STUDY OF HEMATOLOGICAL FINDINGS AND CHARACTERISATION OF HEMOGLOBIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN CASES OF HEMOGLOBINOPATHIES

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Hemoglobinopathies, HPLC

ABSTRACT

Introduction:
Hemoglobinopathies including sickle cell disease and thalassemia are prevalent genetic disorders affecting hemoglobin molecule. Hematological parameters, blood smear examination and HPLC together allow for effective differentiation among various hemoglobinopathies, facilitating timely intervention, genetic counseling.

Objective:
This study aimed to study the haematological and HPLC findings in various cases of hemoglobinopathies.

Materials And Methods:
A retrospective analysis was conducted on 585 individuals diagnosed with hemoglobinopathies at the Tertiary Health Centre over one year. All the confirmed cases of hemoglobinopathies by HPLC were retrospectively analysed for Hemoglobin %, RBC indices, and detailed HPLC reports. Data were collected and analysed using SPSS.

Results:
Among the 585 cases, 47.35% were aged 15-30, with a predominance of females (67.18%). Sickle cell trait was the most common (392 cases), followed by beta thalassemia trait (83 cases). Additionally, 4 rare variants of haemoglobin were identified. Beta-thalassemia major had a severe reduction in Hb%. HPLC revealed distinct haemoglobin profiles across disorders, with significant variations in HbA, HbA2, and HbF levels along with the appearance of variant Hb window.

Conclusion:
Haematological parameters help in the effective screening of hemoglobinopathies. With its high sensitivity and specificity, HPLC enables precise identification and quantification of abnormal haemoglobin variants. These diagnostic tools allow effective differentiation among various hemoglobinopathies, facilitating timely intervention, genetic counselling, and family planning guidance.

INTRODUCTION

Hemoglobin, a principal constituent of red blood cells (RBCs), is responsible for transporting and delivering oxygen to tissues. Hemoglobinopathies, which include sickle cell disease and thalassemia, are autosomal recessive disorders of the haemoglobin (Hb). (1) In thalassemia, the quantity of haemoglobin production is affected, while in sickle cell anaemia, the quality of haemoglobin is affected. (1) In cases of insufficient α-globin chain synthesis, there is an increased level of β-globin chains, resulting in α-thalassemia. Conversely, insufficient synthesis of β-globin chains leads to an elevated production of α-globin chains, which manifests as β-thalassemia. Furthermore, there exist rare hemoglobinopathies, including Hb E, Hb C, and Hb D disease. World Health Organization (WHO) estimated that approximately 5% of the global population carries genetic traits associated with hemoglobinopathies, primarily sickle-cell disease and thalassemia. (2) Hemoglobinopathies have a high prevalence in India and are a significant public health problem. India is home to more than 40 million carriers of β-thalassemia, leading to an estimated 10,000 to 12,000 affected births annually. (3)

As most hemoglobinopathies show recessive inheritance, carriers are usually clinically silent. Detection of carriers is essential for effectively managing and controlling this disease. (4) Carrier state is usually diagnosed with a routine Complete Blood Count (CBC) test, followed by a sickling or solubility test and High Performance Liquid Chromatography (HPLC) for confirmation.

We thus aimed to study the haematological and HPLC findings in various cases of hemoglobinopathies.

MATERIALS AND METHODS

This study was conducted at Teritiary Health Centre in India. A retrospective data analysis of 585 cases diagnosed in 1 year for various hemoglobinopathies by HPLC was done. Demography, HPLC data and hemogram parameters were noted for analysis from records. The haemoglobin percentage (Hb%), complete blood count (CBC), and red blood cell indices—including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and red cell distribution width (RDW) were quantitatively assessed utilising an automated cell counter. For the suspected cases of hemoglobinopathies, Solubility test results were performed. Subsequently, the samples were tested using CE-HPLC (Capillary Electrophoresis-High Performance Liquid Chromatography). All samples were assessed using the BIO-RAD HPLC variant haemoglobin testing system. The β-thalassemia short program recorder pack was used with this system to quantify the levels of Hb A, Hb A2, Hb F, and other haemoglobin variants. The samples whose hemoglobinopathy diagnosis was confirmed with HPLC were included in the study for evaluation. The data were meticulously collected, compiled, and analysed utilising SPSS. Qualitative variables were expressed in percentages, while quantitative variables were categorised and represented as percentages or as means with standard deviations.

RESULTS

In the present study, a total of 585 cases were analysed. A total of 277 cases, accounting for 47.35%, fell within the age group of 15 to 30 years, making it the largest group. Additionally, 164 cases, or 28.03%, belonged to the age group < 15 years. The age group of 30 to 45 years accounted for 118 cases, or 20.17%. Lastly, 26 cases, or 4.44%, were classified in the age group exceeding 45 years. (Table 1)

393 cases (67.18%) were identified as female, whereas 192 cases (32.82%) were male, indicating a notable predominance of females. Additionally, 324 cases (55.38%) were classified as married, while 261 cases (44.62%) were classified as unmarried. The largest group comprised individuals with sickle cell trait (SCT), totalling 392 cases, followed by 83 cases of β-thalassemia trait. We also identified 78 cases of sickle cell disease (SCD) and 21

double heterozygous cases for both sickle cell disease and β-thalassemia. There were 7 cases of β-thalassemia major. In addition to beta-thalassemia, four additional rare variants were identified. These variants include one case of HbE trait, one case of homozygous HbE, one case of double heterozygosity for HbS and HbD, and one case of double heterozygosity for HbS and HbC.

Sickle Cell Trait had the highest mean haemoglobin (Hb) at 11.34 ± 2.50 g/dl and mean corpuscular volume (MCV) at 79.89 ± 11.23 fl, along with a moderate red cell distribution width coefficient (RDW-CV) of 18.21 ± 18.56%. In contrast, β thalassemia major, with seven individuals, showed the lowest mean Hb (5.62 ± 2.52 g/dl) and MCV (62.80 ± 2.17 fl) but a higher RDW-CV of 31.70 ± 7.69%. SCD had Hb levels of 7.30 ± 2.68 g/dl and an MCV of 69.51 ± 15.18 fl, with a lower RDW-CV (19.00 ± 4.45%). Double heterozygous for sickle and beta-thalassemia condition had similar Hb levels (8.59 ± 1.86 g/dl) but lower MCV and mean corpuscular haemoglobin (MCH). Additionally, HbE Homozygous showed distinct characteristics, with lower MCV (50.6 fl) and MCH (14.4 pg). (Table 2)

Table 3 compares haematological parameters across various hemoglobinopathies, highlighting distinct patterns in HbA0, HbA2, HbF, and variant haemoglobin (HbS/HbC/HbD) levels.

Sickle Cell Trait showed a significant presence of variant hemoglobin HbS (HbS- 35.25 ± 6.66) and HbA0 of 53.74 ± 8.00. β-thalassemia trait exhibited a dominant HbA0 (83.26 ± 6.96) with a moderate HbA2 (5.22 ± 0.73) and minimal HbF (1.28 ± 1.15). β-thalassemia major had a significantly reduced HbA0 (26.91 ± 15.34) and elevated HbF (58.56 ± 16.79). Sickle Cell Disease had markedly lower HbA0 (7.71 ± 11.21) and higher HbS levels (HbS- 69.17 ± 9.83), which is a hallmark of the disease. The Double Heterozygous for β-thalassemia and Sickle Cell Disease showed lower HbA0 levels of 12.20 ± 17.27 with an elevated HbS (HbS- 68.30 ± 8.11) and HbF (14.99 ± 5.55), reflecting the combined effects of both conditions. (Fig 1)

Double Heterozygous for HbS and HbC displayed HbS levels of 37.7 and HbC levels of 0.6. Double Heterozygous for HbS and HbD displayed HbS levels of 24.7 with a D window of 41.1, indicating distinct variant haemoglobin profiles. (Fig 2)

HbE Homozygous and HbE Traits showed very low HbA0 (3.2 and 3.9, respectively) and high HbA2 levels (90.7 and 36), with minimal HbF. (Fig 3,4) The raised HbA2 levels are characteristic of HbE-related disorders. The retention time alone or in conjunction with %Hb could identify all the rare cases of haemoglobin variants. Family HPLC screening also helped in some cases. Overall, these results emphasise the variability in haemoglobin profiles across different hemoglobinopathies.

DISCUSSION

Thalassemia and other hemoglobinopathies are autosomal recessive disorders affecting the haemoglobin. Hemoglobinopathies represent a significant health concern in India, resulting in various financial, social, psychological, and medical challenges. Timely and accurate diagnosis of these conditions is imperative to protect future generations from their potential impact. For this purpose, various methods can be used like HPLC. Nowadays, HPLC is the most common diagnostic procedure for haemoglobinopathies in India as it is more accurate and rapid.

In the present study, we found that the majority of the cases (47.35%) fell within the age group of 15 to 30 years, followed by the age group of <15 years which comprised 28.03% of cases. Similar observations were found in a study conducted by Pathak V et al. [(1)]. In a study conducted by B Ganesh et al. [(14)], the majority of the cases (46.5%) belonged to the age group of <15 years. There was a notable prevalence of females (67.18%) in our study, which is in concordance with a study done by Pathak V et al. [(1)] and Anusha R et al. [(11)]. Contrary to our

findings, a study done by Kumar et al. [7], Chopra et al [8], and Patel et al. [15] found male predominance in their research while Uddin et al [13] found equal predominance of males and females in their study.

Several studies indicate that the β-thalassemia trait is the most prevalent haemoglobin disorder in various regions of India (Pathak V et al. [1], Mondal et al. [5], Biswas AK. et al. [6]). In contrast, our research identified the Sickle cell trait as the most commonly detected hemoglobinopathy, followed by β-thalassemia trait which is in concordance ton a study done in Orissa (Balgir RS et al. [9]). This discrepancy may be attributed to regional variations in prevalence. We also found four rare variants of HbE trait, HbE homozygous, double heterozygous for HbS and HbD, and double heterozygous for HbS and HbC in the present study.

In the present study, Thalassemia major cases and Hb E homozygous had the lowest Hb, MCV, MCH and higher RDW values as compared to the other hemoglobinopathies. In contrast, the cases of Sickle cell trait and Hb E trait had slightly decreased Hb with near normal MCV, MCH and RDW values. Similarly, β-thalassemia trait cases and double homozygous for sickle and β-thalassemia cases had a reduction of Hb, MCV and MCH. Similar observations were found by Mondal et al. [5], Anusha R et al. [11] and Venkataswany C et al. [12]. Kumar U et al. [7] found lower mean Hb, MCV and MCH values as compared to the present study.

HPLC has been established as a precise, specific, and sensitive method for identifying and quantifying various Hb fractions. In a standard adult HPLC analysis, the predominant haemoglobin species detected is haemoglobin A (HbA). This is accompanied by a minor percentage of haemoglobin A2 (HbA2), typically below 3.9%, and trace levels of haemoglobin F (HbF), occurring at less than 1%. In the present study, the β-thalassemia trait cases showed slightly higher mean Hb A2 values (5.22 ± 0.73%). Thus, borderline HbA2 levels should be carefully evaluated as they can point towards β-thalassemia trait cases. β-thalassemia major cases had elevated Hb F levels (58.56%). Similarly, Khera R et al. [10] in their study had a mean Hb F of 65.8%. Hb S was detected in cases of Sickle cell trait (mean HbS =35.25 ± 6.66%), Sickle cell disease (mean HbS =69.17 ± 9.83%), Double Heterozygous For β Thalassemia and Sickle Cell Disease cases (mean HbS= 68.30 ± 8.11%), Double Heterozygous for HbS and HbC (HbS= 37.7%) and Double Heterozygous for HbS and HbD (mean Hb S = 24.7%)

The solubility test was positive in all the cases. Additionally, the C window and D window were seen in cases of Double Heterozygous for HbS and HbC and Double Heterozygous for HbS and HbD, respectively. Similar observations were found in other studies. (Khera R et al. [10], Mondal et al. [5], Biswas AK et al. [6]. HbE is the most prevalent haemoglobin variant in Asia, second only to HbS. This variant arises from the substitution of a lysine residue for a glutamic acid residue at position 26 of the beta globin chain. During HPLC analysis, HbE is typically observed to elute within the A2 window. [6] HbE homozygous cases have HbA2 values between 60 and 90% and Hb E trait have HbA2 values between 20 and 40%. In the present study, the HbA2 in Hb E homozygous was 90.7%, and it was 36% in Hb E trait.

CONCLUSION

In conclusion, haematological parameters, along with peripheral blood smears, play an essential role in the screening of hemoglobinopathies. It provides valuable initial clues, helping to narrow down potential haemoglobin disorders. HPLC, with its high sensitivity and specificity, complements these findings by enabling precise identification and quantification of abnormal haemoglobin variants. These diagnostic tools allow for effective differentiation among various hemoglobinopathies, facilitating timely intervention, genetic counselling, and family planning guidance. Advancements in HPLC technology and a deeper understanding of haematological markers will continue to enhance the detection and management of

hemoglobinopathies, ultimately improving patient outcomes and reducing the disease burden on affected populations.

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Table 1. Demographic characteristics in the study population

Variable		Frequency	Percentage
Age (years)	< 15	164	28.03
	15-30	277	47.35
	30-45	118	20.17
	> 45	26	4.44
Gender	Male	192	32.82
	Female	393	67.18
Marital Status	Married	324	55.38
	Unmarried	261	44.62

Table 2. Hematological parameters in various hemoglobinopathies in the study population

Condition	Frequency (%)	Hb (g/dl)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW-CV
Sickle Cell Trait	392 (67)	11.34 ± 2.50	79.89 ± 11.23	26.82 ± 10.70	30.52 ± 2.41	18.21 ± 18.56
β Thalassemia Trait	83 (14.19)	9.75 ± 2.84	70.09 ± 13.85	22.61 ± 6.15	29.76 ± 1.70	19.28 ± 5.75
Sickle Cell Disease	78 (13.33)	7.30 ± 2.68	69.51 ± 15.18	27.66 ± 9.95	30.77 ± 2.47	19.00 ± 4.45
Double Heterozygous For β Thalassemia and Sickle Cell Disease	21 (3.59)	8.59 ± 1.86	71.16 ± 15.91	22.33 ± 4.88	29.84 ± 3.37	19.44 ± 3.55
β Thalassemia Major	7 (1.2)	5.62 ± 2.52	62.80 ± 2.17	22.77 ± 2.30	29.77 ± 1.91	31.70 ± 7.69
Double Heterozygous for HbS and HbC	1 (0.17)	12	72.7	24.2	33.3	14.7
Double Heterozygous for HbS and HbD	1 (0.17)	11	71.3	23.2	32.3	17.5
HbE Homozygous	1 (0.17)	6.7	50.6	14.4	28.5	33.9
HbE Trait	1 (0.17)	12.1	84.2	24.6	29	18.3

Table 3. Haemoglobin characterization by HPLC in various hemoglobinopathies in the study population

Condition	HbA0 % (Mean ± SD)	HbA2 % (Mean ± SD)	HbF % (Mean ± SD)	Variant Hb % (HbS/HbC/HbD)
Sickle Cell Trait	53.74 ± 8.00	2.83 ± 3.08	1.16 ± 1.46	HbS- 35.25 ± 6.66
Beta Thalassemia Trait	83.26 ± 6.96	5.22 ± 0.73	1.28 ± 1.15	-
Sickle Cell Disease	7.71 ± 11.21	2.94 ± 1.17	7.74 ± 4.53	HbS- 69.17 ± 9.83
Double Heterozygous for β-thalassemia and Sickle Cell Disease	12.20 ± 17.27	4.82 ± 0.81	14.99 ± 5.55	HbS- 68.30 ± 8.11
B Thalassemia Major	26.91 ± 15.34	3.64 ± 1.18	58.56 ± 16.79	-
Double Heterozygous for HbS and HbC	50.4	3.5	-	HbS- 37.7, HbC- 0.6
Double Heterozygous for HbS and HbD	3.4	1.7	20.6	HbS- 24.7, HbD- 41.1
HbE Homozygous	3.2	90.7	3.5	-
HbE Trait	3.9	36	1.3	-

Figure 1. Double heterozygous for HbS and thalassemia showing increased Hb A2, Hb F and Hb S window

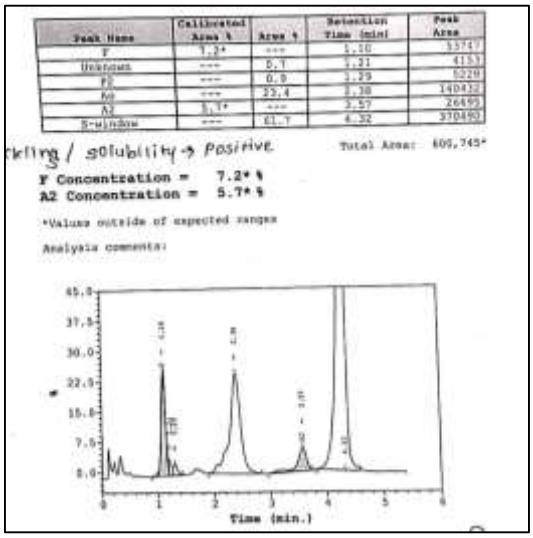
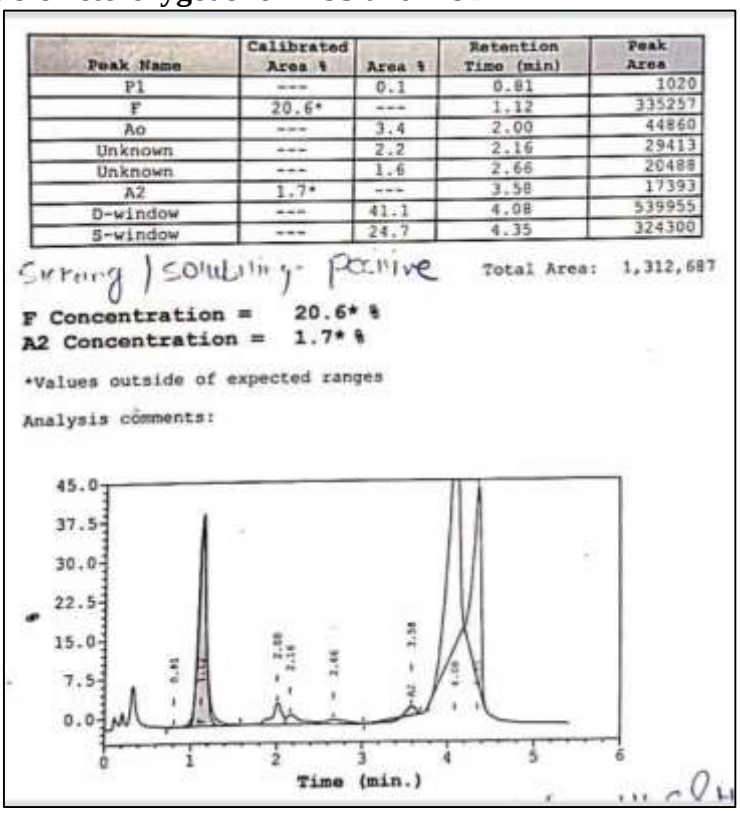


Figure 2. HPLC chromatogram showing both S and D windows in with demarcation line in case of double heterozygous for HbS and HbD



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.81	1020
F	20.6*	---	1.12	335257
Ao	---	3.4	2.00	44860
Unknown	---	2.2	2.16	29413
Unknown	---	1.6	2.66	20488
A2	1.7*	---	3.58	17393
D-window	---	41.1	4.08	539955
S-window	---	24.7	4.35	324300

Sickling | solubility: Positive

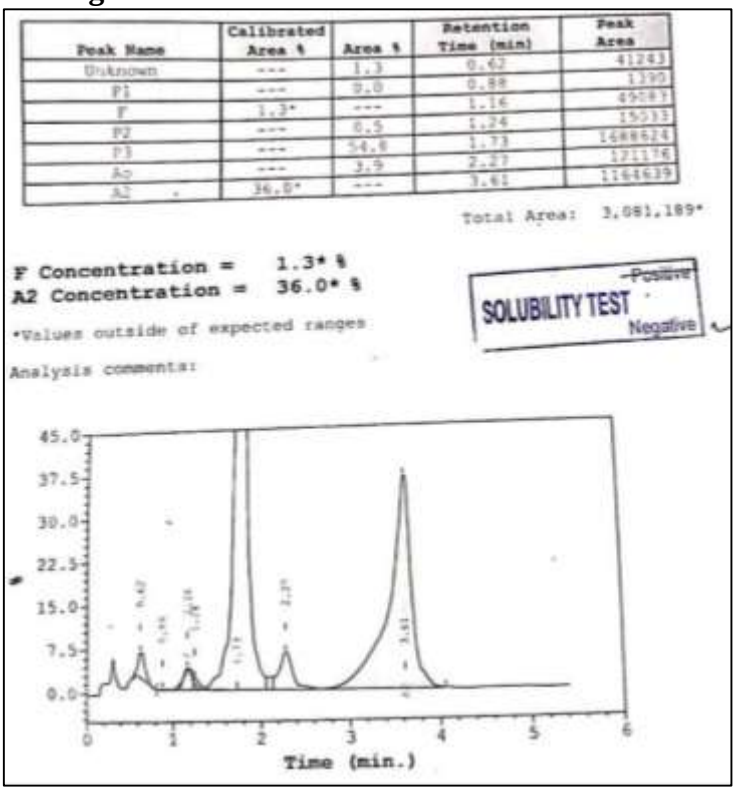
Total Area: 1,312,687

F Concentration = 20.6* %
A2 Concentration = 1.7* %

*Values outside of expected ranges

Analysis comments:

Figure 3. HPLC showing HbE trait with elevated HbA2 levels of 36%



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	1.3	0.42	41243
P1	---	0.0	0.88	1390
F	1.3*	---	1.16	49087
P2	---	0.5	1.24	15033
P3	---	54.8	1.73	1688624
Ao	---	3.9	2.27	121176
A2	36.8*	---	3.61	1164639

Total Area: 3,081,189*

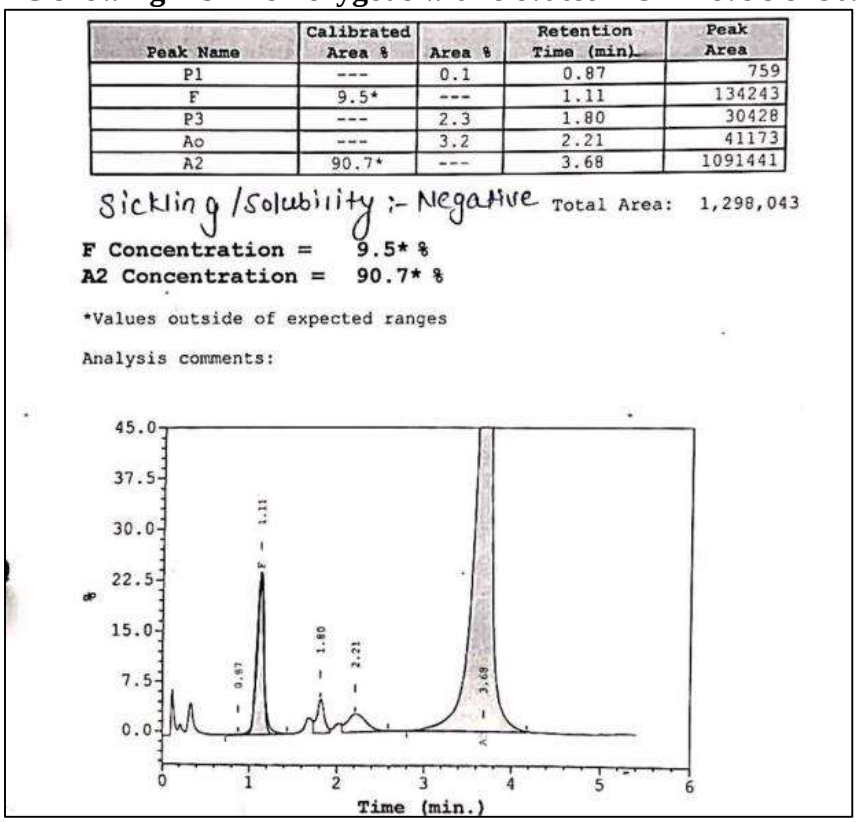
F Concentration = 1.3* %
A2 Concentration = 36.0* %

SOLUBILITY TEST Positive Negative

*Values outside of expected ranges

Analysis comments:

Figure 4. HPLC showing HbE homozygous with elevated HbA2 levels of 90.7%



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.87	759
F	9.5*	---	1.11	134243
P3	---	2.3	1.80	30428
Ao	---	3.2	2.21	41173
A2	90.7*	---	3.68	1091441

Sickling/Solubility :- Negative Total Area: 1,298,043

F Concentration = 9.5* %
A2 Concentration = 90.7* %

*Values outside of expected ranges

Analysis comments: