Original Research Article

Antenatal Screening for Haemoglobinopathies by Using High Performance Liquid Chromatography

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Abstract

Clinical manifestation of Haemoglobinopathies may vary from asymptomatic state to severe, lifelong, transfusion dependant anemia and reduced life expectancy. About 1.1% of couples around the world are at risk for having children with a hemoglobin disorder of which 2.7 per 1000 conceptions are actually affected. Screening of Antenetal cases for haemoglobinopathies has a very vital role in preventing thalassaemia births. In this study 674 antenatal cases who were registered at their first visit were screened for Haemoglobinopathies. Clinical history, haematological parameters using LH750 (Beckman Coulter) and Peripheral blood smears were recorded. Whole blood EDTA samples were run on BioRad Variant II Hb-HPLC system (considered as gold standard for haemoglobinopathy secreening) for Hb F and Hb A2 quantification and detection of variant haemoglobins. The results were interpreted based on% of the eluted Haemoglobins and CBC data. Out of 674 cases, 307 (45.55%) showed Normal study, 309 (45.85%) were found to have Hb HPLC Normal Pattern with abnormal RBC indices suggestive of iron deficiency, 45 (6.68%) were found to be Heterozygous for Beta Thalassaemia, 5 (0.74%) Heterozygous for Hb sickle, 3 (0.45%) Heterozygous for Hb sickle with Alpha Thalassemia, 2 (0.30%) Heterozygous Delta Beta Thalassemia, 1 (0.15%) Heterozygous for Hb E, 1 (0.15%) Heterozygous for Hb D coinheritance of Beta Thalassemia to be investigated, 1 (0.15%) Heterozygous for Hb-HOFU. In this study, heterozygous beta thalassemia was the commonest haemoglobin disorder, followed by heterozygous Hb- Sickle and other Hb variants (Fig. 1). We recommend Antenatal screening for haemoglobinopathies in all ANC to rule out haemoglobinopathies because carriers haemoglobinopathies are asymptomatic. With effective screening of the spouse of the carrier women and genetic counseling will greatly help in preventing the disease. Cases with associated deficiency anaemias need further follow up after complete course of haematinics

Keywords: ANC; HPLC; Haemoglobinopathies

Introduction

Anaemia is a major public health problem in developing countries, and is a cause of serious concern. It is also one of the most commonly encountered medical disorders during pregnancy. Iron deficiency is one of the important causes whereas deficiencies of other nutrients such as folic acid, protein, vitamin B-12, vitamin A and copper also contribute. The other major cause is presence of "Haemoglobinopathies" - the inherited disorders of haemoglobin, resulting in the spectrum ranging from mild anaemia to severe transfusion dependent anaemia presenting in infancy.

Haemoglobinopathies are divided in to two main groups as follows:

- Thalassemia syndromes-quantitative disorders with reduced number of globin chains

- Structural haemoglobin variants – qualitative disorders of globin chains

The haemoglobinopathies result from the genetic defects in the globin genes.[1–3] There exist many cases with combinations of structural variants and Thalassemias. Amongst all Haemoglobinopathies, Sickle cell syndrome and beta Thalassemias are the major constituents of public health problems.

The frequency of beta-thalassemia heterozygous state (trait -βTT) in India has been reported to around of 3.3%.[4] The average frequency of Sickle cell disease is 4.3%.[5] Hemoglobin E is the second most prevalent haemoglobin variant worldwide and is commonly seen in the north-east region of India.[6]

Beta Thalassemia is detectable in almost every Indian population, most commonly seen in Sindhis, Gujaratis, Muslims, Punjabis and Bengalis. HbS is predominantly found in central India i.e., Vidarbh in Maharashtra, Madhya Pradesh, Orissa, Andhra Pradesh, Gujarat and to a lesser extent in Tamil Nadu, Karnataka, Kerala. HbE is widely distributed in north-eastern parts of India. HbD is predominantly seen in Punjab, Uttar Pradesh, Gujarat and Jammu and Kashmir.[7]

Clinical manifestations of these Haemoglobinopathies can vary from asymptomatic state to severe, lifelong, transfusion dependant anaemia and reduced life expectancy.

About 1.1% of couples around the world are at risk for having children with a hemoglobin disorder of which 2.7 per 1000 conceptions are actually affected. Thus it is estimated that the cost of treatment of an average 4 year thalassemia child is around one lacs per year and stem cell transplantation - a curative treatment costs up to 16 lacs which is out of reach for majority of families. [8]

It is highly recommended to screen the population at large before marriage/pregnancy– the target population mainly being senior college students, premarital age group, newly wed couples (Mass Screening). However, if this is not possible, it becomes important that the presence of haemoglobinopathies in the expectant parents is detected in a timely manner. During the antenatal period, the screening needs to be offered early in pregnancy – at the first antenatal visit/registration, to allow time for fathers to be screened so that the results of the screening tests and any prenatal diagnosis (PND) are available sufficiently early for couples to be able to make timely informed choices.

Antenatal screening for Haemoglobinopathies done by performing a routine Haemogram and to determine the red blood cell indices along with specific testing-such as Hb-HPLC or Capillary Electrophoresis.

Some simple calculation tools based on the MCV and RBC count, such as 'Mentzer index' may help to differentiate iron deficiency anaemia from heterozygous β-thalassemia-since both present as microcytic hypochromic blood picture. The structural haemoglobin variants, however, cannot be suspected based on CBC alone.

Diagnosis of haemoglobinopathies is effectively achieved by using Haemoglobin-high performance liquid chromatography [Hb-HPLC], is considered to be the gold standard. Hb -HPLC is of great utility due to the superior resolution of Hemoglobin variants, rapid assay time and accurate quantification of Hemoglobin variants.

Materials and Methods

Procedure

All the antenatal cases registered at their first visit during the period of 18 months were included in this study.

Two ml of peripheral blood samples were collected, in EDTA vacuum containers. Samples were initially subjected to CBC, peripheral blood smear examination. CBC was performed on fully automated 5 part differential analyzer (LH 750 from Beckman Coulter). The results of haemoglobin, MCV, MCH, MCHC, RBC count and RDW were

correlated with peripheral smear examination(Stain–Leishman). MENTZER Index for Thalassemia was calculated for all the results. Index of <13 was considered indicative of Beta Thalassaemia trait.

Hb–HPLC on BIO–RAD VARIANT II using 'beta thalassemia short program' was subsequently performed on all the samples. CBC and peripheral smear were correlated with HPLC results and analyzed with statistical tools.

The status of Thalassaemias and other haemoglobinopathies were determined depending upon the percentage of HbF and HB A2.

The data was analyzed using SPSS (statistical package for social sciences) Version 20.0 software. The results were presented in tabular and graphical format. For qualitative data various rates, ratios and percentages (%) were calculated.

For quantitative data the mean SD, mean etc. was calculated. If applicable for qualitative data, tests like chi-square test and for quantitative data test like, t-test/ anova were used for comparison of variables. A two tailed test with p-value <0.05 was considered as significant.

Results and Discussion

In the present study, Heterozygous for Beta Thalassaemia (6.68%) (Fig. 4), was found to be the commonest disorder. Other haemoglobinopathies diagnosed were Heterozygous for Hb Sickle (0.74%) (Fig. 5), Heterozygous for Hb Sickle associated with alpha Thalassaemia (0.45%) (Fig. 6), Heterozygous delta-beta Thalassaemia (0.30%) (Fig. 9) and few other haemoglobinopathies (Fig. 7, 8, 10).

Measurement of Serum Ferritin levels were advised to the patients who had normal (Fig. 2) or borderline Hb A2 levels without any variant haemoglobin (Hb-HPLC Pattern normal) but showed abnormal RBC indices (Fig. 3). A review of CBC, PBS and Hb-HPLC after complete course of haematinics was suggested to assess the improvement in their RBC parameters and their Hb A2 levels. In our study, 5.63% cases were positive by Mentzer index of which 3.41% cases were turned to be Hb-HPLC Normal Pattern, 1.63% cases were Heterozygous for Beta Thalassaemia and 0.29% cases were Heterozygous Delta Beta Thalassaemia (Table 1).

Table 1:

	Hb (g/dl) Mean	RBC count (million/cumm) Mean	MCV (fL) Mean	MCH (pg) Mean	MCHC (g/dL) Mean	Mentzer's index		HbF (%) Mean	Hb A (%) Mean	Hb A2 (%) Mean	Variant Hb (%) Mean
						<13	>13				
Normal study (n=307)	11.29	4	84.52	28.28	33.5	0	307	0.46	86.96	2.73	Nil
Normal pattern (n=309)	9.01	4.12	69.19	21.97	31.52	23	286	0.35	86.92	2.50	Nil
Hetrozygous for Beta Thalassemis (n=45)	9.03	4.53	64.07	20.0	31.2	11	34	0.95	84.2	4.9	Nil
Heterozygous for Hb Sickle (n=5)	12.88	4.66	83.04	30.36	31.78	0	5	0.78	51.62	2.86	Hb S – 38.66
Heterozygous for Hb Sickle with associated alpha thalassemia (n=3)	10.53	4.8	69.77	21.87	31.37	0	3	0.90	56.07	2.77	Hb S – 34.33
Heterozygous Delta-Beta Thalassaemia (n=2)	11.0	5.4	65.2	20.4	31.40	2	0	24.45	65.20	2.05	Nil

Our findings support that Hb-HPLC is an excellent, diagnostic tool for identification of haemoglobin variants with a high degree of precision in the quantification of Hb A2, HbF, haemoglobin variants.

We also endorse that antenatal screening for haemoglobinopathies is an effective method to diagnose those and with further counseling and spouse's testing would help prevention of birth of children with transfusion dependent anaemias. Our society has a significant incidence of beta thalassemia major and other transfusion dependent disorders; while the government support systems for such patients still fall short. We are sure that the education of the patients, society at large, medical fraternity along with effective counseling, that we tried to achieve through this project, will go long way in achieving the 'thalassemia free society.
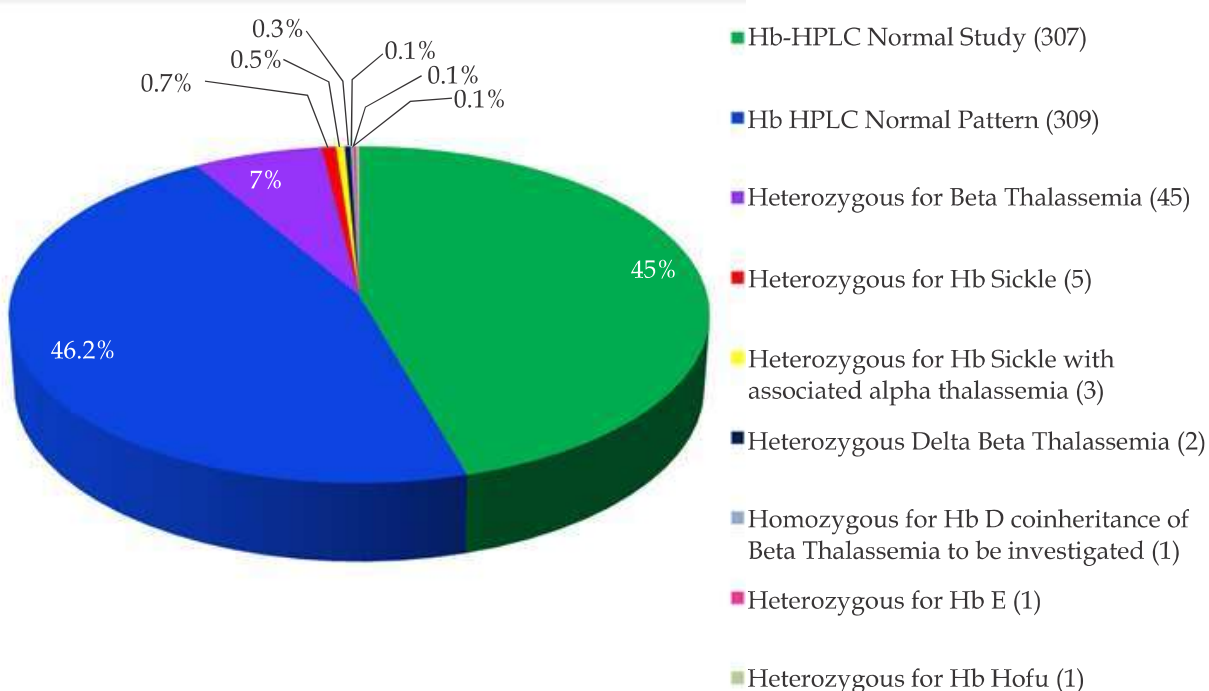

Fig. 1: Distribution of cases

Legend:
- Hb-HPLC Normal Study (307)
- Hb HPLC Normal Pattern (309)
- Heterozygous for Beta Thalassemia (45)
- Heterozygous for Hb Sickle (5)
- Heterozygous for Hb Sickle with associated alpha thalassemia (3)
- Heterozygous Delta Beta Thalassemia (2)
- Homozygous for Hb D coinheritance of Beta Thalassemia to be investigated (1)
- Heterozygous for Hb E (1)
- Heterozygous for Hb Hofu (1)

Table 2: Case 1

Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
Unknown	–	0.0	0.97	694
F	0.3	–	1.06	6099
Unknown	–	1.0	1.23	20257
P2	–	4.0	1.34	82406
P3	–	4.9	1.75	101611
A0	–	87.1	2.49	1817966
A2	2.8	–	3.62	57177

Analysis comments:

Analysis Date: 02/01/2017

Total Area: 2,086,210
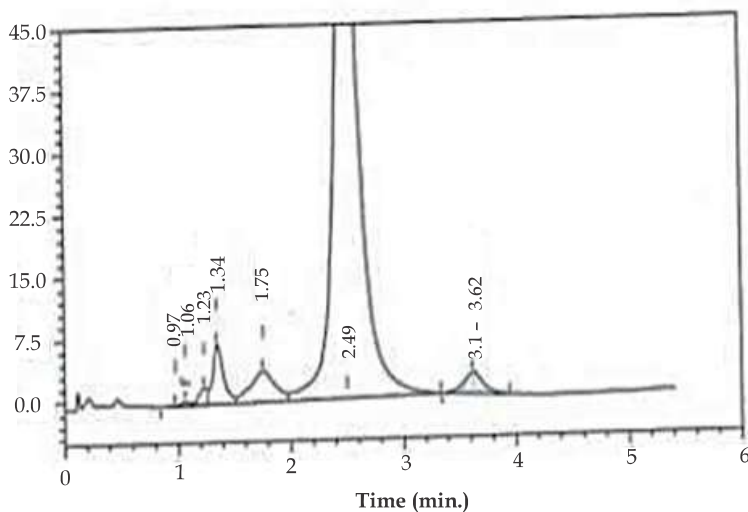
F Concentration = 0.3%

A2 Concentration = 2.7%

Fig. 2: Normal study (Case 1).

Table 3: Case 2

Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
Unknown	–	0.0	0.92	16630
F	0.3	–	1.10	20775
P2	–	4.0	1.26	86757
P3	–	4.8	1.73	105901
A0	–	86.8	2.40	1903085
A2	2.5	–	3.62	58584

Analysis comments:

Analysis Date: 02/01/2017

Total Area: 2,191,732

F Concentration = 1.0%
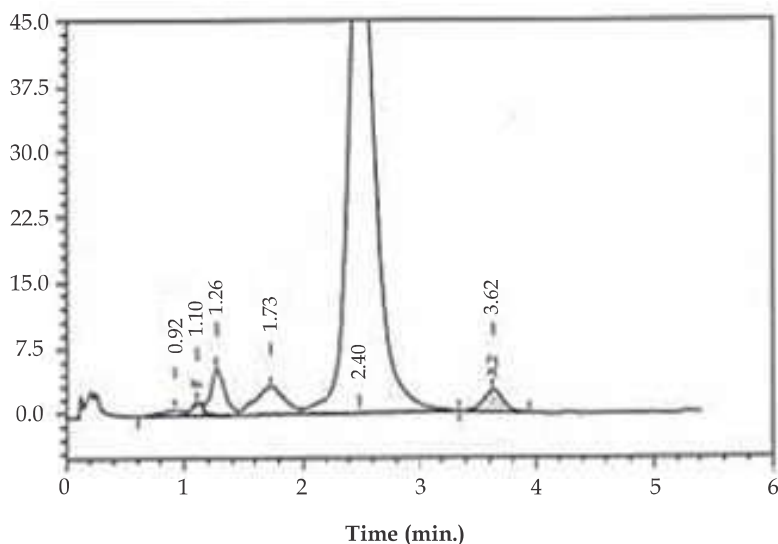
A2 Concentration = 2.5%

Fig. 3: Normal pattern iron studies suggested with follow up (Case 2)

Table 4: Case 3

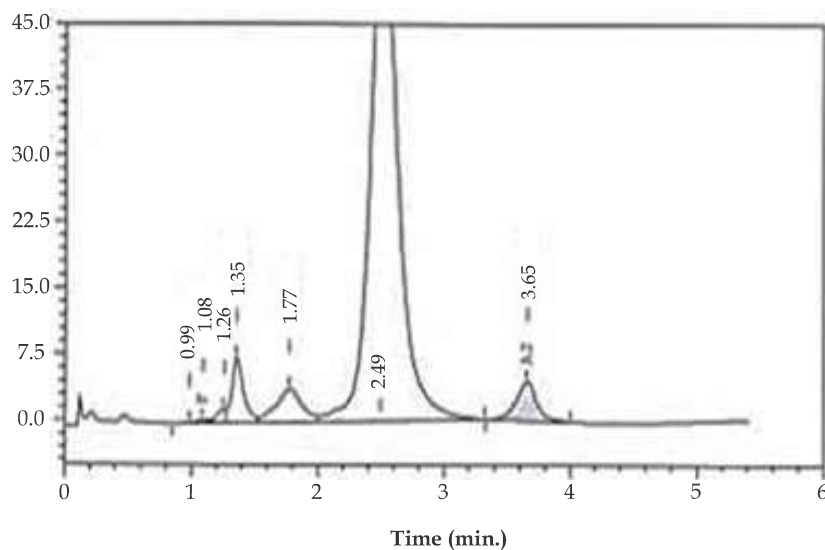| Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
Unknown	–	0.0	0.99	1209
F	0.2	–	1.08	4991
Unknown	–	0.8	1.26	17481
P2	–	4.4	1.35	96472
P3	–	5.1	1.77	110983
A0	–	84.6	2.49	1855935
A2	4.5*	–	3.65	105729

Analysis comments:

Analysis Date: 02/01/2017

Total Area: 2,192,802

F Concentration = 0.2%

A2 Concentration = 4.5*%

Fig. 4: Heterozygous beta thalassaemai (Case 3)

Table 5: Case 4

Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
F	1.0	–	1.11	21811
Unknown	–	1.0	1.231	26365
P2	–	4.0	1.36	44974
P3	–	4.9	1.74	57024
A0	–	87.1	2.54	1084659
A2	2.6	–	3.65	57639
S-window	–	39.0	4.41	825513

Analysis comments:

Analysis Date: 02/01/2017

Total Area: 2,117,984
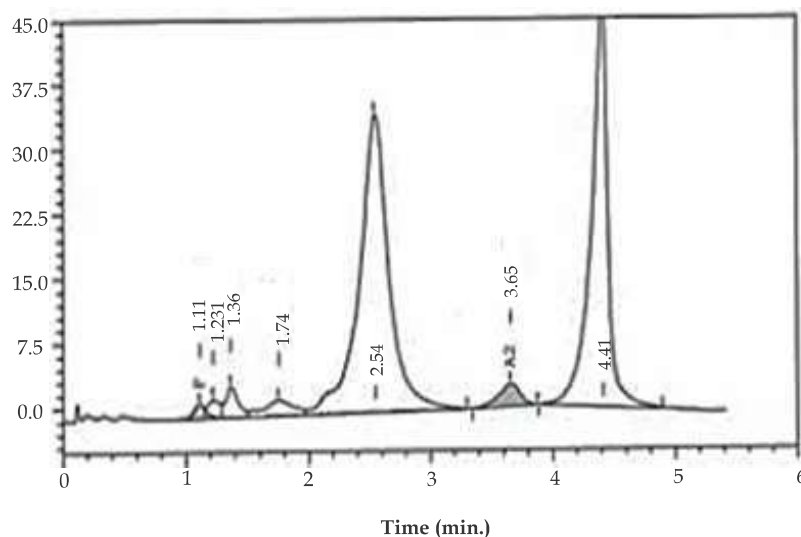
F Concentration = 1.0%

A2 Concentration = 2.6%

Fig. 5: Heterozygous Hb Sickle (Case 4)

Table 6: Case 5

Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
Unknown	–	0.0	0.96	640
F	0.5	–	1.08	13660
Unknown	–	0.8	1.26	18500
P2	–	2.5	1.38	55926
P3	–	3.0	1.74	68297
A0	–	67.6	2.53	1307313
A2	2.8	–	3.66	67211
S-Window	–	32.6	4.40	740315

Analysis comments:

Analysis Date: 17/08/2018

Total Area: 2,269,862

F Concentration = 0.5%

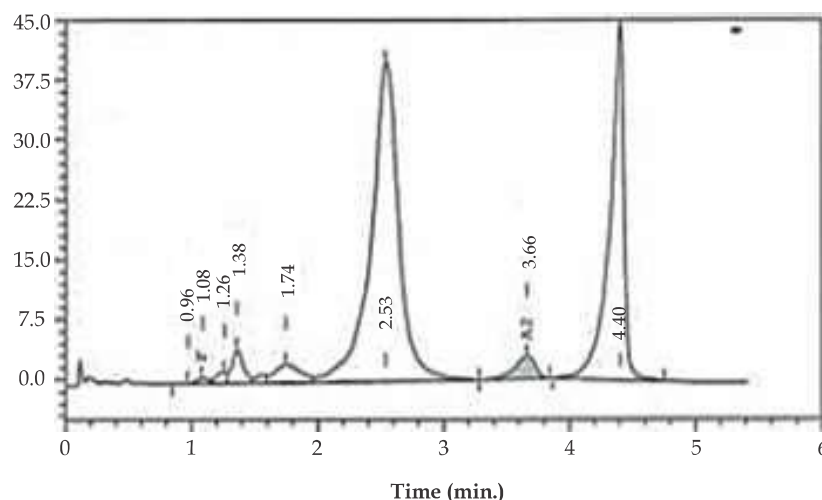A2 Concentration = 2.8%

Fig. 6: Heterozygous Hb Sickle with Alpha thalassaemia (Case 5)

Table 7: Case 6

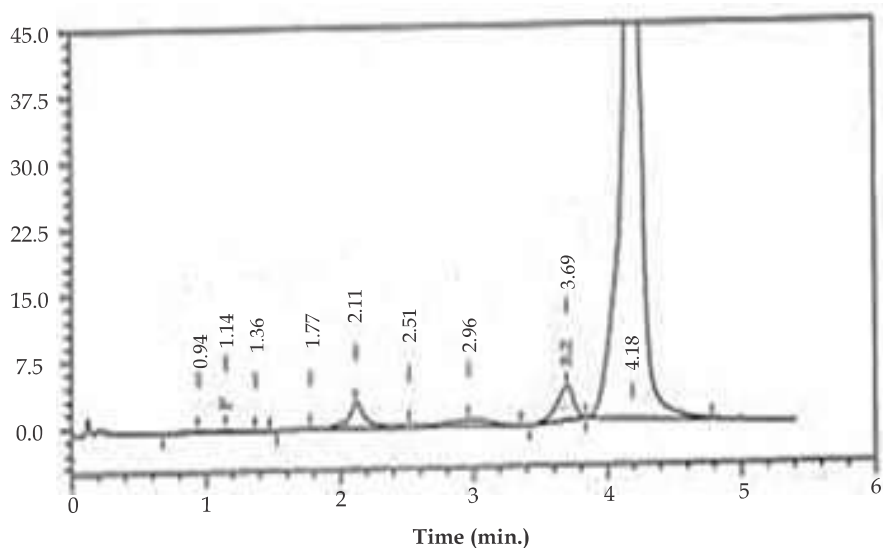| Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
Unknown	–	0.4	0.94	8059
F	0.3	–	1.14	6605
P2	–	0.0	1.36	947
P3	–	0.2	1.77	5379
A0	–	3.6	2.11	80508
Unknown	–	0.8	2.51	17342
Unknown	–	2.5	2.96	55433
A2	3.8	–	3.69	95783
Unknown	–	88.0	4.18	1982290

Analysis comments:

Total Area: 2,252,346

Analysis Date: 08/02/2017

F Concentration = 0.3%

A2 Concentration = 3.8%

Fig. 7: Homozygous for Hb D coinheritance of Beta Thalassemia to be investigated (Case 6)

Table 8: Case 7

Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
F	0.2	–	1.06	4115
Unknown	–	0.7	1.22	14953
P2	–	2.6	1.35	55132
Unknown	–	1.8	1.74	38332
P3	–	2.0	1.84	41785
A0	–	67.3	2.52	1437659
A2	24.6	–	3.70	543235

Analysis comments:

Total Area: 2,135,211

Analysis Date: 01/03/2018

A2 Concentration = 24.6%
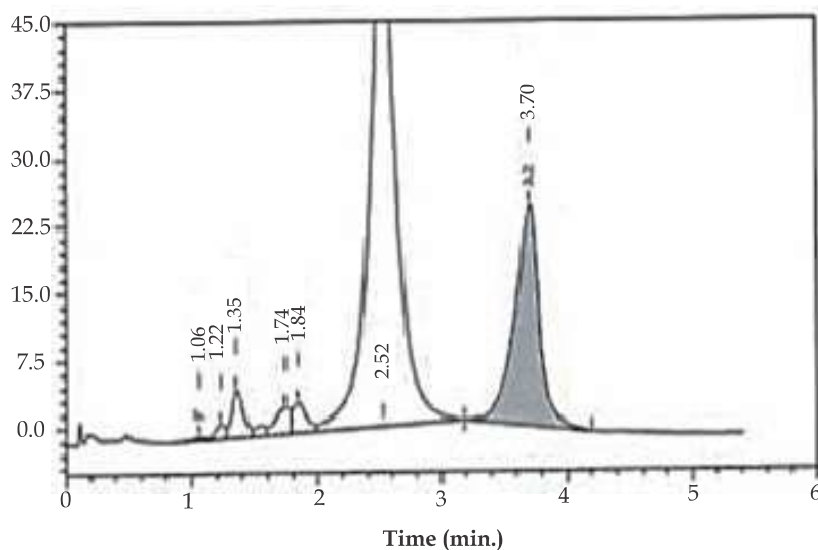
Fig. 8: Heterozygous Hb E (Case 7)

Table 9: Case 8

Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
P 1	–	0.1	0.87	5020
F	25.5*	–	1.21	904971
P2	–	4.7	1.34	164549
P3	–	3.1	1.77	110671
A0	–	64.3	2.48	2264836
A2	2.0*	–	3.67	73118

Analysis comments:

Total Area: 3,523,166

Analysis Date: : 05/01/2018
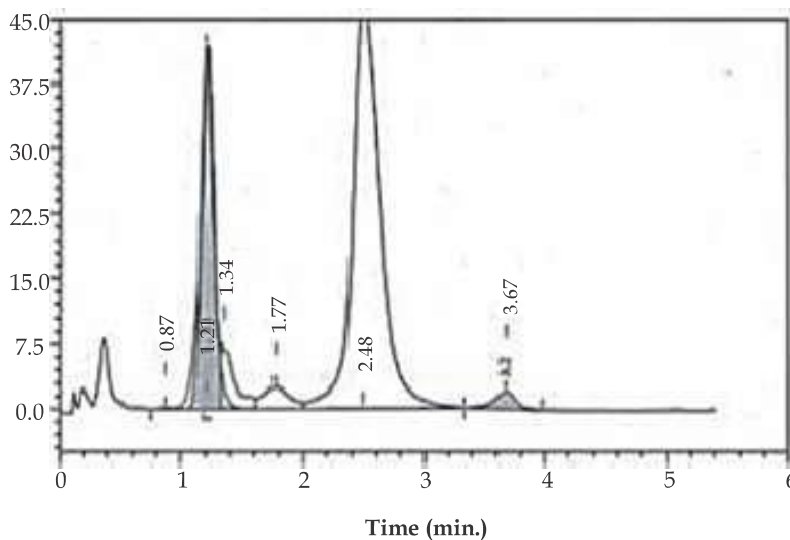
F Concentration = 25.5*%

A2 Concentration = 2.0*%

Fig. 9: Heterozygous delta beta thalassaemia (Case 8)

Table 10: Case 9

Peak Name	Calibrated Area%	Area%	Retention Time (min)	Peak Area
Unknown	–	0.1	0.97	2671
F	0.7	–	1.09	18711
Unknown	–	1.9	1.23	50102
P2	–	2.6	1.36	68229
Unknown	–	2.0	1.55	53458
P3	–	3.6	1.76	93512
Unknown	–	34.8	2.27	909220
A0	–	50.8	2.53	1329631
A2	3.3	–	3.67	90373

Analysis comments: Analysis Date: 17/08/2018   Total Area: 2,615,808

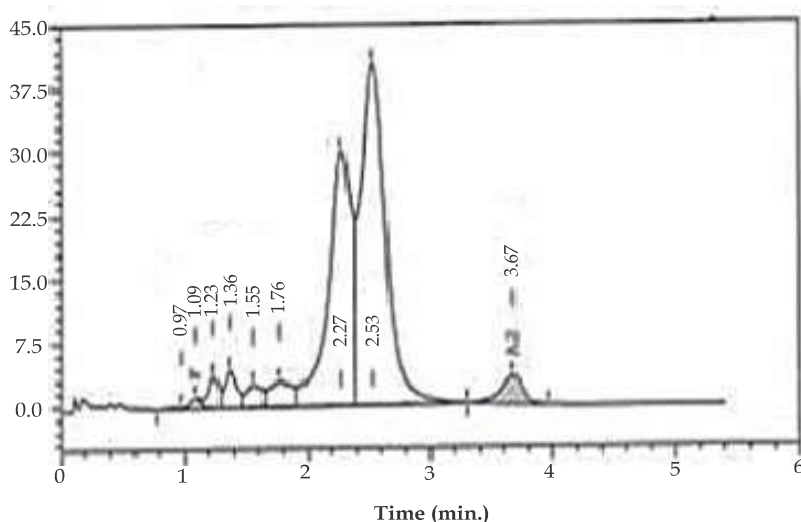F Concentration = 0.7%   A2 Concentration = 3.3%

Fig. 10: Heterozygous for Hb-Hofu (Case 9)

Late antenatal registration, non-cooperation of the spouse, unwillingness to undergo the tests by apparently normal population and refusal for prenatal diagnosis are still the hurdles in the diagnoses of haemoglobinopathies and their prevention program.

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