

Comparison of two analytical methods (electrophoresis and HPLC) to detect thalassemias and hemoglobinopathies

Monica Dogaru^{1*}, Daniel Coriu¹, Trefor Higgins²

1. Immunochemistry Laboratory, Clinical Hematology, Fundeni Clinical Institute, Bucharest

2. Dynacare Kasper Medical Laboratories, Edmonton, Canada

Abstract

The role of the laboratory in the diagnosis of thalassemia and hemoglobinopathies is crucial. The objective of our study was to compare two common methods used in hemoglobinopathy and thalassemia investigation. Hemoglobin electrophoresis and HPLC (high performance liquid chromatography) were used to investigate patients suspected with thalassemia. A total of 301 adult and child blood samples were examined for routine thalassemia screening with both methods. The HPLC method and electrophoresis methods gave similar results for patients without thalassemia. HbH disease was readily noted on HPLC but was difficult to detect on electrophoresis. There were some changes in classification for patients with possible β thalassemia minor. For patients with β thalassemia the HPLC gave better results for low HbA₂ concentrations.

Key words: thalassemia, hemoglobinopathy, high performance liquid chromatography

Introduction

Thalassemias and hemoglobinopathies are a group of genetic disorders resulting from mutation or deletion of one of the globin genes of hemoglobin. Thalassemias are caused by the defective or absent production of one or more of the globin chains. Deficient α chain production lead to α thalassemia and decreases in β globin production leads to β thalassemia. The hemoglobinopathies are those hemoglobins that are structurally abnormal due to an alteration in the amino acid sequence of the globin chains. These hereditary hemoglobinopathies represent

the most frequent genetic disorders affecting approximately 4.5% of the world population. Each year about 300.000 affected homozygotes are born, equally divided between sickle cell disorders and thalassemia syndromes (WHO Scientific Group, 1996)³. Diagnosis of these diseases requires the use of methods which allow for precise and accurate quantification of different hemoglobin fractions.

The preliminary selection of individuals at risk of being heterozygous for a form of thalassemia is based on the determination of MCV and MCH. Thalassemic individuals have reduced MCV and MCH, an MCV of 72 fL or

*Corresponding author: Monica Dogaru, Immunochemistry Laboratory, Clinical Hematology, Fundeni Clinical Institute, Bucharest, Romania
Phone +40 723 610589, E-mail monica2982003@yahoo.com

less and MCH of less than 27pg are suggestive of a presumptive diagnosis of thalassemia⁵.

All β thalassemias are characterized by an increase in HbA₂ concentration⁸. At the molecular level the β thalassemias are very heterogeneous with more than 200 point mutations and deletions of different severity⁴. The degree of severity generally correspond to the magnitude of residual output by the defective β globine gene, accordingly the β thalassaemia mutations are classified into severe, mild and silent.

The laboratory plays an important role in the investigation of the β thalassemias¹. The technical facilities, infrastructure and available financial resources play a very important part in selecting the most suitable laboratory methods for carrier identification. In several countries of the world there are screening programs with the aim of identifying carriers of hemoglobin disorders in order to assess the risk of a couple having a severely affected child and to provide information on the options available to avoid such an eventuality. The laboratory methods involved in diagnosis and screening of thalassemia are relatively expensive (electronically determined RBC indices, HPLC analysis of hemoglobin) and the flowchart for identification of carriers is complex and may include methods such as globin chain analysis and DNA analysis especially in populations where both α and β -thalassemia are common and where interactions of α and β -thalassemia could lead to missed diagnoses due to the normalization of red cell indices.

In this study two methods were used to quantitate hemoglobin fractions that may be used in the diagnosis of thalassemia. These methods were hemoglobin electrophoresis and HPLC (High Performance Liquid Chromatography). In Romania, hemoglobin electrophoresis has been the method of choice for the identification and quantification of different hemoglobins.

Methods and materials

Sebia (Sebia, France) hemoglobin electrophoresis at pH 8.4 on agarose gel was used as the initial electrophoresis with visualization using Amido Black. The order of migration of the hemoglobin bands was (from cathode to anode) HbC, HbS, HbF, HbA, HbJ, HbI, HbN, HbH. Hemoglobins D and HbG co-migrate with HbS and hemoglobins E and O co-migrate with HbC. When an abnormal band is observed in electrophoresis at alkaline pH then hemoglobin electrophoresis at pH 6.2 utilizing a citrate buffer was used for confirmation.

For the second method an automated HPLC (VARIANTTM, Bio-Rad) system with the Beta-thalassemia Short Program was used. The Beta-thalassemia Short Program is applicable to the diagnosis of α -thalassemia and β -thalassemia disorders including HbH and HbBart's disease in adults, newborns and fetuses. This program has been designed to separate and quantify the hemoglobins HbA, HbA₂, HbF, HbS, HbC from other hemoglobins in a 6.5 min run. The system cannot quantify accurately HbH and Hb Bart's⁹.

The Beta-thalassemia Short Program utilizes the principles of cation-exchange high pressure liquid chromatography in which a mixture of molecules with a net positive charge is separated into their components by absorption onto a negatively charged stationary phase in a chromatography column, followed by their elution by a mobile phase. Specifically the Beta – thalassemia Short program uses a 3cm×0.46cm cartridge packed with a 5 μ m silica-based weak cation exchange material. Samples of whole blood (5 μ l in 1 ml of buffer) are hemolyzed before injection. The analytes are eluted at a flow rate of 2ml/min using a step gradient of two phosphate buffers with different pH and ionic strengths. As the ionic strength of the mixture increases, more strongly retained hemoglobins elute from analytical cartridge. A dual wavelength filter photometer (415nm and 690 nm)

monitors the hemoglobin elution from the cartridge, detecting absorbance changes at 415 nm. The 690 nm secondary filter corrects the baseline for effects caused by mixing buffers with different ionic strengths. Changes in absorbance are monitored and displayed as a chromatogram of absorbance versus time. There are established windows with specific retention times for the most frequently occurring hemoglobins⁸.

At the beginning of each run the HbA₂/HbF Calibrator was analyzed to provide calibration factors for both hemoglobin A₂ and F. These factors are used in the calculation of area percentages for HbA₂ and F in all subsequent analyses in the run⁸. The Lyphocheck Hemoglobin A₂ Control (Levels 1 and 2 from Bio-Rad) was also analyzed establishing that the concentration values of HbA₂ and HbF were within acceptable limits.

The age, ethnic origin, family history of the patient plus laboratory data including serum iron and red cell morphology was included. The erythrocyte indices MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), RBC (red blood cell count), RDW (red cell distribution width)⁵ and hemoglobin were determined using the Sysmex NE1500 automatic cell counter.

This study was undertaken during a 12 month period at the Immunochemistry Labora-

tory, Clinical of Hematology, Fundeni Clinical Institute. A total of 301 adult and child blood samples were examined for routine thalassemia screening with both methods. The patients were guided to our department with the presumptive diagnosis of thalassemia.

Results

Table 1 summarizes the results obtained in this study.

Both methods (hemoglobin electrophoresis and HPLC) gave almost similar results.

Hemoglobin electrophoresis and HPLC must be repeated after the iron deficiency is ruled out.

There were no clinical and hematological information about the patients.

The diagnosis was made by HPLC, but could not be established by hemoglobin electrophoresis.

Eight patients had high levels of HbA₂ by using hemoglobin electrophoresis but normal levels of HbA₂ by using HPLC, erythrocyte indices were also normal. The diagnosis of these patients was negative for thalassemia.

Two patients had normal levels of HbA₂ by using hemoglobin electrophoresis but high levels of HbA₂ by using HPLC, erythro-

Table 1. Results obtained by using hemoglobin electrophoresis and HPLC

Diagnosis	Number of patients	%
Major beta thalassemia ³	4	1.32
Intermedia beta thalassemia ³	2	0.66
Minor beta thalassemia ³	132	43.8
Beta-delta talasemia ³	3	0.99
Normal ³	102	33.88
Anemia+ possibly thalassemia ⁵	32	10.63
Uncertain diagnosis ⁸	14	4.65
Hemoglobin H ⁹	1	0.33
Minor alpha thalassemia ³	1	0.33
Minor beta thalassemia by using electrophoresis and normal by using HPLC ¹	8	2.65
Normal by using electrophoresis and minor beta thalassemia by using HPLC ⁷	2	0.66

Table 2. The erythrocyte indices and levels of HbA and HbA₂ using hemoglobin electrophoresis and HPLC in 10 patients

Number	HbA% ELF	HbA ₂ % ELF	HbA% HPLC	HbA ₂ % HPLC	RBC	Hb	MCV	MCH	RDW	Iron
1	96.3	3.7	82.9	2.9	5.1	14.1	80	27.7	12.7	176
2	97.2	3.7	88.8	2.9	3.85	12.97	92.07	33.6	14.84	271
3	94.8	4	84.1	2.8	4.54	13.09	76.02	28.84	13.57	60
4	94.5	4.8	86	3.1	4	12.4	85.22	30.73	12.3	106
5	95.6	4.1	87.3	2.8	4.1	12.64	83.88	30.74	15.85	79
6	95.7	4.3	85.9	3.2	4.79	14.25	83.66	29.74	15.01	96
7	95.5	3.8	86.2	2.7	4.9	15.39	88.06	30.99	16.25	67
8	96.3	3.7	82	2.8	3.19	9.67	82.18	30.29	15.08	
9	97	3	84	4		13.4	69	22.6	14.3	
10	96.7	3.3	83.4	4.5	4.63	9.7	70	21	13.9	80

cyte indices were low. The diagnosis in these patients was β -thalassemia minor.

In Table 2 are presented the erythrocyte indices and levels of HbA and HbA₂ using both methods in the 10 patients where HbA₂ by the two methods did not agree with respect to the reference range.

Patients classified as normal by both methods

One hundred and two patients (33.88 %) were confirmed to have no hemoglobin disorders by using hemoglobin electrophoresis and HPLC.

In Figure 1 is shown the chromatogram and electrophoretic migration of a healthy sub-

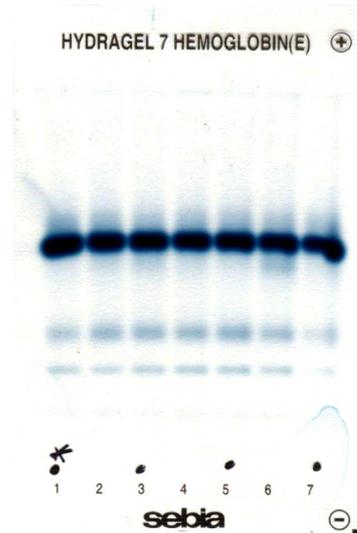
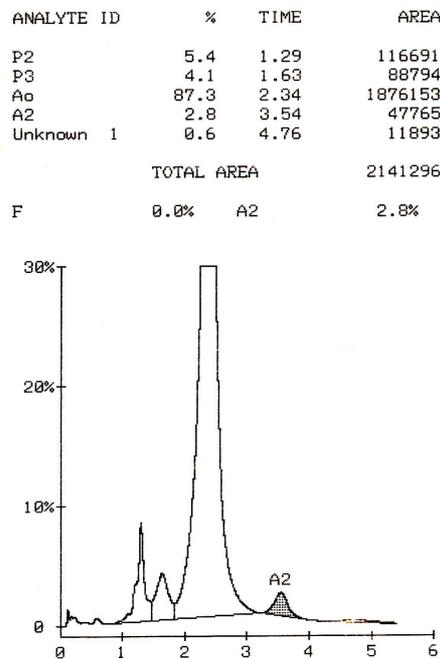


Figure 1. HPLC chromatogram and electrophoresis at alkaline pH for a normal subject (lane 1)

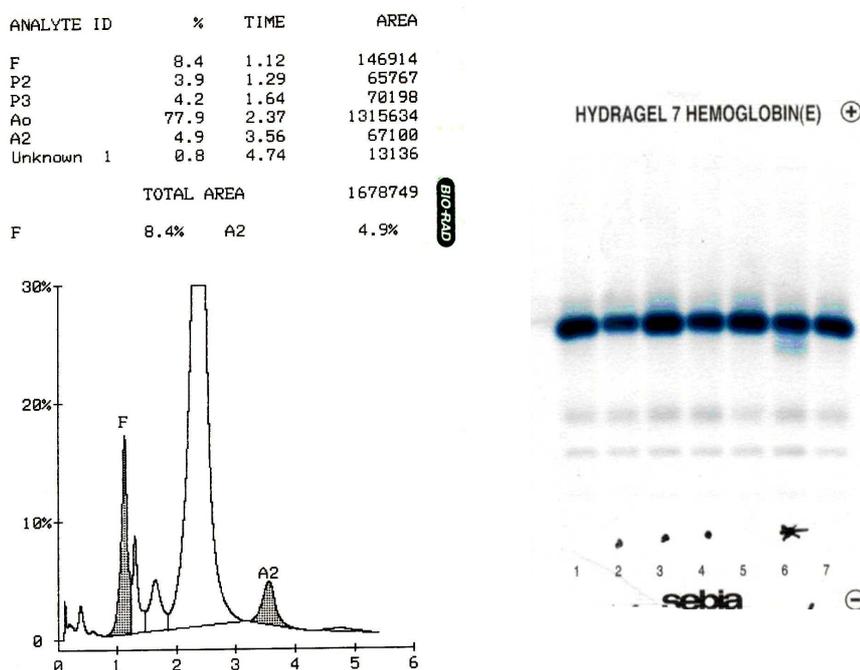


Figure 2. HPLC chromatogram and electrophoresis at alkaline pH for a subject with β thalassemia minor (lane 6)

ject. By densitometric scanning the following values were obtained HbA-97.2%, HbA₂- 2.8%, HbF obtained by the method of alkaline-resistance was 0.1%. The HPLC showed the following values: HbA-87.3%, HbA₂-2.8%, HbF-0.0%.

The erythrocyte indices on this patient were: RBC 4.5 X 10⁶ / μ L (normal 3.50-6.00 X 10⁶), Hb 13.9g/dL (normal 12-18g/dL), HCT 38.25% (normal 36-54%), MCV 83.98 fL (normal 80-100fL), MCH 30.51pg (normal 27-34 pg), MCHC 36.33 g/dL (normal 33-35g/dL), RDW 13.68 % (normal 11.5-14.5%), iron 80 μ g/dL (normal 37-145 μ g/dL).

Patients with β thalassemia minor

132 patients (43.8%) were confirmed to have β thalassemia minor. In all these cases the hemoglobin electrophoresis showed high levels of HbA₂ greater than 3.5% (normal 1.5-3.5%) with a mean HbA₂ concentration of 5.8% (range 3.9-8.3%) and the HPLC showed levels greater

than 3.1% (normal: 2.1-3.1%) with a mean of 5.3% (range 3.3%-6.1%). High levels of HbA₂ (greater than 7%) obtained by electrophoresis were not confirmed by the HPLC.

Figure 2 shows the chromatogram and hemoglobin electrophoresis of a 31 year old patient with a diagnosis of β thalassemia minor. The following values were obtained by densitometric scanning HbA 90.1%, HbF 5.3%, HbA₂ 4.6%. HbF obtained by the method of alkaline-resistance was 6%. The HPLC showed the following values: HbA-77.9%, HbA₂-4.9%, HbF-8.4%. The erythrocyte indices were: RBC 4.28x 10⁶/ μ L, Hb-11.25g/dL, HCT 30.28%, MCV 70.61fL, MCH 26.24pg, MCHC 37.16g/dL, RDW 16.58%, Rt 7%. Red cell morphology shows anisocytosis, poikilocytosis, target cells, hypochromia and microcytosis. The iron was of 169 μ g/dL.

Patients with β thalassemia major

4 patients (1.32%) were reported with β

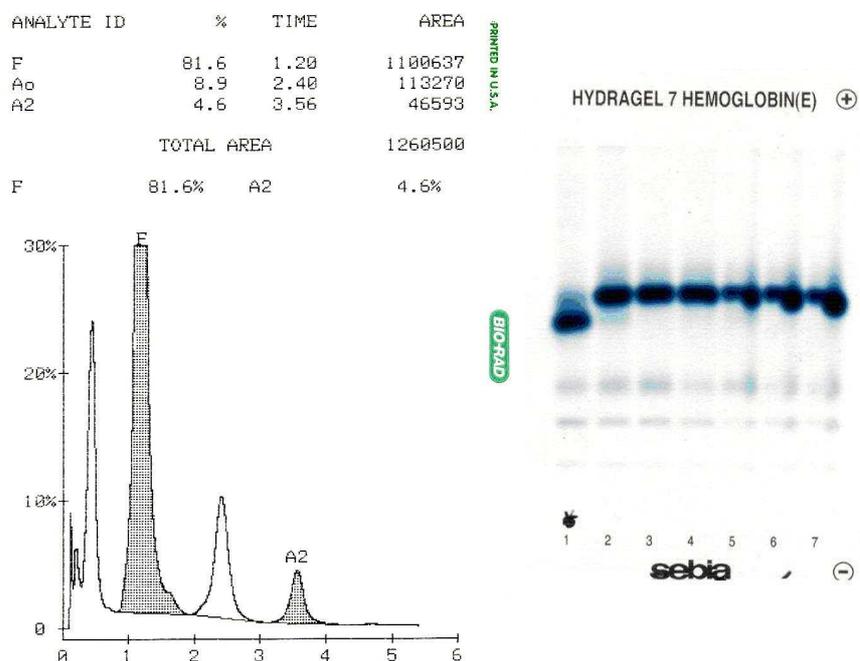


Figure 3. HPLC chromatogram and electrophoresis at alkaline pH for a subject with β thalassemia major (lane 1)

thalassemia major.

Figure 3 shows the chromatogram and hemoglobin electrophoresis of a 39 years old patient with a diagnosis of β thalassemia major. The following erythrocyte indices were obtained on this patient; RBC $3.6 \times 10^6/\mu\text{g}$, Hb 8.6 g/dL, HCT 24.2%, MCV 65.8 fL, MCH 23.5pg, MCHC 35.7g/dL, RDW 33%, Rt 5.8%. Red cell morphology shows anisocytosis, poikilocytosis, target cells, hypochromia and microcytosis. The iron was of 99 $\mu\text{g}/\text{dL}$, total bilirubin was of 2.37mg/dl (normal: 0-1.2mg/dl). Also the patient presented with an enlarged liver and spleen (25 cm).

Beta thalassemia intermedia

The results of 2 patients (0.66%) were reported with β thalassemia intermedia are summarized in Table 3.

Patients with HbH disease

A patient, age 43, was reported with hemoglobin H.

The erythrocyte indices in this patient were: RBC $4.28 \times 10^6/\mu\text{L}$, Hb 8.87g/dL, HCT 28.37%, MCV 66.28fL, MCH 20.71pg, MCHC 31.25 g/dL, RDW 21.39%, Rt 13 %.

Red cell morphology shows marked anisocytosis, marked poikilocytosis, target cells, hypochromia and microcytosis. The iron

Table 3. The results of 2 patients reported with β thalassemia intermedia

Nr.	HbA% ELF	HbA ₂ % ELF	HbF% ELF	HbA% HPLC	HbA ₂ % HPLC	HbF% HPLC	RBC	Hb	MCV	MCH	RDW
1	67.3	1.6	31.1	54.8	3.5	38.9	3.15	8.9	77.7	28.3	22.6
2				50.3	5.7	41.4	3.77	7.6	69.2	20	34

ANALYTE ID	%	TIME	AREA
F	1.7	1.10	20405
Unknown 1	1.2	1.20	14804
P2	2.5	1.30	29676
P3	9.3	1.63	111470
A0	83.1	2.35	993051
A2	1.3	3.54	12609
Unknown 2	1.0	4.72	11711
TOTAL AREA			1193726
F	1.7%	A2	1.3%

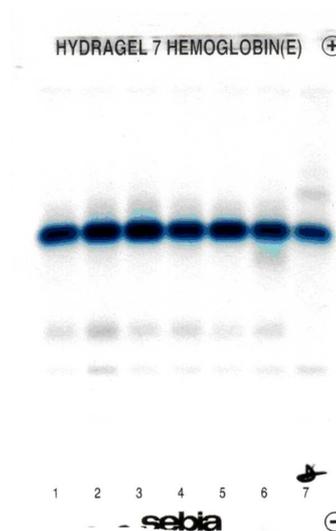
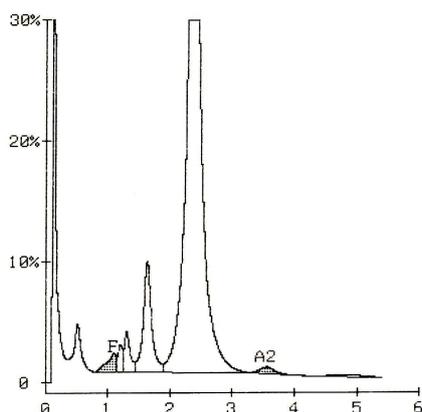


Figure 4. HPLC chromatogram and electrophoresis at alkaline pH for a subject with HbH (lane 7)

was of 97 µg/dL, total bilirubin 3 mg/dL. The patient presented with enlarged liver and spleen (15 cm).

The electrophoresis of hemoglobin showed an abnormal band which migrated faster than HbA closer to anode. The HbA₂ band was poorly visible. The chromatogram showed a sharp, abnormal peak at the point of injection and the Hb values were HbA-83.1%, HbA₂-1.3%, HbF-1.7% (Figure 4).

The beta-thalassemia Short Program cannot measure the concentration of HbH and the diagnosis of HbH disease must be confirmed by the findings of erythrocytes containing inclusion bodies.

Patients with α thalassemia minor

A presumptive diagnosis was made for α-thalassemia minor (Figure 5) when the patient, 18 years old, had a low mean corpuscular volume. Her mother was diagnosed with HbH disease.

The erythrocyte indices are: RBC 5.59×⁶10/^µL, Hb 12.47g/dL, HCT 36.07%, MCV 64.45fL, MCH 22.27pg, MCHC 34.55g/dL, RDW 14.67%. Red cell morphology shows anisocytosis, poikilocytosis, target cells, hypochromia and microcytosis. The iron was of 48 µg/dL.

The electrophoresis showed the following: HbA-97.1%, HbA₂-2.9%, HbF obtained by the method of alkaline-resistance was 1%. The HPLC showed the following values: HbA-76.4%, HbA₂-2.4% and HbF-1.9% .

In this case, additional methodologies, such as DNA analysis are needed to confirm the diagnosis of α- thalassemia minor.

Hb Lepore

Three cases of hemoglobin Lepore, all from the same family: mother, son and daughter, were identified. For all three cases hemoglobin electrophoresis at alkaline and acid pH were performed to confirm the presence of

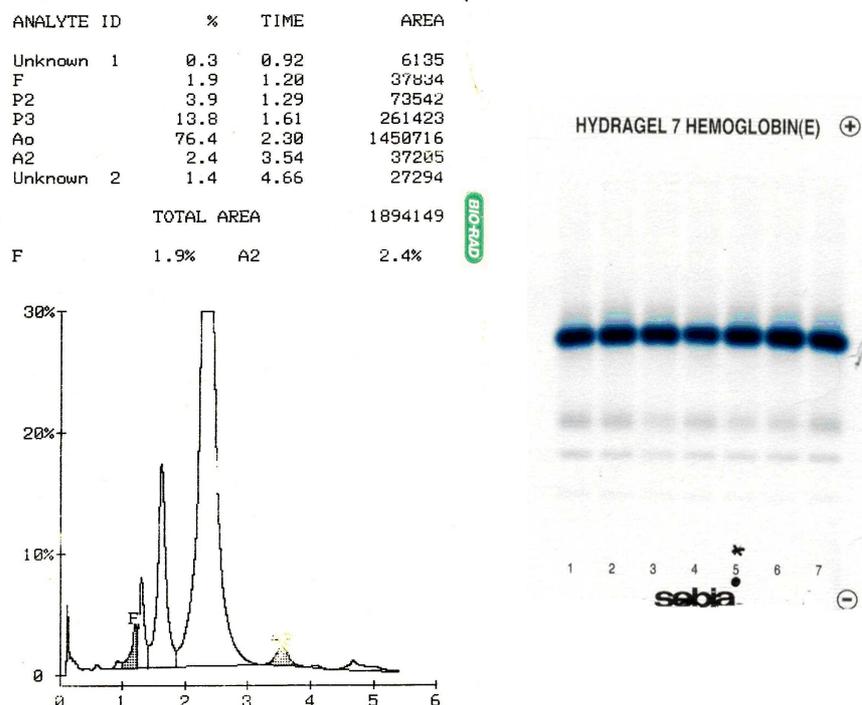


Figure 5. HPLC chromatogram and electrophoresis at alkaline pH for a subject with α -thalassemia minor (lane 5)

hemoglobin Lepore and rule out a possible existence of hemoglobin S (on alkaline electrophoresis HbS and Hb Lepore have the same migration but on acid electrophoresis where HbS migrates differently from Hb Lepore). In *Figure 6* are presented two cases with hemoglobin Lepore and electrophoretic migration at alkaline and acid pH with one control positive for HbS and HbC and one normal control.

Discussion

Hemoglobin electrophoresis is a labor intensive and time consuming method and is not that efficient when quantifying low concentrations of HbA₂ and HbF or detecting HbH or HbBart's.

The HPLC (High Performance Liquid Chromatography) method is a sensitive and precise method and has become the preferred method for thalassemia screening because of its

simplicity, superior resolution, rapid assay time and accurate quantification of Hb fractions⁷.

Despite technical advances and the large amount of accumulated knowledge several problems in carrier identification remain. The most common problem is the presence of microcytosis with HbA₂ and HbF concentrations within the reference range. This may be due to iron deficiency or α -thalassemia trait. Iron deficiency anemia produces a wide range of red cell abnormalities (reduction of MCV, MCH and hemoglobin levels and normal or lowered RBC) depending on the severity at the time of hematological analysis. For this reason iron deficiency anemia can be easily mistaken for some forms of heterozygous thalassemia⁴. On the other hand a raised RBC with low MCV and MCH is more consistent with α thalassemia trait. It is mandatory that testing for iron deficiency accompany all requests for thalassemia analysis. There are a number of calculations based on the red cell indices that are helpful in differentiat-

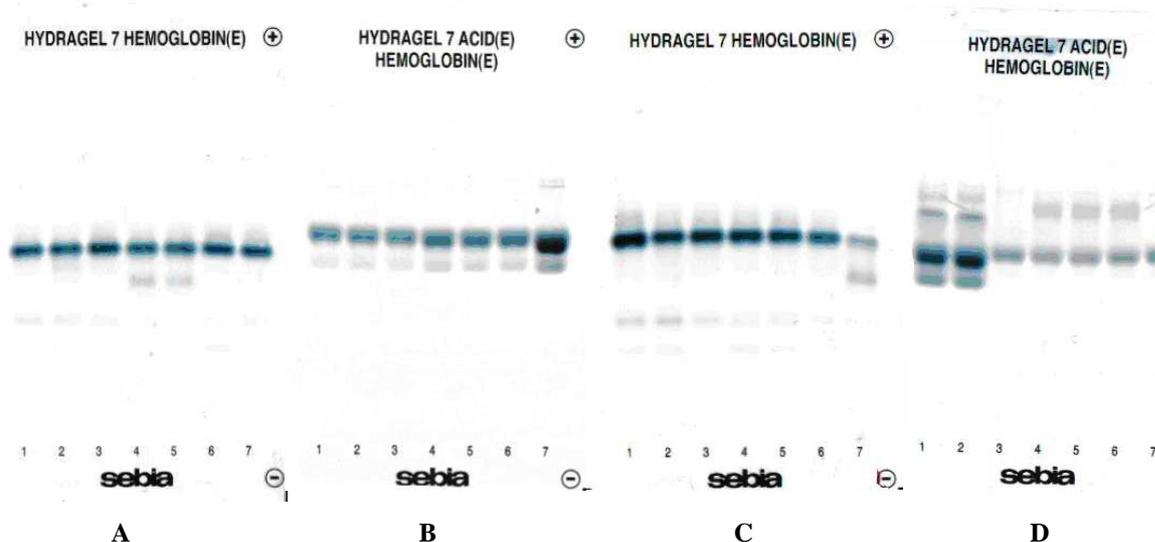


Figure 6. Electrophoretic migration at alkaline (A) and acid (B) pH for two subjects with hemoglobin Lepore and electrophoretic migration at alkaline (C) and acid (D) pH with one control positive for HbS and HbC and one normal control.

ing iron deficiency from thalassemia⁶.

It is possible that with a very severe iron deficiency in β -thalassemia carriers the HbA₂ levels can fall to within the normal range². In practice, if an individual has very severe iron deficiency anemia with normal HbA₂, it is preferable to correct the anemia before repeating tests to determine HbA₂ levels. Family studies may also be useful for distinguishing iron deficiency anemia from the thalassemia traits.

In Romania, hemoglobin electrophoresis represents the only readily available method for the diagnosis of hemoglobinopathies. It should be pointed out that densitometric evaluation of HbA₂ after electrophoresis is not an accurate and reliable method for quantifying HbA₂ minimizing the usefulness to detect patients with β thalassemia minor.

An effective health education programme should aim to provide reliable, accurate and up-to date information on all aspects of the prevention and clinical care of thalassemia major, in a clear, accessible format. The key to successful control programmes is health educa-

tion along with screening, genetic counseling and prenatal diagnosis.

Conclusion

Premarital testing should be introduced in Romania. In other Mediterranean countries such as Greece, Italy and Cyprus it is already in place. In Cyprus the church requires couples to produce a certificate of carrier testing before they can be married.

Inductive testing (also known as extended family testing) involves the testing of relatives of identified carriers and is considered as a way of improving the efficiency of carrier identification.

If a silent carrier is suspected on the basis of borderline red cell indices and/or borderline HbA₂ levels, definitive diagnosis may be obtained using characterization of the mutation by DNA analysis. Because of their silent phenotype these carriers may escape identification in a routine screening programme.

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