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Hemoglobin Typing by High Performance Liquid Chromatography

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Abstract

To evaluate the potential application of high performance liquid chromatography (HPLC) in performing hemoglobin typing, comparison between this technique and routine ones was done. The blood specimens from Pediatrics 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 statistical significant different but correlated well. There was no difference but very good correlation found between Hb levels from HPLC compared with those from microcolumn chromatography. Hb E level determined by HPLC and microcolumn chromatography were statistical significant different with good correlation. There was 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 in performing hemoglobin typing provided that Hb F was 10% or more, by calculating from the equation: Alk F = [0.83 x Hb F by HPLC] - 0.98. If Hb F, by HPLC, was less than 10%, the value was unreliable.

Key words : Hemoglobin typing, high performance liquid chromatography

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Introduction

Hemoglobin is a globular protein comprising of four polypeptide chains of which two of one kind and two of another. Different types of hemoglobin contain different polypeptide chains. Basically, different polypeptide chains bear different net electrostatic charge and this is the same for hemoglobins which is the basic for their separation.

The separation of hemoglobin is accomplished by two main principles: electrophoresis and chromatography. Routinely, hemoglobin separation and quantitation are done by the technique of cellulose acetate electrophoresis.[1] Hemoglobin F quantitation is done by the technique of Betke alkaline denaturation test.[2] Besides the separation on cellulose acetate, Hb A_2 may be separated and quantitated by the technique of microcolumn chromatography.[3] Practically, these mentioned techniques require several steps to complete the analysis. Mass hemoglobin analysis are not possible by these routine procedures.

Since 1950, the separation and quantitation of hemoglobin by the technique of chromatography was introduced.[4] High performance liquid chromatography (HPLC) was used for the same purpose shortly thereafter.[5-8] The advantage of HPLC over routine procedures for hemoglobin typing was the ease of performing, requirement of smaller amount of sample, mass hemoglobin typing could be done within shorter analysis time.

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

Materials and Methods

1. Subjects

The subjects were the patients attending Pediatrics Hematology Unit, Research center, Faculty of Medicine Ramathibodi Hospital. Four millilitres of EDTA blood was collected in which 3 mL was for hemolysate preparation, 1 mL was for assay by HPLC.

2. Routine Hemoglobin Typing

Cellulose acetate electrophoresis (CAE) was employed to separate and quantitate hemoglobins.[1,9] Two types of cellulose acetate membrane were used. Cellulose acetate plate (Titan III-H, Helena Laboratories) was used to identify hemoglobin patterns. Cellulose acetate strips (Gelman) was used to quantitate hemoglobin fractions. Microcolumn chromatography (MC, Bio-Rad Laboratories) was also used to quantitate Hb A_2 and Hb E. Betke alkaline denaturation test (Alk. F) was employed for Hb F quantitation.

3. High Performance Liquid Chromatography (HPLC)

The Bio-Rad eqiupment (Model 2800) for ion exchange HPLC and Bio-Gel MA7C weak cation exchanger (50x7.8 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 Science) membrane filter with 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 min, 10% B for 2 min; from 10 to 0% B in 0.5 min, 0% B for 2 min. The elution flow rate through out the analysis was 2.5 mL/min. The varieties of chromatogram were shown on Figure 1.

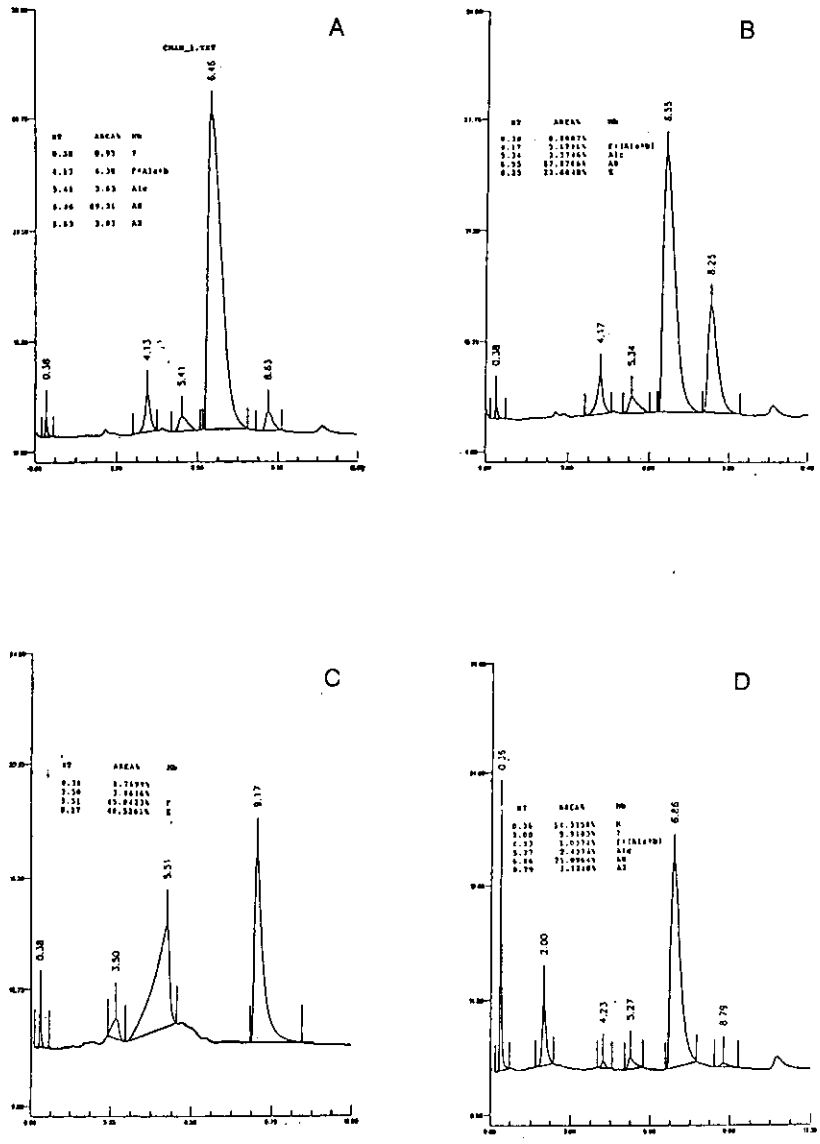

Figure 1 Hb chromatograms (A=Normal, B=Hb E heterozygote, C = β thalassemia/Hb E disease, D = Hb H disease)

4. Statistic evaluation of data

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

Results

1. Evaluation of the difference between HPLC and routine procedures.

It was found that Hb A_2 level determined by HPLC was statistically significant different from that determined by CAE ($p < 0.05$) with a good correlation ($r = 0.43$, $p < 0.05$) but it was not different from that determined by MC ($p = 0.33$) with good correlation ($r = 0.41$, $p < 0.05$). It was also found that Hb E level determined by HPLC was not different from that determined by CAE ($p = 0.98$) with very good correlation ($r = 0.96$, $p < 0.05$) but the statistically significant different was encountered when Hb E level determined by HPLC was compared with that determined by MC ($p < 0.05$). However, Hb E level determined by the last two methods correlated well ($r = 0.85$, $p < 0.05$). (Table 1,2 and Figure 2,3)

Table 1 The comparison of Hb A_2 and Hb E levels determined by HPLC and CAE.

	Hb A_2		Hb E	
	HPLC	CAE	HPLC	CAE
Mean	3.1	3.6	48.8	48.9
S.D.	1.2	1.4	22.0	24.1
n	44	44	15	15
p-value	<0.05		0.98	

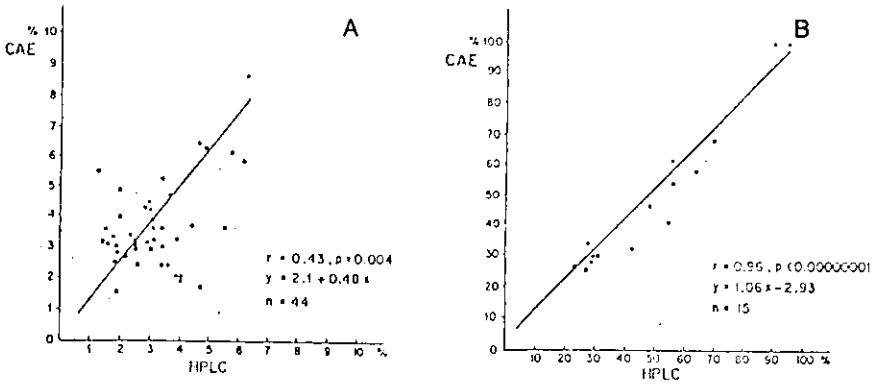

Figure 2 Correlation of Hb A_2 (A) and Hb E (E) levels determined by HPLC and CAE.

Table 2 The comparison of Hb A_2 and Hb E levels determined by HPLC and MC.

	Hb A_2		Hb E	
	HPLC	MC	HPLC	MC
Mean	2.7	2.9	32.3	26.7
S.D.	0.9	1.0	9.7	4.9
n	32	32	15	15
p-value	0.33		< 0.05	

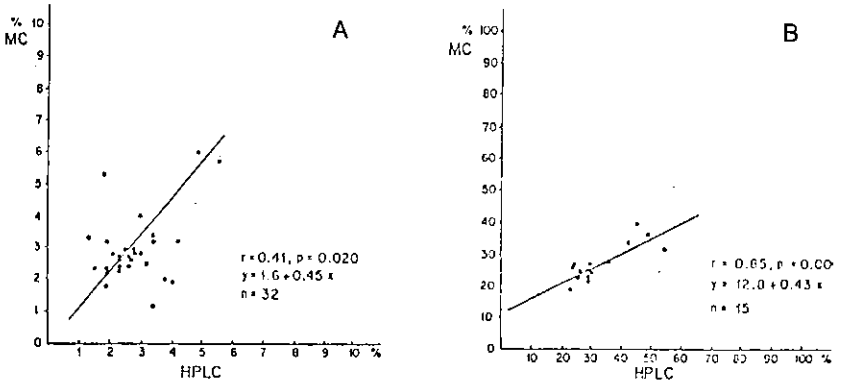

Figure 3 The correlation of Hb A_2 (A) and Hb E (B) levels determined by HPLC and MC.

For Hb F quantitation, Hb F level determined by HPLC was statistically significant different from that determined by an alkaline denataturation method with $p < 0.05$. No correlation was obtained from these two methods when Hb F level was less than 2.0% ($r = -0.42$, $p = 0.12$) but when Hb F level was more than 2.0%, the values from these two methods correlated well ($r = 0.94$, $p < 0.05$). (Table 3 and Figure 4)

Table 3 Comparison of normal and abnormal Hb F levels determined by HPLC and Alk.F.

HbF Level	< 2.0%		> 2.0%	
	HPLC	Alk.F.	HPLC	Alk.F.
Mean	4.3	0.9	19.7	15.5
S.D.	1.5	0.5	17.4	15.3
n	34	34	26	26
p-value	< 0.05		< 0.05	

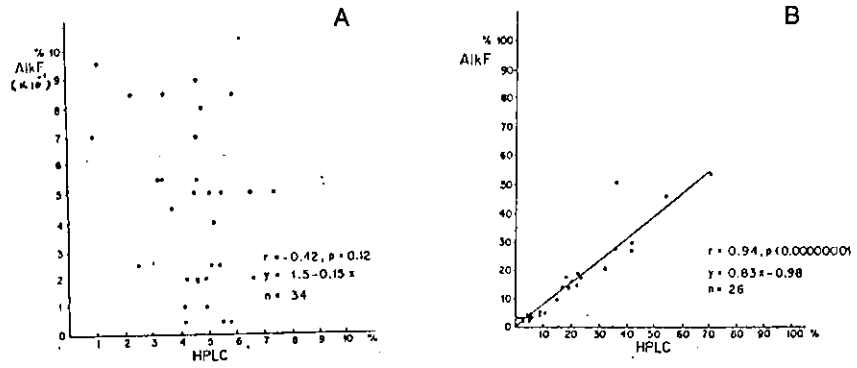

Figure 4 The correlation of A. normal (<2%) and B. abnormal Hb F (>2%) level determined by HPLC and Alk.F.

2. Evaluation of the precision of HPLC.

Coefficient of variance (C.V.) was performed among low, normal and high levels of Hb A_2. It was found that the precision of HPLC in hemoglobin typing was very high (C.V. = 0.2%, 0.008%, 0.1%, respectively). For Hb E, high precision were also found among both low (less than 30%) and high (more than 30%) levels (C.V. = 0.6%, 1.1%, respectively). (Table 4)

Table 4 Precision of HPLC in determining different levels of Hb A_2 and Hb E.

	HbA_2			Hb E	
	Low	Normal	High	< 30%	> 30%
Mean	1.7	1.5	4.3	29.8	47.7
S.D.	0.4	0.09	0.4	0.7	1.1
C.V.	0.1	0.008	0.2	0.6	1.1
n	20	20	20	20	20

Discussion

The routine hemoglobin typing was accomplished by several steps requiring several analytical techniques[1,2,10] and various types of instrument. Some chemicals used were poisonous and might cause hemoglobin degradation especially unstable ones. The reproducibilities were fluctuated depending on many factors such as skill of performer, stability of reagents and age of hemoglobin solution.

In case of separation and quantitation by electrophoresis, hemoglobin bands must be cut out from each other 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 was not completely elimi-

nated, it would co-migrate with hemoglobin in electric field. This event was the cause of unreliable high level of hemoglobins.

When commercial kit of microcolumn chromatography was employed, as one used in this study, the elution pattern given by manufacturer was suitable for only Hb A_2. Hb E was not completely eluted out of column. This might be the reason 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. However, underestimation might occur. When the level of Hb F was more than 50% by electrophoresis, it was always lower than 50% by alkaline denaturation test.[11]

High performance liquid chromatography (HPLC) has advantages over routine hemoglobin typing techniques. It needs much less amount of blood and sample preparation is very simple. The analytic period per case is short. Hemoglobins of all kinds are separated and quantitated simultaneously.[6,12,13] The result in this study revealed that Hb F from HPLC was higher than that from alkaline denaturation test which might be due to the co-elution of other hemoglobins with Hb F.

Conclusion

Hemoglobin typing could be successfully performed by HPLC. All kinds of hemoglobin were separated and quantitated simultaneously under single operation. However, the value was unreliable if Hb F level, by HPLC, was less than 10%. If Hb F level from HPLC was 10% or more, the equation; $Y = (0.8X) - 0.98$ was employed while Y was Hb F level from alkaline denaturation test and X was Hb F level from HPLC.

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