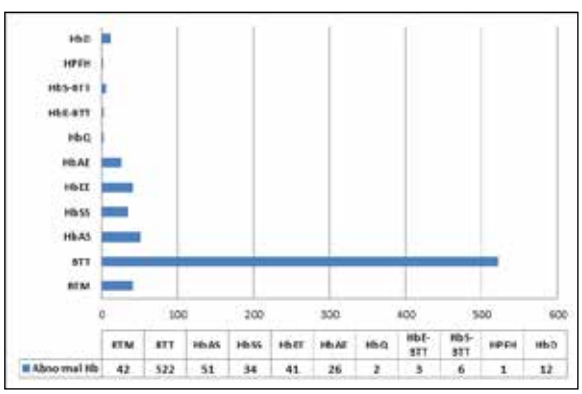

Figure 2: Normal patient hemoglobin (Hb) AA

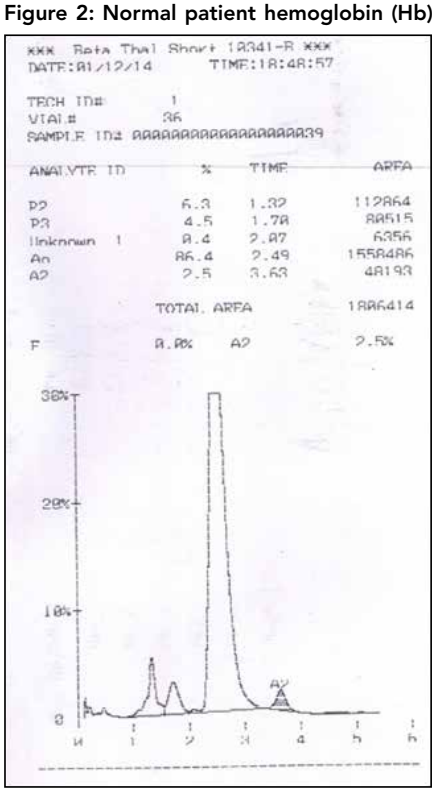

Figure 3: Beta thalassemia trait with A2 fraction 6.2%

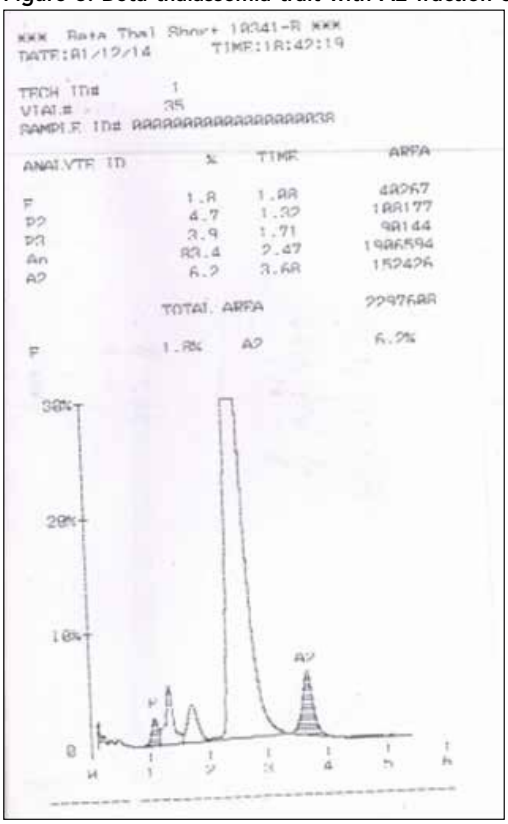

Figure 4: Beta thalassemia homozygous with HbF 98.3%

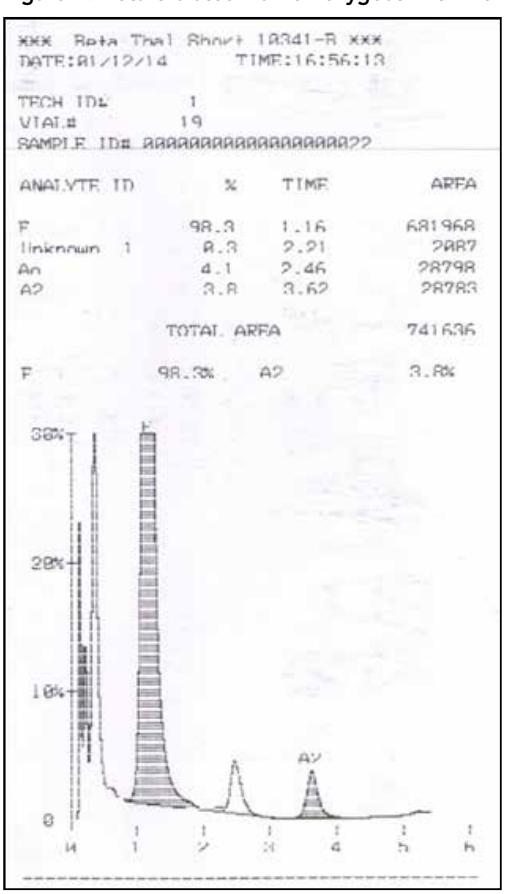

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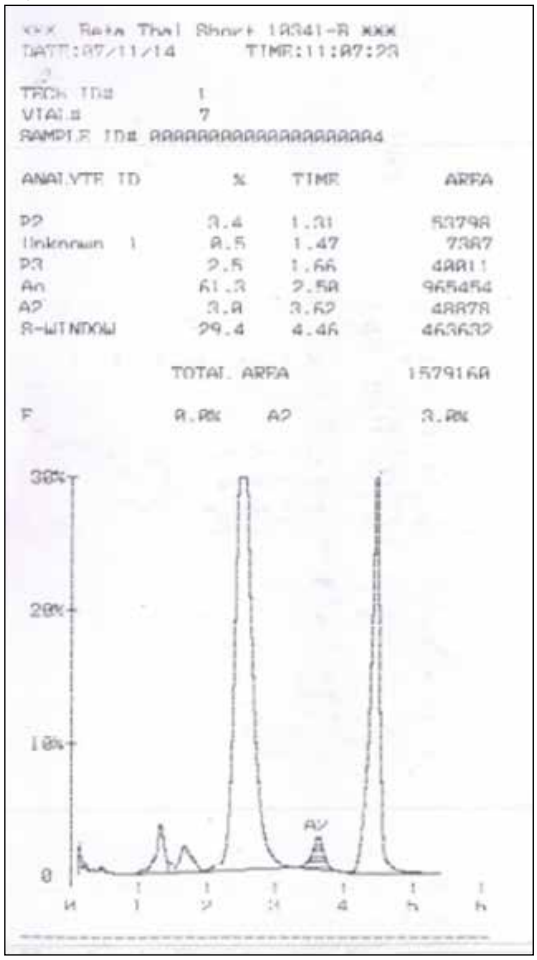
Figure 5: Sickle cell trait with HbS 29.4%



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