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Original Article

Utility of HPLC in the Evaluation of Hemoglobinopathies in Microcytic Hypochromic Anaemia

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ABSTRACT

Background: Microcytic hypochromic anaemia is a common hematological condition with diverse etiologies, including iron deficiency and hemoglobinopathies. Accurate differentiation is essential for appropriate management. High-performance liquid chromatography (HPLC) has emerged as a reliable diagnostic tool for identifying hemoglobin variants.The study was conducted with the aim of evaluating the diagnostic utility of High-Performance Liquid Chromatography (HPLC) in detecting hemoglobinopathies in patients with microcytic hypochromic anemia and studying their spectrum and distribution.

Materials and Methods: This prospective observational study included 380 patients with microcytic hypochromic anaemia. Complete blood counts and peripheral smears were performed, followed by HPLC analysis using the Bio-Rad Variant II system. Hemoglobin fractions were analyzed based on retention time and percentage values. Statistical analysis was done using SPSS version 23.

Results: Among 380 cases, 53 (13.9%) showed abnormal HPLC findings. β-thalassemia trait was the most common abnormality (60.3%), followed by β-thalassemia major, HbE trait, HbD Punjab variants, and other rare hemoglobinopathies. Significant variations were observed in HbA_0, HbA_2, and HbF levels ($p < 0.001$). HbA_2 was elevated in β-thalassemia trait, while HbF was markedly increased in β-thalassemia major. D-window and S-window analyses showed high specificity for HbD and HbS detection, respectively. The majority of patients were females (60%) and belonged to younger age groups.

Conclusion: HPLC is a rapid, reliable, and effective diagnostic modality for detecting both common and rare hemoglobinopathies in microcytic hypochromic anaemia. Early identification facilitates appropriate management, genetic counseling, and prevention strategies.

Keywords: *HPLC, Hemoglobinopathies, Microcytic hypochromic anaemia, β-thalassemia, Hb variants.*

INTRODUCTION

Anaemia refers to a decrease in Hb levels below the lower reference limit of normal for the given age and gender.[1]

Anaemia can be morphologically classified according to MCV (Mean Corpuscular Volume)[2]
1) Normocytic – MCV 80-100 fL
2) Macrocytic – MCV >100 fL
3) Microcytic – MCV <80 fL

Mean corpuscular haemoglobin (MCH) represents the average haemoglobin content per red blood cell. Normal value of MCH is between 27 to 33 pg. Microcytic hypochromic anaemia presents significant diagnostic challenges as its

differential diagnosis encompasses iron deficiency anaemia, thalassemias, various hemoglobinopathies, anaemia of chronic disease and sideroblastic anaemia.[3]

Hemoglobinopathies are inherited disorders affecting haemoglobin structure or synthesis, representing the most prevalent hereditary single gene conditions globally with approximately 300,000-400,000 affected infants born annually.[4]

About 5% of people worldwide are carriers of hemoglobinopathies.[5] HPLC has become a preferred diagnostic technique for the detection of Hb variants due to its high resolution, reproducibility, and accuracy.[6]

The Bio-Rad Variant system operates on the principle of cation exchange high-performance liquid chromatography, separating haemoglobin fractions according to their charge-based interactions with the stationary phase. Following automated haemolysis and dilution of EDTA-anticoagulated blood, samples are injected into the column, where hemoglobin variants are eluted at specific retention times using a programmed buffer system in which the salt concentration is gradually increased, and the entire analysis is generally completed within about 5–6 minutes.[7]

The present study was conducted with the aim of evaluating the spectrum of hemoglobinopathies using High-Performance Liquid Chromatography (HPLC) in patients presenting with microcytic hypochromic anemia, and to correlate the haematological parameters with the clinico-haematological characteristics of the identified hemoglobinopathies.

MATERIALS AND METHODS

The study included patients with a complete blood count (CBC) suggestive of microcytic hypochromic anemia, defined by mean corpuscular volume (MCV) ≤80 fL and mean corpuscular hemoglobin (MCH) ≤27 pg, along with supportive findings on peripheral smear. Patients who were refractory to conventional treatment for microcytic hypochromic anemia or had unexplained anemia were also included. Exclusion criteria comprised patients with peripheral smear findings other than microcytic hypochromic anemia, a history of blood transfusion within the past four weeks, age less than one year, presence of known comorbid causes of microcytic hypochromic anemia, and those diagnosed with iron deficiency anemia.

A total of 5 mL of venous blood was collected in an EDTA vacutainer from each patient. Complete blood count (CBC) was performed using the Med Source Alpha Count 60 automated cell counter. A peripheral blood smear was then prepared. Samples showing MCV ≤80 fL, MCH ≤27 pg, and peripheral blood film findings suggestive of microcytic hypochromic anemia were further analyzed using high-performance liquid chromatography (HPLC) on the Bio-Rad Variant II system for the detection of various hemoglobinopathies. Reports and chromatograms generated were studied and interpreted by observing HbA_2 and Hb F concentrations for β-thalassemia, and by assessing retention time and area percentage of other peaks and windows for structural variants. Each chromatogram showed peaks of Hb A_0, A_2, and Hb F along with the C window, D window, S window, and two minor peaks, P2 and P3. Several hemoglobin variants eluted in the same window; they were provisionally diagnosed based on retention time and area percentage. The sickling test was carried out in a case of sickle cell trait with β- thalassemia trait. The Kleihauer–Betke test was performed in cases of HPFH (Hereditary Persistence of Fetal Hemoglobin) based on the principle of acid elution, wherein adult hemoglobin is eluted by acid while fetal hemoglobin remains resistant. The data was analyzed using SPSS version 23 and Microsoft Excel.

RESULTS AND OBSERVATIONS

The total study population comprised 380 patients, all of whom had microcytic hypochromic anemia. The majority of patients belonged to the 0–15 years age group (44.2%), followed by 16–30 years (27.1%), 31–45 years (19.7%), 46–60 years (7.4%), and 61–75 years (1.6%). There was a female predominance in the study population, with females accounting for 60.0% and males 40.0% of the cases. In terms of severity, most patients presented with moderate anemia (41.1%), followed by mild anemia (39.2%), while severe anemia was observed in 19.7% of the patients.

Out of the total 380 patients with microcytic hypochromic anemia, 327 (86.1%) showed normal HPLC findings, while 53 (13.9%) exhibited abnormalities. Among 53 abnormal cases:

- β-thalassemia trait (BTT): 32 cases (60.3%) – most common
- BTT with Iron Deficiency Anaemia: 4 cases
- β-thalassemia major: 4 cases
- HbE trait: 4 cases
- HbD Punjab (heterozygous): 3 cases
- HPFH (heterozygous): 2 cases
- Rare variants: HbD Iran, HbJ Meerut, sickle cell trait with BTT, HbD Punjab Homozygous(1 case each)

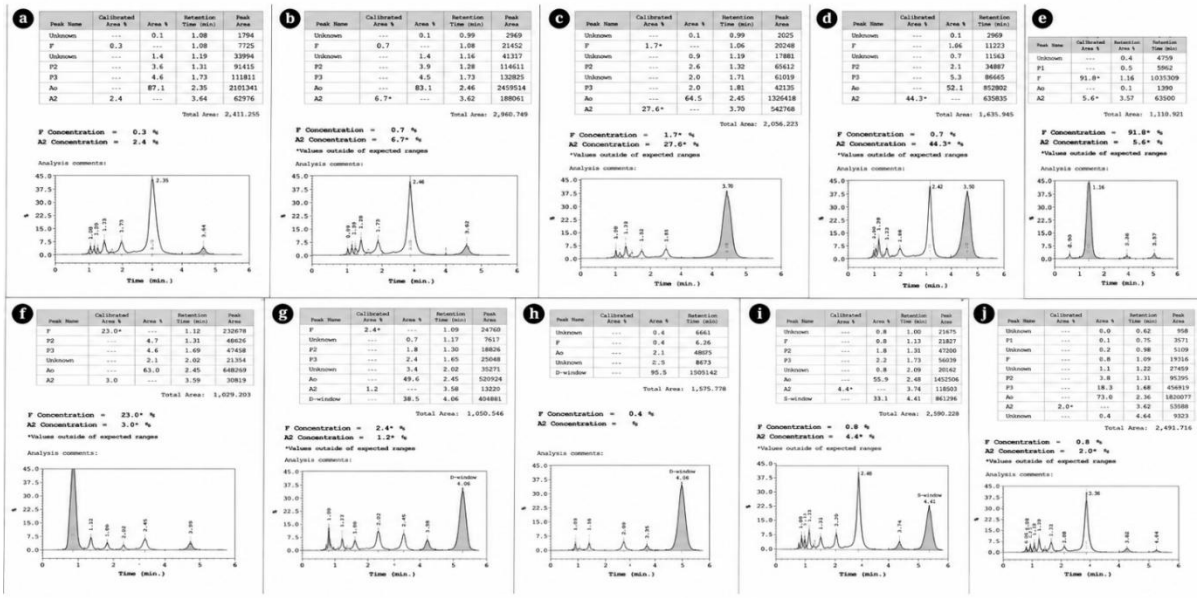

IMAGE 1:HPLC Chromatogram showing :a)Normal Study, b) BTT, c) HbE Trait,d) HbD Iran, e) β Thalassemia Major,f) HPFH Heterozygous,g) HbD Punjab Heterozygous,h) HbD Punjab Homozygous,i) Sickle Cell Trait with BTT, j) HbJ Meerut .A total of 53 cases with abnormal HPLC findings were analyzed for age-wise distribution. The maximum cases were observed in the 16–30 years age group (21 cases), followed by the 0–15 years (16 cases) and 31–45 years (14 cases), whereas one case each was seen in the 46–60 and 61–75 years age groups.All cases of β Thalassemia Major, HbD Punjab homozygous, and HPFH heterozygous were diagnosed exclusively in the 0– 15 years age group, highlighting their early clinical presentation.

Among the 53 cases with HPLC abnormalities, females (33) outnumbered males (20), with β-thalassemia trait being the most common finding and showing female predominance.

Among the 53 cases with HPLC abnormalities, the majority were from Punjab, which is expected as the study was conducted in this region, leading to higher case representation. A statistically significant association was observed between hemoglobinopathies and religion in Punjab ($\chi^2 = 27.292$, $p = 0.038$). β-thalassemia trait was the most common abnormality, predominantly seen in Hindus and Sikhs, with a higher frequency among Hindus. Other conditions, including BTT with IDA, β-thalassemia major, HbD Punjab variants, and HPFH, were also largely distributed within these groups, indicating a pattern influenced by regional and community distribution.

Bihar (n=5) showed cases exclusively in Hindus, including BTT, β-thalassemia major, HbE trait, and HPFH heterozygous, while Haryana, Himachal Pradesh, and Rajasthan each reported isolated cases predominantly of BTT or BTT with IDA in Hindus.

In Uttar Pradesh (n=5), BTT was the most common abnormality among Hindus, along with isolated cases of HbJ Meerut (Hindu) and HbE trait (Muslim).

β-thalassemia trait was most commonly associated with fatigue and detection through family screening, while β-thalassemia major presented with pediatric symptoms like excessive crying and poor feeding.

Overall, fatigue was the predominant complaint, and a statistically significant association was observed between hemoglobin abnormalities and presenting symptoms ($\chi^2 = 128.80$, $p = 0.005$).

The assessment of hemoglobinopathies based on past history revealed that a substantial proportion of cases (45.3%) had a positive past history of anemia or blood transfusion while 54.7% had no significant past history.

In the study all 53 cases (100%) had microcytic hypochromic red cell morphology on PBS as per the inclusion criteria. Among additional morphological features, tear drop cells were observed in 2 cases, predominantly in BTT with IDA and β thalassemia major, while elliptocytes were seen in 1 case of BTT with IDA. Target cells were noted in 5 cases, mainly associated with thalassemia major and HPFH, reflecting disordered hemoglobin synthesis. nRBCs were seen in 1 case, and polychromasia was noted in 5 cases, particularly in thalassemia major and HPFH, indicating increased erythropoietic activity. A single case of sickle cells was identified in the case of sickle cell trait with beta thalassemia

TABLE 1: DISTRIBUTION AND COMPARISON OF VARIOUS HEMOGLOBIN ABNORMALITIES WITH RED CELL INDICES AND HEMOGLOBIN LEVELS

Abnormalities	N	MCV(fL)	MCH(pg)	MCHC(g/dL)	RDW-CV(%)	HB(g/dL)
BTT	**32**	66.33 ± 8.90	20.03 ± 3.16	29.72 ± 3.30	15.91 ± 2.58	8.83 ± 1.89
BTT with IDA	**4**	69.03 ± 5.42	20.73 ± 2.35	29.65 ± 1.30	14.90 ± 0.88	8.65 ± 1.05
β Thalassemia Major	**4**	63.25 ± 0.96	22.15 ± 2.75	31.83 ± 2.62	19.43 ± 6.16	7.20 ± 2.65
HbE Trait	**4**	76.48 ± 2.48	24.75 ± 2.08	31.05 ± 1.4	14.40 ± 1.64	10.75 ± 0.06
HbD Punjab Heterozygous	**3**	77.67 ± 1.53	24.27 ± 0.25	28.97 ± 0.91	16.20 ± 2.43	9.30 ± 1.92
HPFH Heterozygous	**2**	78.10 ± 0.14	25.40 ± 1.98	30.55 ± 0.35	20.55 ± 1.20	10.45 ± 0.49
HbD Iran	**1**	68.00 ± 0.00	21.50 ± 0.00	27.40 ± 0.00	15.40 ± 0.00	8.00 ± 0.00
HbD Punjab Homozygous	**1**	63.00 ± 0.00	26.10 ± 0.00	33.00 ± 0.00	17.30 ± 0.00	6.50 ± 0.00
HbJ Meerut	**1**	79.00 ± 0.00	26.40 ± 0.00	31.10 ± 0.00	14.50 ± 0.00	10.80 ± 0.00
Sickle Cell Trait with BTT	**1**	76.00 ± 0.00	26.80 ± 0.00	30.00 ± 0.00	16.50 ± 0.00	9.40 ± 0.00
P value		0.047	0.005	0.840	0.185	0.224
Significance		S	HS	NS	NS	NS

HbA_0 levels showed a statistically significant variation across hemoglobinopathies ($p < 0.001$). β-thalassemia trait exhibited high HbA_0 levels (83.62 ± 1.63%), with slightly higher values in cases with coexisting iron deficiency (86.50%). In contrast, markedly reduced HbA_0 levels were observed in β-thalassemia major (7.93%) and HbD Punjab homozygous state (3.10%), while HbE trait demonstrated intermediate levels (~62%). These findings indicate that HbA_0 is significantly reduced in severe disorders and reflects replacement by variant hemoglobin fractions.

HbA_2 levels showed a statistically significant variation across hemoglobinopathies ($p < 0.001$). β-thalassemia trait demonstrated elevated HbA_2 levels (5.26 ± 0.67%), while cases with coexisting iron deficiency showed borderline values (~3.5%). Markedly high HbA_2 levels were observed in HbE trait (~27–28%) and HbD Iran (~44.3%) due to co-elution in the A_2 window, whereas HbD Punjab showed reduced or absent HbA_2. These findings confirm HbA_2 as a key diagnostic marker for β-thalassemia trait, although its interpretation may be affected by iron deficiency and variant hemoglobins.

HbF levels showed a statistically significant variation across hemoglobinopathies ($p < 0.001$). β-thalassemia major demonstrated markedly elevated HbF levels (~92.65%), while HPFH showed moderately increased levels (~24%). In contrast, β-thalassemia trait exhibited only mild elevation (~1.3%), and other variants had near-normal HbF levels. These findings highlight the utility of HbF estimation in distinguishing severe forms from trait conditions.

D-window analysis demonstrated a distinct and highly specific pattern for HbD variants. HbD Punjab heterozygous cases showed values of approximately 30–40%, while the homozygous state exhibited markedly elevated levels around 95%. In contrast, all other hemoglobinopathies showed negligible or absent D-window peaks. These findings establish the D-window as a highly specific diagnostic marker for HbD variants.

S-window analysis revealed that only the case of sickle cell trait with β-thalassemia trait showed a significant elevation (~33%), while all other hemoglobinopathies demonstrated negligible or absent values. This finding confirms the high specificity of the S-window for detection of HbS. Sickle cell with BTT showed positive sickling test.

HbE trait cases showed elevated HbA_2/E (~27–28%) with mild anaemia.
HbD Iran demonstrated falsely elevated HbA_2 due to co-elution .
HbJ Meerut presented with P3 peak elevation.

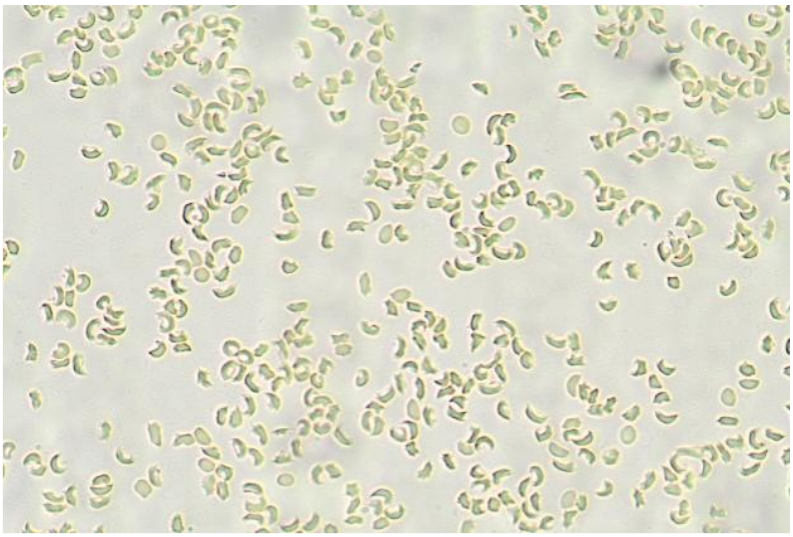

IMAGE 2: Positive Sickling Test (400X)

DISCUSSION

In the present study, microcytic hypochromic anaemia was predominantly observed in younger age groups, with 44.2% of cases occurring in individuals aged 0–15 years, followed by 16–30 years (27.1%). This trend is consistent with previous studies by Singh et al. [8]and Gupta et al.[9], which also reported a higher burden of anaemia in individuals below 30 years. The increased prevalence in younger populations may be attributed to higher nutritional demands, dietary deficiencies, and early manifestation of inherited hemoglobinopathies.

A clear female predominance (60%) was observed, aligning with findings from Singh et al.[8] and Gupta et al[9]. This can be explained by increased iron requirements, menstrual blood loss, pregnancy, and higher screening rates in females, particularly during antenatal care.

Most patients presented with mild to moderate anaemia (80.3%), while severe anaemia constituted a smaller proportion (19.7%). Similar observations have been reported in previous studies, suggesting that early detection and routine screening contribute to identification at less severe stages.

The prevalence of abnormal HPLC findings in this study was 13.9%, comparable to Mondal et al.[10] (12.17%) and Sachdev et al. [11](12.6%), indicating a consistent burden of hemoglobinopathies in Indian populations. However, a higher prevalence reported by Singh et al.[8] (20.12%) may reflect regional variation and larger sample size.

β-thalassemia trait emerged as the most common abnormality, followed by structural variants such as HbE and HbD, consistent across all studies.

Although α-thalassemia is known to be prevalent in the Indian population, as demonstrated by Nadkarni et al.[12], its diagnosis cannot be reliably established on HPLC alone due to the absence of definitive HbA_2 or HbF alterations, as highlighted by Khera et al.[13]

In the present study, among 53 cases with abnormal HPLC findings, the majority were observed in the 16–30 years age group (39.6%), followed by 0–15 years (30.2%) and 31–45 years (26.4%), with very less cases in more than 45 years of age. Rao et al.[14]mentioned that almost 65% of cases showed up before 25 years of age.
These findings reinforce the utility of HPLC as a reliable and sensitive diagnostic tool for screening hemoglobinopathies.

Overall, the study highlights the importance of early screening, especially in high-risk populations, and emphasizes the need for careful interpretation of HPLC findings in conjunction with clinical and hematological parameters.

CONCLUSION

The present study demonstrates that High-Performance Liquid Chromatography (HPLC) is a rapid, reliable, and efficient method for the detection and characterization of hemoglobinopathies in patients with microcytic hypochromic anemia, with abnormal hemoglobin patterns identified in 13.9% of cases. Detected abnormalities included β-thalassemia trait, β-thalassemia major, HbE trait, HbD variants, hereditary persistence of fetal hemoglobin, HbJ Meerut, and sickle cell trait with β-thalassemia trait, highlighting the wide spectrum of variants identifiable by HPLC. The technique proved effective in detecting both common and rare hemoglobin variants, supporting its role as an ideal screening tool in the Indian

population. Molecular studies may further aid in definitive diagnosis, carrier detection, prenatal diagnosis, and genetic counseling. However, HPLC has limitations in diagnosing conditions such as α-thalassemia and certain rare variants without molecular confirmation, and interpretation may require correlation with clinical and hematological findings. Additionally, as this was a hospital-based study, the possibility of selection bias exists, and the findings may not represent the true population prevalence of hemoglobinopathies.

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