# Evaluation of (HPLC) Patterns of Sickle Cell Anaemia Patients in Comparison with Apparently Healthy Individuals

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## 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:*** 1- 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.

2- To study the Hb chromatographic patterns according to the gender, age and blood groups.

3- To evaluate the efficiency of variant Hb testing system in detection of HbS type of sickle cell anaemia patients.

***Methods:*** A total of eighty four sickle cell anemia patients who were attending to the Ibn Al-Baladi pediatrics hospital (in Baghdad) and Kerbala teaching pediatrics hospital, the samples were (39) males and (45) females , from April of 2006 to February of 2007 . And thirty seven 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 the separation and quantification of Hb types by high performance liquid chromatography technique.

***Results:*** The study of Hb chromatographic patterns of samples revealed that there were no significant differences ($p \geq 0.05$) between the values of HbA2 for the patients and control groups and there were significant differences ($P \leq 0.05$) for HbF and highly significant differences ($P \leq 0.01$) for HbA 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 ($p \geq 0.05$) 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 heterogeneous results with highest HbF, HbA, and $HbA_2$ percentages of patients were (10.83±3.32, 57.6±7.33, and 4.22 ±1.88) respectively in age group less than five years old, while it was (54.33±8.9) for HbS type in age group (6-10) years old. As for control group, the highest HbF percentages was (8.2±4.09) in age group less than five years old, for HbA was (87.22±5.86) in age group (6-10) years old, for $HbA_2$ was (3.6±0.23) in age group (11-15) years old, and for HbS was (0.3±0.05) in age group less than five years old. Finally, the results also showed that there were no significant differences at ($P \geq 0.05$) for Hb chromatographic patterns of

different Hb types percentages according to the blood groups of studied samples within group (patients or controls).

***Conclusion:***

1- The study of Hb chromatographic patterns is useful for the diagnosis of sickle cell anaemia.
2- 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.
3- HPLC is an excellent, powerful diagnostic tool for the direct identification of HbS.

***Key words:*** Iraq, Sickle cell anemia, HbS, HPLC, Variant Hb testing system

**الخلاصة**

**موطئة:** فقر الدم المنجلي مرض ناتج عن وجود الطفرات الوراثية المتنحية في الجين الموجود على الكروموسوم الجسمي رقم ( 11) والمسؤول عن انتاج السلاسل متعدد الببتيد نوع بيتا في جلوبين الدم , وتؤدي هذه الطفرات الى تغيير الحامض الأميني السادس الكلوتامين الى فالين مما يؤدي الى التحلل السريع لكريات الدم الحمراء والذي ينتج عنه فقر الدم. ويعتبرالفصل باستخدام كروماتوغرافيا السائل عالي الأداء ( HPLC) لمختلف انواع خضاب الدم من الفحوصات المختبرية الدقيقة شائعة الأستعمال في تشخيص فقر الدم المنجلي من خلال تحديد كميات نوع جديد من بروتينات الدم يسمى خضاب الدم المنجلي (HbS).

**الهدف:** تهدف الدراسة الى تقييم انماط الفصل باستخدام كروماتوغرافيا السائل عالي الأداء لمختلف انواع خضاب الدم ( HbA, HbF ، HbS , HbA2 ) لمرضى فقر الدم المنجلي بالمقارنة مع عينات الأصحاء مظهريا . ودراسة علاقة انماط الفصل لبروتينات الدم بالجنس والعمر وفصائل الدم.

**طرائق العمل:** شملت الدراسة ( 84) مريضا مصابا بفقر الدم المنجلي حيث جمعت العينات من مستشفى ابن البلدي (في بغداد) ومستشفى الأطفال التعليمي في كربلاء للفترة من نيسان 2006 ولغاية شباط 2007, وضمت العينة (39) مريضا من الذكور و ( 45) مريضا من الأناث, وشملت الدراسة ايضا ( 37) شخصا تم اختيارهم عشوائيا من الأصحاء مظهريا والمطابقين بالجنس والعمر كمجموعة سيطرة , تم استخدام تقنية الفصل الكروماتوغرافي السائل عالي الأداء لخضاب الدم لكلا المجموعتين لتحديد الأنواع المختلفة لخضاب الدم باستخدام جهاز ( Variant haemoglobin testing system ) .

**النتائج:** أظهرت دراسة انماط الكروماتوغرافيا لخضاب الدم عدم وجود فرق احصائي معنوي بين قيم HbA2 مع وجود فرق احصائي مهم لنسب HbA، HbF و HbS للمرضى مقارنة بالأصحاء مظهريا , وان دراسة انماط الكروماتوغرافيا للعينات طبقا للجنس اظهرت عدم وجود فرق احصائي مهم بين الذكور والأناث ضمن مجموعتي المرضى والأصحاء مظهريا. وطبقا للعمر اظهرت النتائج تفاوت نسب انواع خضاب الدم وتركزت القيم الأعلى ضمن الفئة العمرية اصغر من خمس سنوات للأنواع HbA، (HbF و HbA2) في حين كانت اعلى قيمة لخضاب الدم نوع S في الفئة العمرية ( 6-10) سنوات . وأخيرا أظهرت النتائج ايضا عدم وجود فرق احصائي معنوي لنسب انواع خضاب الدم طبقا لفصائل الدم للعينات المدروسة في كلتا مجموعتي الدراسة .

**الأستنتاجات:**

**1**- ان دراسة انماط الكروماتوغرافيا لخضاب الدم مفيدة في تشخيص فقر الدم المنجلي .

**2**- لا توجد تأثيرات معنوية لكل من الجنس وفصائل الدم على انماط الفصل الكروماتوغرافي لمختلف انواع خضاب الدم لمرضى فقر الدم المنجلي عند مقارنتهم بالأصحاء مظهريا.

3- ان تقنية الفصل باستخدام كروماتوغرافيا السائل عالي الأداء ( HPLC ) تعتبر اداة تشخيصية مهمة للتحديد المباشر لمستوى خضاب الدم نوع S في مرضى فقر الدم المنجلي .

## 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.[1,2,] 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 hemoglobin S to form polymers and produce a red corpuscle shaped as a sickle. Vaso-occlusion is hereby provoked, as well as the release of the hemo group, which interacts with the membrane of red blood cells and causes hemolysis and the consequent anemia.[2] Life expectancy is shortened, with studies reporting an average life expectancy of 42 and 48 years for males and females, respectively[3].

**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.[4]

**HPLC :**

More than 900 hemoglobin (Hb) variants are currently known. Worldwide, an estimated 150 million people carry Hb variants [1] and hemoglobinopathies are the commonest inherited disorders, constituting a significant healthcare problem. [4]

Therefore, reliable detection and identification methods are essential. Common techniques used in Hb analysis are electrophoretic and chromategraphic assays. Numerous automated HPLC systems are now commercially available, and evaluations have been published. [5,6] 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 [5,7,8].

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.

## Patients and Methods

***Patients:*** A total of eighty four SCA patients who were attending to Ibn Al-Baladi pediatrics hospital (in Baghdad) and Karbala teaching pediatrics hospital, the samples were 39 males and 45 females ,from April of 2006 to February of 2007.

***Control group:*** Thirty seven of case controls with matched age and sex were randomly selected from apparently healthy individuals.

***Variant Hb testsing system:*** The blood samples from all participants were collected in labeled 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 (USA), variant Hb testing system for the separation and determination of HbA, HbF, $HbA_2$, and as an aid in identification of abnormal Hb in whole blood according to manufacturer's instructions using the high performance liquid chromategraphy 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.

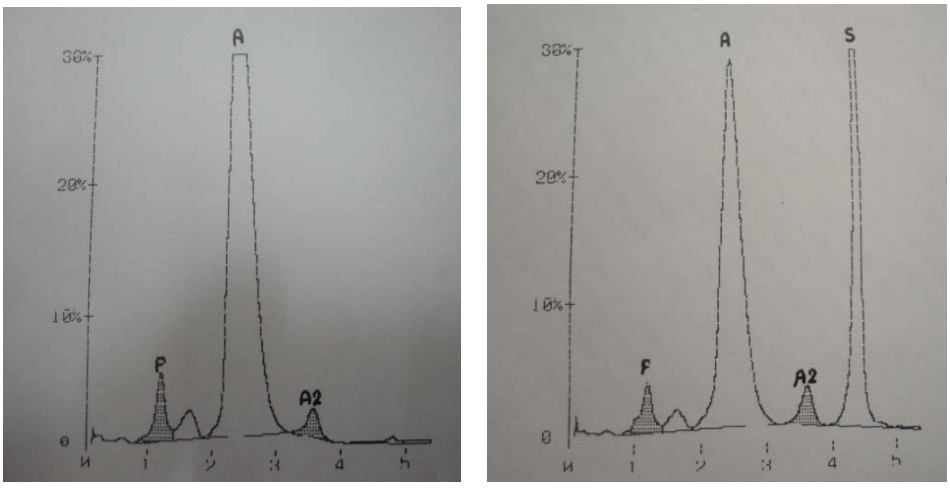
## Results

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 (7.78 ± 6.89) , HbA (43.03± 26.65), HbA2 (3.78 ± 1.17) , and HbS (37.28±23.81) , while for control group, the mean of HbF , HbA , HbA2 , and HbS percentages were (5.72±2.93, 82.14±4.73, 3.84±1.41, and 0.11±0.26) respectively The statistical analysis showed that there were no significant differences (p ≥ 0.05) between the values of HbA2 for the patients and control groups and there were significant differences (P≤ 0.05) for HbF and highly significant differences (P≤ 0.01) for HbA and 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 (7.84±6.95%), HbA (42±26.27%), HbA2 (3.89 ±1.3 %) and HbS (38.19 ±23.97 %) while for female patients were HbF (7.73 ±6.93%), HbA (43.71 ±27.19%), HbA2 (3.7 ±1.07%) and HbS (36.59 ±23.91 %).

As for the control group, the mean of HbF, HbA ,HbA2 and HbS percentages of males were (5.76±3.45, 81.23±5.4, 4.12±1.61 and 0.14±0.31%) ,respectively; whereas for females were (5.66 ±2.27%,83.21 ±3.67% , 3.53 ±1.1 % and 0.08 ±0.19 ) , respectively.The statistical analysis showed that there are no significant differences at (p≥ 0.05) between males and females within patient and control groups.




(A) (B)

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

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 | 84 | 7.78 ± 6.89 | 43.03 ± 26.65 | 3.78 ± 1.17 | 37.28 ± 23.81 |
| Control | 37 | 5.72 ± 2.93 | 82.14 ± 4.73 | 3.84 ± 1.41 | 0.11 ± 0.26 |
| Significance (p – value) | 121 | *Significance (0.01137) | **Highly significance (5.1273E-23) | No significance (0.3983) | **Highly significance (2.0144E-24) |

*Significant differences (P≤ 0.05). ** Highly significant differences (P≤ 0.01).

Table 2. Haemoglobin chromatographic pattern according to the gender.

| Group / Gender | Patients | | | | | Control | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No | Haemoglobin type (%) (Mean ± SD) | | | | No | Haemoglobin type (%) (Mean ± SD) | | | |
| | | HbF | HbA | HbA2 | HbS | | HbF | HbA | HbA2 | HbS |
| Male | 39 | 7.84 ± 6.95 | 42 ± 26.27 | 3.89 ± 1.3 | 38.19 ± 23.97 | 20 | 5.76 ± 3.45 | 81.23 ± 5.4 | 4.12 ± 1.61 | 0.14 ± 0.31 |
| Female | 45 | 7.73 ± 6.93 | 43.71 ± 27.19 | 3.7 ± 1.07 | 36.59 ± 23.91 | 17 | 5.66 ± 2.27 | 83.21 ± 3.67 | 3.53 ± 1.1 | 0.08 ± 0.19 |
| *Significance (p- value) | 84 | (-) 0.4695 | (-) 0.2382 | (-) 0.3941 | (-) 0.3806 | 37 | (-) 0.4602 | (-) 0.1007 | (-) 0.0971 | (-) 0.2242 |

*No significant differences (P≥ 0.05) between males and females.

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 HbF HbA, and $HbA_2$ percentages of patients were (10.83±3.32, 57.6±7.33 , and 4.22 ±1.88 ) respectively in age group less than five years old, while it was(54.33±8.9) for HbS type in age group (6-10) years old. As for control group, the highest HbF percentages was (8.2±4.09) in age group less than five years old, for HbA was (87.22±5.86) in age group (6-10) years old, for $HbA_2$ was (3.6±0.23) in age group (11-15) years old, and for HbS was (0.3±0.05) in age group less than five years old.

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 (31) and O (29) , and for control blood groups were O (15) and A (12), 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. Haemoglobin chromatographic pattern according to the age.

| Group / Age/ Years* | Patients | | | | | Control | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. | Haemoglobin type (%) (Mean ± SD) | | | | No. | Haemoglobin type (%) (Mean ± SD) | | | |
| | | HbF | HbA | HbA2 | HbS | | HbF | HbA | HbA2 | HbS |
| ≤ 5 | 49 | 10.83 ±3.32 A | 57.6 ±7.33 A | 4.22 ±1.88 A | 32.5 ±13.8 A | 18 | 8.2 ±4.09 A | 84.7 ±7.22 A | 3.2 ±0.68 A | 0.3 ±0.05 A |
| 6-10 | 23 | 2.8 ±1.43 B | 39.3 ±4.56 B | 3.8 ±1.2 A | 54.33 ±8.9 B | 9 | 2.9 ±1.2 B | 87.22 ±5.86 A | 2.2 ±1.03 A | 0 A |
| 11-15 | 8 | 2.2 ±1.01 B | 32.2± 4.78 B | 3.33 ±0.73 A | 43.7 ±4.79 AB | 9 | 3.3 ±1.87 B | 79.9 ±8.56 A | 3.6 ±0.23 A | 0.1 ±0.02 A |
| ≥ 16 | 4 | 1.88 ±1.2 B | 27.88 ±3.44 B | 2.84 ±0.62 A | 38.9 ±3.3 AB | 1 | 2.5 B | 85.4 A | 2.7 A | 0 A |

*Similar capital letters (Columns) denote no significant differences ($p \geq 0.05$) and different capital letters (columns) denote significant differences ($p \leq 0.05$).

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

| Group / Blood Group* | Patients | | | | | Control | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. | H]aemoglobin type (%) (Mean ± SD) | | | | No. | Haemoglobin type (%) (Mean ± SD) | | | |
| | | HbF | HbA | HbA2 | HbS | | HbF | HbA | HbA2 | HbS |
| A | 31 | 7.85 ± 6.99 A | 43.28 ± 26.26 A | 3.83 ± 1.34 A | 36.38 ± 24.08 A | 12 | 5.9 ± 3.31 A | 81.63 ± 5.25 A | 4.19 ± 1.51 A | 0.1 ± 0.27 A |
| B | 15 | 7.17 ± 7.37 A | 37.48 ± 26.5 A | 3.92 ± 0.9 A | 41.68 ± 23.81 A | 7 | 5.77 ± 4.13 A | 80.09 ± 6.14 A | 3.99 ± 1.99 A | 0 |
| AB | 9 | 8.91 ± 7.22 A | 51.4 ± 27.79 B | 3.12 ± 0.5 A | 32.28 ± 22.77 A | 3 | 5.37 ± 1.35 A | 84.57 ± 0.51 A | 3.33 ± 0.67 A | 0 |
| O | 29 | 7.66 ± 6.83 A | 43.05 ± 27.49 A | 3.86 ± 1.22 A | 37.5 ± 24.65 A | 15 | 5.61 ± 2.41 A | 83.04 ± 3.89 A | 3.61 ± 1.55 A | 0.21 ± 0.2 B |

*Similar capital letters (Columns) denote no significant differences ($p \geq 0.05$) and different capital letters (columns) denote significant differences ($p \leq 0.05$).

## Discussion

Figure (1) revealed the presence of HbF, HbA, and HbA2 as a normal Hb types in the apparently healthy individual (A), 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 hemoglobin-

pathies, by comparing them with normal values, thus permitting the determination of phenotypes [7,9,10]. 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 [11].

The result of Hb chromatographic pattern of sickle cell anemia patients in comparison to the apparently healthy individuals (table 1) revealed highly significant differences ($P \leq 0.01$) 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 zygosity of the beta globin gene on chromosome 11 of the studied samples[4]. 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 equals and there were no significant differences ($p \geq 0.05$) between males and females within patient and control groups, this reflect a fact that SCA is an autosomal recessive disease caused by abnormallities in the β-globin gene located on chromosome 11; so there is no sex linked disease. Since this disease is unaffected by sex variable, both sex are equally affected with SCA [4, 13].

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, this 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 newborns infants which lead to the most significantly 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 [11, 12, 14].

Finally, the study of chromategraphic pattern according to the blood groups for all samples (table 4) showed no significant differences between most blood groups with an exception for HbA in blood group AB , and HbS in blood group O, these finding revealed that there were no effects for the type of blood groups on the chromatographic pattern of Hb types for all studied samples taken in consideration this results may be due to the chances of sampling, and small number of examined samples of excepted groups

These findings revealed that SCA may affect patients of different blood group. 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 [4,14].

## 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 haemoglbinopathies.

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