

# HPLC Studies in Thalassemia and Other Hemoglobinopathies

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**ABSTRACT:** Hemoglobinopathies are serious public health problem in the world. The cumulative gene frequency of these disorders is 4.2% in India. The present study was aimed to evaluate high performance liquid chromatography for the identification of different hemoglobinopathies. A total of 142 cases of different hemoglobinopathies were enrolled in this study. Variant II  $\beta$ -thalassemia short program (Bio Rad, Hercules CA) was used for the identification of abnormal hemoglobins. Mean retention time and mean percent hemoglobin (HbF, HbA<sub>2</sub>, HbS and HbE) were compared between cases of different hemoglobinopathies. It was found that HPLC detect all abnormal hemoglobin with high accuracy. It has been concluded that HPLC found to be fast, cost effective and reliable for the diagnosis of different hemoglobinopathies especially in areas with high frequency of hemoglobin variants.

**KEYWORDS:** Thalassemia, Sickle cell anemia, hemoglobin variants, HPLC, retention time

## I. INTRODUCTION

Hemoglobinopathies are major public health problem affecting millions of people worldwide. In India, the cumulative gene frequency of hemoglobinopathies is 4.2% (1). The prevalence of hemoglobinopathies is quite heterogeneous with the geographic locations & ethnic groups. Among the most common hemoglobinopathies (HbS, HbD, HbE and thalassemia), the prevalence of HbS is widely spread in the tribal population of north Maharashtra Khandesh region (2), followed by thalassemia, HbD Punjab and HbE (3). Carrier identification & genetic counseling are the essential steps for the prevention and control of the hemoglobinopathies. Diagnosis of variant hemoglobin could be carried out by conventional methods including red cell indices, red cell morphology, Hb electrophoresis, quantification of HbA<sub>2</sub>, HbF and HbE (4, 5). However cellulose acetate electrophoresis and elution (6) and microcolumn chromatography for the measurement of HbA<sub>2</sub> and HbE are reproducible and accurate but are time consuming and labor intensive.

HPLC on the other hand is a sensitive, precise and widely used method for detection of abnormal Hbs and thalassemia (7) because of its speed and reliability. The main objective of the present study was to evaluate this system on conventional techniques and to determine the range of abnormal hemoglobins in different hemoglobinopathies.

## II. MATERIALS AND METHODS

This study was performed during four year tenure of extended family screening for carrier identification in the families of  $\beta$ -thalassemia major patients. A total of 1702 participants were screened for their carrier state. Of which selected 142 diagnosed cases of different hemoglobinopathies were enrolled in this study. Variant II  $\beta$ -thalassemia short program (Bio Rad, Hercules CA) was used for the identification of abnormal hemoglobins at the laboratory of hematology Shree Bhausahab Hire Medical College, Dhule.

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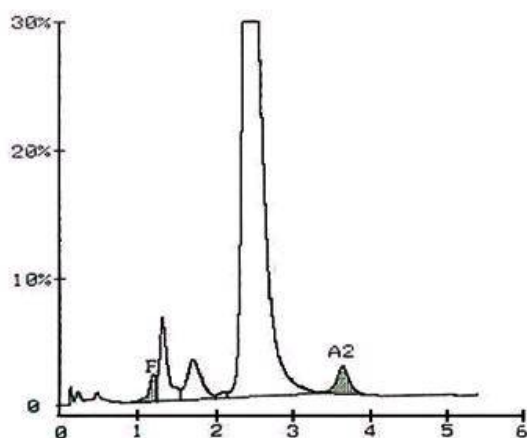
### III. RESULTS

The VARIANT II  $\beta$ -thalassemia Short program (Bio-Rad) performed quantitation of HbA, HbA<sub>2</sub>, HbF, HbS and HbE. The chromatogram of normal subjects with HbA<sub>2</sub> and HbA is shown in **Fig. 1**. Three peaks were reported for normal subjects, HbF in F-window, HbA in A<sub>0</sub>-window and HbA<sub>2</sub> in A<sub>2</sub>-window. **Table 1** represents the mean retention time and percent hemoglobin with standard deviation. The mean retention time of HbF was 1.10 min  $\pm$  0.52, HbA was 2.48 min  $\pm$  0.06 and HbA<sub>2</sub> was 3.63 min  $\pm$  0.02; the total analysis time was 6.9 min  $\pm$  0.5. The mean value of HbA was 86.7  $\pm$  6.9%, HbF was 0.5  $\pm$  0.9% and HbA<sub>2</sub> was 2.6  $\pm$  0.4%.

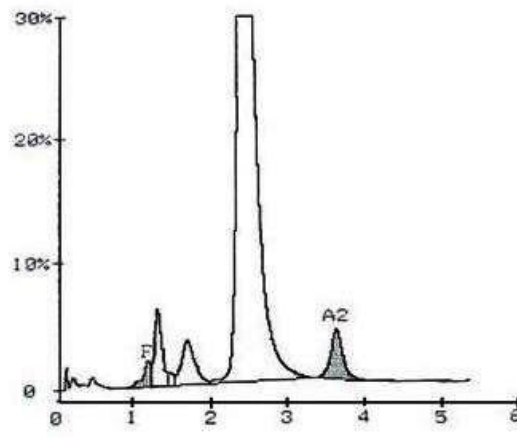
**Table 1** Hemoglobin analysis by HPLC of different hemoglobinopathies with mean percent hemoglobin and retention time (RT)

Phenotype	HbF		HbA		HbA <sub>2</sub> /E		HbS	
	RT min	% Hb	RT min	% Hb	RT min	% Hb	RT min	% Hb
Normal (20)	1.10 (0.52)	0.5 (0.9)	2.48 (0.06)	86.7 (6.9)	3.63 (0.02)	2.6 (0.4)	—	—
$\beta$ -thalassemia trait (90)	1.09 (0.30)	2.2 (0.9)	2.46 (0.035)	87.4 (3.7)	3.66 (0.036)	6.2 (1.5)	—	—
$\beta$ -thalassemia major (21)	1.15 (0.02)	69.5 (13.3)	2.36 (0.15)	25.0 (13.5)	3.64 (0.02)	5.2 (1.3)	—	—
Sickle/ $\beta$ -thalassemia (18)	1.11 (0.02)	9.3 (4.0)	2.66 (0.35)	25.2 (7.9)	3.68 (0.02)	6.4 (1.0)	4.46 (0.01)	58.0 (7.4)
HbE/ $\beta$ -thalassemia (2)	1.13, 1.15	8.2, 9.6	2.47, 2.45	40.2, 38.9	3.71 (0.09)	48.8, 51.3	—	—
HbE trait (6)	1.05 (0.12)	1.8 (0.4)	2.49 (0.08)	68.7 (1.7)	3.73 (0.06)	28.9 (1.4)	—	—

In samples with  $\beta$ -thalassemia trait, the chromatogram is similar to that of the non-diseased sample (normal), but the concentration of HbA<sub>2</sub> was increased 6.2  $\pm$  1.5% (**Fig. 2**). The peaks were reported in the same window as in the normal subjects. The mean retention time of HbF was 1.09  $\pm$  0.30 min; HbA was 2.46  $\pm$  0.035 min and HbA<sub>2</sub> was 3.66  $\pm$  0.036 min. The mean value of HbA was 87.4  $\pm$  3.7% and HbF was 2.2  $\pm$  0.9%.



**Fig. 1** Chromatogram in normal subjects



**Fig. 2** Chromatogram in  $\beta$ -thalassemia trait

Mutations on  $\beta$ -globin gene are responsible for abnormal production of  $\beta$ -globin chains that ranges from minimal ( $\beta^+$ -thalassemia) to a complete absence ( $\beta^0$ -thalassemia). In homozygous conditions of  $\beta$ -thalassemia, the chromatogram showed the prominence of HbF; the absence or minor amounts of HbA, depending on the genotype of  $\beta$ -thalassemia ( $\beta^0$ -thalassemia or  $\beta^+$ -thalassemia respectively); and an unaffected or increased HbA<sub>2</sub> concentration (**Fig.**

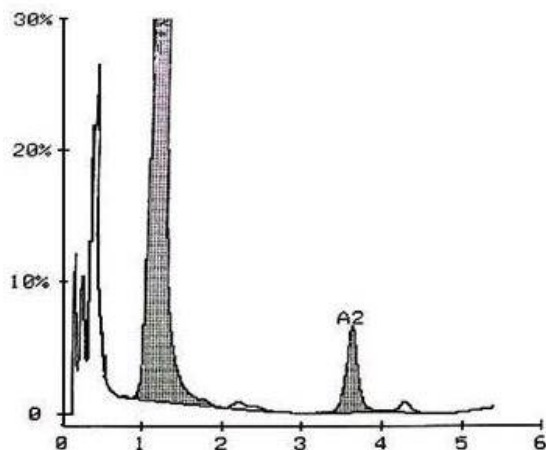
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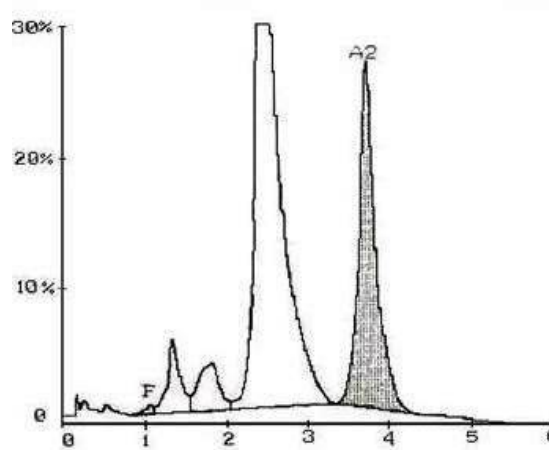
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3). The average retention time of HbF was  $1.15 \pm 0.02$  min; HbA was  $2.36 \pm 0.15$  min and HbA<sub>2</sub> was  $3.64 \pm 0.02$  min. These peaks were reported in the respective windows as in normal and  $\beta$ -thalassemia traits. However, these samples consistently had higher HbF percentage  $69.5 \pm 13.3\%$ . The concentration of HbA<sub>2</sub> was fluctuating; the overall mean of HbA<sub>2</sub> was  $5.2 \pm 1.3$ . The mean percentage of HbA was decreased  $25.0 \pm 13.5\%$ .

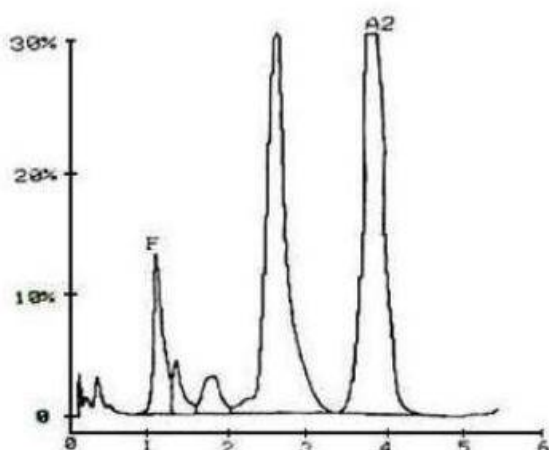
HbE was eluted at the same retention time in HbE traits or disease as HbA<sub>2</sub> in normal samples or samples with the  $\beta$ -thalassemia trait. A hemoglobin concentration  $> 10\%$  at the position of HbA<sub>2</sub> were assumed HbE because HbE peak was reported in the A<sub>2</sub>-window. Usually 25–30% was detected in HbE heterozygotes, and almost 100% was detected in HbE homozygotes. The chromatogram of HbE heterozygote showed the presence of HbA (60–70%), HbF (0–2%) peaks (**Fig. 4**). The average retention time of HbF was  $1.05 \pm 0.12$  min, HbA was  $2.49 \pm 0.08$  min and HbE was  $3.73 \pm 0.06$  min. In the two cases of double heterozygous for HbE/ $\beta$ -thalassemia, chromatogram showed three peaks HbA, HbF and HbE (**Fig. 5**). The concentrations of HbA in these two cases were 40.2%, 38.9%, HbF were 8.2%, 9.2% and HbE were 48.8%, 51.3%.



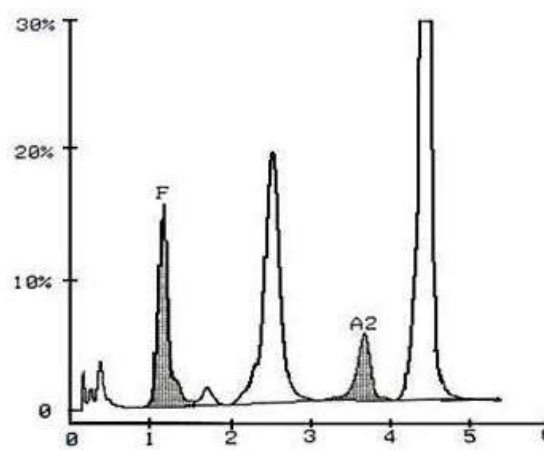
**Fig. 3** Chromatogram in  $\beta$ -thalassemia major



**Fig. 4** Chromatogram in HbE trait



**Fig. 5** Chromatogram in HbE/ $\beta$ -thalassemia



**Fig. 6** Chromatogram in Sickle/ $\beta$ -thalassemia

The chromatogram of double heterozygous sickle/ $\beta$ -thalassemia patients showed four peaks (**Fig. 6**). These are HbF, HbA, HbA<sub>2</sub> and one abnormal peak (HbS). HbS peak was reported in S-window at the retention time of  $4.46 \pm 0.01$  min, after HbA<sub>2</sub> peak. The concentration of HbA<sub>2</sub> and HbF increased with mean values of  $6.4 \pm 1.0\%$  and  $9.3 \pm$

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4.0% respectively, while the concentration of HbA was decreased  $25.2 \pm 7.9\%$ . The mean concentration of HbS was  $58.0 \pm 7.4\%$ . The average retention time of HbF was  $1.11 \pm 0.02$ ; HbA was  $2.66 \pm 0.35$  and HbA<sub>2</sub> was  $3.68 \pm 0.02$ .

## IV. DISCUSSION

High performance liquid chromatography (HPLC) has emerged as a reliable technique over conventional procedures which were used for hemoglobinopathy screening programs for the identification of Hb variants. With the development of rapid, well-resolving and fully automated analyzers its use has been expanded and in past decade, it has appeared to be an appropriate tool for direct identification and quantification of hemoglobin variants (8-11). The Bio-Rad Variant is an automated cation exchange HPLC system well suited for the rapid and accurate quantification of HbA<sub>2</sub> and HbF (7). Chromatography of each sample is completed in 6 min. The system can also be used to identify common Hb variants (HbS and C) (12).

During the study, samples were analyzed in the Hematology Department of Shree Bhausaheb Hire Govt. Medical College, Dhule for quantification of hemoglobin fractions and screening for hemoglobin variants. Some abnormal Hbs, such as HbE, Hb Tak, HbD and Hb Lepore, also co-eluted with HbA<sub>2</sub>. Therefore, samples that have >10% HbA<sub>2</sub> should be tested for the possible presence of other Hb variants. Different concentrations of Hbs may distinguish each abnormal Hb. For example, the percentage of HbD eluted at the HbA<sub>2</sub> peak in the HbD heterozygote was 38.9%, which was higher than HbE or HbA<sub>2</sub> in the usual cases for the HbE heterozygote (25–30%) or for the  $\beta$ -thalassemia heterozygote (4–7%).

An increased HbA<sub>2</sub> in the range of 4–7% is specific for the  $\beta$ -thalassemia trait in almost all cases (13, 14). The present study confirmed the identification of the  $\beta$ -thalassemia trait, using the concentration determined by HPLC (Table 1). The HbF concentration was increased in majority of the cases of  $\beta$ -thalassemia trait are in agreement with previous finding (7). Although the HbF concentration is not a useful discriminant for  $\beta$ -thalassemia trait, an increased HbF concentration may be observed in homozygous  $\beta$ -thalassemia variants,  $\delta\beta$ -thalassemia and hereditary persistence of fetal hemoglobin or  $\beta^E/\beta^{\text{thal}}$  (14).

Of the hemoglobin variants which co-migrated with HbA<sub>2</sub>, it is HbE principally which interferes with HbA<sub>2</sub> estimation in Southeast Asian populations. Its clinical importance resides in the fact that while heterozygous or homozygous HbE do not cause clinically important anemia, compound heterozygotes for HbE and  $\beta$ -thalassemia may express a phenotype indistinguishable from homozygous  $\beta$ -thalassemia (15). In practice, HbE carriers may be readily distinguished from  $\beta$ -thalassemia trait by the apparent concentration of HbA<sub>2</sub>. The concentrations of HbE in heterozygotes are typically in excess of 20% (15). Therefore, HbA<sub>2</sub> concentrations in excess of perhaps 10% should suggest co-elution of abnormal hemoglobin such as HbE.

The present study showed that the qualitative demonstration of HbE in the A<sub>2</sub>-window using the VARIANT-II Beta Thalassemia Short (BTS) program aids in the diagnosis of HbE disease, HbE trait and double heterozygotes HbE/ $\beta$ -thalassemia. All cases of HbE variant viz. HbE disease, HbE trait and HbE/ $\beta$ -thalassemia were detected. The BTS program helps to quantitate the amount of HbE and provides a diagnosis for newborns as well as adults with these variants. An increased HbE in the range of 30–40%, 40–60% and 80–100%, confirmed the identification of the HbE trait, HbE/ $\beta$ -thalassemia and HbE disease respectively. This finding correlated with the findings of Fucharoen, et al. (10). The average retention time for HbE was 3.72 min; which is quite comparable with the findings of Joutovsky, et al. (16) who reported 3.69 min. They concluded that the retention time on HPLC used as diagnostic tool is reliable, reproducible and superior to conventional hemoglobin electrophoresis in the detection of many hemoglobin variants.

The mean retention times for the HbF, HbA, HbA<sub>2</sub> and variants hemoglobin viz. HbS and HbE reported in different phenotypes were correlated with the data of Fucharoen, et al. (10) and Joutovsky, et al. (16). Different reports have addressed the precision of the retention times obtained with stored normal samples (9); specimens containing HbS, HbC and HbE (8) and liquid controls (17). Different reports have also tabulated the retention times for various hemoglobin variants (8, 11, 16).

The retention time alone or in combination with either the percent Hb or the peak characteristics could identify the different hemoglobin variants. The percent Hb can generally be the initial predictor whether the detected variant is a  $\alpha$ - or  $\beta$ -variant. The  $\beta$ -variants all had mean percentage Hb values > 34% except for HbE ( $28.9 \pm 1.4\%$ ) in HbE traits, confirming and extending the data of Joutovsky, et al. (16).

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Electrophoresis of hemoglobin variants with similar mobilities has limitations. The identification of variants is dependent on the technical performance of electrophoresis. HPLC, on the other hand, has been shown to have a high degree of reproducibility and precision (18). These data demonstrate that HPLC is an excellent, powerful diagnostic tool for the direct identification of hemoglobin variants with a high degree of precision in the quantification of major and minor, normal and abnormal hemoglobin fractions.

## V. CONCLUSION

HPLC is suitable for the routine investigation of hemoglobin variants and hemoglobinopathies including thalassemia. The technique is reliable and cost effective for screening of large population with high accuracy in countries with huge population like India.

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