HPLC in Characterization of Hemoglobin Profile in Thalassemia Syndromes and Hemoglobinopathies: A Clinicohematological Correlation

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Abstract High-performance liquid chromatography (HPLC) is a technique introduced for the accurate diagnosis of hemoglobinopathies and thalassemias. The advantage of the HPLC system is the excellent resolution, reproducibility & quantification of several normal & abnormal hemoglobin resulting in accurate diagnosis of thalassemia syndromes. The purpose of this study is to evaluate the HPLC technique in diagnosis of thalassemia syndromes and also correlate it with clinicohematological profile in these cases. A total of 110 cases were diagnosed as thalassemias and hemoglobinopathies by Bio- Rad variant II HPLC system by β -thal short program. The retention times, proportion of the haemoglobin (%), and peak characteristics for all hemoglobin (Hb) fractions were recorded. Alkaline Hb electrophoresis was performed in each case. Other tests performed were HbF estimation by Betke's method, brilliant cresyl blue preparation for HbH inclusion bodies, sickling tests using 2 % metabisulphite and serum Ferritin estimation. Family studies were carried out wherever necessary. Of 110 cases included in the study, 87 cases were of thalassemic disorders and 23 cases were of hemoglobinopathies. Four Hb variants were identified including HbD, HbE, HbS, HbJ Oxford. There was a

significant decrease in the level of HbA2 associated with iron deficiency anemia. The mean HbA2 levels in both iron deplete and iron replete groups were clearly $>4\%$, suggesting that HPLC identified nearly all high HbA2 β -thalassemia trait even in spite of iron deficiency.

Keywords Thalassemia · Hemoglobinopathy · HPLC

Introduction

Inherited abnormalities of hemoglobin (Hb) include a myriad of disorders ranging from thalassemia syndromes to structurally abnormal Hb variants/hemoglobinopathies. Detailed clinical history, complete hematologic evaluation along with newer ancillary techniques including High-performance liquid chromatography (HPLC), when used systematically can achieve laboratory diagnosis of thalassemia syndromes and hemoglobinopathies. Family studies can be of immense importance in diagnosing certain problematic cases.

The aim of the present study was to evaluate the role of cation exchange HPLC (CE-HPLC) in the diagnosis of thalassaemia syndromes/hemoglobinopathies and to correlate Hb profile in such cases with clinico-hematological features.

Materials and Methods

This was a prospective study carried out in the Department of Pathology, Maulana Azad Medical College (MAMC), New Delhi, over 18 months duration from October 2008 to April 2010. A total of 110 cases of thalassemia syndromes and hemoglobinopathies were included in the study.

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Consecutive 1,500 blood samples sent for suspected thalassaemia/hemoglobinopathy work-up were analyzed by CE-HPLC (BioRad laboratories, California, USA) using Variant II β -thal short program. This included mainly transfusion requiring children and adults, antenatal cases and their family members. No absolute exclusion criteria were used but for patients requiring blood transfusions, sampling was deferred for at least 4 week after or just before next transfusion. Written consent was taken from all patients for using their sample for diagnostic purpose. About 2 ml of blood sample was collected in EDTA vacutainers and was analyzed in automated cell counter (Sysmex XT-2000i) for complete blood counts. Samples were stored at 4–8 °C and were analyzed in batches within 1 week. Hemolysate preparation was also used for HbF estimation by alkali denaturation test of Betke and run on agarose gel electrophoresis at alkaline pH of 8.6 from the same sample in all the cases. The presence of HbH was confirmed by using brilliant cresyl blue test for HbH inclusions. Presence of HbS was confirmed by sickling test using 2 % sodium metabisulphite wherever required. Serum ferritin levels were performed using chemiluminescence assay, (Serum Ferritin kit; Roche Diagnostics) to assess for iron status in all the cases in order to rule out IDA. Cut off value of iron deficiency was taken as <30 ng/ml in males and <15 ng/ml in females. Continuous variables were expressed as mean $\pm$ SD. Categorical variables were expressed as frequencies and percentages. Two sample *t* test was applied for significance of correlation between continuous variables. All statistical analysis was performed using SPSS 16.0 statistical software.

Results

Of the 110 cases included in the study, 87 cases were of thalassemic disorders and 23 cases were of hemoglobinopathies. Out of 87 cases of thalassemic disorders, there were 62 cases of β -thalassemia trait, 6 cases of thalassemia major, 5 cases of thalassemia intermedia and 4 cases of HbH disease. 10 cases were compound heterozygotes, 2 of which were for HbS/ β thalassemia and 8 for HbE/ β -thalassemia. Hemoglobinopathy cases included 7 cases of HbE trait, 8 cases of HbD Punjab trait, 1 case each of HbS trait, HbD Iran, HbDD, HbSS and HbEE, 2 cases of HbJ Oxford, 1 case was compound heterozygote for HbS and HbE. Mean $\pm$ SD values of hematological parameters in each of these cases is given in Table 1.

Pallor is usually the first presenting sign in thalassemia syndromes, accompanied by splenomegaly of variable size [1]. Our 19 patients of thalassemia syndromes presented with pallor (21.8 %) and splenomegaly was present in 18 (22.9 %) cases (Thal major 6/6, Thal inter 2/5, HbH 3/4

HbE/thalassemia 6/8, HbS thalassemia 1/2). Size of spleen was correlated with HbF concentration in these cases however no significant correlation was found ($p = 0.548$). Growth and development was affected in 50 % of our cases. We found thalassemic facies in 8 (9.1 %) cases, youngest was 4 years old while cases <3 years of age did not show presence of hemolytic facies. It appears that bone changes manifest after the age of 4 years. Various haemoglobin fractions obtained on HPLC leading to presumptive diagnosis in each case are shown in Table 2.

No definite correlation was obtained between HbF and clinical and other hematological parameters in thalassemia major group. Significant positive correlation was found between HbF and spleen size ($p < 0.05$) while significant negative correlation was found between HbF and MCV (<0.05) in thalassemia intermedia group.

Although linear regression analysis of HbF measured by HPLC showed excellent correlation ($r = 0.909$) with values obtained by alkali denaturation method, the mean values of HbF detected by HPLC (61.06 %) were substantially higher than those determined by the alkali denaturation method (45.6 %).

Cases of HbE/ β thalassemia were divided into severe and non-severe groups and a comparison was made between the two groups with respect to various clinical and hematological parameters. No significant difference was found between the two groups with respect to MCV, MCH, RBC count, HbF and HbE. No definite correlation was observed between HbF/HbE and other variables like age, palpable liver and spleen size, Hb, MCV and MCH ($p > 0.05$).

Mean S. ferritin in our cases of β -thalassemia trait was 71.9 ng/ml. 8 out of 62 patients (12.9 %) were found to be iron deficient (mean S.Ferritin 13.1 ng/ml). Hematological parameters in iron deplete and iron replete groups of thalassemia trait are given in Table 3.

However, HbA₂ levels were also found to be significantly lower in the iron deficient group (mean 4.3 %) as compared to iron replete group (mean 6.0 %) ($p < 0.001$). The mean HbA₂ levels in both the groups were clearly >4 %, suggesting that HPLC identified nearly all high HbA₂ β -thalassemia trait even in spite of iron deficiency.

Authors encountered 6 (8 %) cases where HbA₂ ranged between 3.5–3.9 % but these cases were excluded from the study and were not worked up further.

Retention times and proportions of haemoglobin variants

As expected, out of 110 positive cases, β -thalassaemia trait was detected most frequently i.e., in 62 (56.3 %) cases with cut-off value of HbA₂ being >4.0 per cent and β -thalassaemia major/intermedia was seen in 11 (10 %) cases.

Table 1 Hematological parameters (mean $\pm$ SD, range) in Thalassemia Syndromes and Hemoglobinopathies

	No. of cases (%)	Hb (g/dl)	MCV (fl)	MCH (pg)	MCHC (%)	RBC count ($\times 10^6/\mu\text{l}$)	RDW-CV (%)
β -Thal trait	62 (56.3)	9.3 $\pm$ 2.7 (3.2–14.4)	62.7 $\pm$ 7.7 (49.8–85.2)	19.56 $\pm$ 2.5 (10.9–27.2)	31.4 $\pm$ 2.3 (20.9–36)	5.18 $\pm$ 2.7 (1.65–7.12)	19.5 $\pm$ 4.4 (14.6–36.7)
β -Thal major	6 (5.4)	5.03 $\pm$ 1.9 (1.5–7.6)	64.9 $\pm$ 7.9 (56.7–79.7)	20.0 $\pm$ 1.9 (17.7–22.3)	31.0 $\pm$ 3.9 (23.8–34)	2.27 $\pm$ 1.2 (0.79–4.3)	26.5 $\pm$ 6.7 (20.2–36.2)
β -Thal Inter	5 (4.5)	5.9 $\pm$ 1.6 (3.1–6.8)	69.0 $\pm$ 6.8 (62–76.7)	19.5 $\pm$ 1.5 (17.8–21.3)	28.5 $\pm$ 3.3 (23.2–32.5)	2.9 $\pm$ 0.6 (1.63–3.39))	27.1 $\pm$ 5.8 (20–32.8)
HbS/ β -thal	2 (1.8)	8.4 $\pm$ 0.4 (8.1–8.6)	64.4 $\pm$ 5.4 (60.6–68.2)	21.8 $\pm$ 1.8 (20.5–23)	33.8 $\pm$ 0.1 (33.7–33.8)	3.9 $\pm$ 0.2 (2.71–3.96)	19.6 $\pm$ 1.2 (18.5–20.7)
HbE/ β -thal	8 (7.2)	4.9 $\pm$ 1.9 (2.7–8.2)	71.7 $\pm$ 5.8 (64.3–79)	19.6 $\pm$ 1.8 (17.2–22.7)	27.3 $\pm$ 3.3 (22.3–33.8)	2.5 $\pm$ 0.7 (1.57–3.8)	31.9 $\pm$ 5.3 (22.6–33.8)
HbH ds	4 (3.6)	5.8 $\pm$ 2.1 (3.5–7.6)	61.1 $\pm$ 2.7 (58.6–64.9)	17.3 $\pm$ 1.1 (15.6–18)	28.9 $\pm$ 0.7 (27.8–29.3)	3.3 $\pm$ 1.2 (1.94–4.3)	28.1 $\pm$ 7.5 (18.3–33.9)
HbS trait	1 (0.9)	9.9	73.2	23.3	31.8	4.25	16.1
Homo Sickle (HbSS)	1 (0.9)	6.4	64.2	17.4	26.3	4.32	22.7
HbE-trait	7 (6.3)	9.5 $\pm$ 3.5 (3.7–13.6)	70.3 $\pm$ 9.2 (60.7–86.5)	22.3 $\pm$ 2.7 (19.8–27.3)	31.8 $\pm$ 1.1 (30.3–32.9)	4.47 $\pm$ 1.5 (1.65–5.69)	18.8 $\pm$ 7.0 (13.4–33.9)
Homo HbE (HbEE)	1 (0.9)	5.2	58.6	15.6	29.3	2.65	18.3
HbD-Punjab trait	8 (7.2)	10 $\pm$ 3.6 (5.2–15.8)	78.4 $\pm$ 16.8 (47.2–102)	24.9 $\pm$ 6.3 (12.1–30.9)	31.5 $\pm$ 3.3 (25.6–35.8)	4.35 $\pm$ 1.4 (2.2–5.89)	19.0 $\pm$ 4.3 (13.9–26.8)
Homo HbD (HbDD)	1 (0.9)	8.7	71.2	21.8	30.6	3.72	18.9
Double hetero HbSE	1 (0.9)	13.3	76.9	26.0	33.8	5.11	14.7
HbD-Iran trait	1 (0.9)	10.7	71.8	21.7	30.2	4.93	16.8
Hb J Oxford	2 (0.9)	8.3 $\pm$ 3.8 (5.6–11)	62.6 $\pm$ 11.2 (54.6–70.5)	15.8 $\pm$ 3.4 (13.4–18.2)	25.2 $\pm$ 0.8 (24.6–25.8)	5.07 $\pm$ 1.4 (4.1–6.04)	23.5 $\pm$ 2.1 (22–25)

Homo homozygous, *Hetero* heterozygous, *Thal* thalassemia

Table 2 Hemoglobin profile in each case obtained on HPLC (mean $\pm$ SD, range)

Presumptive HPLC diagnosis (no. of cases)	Hb A (%) (range)	Hb F (%)	HbA ₂ (%)	Variant Hb (%)
β -Thal trait (62)	83.6 $\pm$ 2.7 (71.7–89.1)	1.5 $\pm$ 1.2 (0.2–4.9)	5.7 $\pm$ 0.9 (4.0–8.2)	
β -Thal major (6)	25.0 $\pm$ 22.7 (4.6–60.5)	65.8 $\pm$ 29.8 (30.6–91)	2.8 $\pm$ 1.5 (0–4.5)	
β -Thal Intermedia (5)	30.4 $\pm$ 31.6 (0.5–65.9)	62.0 $\pm$ 37 (22–98)	5.5 $\pm$ 2.6 (2.1–8.2)	
HbS/ β -thal (2)	1.6 $\pm$ 2.8 (1.4–1.8)	19.3 $\pm$ 9.4 (14.2–24.3)	5.0 $\pm$ 0.6 (4.9–5.1)	74.4 $\pm$ 7.8 (69.2–79.5)
HbE/ β -thal (8)	24.4 $\pm$ 18.1 (6.5–47.2)	18.7 $\pm$ 22.2 (4.4–33.2)	–	52.8 $\pm$ 17.9 (34.2–60.9)
HbH disease (4)	88.1 $\pm$ 4.9 (81.5–93.3)	0.8 $\pm$ 0.6 (0.2–1.8)	2.6 $\pm$ 0.6 (2–3.4)	
Sickle cell trait (1)	46.3	1.2	6.9	33.8
Homozygous Sickle cell ds (1)	0	9.8	3.1	85.7
HbE-trait (7)	62.3 $\pm$ 2.7 (59.8–67.7)	0.96 $\pm$ 0.7 (0.4–2.3)	0	28.2 $\pm$ 5.0 (17–31.2)
Homozygous HbE ds (1)	4.0	0.7	0	93.7
HbD-Punjab trait (8)	54.5 $\pm$ 8.5 (50.7–77)	0.6 $\pm$ 0.4 (0.2–1.2)	1.4 $\pm$ 0.4 (0.7–1.8)	34.8 $\pm$ 8.0 (13.4–38.5)
Homozygous HbD ds (1)	5.0	1.2	2.1	86.5
Double hetero HbSE ds (1)	1.4	0.4	61(E)	30.7
HbD-Iran trait (1)	45.1	0.8	0	43.3
Hb J Oxford (2)	72.5 $\pm$ 8.3 (66.4–78.6)	2.3 $\pm$ 2 (0.8–3.7)	1.9 $\pm$ 0 (1.9)	18.2 $\pm$ 4 (15.3–21)

Table 3 Hematological parameters in iron deplete and iron replete groups of thalassemia trait

Parameter (No. of cases; n)	Iron deplete group (n = 8)	Iron replete group (n = 54)	P value
Mean Hb(g %)	6.12 $\pm$ 2.3 (3.2–10)	12.83 $\pm$ 2.7(3.2–13.9)	<0.001
Mean MCV(fl)	58.9 $\pm$ 5.7 (52.1–69.7)	63.3 $\pm$ 7.8 (49.8–85.2)	<0.001
Mean MCH(pg)	16.7 $\pm$ 2.6 (10.9–20.5)	19.98 $\pm$ 2.2 (16–27.2)	<0.001
Mean HbA ₂ (%)	5.3 $\pm$ 1.3 (4.0–8.1)	6.0 $\pm$ 0.8 (4.2–8.2)	<0.001

Apart from β -thalassaemia, six additional variants were encountered; HbS, HbE (Fig. 1a) and HbD Punjab were the most common variants present. Other variants included, HbD Iran (Fig. 1b), HbJ Oxford (Fig. 2), HbH disease (Fig. 3a, b).

The retention time alone [$n = 3$ (i.e., HbS, HbD-Punjab, HbH)] or in conjunction with %Hb [$n = 3$ (i.e., HbJ, HbE, HbD-Iran)] could identify all the six haemoglobin variants seen. However, Hb-electrophoresis was resorted to in many doubtful cases to confirm the diagnosis. Family HPLC screening also helped in some difficult cases especially in double heterozygous states like HbE thalassemia and HbS thalassemia. Role of family studies has been emphasized by other studies also [2].

Discussion

HPLC has been shown to be a sensitive, specific, and reproducible alternative to electrophoresis. With automation and quantitative power, it appears to be a sensitive and accurate technique for direct identification and quantification of normal and abnormal haemoglobin fractions [3–8]. Different reports have addressed the precision of the

retention times obtained with stored normal [8] and abnormal samples [5, 6]. There are a few studies from India which evaluated and emphasized the role of HPLC for diagnosis of thalassaemia and various haemoglobinopathies [9, 10].

In β -thalassaemia trait group, in our cases RDW-CV was higher as compared to series of Aslan et al. (14.88 %), Rathod et al. (14.47 %) and Demir et al. (14.91). This was consistent with the degree of anisopokilocytosis (32-mild, 8-moderate) we observed in our cases. It may be because of the fact that 12.9 % of our cases had concomitant iron deficiency.

Iron deficiency may lower the HbA₂ concentration [11]. In most cases, β -thalassaemia trait can be diagnosed in the presence of iron deficiency. In a study, Madan et al. [12], studied iron status in 463 heterozygous beta-thalassemics, 27.2 % patients were iron deficient. Mean HbA₂ was not significantly different in the two groups of patients with the trait and was elevated (>3.5 %) in all but one heterozygote investigated. Elevation of HbA₂ was striking and could be used with reliability in making the diagnosis of BTT even in the presence of iron deficiency.

It appeared that MCV and MCH were almost similar in thalassemia major, thalassemia intermedia and thalassemia

Fig. 1 a and b HPLC chromatogram showing abnormal hemoglobin eluting in A2 window with concentration ~ 30 % in a case of HbE trait. (a) [in contrast to HbD Iran where variant Hb is more than 40 % (b)]

Fig. 2 HPLC chromatogram showing a peak in P3 window of 21 % with retention time 1.58 min indicating presence of HbJ Oxford

trait cases. Red cell count was high in thalassemia trait. Though anisopoikilocytosis was moderate to severe in thalassemia major/intermedia and it was mild in thalassemia trait. It appears difficult to differentiate thalassemia major and intermedia on the basis of red cell morphology alone or with absolute values. Therefore blood findings must be correlated with clinical picture for accurate diagnosis.

In our study, apart from β -thalassaemia, four additional variants were encountered with various incidences; HbS, HbE and HbD-Punjab being the most common variants present. Separation between HbF, HbA₂, HbA, HbS and D-window was clear. In certain difficult cases percentage

Fig. 3 a and b : HPLC chromatogram showing a sharp peak of HbH (arrow) at the start of integration (a), also confirmed by HbH inclusions on brilliant cresyl blue preparation (b)

of Hb and/or band on electrophoresis helped in arriving at a diagnosis. HPLC was also able to separately identify two Hb variants of HbD family; HbD-Iran and HbD-Punjab. Both exhibited identical electrophoretic mobilities but eluted in A₂ and D windows respectively on HPLC. These situations are clinically important because HbD-Punjab produces a significant sickling disorder when present in a double heterozygous form; whereas HbD-Iran is clinically benign [13, 14]. The misdiagnosis of HbD-Iran as HbD-Punjab based solely on Hb- electrophoresis or as HbE based solely on HPLC, where the subtle difference in %Hb or retention time is disregarded, may lead to incorrect genetic counselling in addition to undue anxiety for the family.

HbH elution peak was detected by visual analysis characterized by sharp peak in the first minute of elution just before the start of integration. Such peak obtained on HPLC in association with golf ball inclusions on brilliant cresyl blue preparation helped in identifying cases of HbH disease.

Values more than 40 % in Hb A₂ window with a different retention time made us suspect Hb D Iran which was confirmed by starch agarose gel electrophoresis which revealed a band in S/D/G region. Joutovsky et al. [15] found statistically significant difference amongst the

retention times of different normal and variant haemoglobins within the same retention windows.

The present findings show HPLC as an excellent, powerful diagnostic tool for the direct identification of haemoglobin variants with a high degree of precision in the quantification of normal and abnormal haemoglobin fractions. CE-HPLC (β -thal short program) may be a valuable tool in rapid diagnosis of a varied spectrum of haemoglobinopathies. Retention time and percentage of variant haemoglobin can provide important clues in differentiating variant haemoglobins eluting in the same window. Iron deficiency anaemia causes significant lowering of HbA₂ values. HbA₂ in the borderline range needs further evaluation especially for silent mutations, α -thalassaemia and co-existing nutritional deficiency.

Current guidelines require that abnormal variant Hbs should be confirmed by another independent technique. This is prudent practice, and in most cases (like a sickling test for S-window peaks, electrophoresis for others) cheap and easy. It is especially important while screening pregnant women as the diagnosis has implications for prenatal testing.

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