HEMOGLOBIN TYPING BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract. To evaluate the potential application of high performance liquid chromatography or HPLC in performing hemoglobin typing, comparison between this technique and routine methods was carried out. Blood specimens from Pediatric Hematology Unit, Research Center, Faculty of Medicine, Ramathibodi Hospital were examined by these methods. The level of Hb A_2, Hb E and Hb F were compared. Hb A_2 level determined by HPLC and electrophoresis was statistically significantly different but correlated well. Good correlation was found between Hb A_2 level from HPLC compared with microcolumn chromatography although there was a statistical difference. No difference but very good correlation was found between the level of Hb E from HPLC compared with electrophoresis. Statistical difference was encountered when Hb F level determined by HPLC was compared to that determined by Betke alkaline denaturation test. However, good correlation was observed when the level of Hb F was greater than 2.0%.

In conclusion, HPLC could be an alternative way of performing hemoglobin typing provided that Hb F is 10% or more, by calculating from the equation: Alk F = (0.83 × Hb F by HPLC) - 0.98.

INTRODUCTION

Hemoglobin is globular protein comprised of four polypeptide chains, two of one kind and two of another. Different types of hemoglobin contain different polypeptide chains. Generally, different polypeptide chains bear different net electrostatic charges and this is the same for hemoglobins. This electrostatic property of hemoglobins is basic for their separation.

The separation of hemoglobin chains is accomplished by two main methods: electrophoresis and chromatography. Routinely, hemoglobin separation and quantitation are done by the technique of cellulose acetate electrophoresis (Marengo-Rowe, 1965). Hemoglobin F quantitation is done by the technique of alkaline denaturation (Betke *et al*, 1959). Besides the separation on cellulose acetate, Hb A_2 may be separated and quantitated by the technique of microcolumn chromatography (Huisman *et al*, 1975). Practically, these techniques require several steps to complete the analysis. Mass hemoglobin analysis is not possible by these routine procedures.

Since 1950, the separation and quantitation of hemoglobin by the technique of chromatography was introduced (Schroeder and Huisman, 1980). High performance liquid chromatography (HPLC) was used for the same purpose shortly thereafter (Huisman, 1987; Schroeder, 1986; Wilson *et al*, 1986; Huisman, 1986). The advantage of HPLC over the routine procedures for hemoglobin typing is the ease of performance, requirement of smaller amount to sample, shorter analysis, so that mass hemoglobin typing can be done.

The aim of this study was to evaluate the hemoglobin typing ability of HPLC compared with the routine procedures.

MATERIALS AND METHODS

Subjects

The subjects were patients attending the Pediatric Hematology Unit, Research Center, Faculty of Medicine Ramathibodi Hospital. Four ml of EDTA blood was collected in which 3 ml was for hemolysate preparation, 1 ml was for HPLC.

Routine hemoglobin typing

Cellulose acetate electrophoresis was employed to separate and quantitate hemoglobins (Marengo-Rowe, 1965; ICSH, 1986). Two types of cellulose

acetate membrane were used. Cellulose acetate plate (Titan III-H, Helana Laboratories) was used to identify hemoglobin patterns. Cellulose acetate strips (Gelman) were used to quantitate hemoglobin fractions. Microcolumn chromatography (Bio-Rad Laboratories) was also used to quantitate Hb A_2 and Hb E. Betke alkaline denaturation test was employed for Hb F quantitation.

High performance liquid chromatography (HPLC)

The Bio-Rad equipment (Model 2800) for ion exchange HPLC and Bio Gel MA7C weak cation exchanger (50 × 7.8 mm) HPLC column were used. Chromatography was performed at room temperature. The optical density was recorded at 415 nm. Two developers were employed: developer A containing 20 mM bis-tris, pH 6.30; and developer B containing 20 mM bis-tris, 500 mM NaCl, pH 6.30. The solutions were filtered through FP Vericel™ (Gelman) membrane filter with a pore size of 0.45 μm. The different hemoglobins were eluted by increasing the proportion of developer B. The following gradient was used: from 2 to 10% B in 12 minutes, 10% B for 2 minutes; from 10 to 0% B in 0.5 minutes, 0% B for 2 minutes. The elution flow rate through out the analysis was 2.5 ml/minute. The varieties of chromatogram are shown on Figs 1, 2, 3, 4.

Statistical evaluation of data

Mean, standard deviation, coefficient of variance analysis, Student's *t* test for paired data, Pearson's correlation analysis and simple linear regression analysis were used to evaluate the data.

RESULTS

Evaluation of the difference between HPLC and routine procedures

HPLC *vs* cellulose acetate electrophoresis: For Hb A_2, a statistically significant difference was found ($p < 0.05$) but the correlation was good ($r = 0.43$, $p < 0.05$). See Fig 5 and Table 1 for details. For Hb E, no difference ($p = 0.98$) and good correlation were found ($r = 0.96$, $p < 0.05$). See Fig 6 and Table 2 for details.

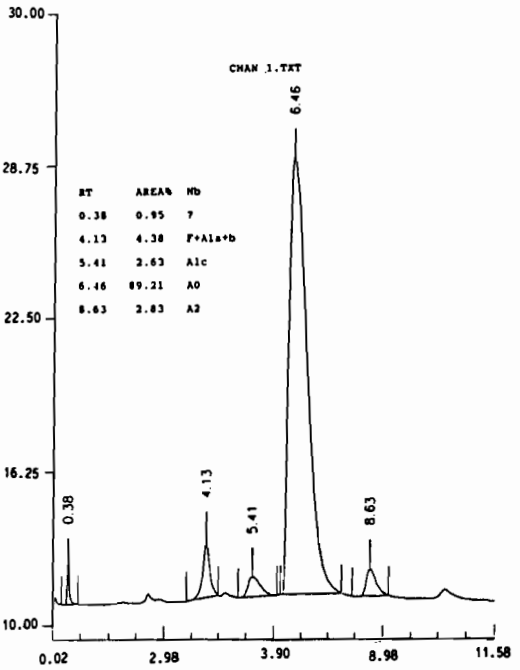

Fig 1–Normal chromatogram.

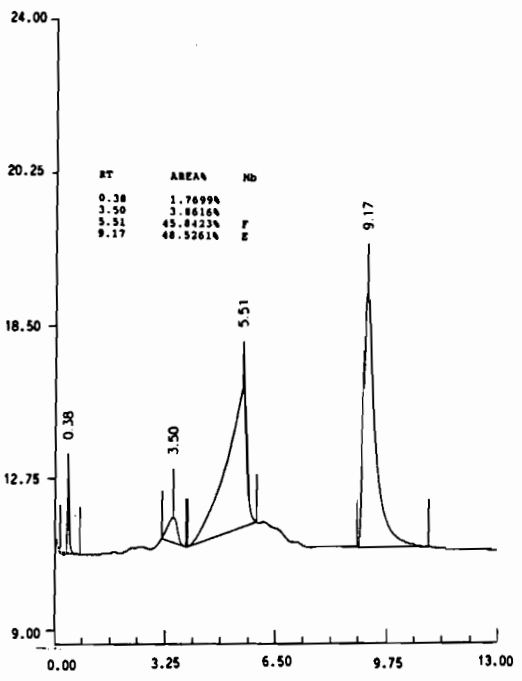

Fig 2–Abnormal chromatogram (Beta-thal/Hb E disease).

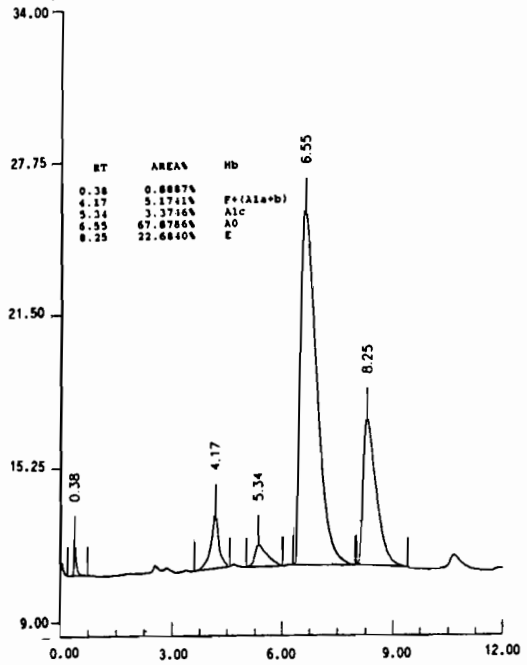

Fig 3–Abnormal chromatogram (Hb E heterozygote).

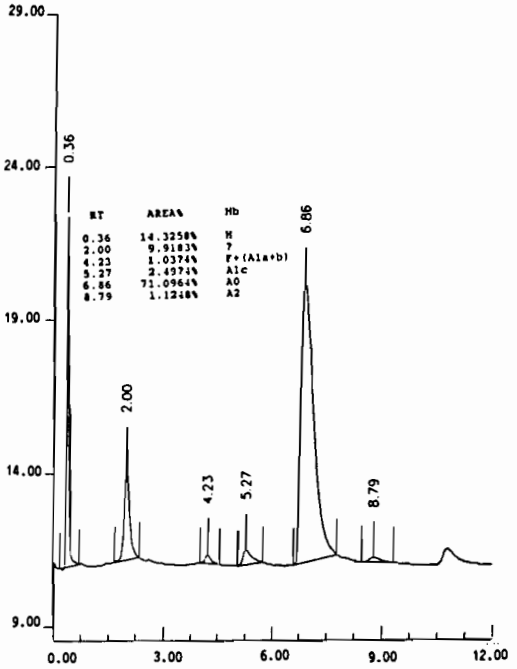

Fig 4–Abnormal chromatogram (Hb H disease).

HPLC *vs* microcolumn chromatography: For Hb A_2, no difference ($p = 0.33$) and good corretion ($r = 0.41$, $p < 0.05$) were found. See Fig 7 and Table 3 for details. For Hb E, a statistically significant difference was found ($p < 0.05$) with good correlation ($r = 0.85$, $p < 0.05$). See Fig 8 and Table 4 for details.

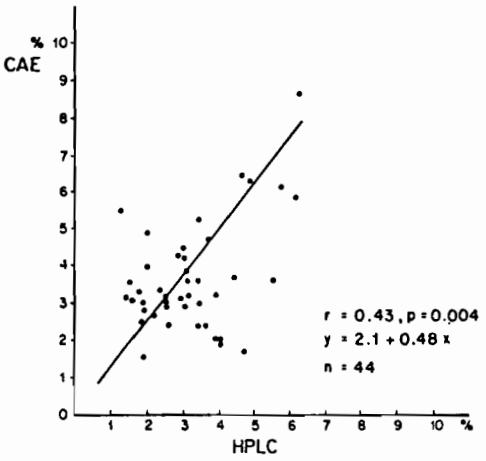

Fig 5–The correlation of Hb A_2 level determined by the two analytic procedures: High performance liquid chromatography; HPLC (X-axis) and cellulose acetate electroresis; CAE (Y-axis).

Table 1

The comparison of Hb A_2 level determined by two analytical procedures: High performance liquid chromatography; HPLC and cellulose acetate electrophoresis; CAE.

	HPLC	CAE
Mean	3.1	3.6
SD	1.2	1.4
n	44	44
p-value	0.014	

HPLC *vs* Betke alkaline denaturation test

A statistically significant difference was found within both low (less than 2.0%) and high (more than 2.0%) levels of Hb F with $p < 0.05$ and $p < 0.05$, respectively. Correlation evaluation was performed and no correlation was obtained at a low level of Hb

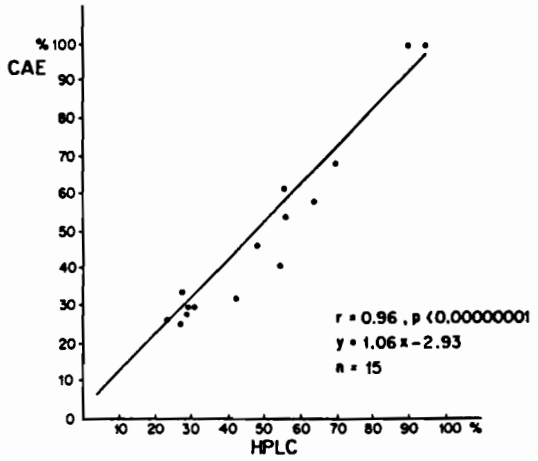

Fig 6–The correlation of Hb E level determined by two analytical procedures: High performance liquid chromatography; HPLC (X-axis) and cellulose acetate electroresis; CAE (Y-axis).

Table 2

The comparison of Hb E level determined by two analytical procedures: High performance liquid chromatography; HPLC and cellulose acetate electrophoresis; CAE.

	HPLC	CAE
Mean	48.8	48.9
SD	22.0	24.1
n	15	15
p-value	0.98	

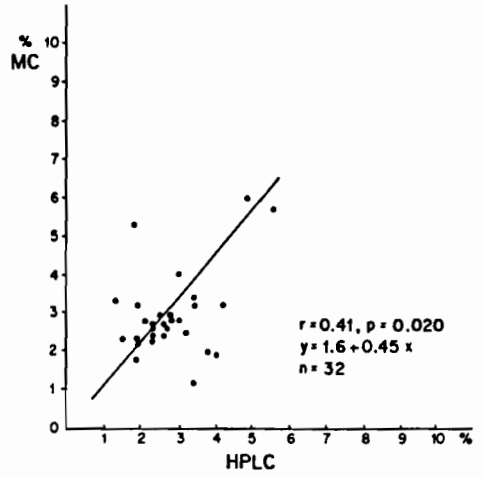

Fig 7–The correlation of Hb A_2 level determined by two analytical procedures: High performance liquid chromatography; HPLC (X-axis) and microcolumn chromatography; MC (Y-axis).

Table 3

The comparison of Hb A_2 level determined by two analytical procedures: High performance liquid chromatography; HPLC and microcolumn chromatography; MC.

	HPLC	MC
Mean	2.7	2.9
SD	0.9	1.0
n	32	32
p-value	0.33	

F ($r = 0.42$, $p = 0.12$). However, the two methods correlated well at a high level Hb F ($r = 0.94$, $p < 0.05$). See Figs 9, 10 and Tables 5, 6, for details.

Evaluation of the precision of HPLC

Coefficient of variance was analyzed among low, normal and high levels of Hb A_2. It was found that the precision of HPLC in hemoglobin typing was very high (CV = 0.2, 0.008, 0.1). For Hb E, high precision was also found among both low (less than 30%) and high (more than 30%) levels (CV = 0.6, 1.1 respectively). See Table 7 for details.

DISCUSSION

Routine hemoglobin typing is accomplished by several steps requiring several analytical techniques (Marengo-Rowe, 1965; Betke *et al*, 1959; Wehinger, *et al*, 1989). Many types of instrument are required. Some chemicals employed are poisonous and may cause degradation of hemoglobins, especially unstable ones. Reproducibility fluctuates, depending on many factors such as skill of the performer, stability of reagents and age of the hemoglobin solution.

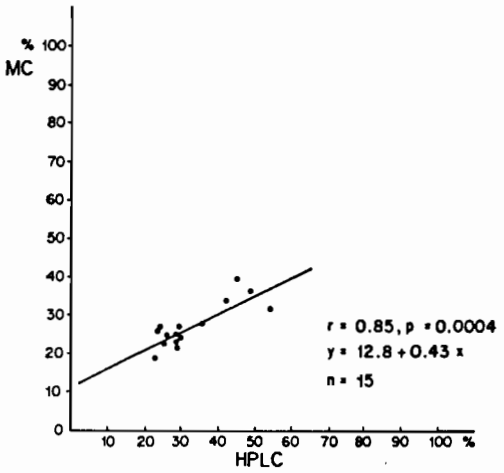

Fig 8–The correlation of Hb E level determined by two analytical procedures: High performance liquid chromatography; HPLC (X-axis) and microcolumn chromatography; MC (Y-axis).

Table 4

The comparison of Hb E level determined by two analytical procedures: High performance liquid chromatography; HPLC and microcolumn chromatography; MC.

	HPLC	MC
Mean	32.3	26.7
SD	9.7	4.9
n	15	15
p-value	0.011	

In case of separation and quantitation by electrophoresis, hemoglobin bands must be cut out for subsequent elution. This technique needs skillful technologists to cut a single band of each hemoglobin. In case of quantitation of hemoglobin by densitometry, if plasma protein is not completely eliminated, it would co-migrate with hemoglobin in the electric field, giving falsely high values.

When a commercial kit of microcolumn chromatography is employed, such as that used in this study, the elution pattern given by the manufacturer is suitable for only Hb A_2. Hb E was not completely eluted from the column. This might be the reason

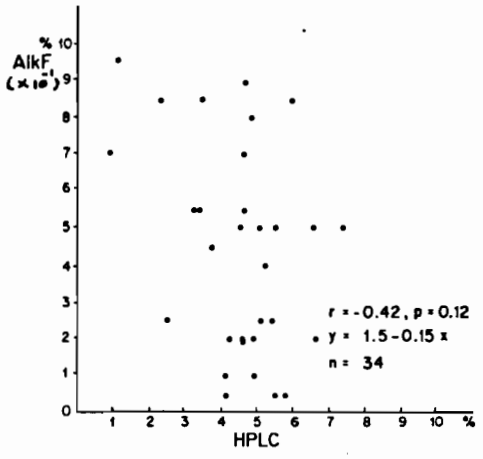

Fig 9–The correlation of normal Hb F level (less than 2.0%) determined by two analytical procedures: High performance liquid chromatography; HPLC (X-axis) and Betke alkaline denaturation test; AlkF (Y-axis).

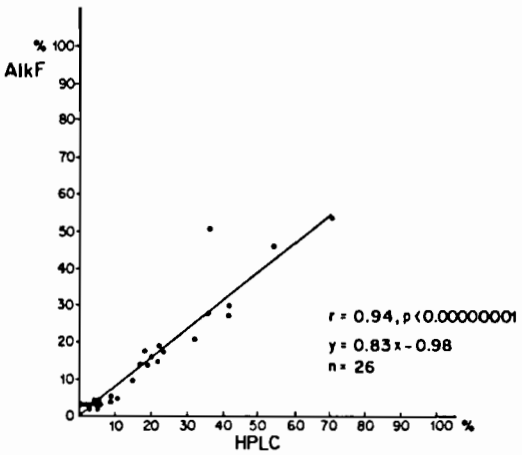

Fig 10–The correlation of high Hb F level (level than 2.0%) determined by two analytical procedures: High performance liquid chromatography; HPLC (X-axis) and Betke alkaline denaturation test; AlkF (Y-axis).

why the level of Hb E from microcolumn chromatography was lower than that from HPLC and electrophoresis.

The estimation of Hb F by Betke alkaline denaturation test needed several steps, and, underestimation may occur. When the level of Hb F was more than 50% by electrophoresis, it was always

Table 5

The comparison of normal Hb F level (less than 2.0%) determined by two analytical procedures: High performance liquid chromatography; HPLC and Betke alkaline denaturation test, AlkF.

	HPLC	AlkF
Mean	4.3	0.9
SD	1.5	0.5
n	34	34
p-value	< 0.00000001	

Table 6

The comparison of high Hb F level (more than 2.0%) determined by two analytical procedures: High performance liquid chromatography; HPLC and Betke alkaline denaturation test, AlkF.

	HPLC	AlkF
Mean	19.7	15.5
SD	17.4	15.3
n	26	26
p-value	0.001	

lower than 50% by alkaline denaturation test (Chanarin, 1989).

HPLC has an advantage over the routine hemoglobin typing techniques. It needs a smaller amount of blood. The sample preparation is very simple. The analytic period per case was short. Hemoglobins of every kind are separated and quantitated simultaneously (Schroeder, 1986; Huisman *et al*, 1983, Kutlar *et al*, 1984). The result in this study showed that Hb F from HPLC was always higher than that from alkaline denaturation test. This might be due to the co-elution of other hemoglobins with Hb F.

Thus, hemoglobin typing could be successfully performed by HPLC. Every kind of hemoglobin was separated and quantitated simultaneously in a single operation. However, if Hb F level, by HPLC, was less than 10%, the value was unreliable. If Hb F level from HPLC was 10% or more, the equation;

Table 7

An evaluation of precision of HPLC in determining different level of Hb A_2 and Hb E.

	Hb A_2			Hb E	
	Low	Normal	High	< 30%	> 30%
Mean	1.7	2.5	4.3	29.8	47.1
SD	0.4	0.09	0.4	0.7	1.1
CV	0.1	0.008	0.2	0.6	1.1
n	20	20	20	20	20

$Y = (0.83X)-0.98$ was employed while Y was Hb F level from alkaline denaturation test and X was Hb F level from HPLC.

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