

## Evaluation of (HPLC) Patterns of Sickle Cell Anaemia patients in comparison with apparently healthy individuals

R Manasa Reddy<sup>1</sup>, Srilatha K<sup>2</sup>

<sup>1,2</sup>Assistant Professor, Department of Pathology, Prathima Institute of Medical Sciences, Nagunur, Karimnagar, Telangana.

Address for correspondence: Dr R Manasa Reddy, Assistant Professor, Department of Pathology, Prathima Institute of Medical Sciences, Nagunur, Karimnagar, Telangana, India.

Email: ravipallymanasa@yahoo.co.in

### ABSTRACT

**Background:** Sickle cell haemoglobin (HbS) results from an autosomal recessively inherited mutation in which the amino-acid glutamine is replaced by valine at position 6 in the beta globin chain of haemoglobin (Hb). Sickle cells have a reduced deformability and are easily destroyed, causing occlusion of the microcirculation and a chronic haemolytic anaemia with a median Hb concentration level of about 9 g/dl. Routine electrophoresis methods and High performance liquid chromatography (HPLC) were used to screen normal and variant Hb, and allowed the verification of the Hb observed with electrophoresis and precise quantification of their proportion.

**Objectives:** This study aimed to evaluate the chromatographic pattern of Hb types (HbA, HbF, HbA2 and HbS) of sickle cell anemia patients in comparison with the apparently healthy individuals and to study the Hb chromatographic patterns according to the gender, age and blood groups. Also to evaluate the efficiency of variant Hb testing system in detection of HbS type of sickle cell anaemia patients.

**Materials & Methods:** A total of Thirty five sickle cell anemia patients who were attending to the Prathima Institute of Medical Sciences, Teaching Hospital, Karimnagar Telangana state, the samples were (25) males and (10) females, from February of 2017 to April of 2017. And Fifteen of case controls with matched age and sex were randomly selected from apparently healthy individuals. High performance liquid chromatography (HPLC) was adopted to determine the different types of Hb for patients and control groups using variant Hb testing system which depend upon these paration and quantification of Hb types by high performance liquid chromatography technique.

**Results:** The study of Hb chromatographic patterns of samples revealed that there were highly significant differences ( $P=0.001$ ) for HbA2, HbA, HbF and HbS percentages of patients in comparison with the control group. The results of Hb chromatographic patterns of samples according to the gender revealed that there were no significant differences at ( $p=0.05$ )

between males and females within patient and control groups. The results of Hb chromatographic patterns of Hb types for patients and control groups according to the age groups revealed that the mean of the highest HbA and HbA2 percentages of patients were ( $8.13\pm7.09$  and  $2.82\pm1.32$ ) respectively in age group (6-10) years, HbF ( $17.32\pm6.63$ ) in 11-15yrs age group, while it was ( $74.55\pm2.35$ ) for HbS type in >16yrs age group. As for control group, the highest HbF percentages was ( $0.78\pm0.18$ ) in age group less than five years old, for HbA was ( $86.5\pm3.69$ ) in age group (6-10) years old, for HbA2 was ( $2.13\pm0.32$ ) in age group (11-15) years old, and for HbS was ( $0.00$ ) in all age groups. Finally, the results also showed that there were no significant differences at ( $P=0.05$ ) for Hb chromatographic patterns of different Hb types percentages according to the blood groups of studied samples within group (patients or controls).

**Conclusion:** The study of Hb chromatographic patterns is useful for the diagnosis of sickle cell anaemia. There are no significant effects of gender and blood groups on the chromatographic patterns of different Hb types of sickle cell anaemia patients in comparison to the apparently healthy individuals. HPLC is an excellent, powerful diagnostic tool for the direct identification of HbS.

**Keywords:** Sickle cell anemia, HbS, HPLC, Variant Hb testing system

### INTRODUCTION

Sickle-cell anemia (SCA) is a genetic life-long blood disorder produced by hemoglobin S (HbS) in its homozygous form, (HbS-HbS) characterized by red blood cells that assume an abnormal, rigid, sickle shape. Sickling decreases the cells flexibility and results in a risk of various complications.<sup>1,2</sup> The sickling occurs because of a mutation in the (HBB gene) hemoglobin gene. This is cause a translocation of the amino acid in position 6 of a normal beta globin, transforming glutamic acid into valine, and thus diminishing protein solubility. This, in turn, causes haemoglobin S to form polymers and produce a red corpuscle shaped as a sickle. Vasoocclusion is hereby provoked, as well as the release of the hemogroup, which interacts with the membrane of red blood cells and

causes hemolysis and the consequent anemia.<sup>2</sup> Life expectancy is shortened, with studies reporting an average life expectancy of 42 and 48 years for males and females, respectively<sup>3</sup>.

#### Inheritance of haemoglobin- S:

SCA is an autosomal recessive genetic disorder caused by a defect in the HBB gene, which codes for hemoglobin. The presence of two defective genes (SS) is needed for SCA. If each parent carries one sickle hemoglobin gene (S) and one normal gene (A), each child has a 25% chance of inheriting two defective genes and having sickle cell anemia; a 25% chance of inheriting two normal genes and not having the disease; and a 50% chance of being an unaffected carrier like the parents.<sup>4</sup>

#### HPLC :

More than 900 hemoglobin (Hb) variants are currently known. Worldwide, an estimated 150 million people carry Hb variants<sup>1</sup> and hemoglobinopathies are the commonest inherited disorders, constituting a significant healthcare problem.<sup>4</sup> Therefore, reliable detection and identification methods are essential. Common techniques used in Hb analysis are electrophoretic and chromatographic assays. Numerous automated HPLC systems are now commercially available, and evaluations have been published.<sup>5,6</sup> HPLC has been shown to have a high degree of reproducibility and precision. HPLC has made hemoglobin abnormality detection much more accurate, faster, and automated<sup>5,7,8</sup>.

This study aimed to evaluate the chromatographic pattern of Hb types (HbA, HbF, HbA2 and HbS) of SCA patients in comparison with the apparently healthy individuals, and study the effects of gender, Age and ABO system on these chromatographic patterns.

#### Materials and Methods

**Variant Hb testing system:** The blood samples from all participants were collected in labelled 5 ml EDTA anticoagulant tubes, using the Hb diluter machine, a specific amount of blood was mixed with diluter buffer, the Hb automated chromatography was adopted using Bio-Rad D-10™(USA), variant Hb testing system for the separation and determination of HbA, HbF, HbA2, and as an aid in identification of abnormal Hb in whole blood according to manufacturer's instructions using the high performance liquid chromatography technique (HPLC). The results are recorded by a diagram of Hb types percentage levels.

**Biostatistical analysis:** The calculation of percentages, mean estimation, standard deviation estimation and t-test statistical

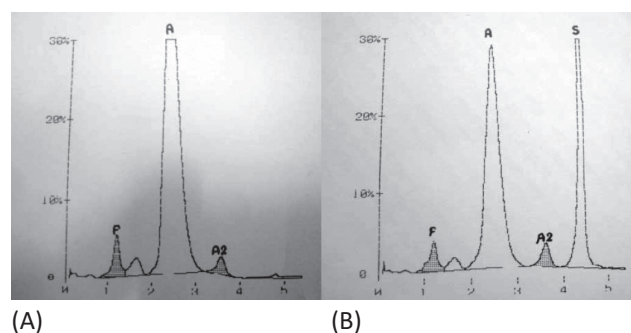
analysis tools which were carried out for the analyses of the data and appropriate p- values of less than 0.05 were considered as statistically significant, and value less than 0.01 was considered to be highly significant.

#### RESULTS

The results of Hb chromatographic pattern using HPLC examination of all studied samples expressed as diagrams to explain the levels of Hb types which analyzed as waves according to the percentages of Hb types in the examined sample (Figure 1).

The study of Hb chromatographic patterns of samples revealed that the mean of Hb percentages of Hb types for patients were HbF (15.32± 6.04) , HbA (4.62± 3.74), HbA2 (2.39 ± 1.20) , and HbS (76.99±4.99) , while for control group, the mean of HbF , HbA , HbA2 , and HbS percentages were (0.76±0.13, 86.24±3.54, 1.81±0.44, and 0.00) respectively [Table 1]. The statistical analysis showed that there highly significant differences (P= 0.01) for HbA,HbA2,HbFand HbS percentages of patients in comparison with the control group. The results of Hb chromatographic patterns of samples according to the gender (Table 2) revealed that the mean of Hb percentages of Hb types for males in patient group were HbF(15.47±5.92%), HbA (4.34±3.76%), HbA2 (2.58 ±1.28 %) and HbS (76.91±23.97 %) while for female patients were HbF (14.97±6.64%), HbA (5.35±3.78%), HbA2 (1.87 ±0.90%) and HbS (76.44 ±5.95 %).

As for the control group, the mean of HbF, HbA ,HbA2 and HbS percentages of males were (0.76±1.50, 86.36±3.64, 1.8±0.49 and absent) ,respectively; whereas for females were (0.76 ±0.089%,86 ±3.74% , 1.84 ±0.36% and absent ) , respectively. The statistical analysis showed that there are no significant differences at (p= 0.05) between males and females within patient and control groups.



**Figure 1. Haemoglobin chromatographic pattern of apparently healthy individual (A) and sickle cell anemia patient (B). The results of Hb chromatographic**

Table 1. Haemoglobin chromatographic pattern of sickle cell anemia patients in comparison to the apparently healthy individuals

| GROUP                  | NO.                | HAEMOGLOBIN TYPE%(MEAN ±SD) |                    |                    |                     |
|------------------------|--------------------|-----------------------------|--------------------|--------------------|---------------------|
|                        |                    | HbF                         | HbA                | HbA2               | Hbs                 |
| Patients               | 35                 | 15.32±6.04                  | 4.62±3.74          | 2.39±1.20          | 76.99±4.99          |
| Controls               | 15                 | 0.76±0.13                   | 86.24±3.54         | 1.81±0.44          | Absent              |
| Significance (p value) | <b>**&lt;0.001</b> | <b>**&lt;0.001</b>          | <b>**&lt;0.001</b> | <b>**&lt;0.001</b> | <b>**&lt;0.0001</b> |

\*Significant differences (P= 0.05). \*\* Highly significant differences (P= 0.01).

Table 2. Haemoglobin chromatographic pattern according to the gender

| GROUP                    | PATIENTS            |                |               |               |                | CONTROLS            |               |                |               |     |
|--------------------------|---------------------|----------------|---------------|---------------|----------------|---------------------|---------------|----------------|---------------|-----|
|                          | HAEMOGLOBIN TYPE(%) |                |               |               |                | HAEMOGLOBIN TYPE(%) |               |                |               |     |
| GENDER                   | (MEAN±SD)           |                |               |               |                | (MEAN±SD)           |               |                |               |     |
|                          | No.                 | HbF            | HbA           | HbA2          | HbS            | No.                 | HbF           | HbA            | HbA2          | HbS |
| MALE                     | 25                  | 15.47<br>±5.92 | 4.34<br>±3.76 | 2.58<br>±1.28 | 76.91<br>±4.66 | 10                  | 0.76<br>±1.50 | 86.36<br>±3.64 | 1.8<br>±0.49  | 0   |
| FEMALE                   | 10                  | 14.97<br>±6.64 | 5.35<br>±3.78 | 1.87<br>±0.90 | 0.76<br>±0.08  | 05                  | 0.76<br>±0.08 | 86.00<br>±3.74 | 1.84<br>±0.36 | 0   |
| *SIGNIFICANCE ( P VALUE) |                     | <b>0.61</b>    | <b>0.91</b>   | <b>0.27</b>   | <b>0.06</b>    |                     | <b>0.06</b>   | <b>0.86</b>    | <b>0.57</b>   | 0   |

\*No significant differences (P = 0.05) between males and female.

Table 3 shows the distribution of studied samples according to the age groups and the Hb chromatographic patterns of Hb types for patients and control groups. The results revealed that the mean of the highest HbA and HbA2 percentages of patients were (8.13±7.09 and 2.82±1.32) respectively in age group (6-10) years, HbF (17.32±6.63) in 11-15yrs age group, while it was (74.55±2.35) for HbS type in >16yrs age group. As for control group, the highest HbF percentages was (0.78±0.18) in age group less than five years old, for HbA was (86.5±3.69) in age group (6-10) years old, for HbA2 was (2.13±0.32) in age group (11-15) years old, and for HbS was

(0.00) in all age groups.

The ABO system was applied to determine the blood groups for all samples. Table (4) shows the distribution of the samples according to the blood groups for both patients and control groups. As for the patients group, the results showed that the most frequent blood groups were blood group A, B<sup>10,11</sup> respectively and O<sup>13</sup>, and for control blood groups were O<sup>4</sup> and A<sup>6</sup>, respectively. Statistically, these results also showed that there were no significant differences (P = 0.05) for different haemoglobin types percentages between blood groups of studied samples within group (patients or controls).

Table 3: Showing distribution of studied samples according to the age groups

| GROUP | PATIENTS  |                |                        |              |               | CONTROLS |               |                        |               |     |
|-------|-----------|----------------|------------------------|--------------|---------------|----------|---------------|------------------------|---------------|-----|
|       | AGE (YRS) | No.            | Hb TYPE (%), (MEAN±SD) |              |               |          | No.           | Hb TYPE (%) (MEAN± SD) |               |     |
| HbF   |           |                | HbA                    | HbA2         | HbS           | HbF      |               | HbA                    | HbA2          | HbS |
| = 5   | 21        | 15.25<br>±6.32 | 4.69<br>±3.84          | 2.4<br>±1.20 | 76.75<br>4.97 | 06       | 0.78<br>±0.18 | 86.00<br>±3.78         | 1.72<br>±0.36 | 0   |

|                                    |    |                |               |               |               |    |               |               |               |          |
|------------------------------------|----|----------------|---------------|---------------|---------------|----|---------------|---------------|---------------|----------|
| 6-10                               | 06 | 16.85<br>±4.74 | 8.13<br>±7.09 | 2.82<br>±1.32 | 74.55<br>2.35 | 04 | 0.7<br>±0.08  | 86.5<br>±3.69 | 2.07<br>±0.25 | 0        |
| 11-15                              | 04 | 17.32<br>±6.63 | 3.1<br>±2.47  | 1.70<br>±1.24 | 78.42<br>6.86 | 03 | 0.8<br>±0.1   | 86.3<br>±4.93 | 2.13<br>0.32  | 0        |
| ≥16                                | 04 | 11.47<br>±6.02 | 4.85<br>±4.59 | 2.38<br>±1.12 | 80.5<br>5.45  | 02 | 0.75<br>±0.07 | 84.3<br>±2.40 | 1.1<br>±0.14  | 0        |
| <b>*Significance<br/>(P value)</b> |    | <b>0.50</b>    | <b>0.31</b>   | <b>0.57</b>   | <b>0.29</b>   |    | <b>0.76</b>   | <b>0.89</b>   | <b>0.01</b>   | <b>0</b> |

\*No significant differences (P= 0.05) in different age groups mentioned above..

Table 4. Haemoglobin chromatographic pattern according to the blood groups.

| GROUP<br><br>BLOOD<br>GROUP        | PATIENTS |                       |               |               |                | CONTROLS |                        |               |               |          |
|------------------------------------|----------|-----------------------|---------------|---------------|----------------|----------|------------------------|---------------|---------------|----------|
|                                    | No.      | Hb TYPE (%) (MEAN±SD) |               |               |                | No.      | Hb TYPE (%) (MEAN± SD) |               |               |          |
|                                    |          | HbF                   | HbA           | HbA2          | HbS            |          | HbF                    | HbA           | HbA2          | HbS      |
| A                                  | 10       | 13.62<br>±5.16        | 4.92<br>±3.14 | 2.54<br>±1.28 | 78.52<br>±5.71 | 06       | 0.76<br>±0.17          | 86.8<br>±3.71 | 1.76<br>±0.39 | 0        |
| B                                  | 11       | 14.95<br>±5.32        | 5.75<br>±4.43 | 2.90<br>1.32  | 76.12<br>±3.97 | 02       | 0.7<br>±0.14           | 87<br>±5.71   | 2.45<br>±0.07 | 0        |
| AB                                 | 01       | 16.3<br>±0.00         | 0.8<br>±0.00  | 0.9<br>±0.00  | 73.4<br>±0.00  | 03       | 0.7<br>±0.00           | 85.6<br>±1.52 | 1.63<br>±0.37 | 0        |
| O                                  | 13       | 16.88<br>7.38         | 3.74<br>±3.53 | 1.96<br>±0.84 | 76.83<br>±5.40 | 04       | 0.82<br>±0.09          | 83.4<br>±1.35 | 1.57<br>±0.43 | 0        |
| <b>*Significance<br/>(P value)</b> |          | <b>0.64</b>           | <b>0.43</b>   | <b>0.14</b>   | <b>0.63</b>    |          | <b>0.62</b>            | <b>0.40</b>   | <b>0.10</b>   | <b>0</b> |

No significant differences (p = 0.05) for different Haemoglobin type percentages .

## DISCUSSION

Figure (A) revealed the presence of HbF, HbA, and HbA2 as a normal Hb types in the apparently healthy individual , and the presence of high level of HbS type as indicated for SCA patient in the Figure B. The mean percentages of Hb obtained by HPLC were shown to be very useful, especially for the identification of Hb variants and demonstrating associations between different hemoglobinopathies, by comparing them with normal values, thus permitting the determination of phenotypes <sup>7,9,10</sup>. In 2004, Joutovsky et al. demonstrated that HPLC is an important analytical tool for the identification of Hb variants, mainly if the information regarding their retention time is used <sup>11</sup>.

The result of Hb chromatographic pattern of sickle cell anemia patients in comparison to the apparently healthy individuals (Table 1) revealed highly significant differences (P= 0.01) between patients and control groups for HbA and HbS

percentages, this is due to the presence of HbS as abnormal Hb type associated with SCA in patient group which lead to decrease the level of HbA within patients in comparison with apparently healthy individuals. Also the results revealed that there were significant differences between patients and control group for HbF percentages, and there were no significant differences for HbA2 percentages, these results may be reflect the homo and hetero zygoty of the beta globin gene on chromosome 11 of the studied samples<sup>4</sup>.

The HbF values found in the subjects with HbS in heterozygosis, although within the range of normality, were statistically higher than those of the control group, due to the presence of the Hb variant and of its possible haplotypes. In the SS group, all samples showed values higher than normal, which may have resulted from the use of medications, hereditary persistence of Hb F or a characteristic of the haplotype. (4,10,12). The study of Hb chromatographic pattern

according to the gender (table 2) showed that the percentages of different types of Hb in both studied groups were nearly equal and there were no significant differences ( $p=0.05$ ) between males and females within patient and control groups, this reflects a fact that SCA is an autosomal recessive disease caused by abnormalities in the  $\beta$ -globin gene located on chromosome 11; so there is no sex-linked disease. Since this disease is unaffected by sex variable, both sexes are equally affected with SCA<sup>4,13</sup>. Table 3 showed the Hb chromatographic pattern according to the age, the results revealed that there were high percentages of HbF values within age group less than five years old in comparison with other age groups for patients and control groups, these findings were due to the high percentages of HbF in children less than one year of age which included with this age group taken in consideration the dominance effect of HbF through the first months of age of newborn infants which lead to the most significant differences showed in the Table 3 due to the incomplete switch from fetal to adult Hb synthesis occurs. Typically, this switch is completed by the sixth month after birth<sup>11,12,14</sup>.

Finally, the study of chromatographic pattern according to the blood groups for all samples (Table 4) showed no significant differences between different blood groups. These findings revealed that there were no effects for the type of blood groups on the chromatographic pattern of Hb types for all studied samples taken.

These findings revealed that SCA may affect patients of different blood groups. Accordingly, there is no association between blood groups and phenotypes; this may be due to gene polymorphism of ABO system, since it is located on chromosome 9 whereas the SCA gene is located on chromosome 11<sup>4,14</sup>.

## CONCLUSION

The study revealed that the detection of Hb chromatographic patterns is useful for the diagnosis of sickle cell anaemia, and there were no significant effects of gender and blood groups on the chromatographic patterns of different Hb types of sickle cell anaemia patients in comparisons to the apparently healthy individuals. And finally the HPLC is an excellent, powerful diagnostic tool for the direct identification of HbS in suspected cases.

## Recommendations

- 1- The use of HPLC for screening newborns with suspected SCA.
- 2- Introduction of PCR based techniques for diagnosis of haemoglobinopathies.

## REFERENCES

- 1- Desai, D. V.; Hire D., (2004). Sickle Cell Disease: History And Origin". *The Internet Journal of Haematology*. ISSN :1540-2649

- 2- Schechter AN, Noguchi CT. Sickle Hg polymer. In: Embury SH, et al., (1994). Sickle cell disease: basic principles and clinical practice. New York: Raven.;33-51.
- 3- Mohandas N, Hebbel R. (1994). Pathogenesis of hemolytic anemia. In: Embury SH, et al., Sickle cell disease: basic principles and clinical practice. New York: Raven:327-334.
- 4- Jord, L.B.; Carey, J.C.; Bamshad, M.J.; and White, R.L. (2004). Medical Genetics. 3rd Ed., Mosby, Elsevier, USA .
- 5- Wild BJ, Stephens AD. (1997). The use of automated HPLC to detect and quantify haemoglobins. *Clin Lab Haematol*. 19: 171-176.
- 6- Clarke GM, Higgins TN., (2000). "Laboratory investigation of haemoglobinopathies and thalassemias: review and update". *Clin. Chem.*:46 (8 Pt 2): 1284-1290.
- 7- Steinberg M. (2007). Sickle cell disease and associated hemoglobinopathies. In: Goldman L, Ausiello D, eds. *Cecil Medicine*. 23rd ed. Philadelphia, Pa: Saunders Elsevier:167.
- 8- Fisher SI, Haga JA, Castleberry SM, Hall RB, et al. , (1997) Validation of an automated HPLC method for quantification of hemoglobin S. *Clin. Chem.*:43: 1667-1669.
- 9- Shokrani M, Terrell F, Turner EA and Aguinaga MD., (2000) Chromatographic measurements of hemoglobin A2 in blood samples that contain sickle hemoglobin. *Ann. Clin. Lab. Sci.*:30: 191-194.
- 10- Riou J, Godart C, Didier H, Mathis M, Bimet C, Bardakdjian-Michau J, et al. (1997) Cation-exchange HPLC evaluated for presumptive identification of hemoglobin variants. *Clin Chem*; 43: 34-39
- 11- Joutovsky A, Hadzi-Nesic J and Nardi MA., (2004) HPLC retention time as a diagnostic tool for hemoglobin variants and hemoglobinopathies: a study of 60,000 samples in a clinical diagnostic laboratory. *Clin. Chem.*:50: 1736-1747.
- 12- Stephen I. F., Jo A. H., Silvia M. C., Robert B. H. and William C. T. (1997) Validation of an Automated HPLC Method for Quantification of Hemoglobin S. *Clin Chem.*:43: 1667-1669.
- 13- Papadea C, Cate JC. (1996) Identification and quantification of hemoglobins A, F, S, and C by automated chromatography. *Clin Chem*;42:57-63.
- 14- Hartwell, L.D., Hood, L., Goldberg, M., Reynolds, A., Silver, L., Veres, R. (2004) Genetics from gene to genome, sec. ed. Mc Graw Hall, US. 987.

**How to cite this article :** Manasa R, Srilatha K. Evaluation of (HPLC) Patterns of Sickle Cell Anaemia patients in comparison with apparently healthy individuals. *Perspectives in Medical Research* 2017;5(3):52-56.

**Sources of Support:** Nil, Conflict of interest: None declared