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Hemoglobin Variants in Patients Attending Aden Diagnostic Center by High Performance Liquid Chromatography

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Abstract

Introduction: High performance liquid chromatography (HPLC) is emerging as the method of choice for initial screening and diagnosis of hemoglobinopathies. The use of alkaline and acid gel electrophoresis may result in incorrect diagnosis of hemoglobinopathies. The aim of the study is to investigate the hemoglobin pattern using the HPLC and to correlate the hematological profile with the types of hemoglobin.

Methods: A cross-sectional study was conducted in Aden Diagnostic Center from July– December 2022. Over a six-month period, 250 samples of patients aged between six months to thirty years, were evaluated by HPLC for detection of hemoglobinopathies using the Lifotronic Hemoglobin Analyzer H9: β-thalassemia Analysis Mode. Red blood cell and red cell indices were determined using automated hematology analyzer.

Results: A total of 152 samples (60.8%) showed different abnormal hemoglobin variants. Sixty-four (25.6%) were diagnosed to have sickle cell anemia, 46 (18.4%) as sickle cell trait, two (0.8) as beta – heterozygous thalassemia based on high level of HbA2 (>3.9%), four (1.6%) as beta – homozygous thalassemia (HbF 25–91%), 18 (7.2%) as compound heterozygous state of sickle - β+ thalassemia, 17 (6.8%) as beta thalassemia trait, and one (0.4%) sample had HbD variant on HPLC was diagnosed as HbD trait.

Conclusion: H9-HPLC is suitable for the routine investigation of hemoglobinopatheis because it is very powerful tool in the evaluation of Hb variants, rapid assay time and accurate quantification.

Keywords: Hemoglobinopathy, High Performance Liquid Chromatography, Thalassemia.

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تحليل متغيرات الهيموجلوبين عند مرضى مركز عدن التشخيصي باستخدام كروماتوغرافيا السائل عالي الدقة

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ملخص الدراسة

المقدمة: يظهر التحليل اللوني السائل عالي الأداء (HPLC) كطريقة مفضلة للفحص الأولي وتشخيص اعتلالات الهيموغلوبين. قد يؤدي استخدام الفصل الكهربائي القلوي والحمضي إلى تشخيص غير صحيح لاعتلال الهيموغلوبين. الهدف من الدراسة هو دراسة نمط الهيموجلوبين باستخدام التحليل الكروماتوجرافي السائل عالي الأداء (HPLC) وربط المظهر الدموي بأنواع الهيموجلوبين.

المنهجية: تم إجراء دراسة مقطعية في مركز عدن التشخيصي في الفترة من يوليو إلى ديسمبر 2022. وعلى مدى ستة أشهر، تم تقييم 250 عينة لأشخاص تتراوح أعمارها بين ستة أشهر إلى ثلاثين عامًا، بواسطة تحليل كروماتوغرافي سائل عالي الأداء للتبادل الأيوني (HPLC) لتشخيص اعتلالات الهيموجلوبين باستخدام محلل الهيموجلوبين. كما تم تحديد مؤشرات خلايا الدم الحمراء والخلايا الحمراء باستخدام محلل أمراض الدم الآلي.

النتائج: أظهر 152 من المرضى (60.8٪) متغيرات مختلفة من الهيموجلوبين غير الطبيعي. تم تشخيص 64 (25.6٪) على أنهم مصابون بفقر الدم المنجلي، و46 (18.4٪) على أنهم من سمة الخلايا المنجلية، واثنان (0.8) على أنهم ثلاسيميا بيتا متغاير الزيجوت على أساس ارتفاع مستوى HbA2 (> 3.9٪)، وأربعة (1.6٪) كبيتا - ثلاسيميا متماثل الزيجوت((91٪ - 25 HbF، 18 7.2٪)) كحالة متغايرة الزيجوت المركبة للمنجل - β+ ثلاسيميا، 17 (6.8٪) كصفة ثلاسيميا بيتا، وعينة واحدة (0.4٪) بها متغير HbD على HPLC تم تشخيصه على أنه سمة HbD.

الخلاصة: إن بساطة تحضير العينة، والقياس الدقيق لتركيز الهيموجلوبين، والأداة القوية جدًا في تقييم متغيرات الهيموجلوبين ووقت الفحص السريع، تجعل هذه المنهجية مناسبة لتشخيص اعتلالات الهيموجلوبين.

كلمات مفتاحية: اعتلالات الهيموجلوبين، كروماتوغرافيا السائل عالي الأداء، ثلاسيميا

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Introduction

High-performance liquid chromatographic methods with high sensitivity and specificity have recently been developed for both screening and confirmation of hemoglobinopathies in newborns [1,2]. It is a highly sensitive, specific, quick but more expensive method for diagnosis [3].

Inherited hemoglobin disorders affect an estimated 7% of the population worldwide and are considered the most common monogenic disease, with 300,000–500,000 infants born each year with symptomatic conditions [4]. In analyzing samples for the purpose of hemoglobinopathy diagnosis it is important to measure the hemoglobin level, red cell count, mean cell hemoglobin, mean cell volume and red cell distribution width as these will indicate if anemia is present and distinguish between normochromic, normocytic and hypochromic, microcytic conditions [5].

Thalassemia being the major concern. Quantitation of HbA2 and HbF levels by HPLC is of prime importance in a laboratory where facilities for genetic studies are not available. The lifotronic H9 "Beta Thalassemia Mode" program uses ion exchange HPLC to separate and elucidate the relative percentages of hemoglobin variants in whole blood. This study which was performed for the first time in Aden aimed to investigate the hemoglobin pattern using the HPLC and to correlate the hematological profile with the types of hemoglobin.

Methods

Study design and setting

The present study is a cross-sectional study carried out in Aden Diagnostic Center at AL-Mansoura district in Aden, Yemen, for six months July–December 2022. A total of 250 patients attending the center with suspected hemoglobinopathies were diagnosed by H9- Hemoglobin Analyzer HPLC system by β -thalassemia analyzer program, under the experimental conditions specified by the manufacturer [6]. The retention times, proportion of the hemoglobin (%) for all hemoglobin (Hb) fractions were recorded. Complete blood count, red blood cell (RBC) indices and red cell distribution width (RDW-cv) were done in all cases by system hematology analyzer (Sysmex KX-21N).

The reason of requesting the lab test was included (anemic patients with hemoglobinpathies suspecting, patients and relatives with family history of hemoglobinopathies, and premarital screening). Patients with recent blood transfusion (63 cases), i.e., within 3 months before sample collection, were excluded from the study.

Sample collection and preparation

A sample of 2.5 ml of whole blood was collected via vena puncture from patients with the help of a disposable syringe, specimens were drawn into tubes containing dipotassium EDTA (ethylene diamine tetra acetic acid), after collection, the samples were stored at 2-8°C and tested within one week of collection. No preparation was required, but in such case, sample was manually prediluted. Predilution was carried out by mixing 1.5 mL

wash/diluents (agent L) with 10uL of whole blood sample [6].

Reagents

- A Eluent: Used to elute HbA1a 、HbA1b and HbF those are less positively charged.
- B Eluent: Used to elute LA1c and HbA1c those are well positively charged.
- C Eluent: Used to elute HbA0 that is most positively charged.
- L-Hemolytic Agent: Used to lyse erythrocytes and release glycosylated hemoglobin.

Principle of HPLC

The H9 Hemoglobin Analyzer (HPLC) Beta Thalassemia Mode Program utilizes principles of ion-exchange high-performance liquid chromatography (HPLC). The samples were automatically mixed and diluted on the sampling position and injected to the analytical column. When hemoglobin passing through cation-exchange column, it will be absorbed by resin which was balanced by acidic buffer. The less cation of hemoglobin means the less absorbing force; the separated hemoglobin then it will be absorbed by photocell after passing through a 415nm band pass filter. Through amplifying and acquiring photocell signal by computer, measure real-time absorbance of the eluates continuously. For each sample, a sample report and a chromatogram are generated by LCD screen showing all hemoglobin fractions eluted, their retention times, the area of the peaks, and values of the fractions [6]. Regarding the cut of value of HbA2 for beta thalassemia trait, different authors have established different cut of values for HbA2 for diagnosis of beta thalassemia trait, which ranges from 3.5 to 4%, although it has been recommended that, each laboratory needs to establish their own normal ranges [7, 8]. In our laboratory the recommended values for HbA2 > 3.5% are used.

Interpretation of rreports

Reports and chromatograms generated were studied and interpreted by observing hemoglobin concentration, retention time, area percentage of peaks and windows for structural variants. Each chromatogram shows peaks of Hb A0, A2, and Hb F along with C window, D window, S window, and two minor peaks, P2 and P3. Several hemoglobin variants elute a/the same window; they were provisionally diagnosed by retention time and area percentage keeping in mind the ethnicity of the patients.

Statistical analysis

Data analysis was performed using IBM SPSS 22 software, the statistical significances was calculated by using P value < 0.05

Ethical considerations

Ethical approval of the study protocol was obtained from the Research and Ethics Committee, Faculty of Medicine and Health Sciences, University of Aden. Oral informed consent was obtained from the patients after explaining the aim of the study.

Results

Two hundred and fifty blood samples of patient with suspected hemoglobinopathies were screened. The age ranged from 6 months to 30 years. They were 130 males and 120 females. Ninety-eight (39.2%) samples had normal hemoglobin and

152 (60.8%) samples showed different abnormal hemoglobin variants as seen in Table 1**.**

There were 18 samples (7.2%) having HbSS with raised HbA2 > 3.7% and diagnosed as compound heterozygous state of sickle - β+ thalassemia.

In the current study, there were two (0.8%) samples with elevated Hb A2 level (4.1−4.8%) with moderate anemia, MCV (62.5 – 63.9 fl), MCH (17.0 – 17.4 pg), diagnosed as heterozygous β thalassemia.

Beta–homozygous thalassemia (HbFF) was seen in only 4 (1.6%) of studied samples HbF (97.4-98.6%), were shown to have severe anemia (Hb < 7.0%). There was statistical significance with HbA2 level, $P < 0.05$.

There was one case of rare variant in Aden. It had elevation peak of Hb D concentration=16.0% (RT=252.0 sec), with hemoglobin concentration of A0= 58.3%, reduce HbA2= 1.2% and slightly elevated of HbF= 2.1%, with IDA. It was provisionally diagnosed as HbD trait after further confirmation by negative sickling test and alkaline electrophoresis.

Among thirty-four cases (13.6%) who came for premarital checkup, 21 cases (8.4%) were recording with family history of hemoglobinobathies. Of them, 8 cases had state of heterozygous trait HbAS & HbAF.

Table 1: Type of Hemoglobin Pattern among the Study Subjects (n= 250)

Hemoglobin type	**No.**	**%**
Normal Hb (HbAA)	98	39.2
Sickle cell anemia (HbSS)	64	25.6
Sickle cell anemia (HbAS)	46	18.4
Sickle - β+ thalassemia	18	7.2
β thalassemia trait (HbAF)	17	6.8
β homozygous thalassemia (HbFF)	4	1.6
Heterozygous β thalassemia (High HbA2	2	0.8
HbD trait	1	0.4

Identification of hemoglobin variants was made primarily by retention time (RT) windows and area percent. The data were processed and the report gave the chromatogram of time vs. absorbance where the different peaks were identifying in defined windows and their retention as demonstrated in Table 2.

Table 2: Retention Time of Hb Variants of Studied Samples on H9- HPLC System (n= 250)

Window of Hb variants	Retention time		
	Mean±SD	Range (second)	Range (min)
HbF	31.0±0.69	30.0 – 32.4	0.50 – 0.54
HbA1c	30.3±27.4	50.6 - 58.5	0.84 – 0.97
HbA_0	86.0±58.6	121.9 – 130.8	2.03 – 2.18
HbA_2	190.3±11.4	112.7 – 214.4	1.87 – 3.57
HbD	252.0	252.0	4.20
HbS	132.3±130.5	255.8 - 269.5	4.26 – 4.49

Out of 98 samples with normal hemoglobin, 69 were suffering from iron deficiency anemia (IDA), which had low MCV (< 80.0 fl), MCH (< 27.0 pg) and high RDW-cv (> 15.0 %), fifty-five of these showed decrease in the level of HbA2 (< 2.0%). Forty-six samples (18.4%) were diagnosing as sickle cell trait, as their HbS values ranged from 21-42.8% and HbS was less than HbA values as shown in Table 3.

Table 3: Mean Values (mean ± SD) of hemoglobin fractions in sickle cell trait detected by H9-HPLC (n= 250)

Hemoglobin type	Mean	±SD	Ranges	
			Minimum	Maximum
HbA	58.04	±6.00	43.8	69.0
HbA2	2.44	±0.80	1.0	4.4
HbS	33.63	±4.98	21.0	42.8
HbF	2.31	±2.51	0	11.5
N= 46				

In Table 4; sixty-four samples (25.6%) had HbS values with no HbA. Of these, fifty-nine samples (23.6%), had normal HbA2 with increased HbF (16.5 ±9.64%).

Table 4: Mean Values (Mean ± SD) of Hemoglobin Fractions in Sickle Cell Anemia Detected by H9-HPLC (n= 250)

Hemoglobin type	Mean	±SD	Ranges	
			Minimum	Maximum
HbA2	2.63	±0.81	0.7	3.5
HbF	16.49	±9.64	0.7	48.0
HbS	80.62	±9.29	49.7	96.2
N= 64				

Table 5 demonstrates the hematological parameters (mean ±SD, range) in different types of hemoglobin and its correlation. There was statistical significance association between all types of hemoglobin with Hb, RBC, MCHC and RDW, while MCV and MCH showed no association (*P*> 0.05).

Table 5: Hematological Parameters (Mean ±SD, Range) in Different Types of Hemoglobin and its Correlation (n= 250)

Hemoglobin type	**Hematological patterns (mean ± SD, range)**					
	HB g/dl	**RBC ×106 cumm**	**MCV fl**	**MCH pg**	**MCHC g/dl**	RDW-CV %
Normal AA	9.87± 2.37 (4.8-15.4)	4.28±0.85 (2.07-6.09)	73.6±11.6 (51.2-103.4)	23.5±4.86 (11.0-32.8)	29.47±3.0 (22.0-35.1)	17.12±3.63 11.7-25.6)(
Sickle cell trait	9.48 ± 2.02 (5.0-14.2)	4.37±0.92 (2.70-7.93)	71.1±11.2 (45.0-88.5)	22.05±4.4 (12.2-30.5)	29.80±2.7 (22.0-34.0)	16.27±1.38 (14.1-19.4)
Sickle cell anemia	7.94 ± 1.33 (5.2-11.1)	3.28±0.77 (1.70-5.50)	75.7±10.12 (56.0-100.0)	24.8±4.04 (17.0-34.0)	30.64±2.87 (23.0-36.0)	20.6v2.62 (16.3-26.1)
Sickle - β+ thalassemia	7.71 ± 1.72 (4.2-10.7)	3.24±0.76 (1.71-4.52)	74.10±10.2 (58.6-93.2)	24.35±4.3 (18.3-34.5)	30.61±2.74 (24.1-34.3)	21.01±2.01 (17.9-24.3)
Heterozygous β+ thalassemia	7.70±2.26 (6.1-9.3)	3.89±2.22 (2.32-5.46)	63.2±0.98 (62.5-63.9)	17.20±0.28 (17.0-17.4)	27.20±0.84 (26.6-27.8)	16.75±0.49 (16.4-17.1)
β homozygous thalassemia	6.82 ± 2.55 (4.3-6.9)	3.20±0.97 (2.38-3.61)	70.5±4.87 (66.4-69.1')	20.6±1.31 (18.1-21.8)	29.6±1.93 (27.2-30.4)	20.17±2.33 (18.0-23.4)
β thalassemia trait	9.32±1.25 (6.5-11.5)	3.442±0.40 (2.63-4.22)	71.8±12.6 (55.9-94.9)	22.1±5.1 (16.3-31.2)	29.3±2.60 (22.0-32.9)	15.08±1.23 (13.1-17.4)
HbD trait	9.50	3.36	77.50	28.20	27.40	13.3
P	**0.000**	**0.001**	**0.393**	**0.061**	**0.012**	**0.000**

Discussion

The hemoglobinopathies and thalassemias, both are common disorders in Yemen. In the current study, three Hb variants were identified including HbD, HbS, HbF. No another variant of thalassemia or hemoglobinopathy was identified in this study, such as, HbC or HbE which might indicate very low prevalence in Yemen.

Sickle cell anemia (SCA) patients and carriers are most prevalent, whereas, beta – homozygous thalassemia and carries are seen in lower percentile. This low incidence of thalassemia state may be either because of low incidence of the disease due to effective premarital diagnosis between carrier couples or may be the affected patients' group did not attend the center where this study was carried out. There were fifty-nine patients who had SCA with increased HbF (16.5 ±9.64%). This finding is inconsistent with the result found by Steinberg *et al*, who reported that high HbF levels (20.7 ± 8.2%) were present in patients with SCA [9]. Increased HbF synthesis is beneficial in patients with SCA and SCD, the fetal hemoglobin (HbF) is modulate the phenotype of SCA by inhibiting deoxy sickle hemoglobin (HbS) polymerization [9].

Fifty-five samples (22.0%) with associated IDA showed decrease in the level of HbA2<2.0%. IDA is a very common occurrence in most of the screening populations, school children and pregnant women in Yemen. Iron deficiency may lower the HbA2 concentration [10]. There are several studies discussed the impact of iron deficiency on HbA2 level and showed controversy over its significance in screening of beta thalassemia trait [7,11,12].

Preventing sickle cell and thalassemia diseases by premarital carrier screening to reduce the number of affected birth is recommended [13]. So, according to precision of retention time [14], the study found the separation between HbF, HbA2, HbA, HbS and D-window was clear. Minor changes in retention times can result in peaks being wrongly identified. The fact that some variant hemoglobins often elute with the same retention time [15]. The study found this problem in two samples with fraction of HbD elute in the same peak of HbS fraction. The problem was solved by checking up the buffers of mobile phase, with the amount of eluent A reagent was very low also after confirmation by positive sickling test. Ching-Nan and Cheryl from America, observed that pH of mobile phase A has a stronger effect on the retention time of fast-eluting hemoglobin [16]. For this reasons, the initial identification and/or quantitation of peaks may be incorrect and if not detected can lead to diagnostic errors.

Conclusion

HPLC achieves good separation and quantification of HbF and HbA2. The study concluded that SCA and sickle cell trait are major hemoglobinopathies. Clearly, there is a significant decrease in the level of HbA2 in samples which associated with low Hb, MCV, MCH and high RDW levels, that suspected as IDA.

Low amount of buffer A can lead to misidentification of correct hemoglobin, because the amount of buffer A has effect on the retention time of fast-eluting hemoglobin with specific flow rate of a liquid (mobile phase).

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