วารสารเกษตรศาสตร์ เชียงใหม่
Bulletin of Chiang Mai Associated Medical Sciences

Vol. 12 No. 3
October 1979

Quantitation of Abnormal Hemoglobins: An assessment of three methods

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ABSTRACT

An assessment of three methods. A comparison of the values of normal and abnormal hemoglobins obtained by cellogel microelectrophoresis, cellulose acetate electrophoresis and DEAE sephadex column chromatography enabled the hemoglobin A_2 values in normal and B-thalassemia trait subjects to be defined. The percentages of E, F, H, and Bart's hemoglobins obtained from two electrophoretic methods are not found to be statistically different

Quantitation of abnormal hemoglobins can be done by several methods. Electrophoresis and chromatography are commonly employed. The assay by DEAE-sephadex column chromatography is limited to slow-moving hemoglobins and to hemoglobin F[1]. Electrophoresis has been improved by changing the size of electrophoretic cells by new types of supporting membrane, and by improved operational procedures[2,3,4] These factors may alter the hemoglobin values. Comparison of A_2 hemoglobin concentration by electrophoresis on different media have been reported[1,5] Recently the use of cellulose acetate gel as a supporting medium for the quantitation of hemoglobin types was introduced by various investigators[2,3,4] There is an obvious need to compare the hemoglo

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Accepted: August 15, 1979.

bin values determined on different types of electrophoretic membrane.

This paper represents a comparison of one chromatographic and two electrophoretic determinations of hemoglobin types. The results obtained will be useful in the clinical evaluation of thalassemic disease.

Methodology

The DEAE sephadex chromatography follows the method of Huishman et al[5]. Hb A_2, E and F were separated from other hemoglobins on DEAE sephadex column. Quantitation depended on elution of the hemoglobins and subsequent colorimetric determination. The two electrophoretic methods employed were those of Wasi and Pootrakul,[6] and Chindavanig[2]. In Wasi's method, all types of Hb were separated in an electrophosetic cell on cellulose polyacetate and quantitated colorimetrically. The other method was performed in microelectrophoretic cell with cellulose acetate gel as supporting medium. Quantitation was made densitometrically. The procedures were summarized in Table 1.

Collection and preparation of samples,

Hemolysates containing 150 normal and 145 abnormal hemoglobins obtained from the laboratory service of two local hospitals were prepared from EDTA venous blood by the method of Chernoff[7]. All samples were analyzed within 3 days of collection. Three methods were performed in parallel on each series of samples. Hemoglobin A_2, E, and F samples were analysed by DEAE sephadex column chromatography. Hemoglobins A_2, E, F, H, and Bart's were quantitated by two electrophoretic methods.

Results and Discussion

Comparison of methods

Results of three determinations for concentrations of homoglobins A_2 E, F, H and Bart's were shown in Table 2. The A_2 hemoglobin values were compared in Fig. 1. The tests and correlation were shown in Table 3. With the exception of A_2 hemoglobin a good correlation was found between cellulose acetate electrophoresis (CAE) and cellulose acetate gel microelectrophoresis (Figs 3,4, and 5). The A_2 and low E Hb values yielded by the CGME method were higher than those found with DEAE sephadex chromatographic method (DSC).

Normal values of A_2 hemoglobin by cellogel micrcelectrophoresis ranged from 2.3 to 5.8% with a mean of 4.1% and a standard deviation $\pm$ 0.8%. In B-thalassemia trait the range was 6,0

to 12 6% with the mean value of 4.83% and a standard deviation of ± 1.72%. No overlap of Hb A_2 values between normal and B-thalassemia trait persons was noted. Hb A_2 values obtained by electrophoresis on different medium reported by other investigators were compared in Table 4.

Protein interference.

Other proteins which might migrate at the same rate as A_a Hb fraction in the CGME method have been considered. The percentages of hemoglobin A_2 determined by staining and elution were similar. It was concluded that no other proteins migrated in the A_2 fraction with the CGME method.

Influence of instrument and supporting membrane.

By using the same microelectrophoretic cell, cellulose polyacetate membrane is too limited to allow for separation of H and Bart's hemoglobins, as shown in Fig. 2. In. contrast, the gelatinized cellulose acetate membrane proves to be capable of detecting these two hemoglobin variants.

We conclude that cellogel microelectrophoretic method is suitable for quantitative determination of abnormal hemoglobins because it is less time consuming[4] and the processed membrane with densitometric analysis can be preserved of a permanent record. The reproducibility is found to be satisfactory. The method provides highly specific confirmation of hemoglobins A, A_2, E, F, H, and Bart's without previous screening[3].

Table 1 Materials and Methods

	DEAE-Sephadex Chromatography (DSC)	Cellulose acetate Electrpphoresis (CAE)	Cellogel Microelctro-phoresis (CGME)
Equipment for separation	Glass column 70 x 0.09 cm	Toyo electrophoretic system, Model SE-2 Toyo Instrument. Inc. Japan	Microzone electrophoretic system, Beckman Instrument, Inc. Fullerton, Calif. USA,
Supporting	DEAE-Sephadex A-50	Sepraphore III. Gelman	Cellcgel, Chemitron, Milan Italy
Buffer	0.007 M phosphate buffer, pH 8.6 with 100 mg. KCN	Tris-EDTA borate buffer pH 8.6 Tris 5.45 g Boric acid 1.546 g EDTA 0.092 g	Tris glycine buffur pH 8.6 Tris 14. 1 g Glyciue 22. 6 g H_2O to 1.5 L
Quantity of hemolysate	100 ul	0.75-10 ul	0.25 ul
Migration	Eluate buffer for A_2 0.01 M phosphate buffer pH 8.6 with 100 mg KCN Eluate buffer for 0.01 M phosphate buffer with 0.3 M NaCl pH 6.0	60 min, 450 V -	45 min, 250 V -
Quantitation	Eluate of each fraction measured at 415 nm with Beckman DB-G spectrophotometer	Eluate (in H_2O) of each fraction measured at 415 nm with Beckmnn CB-G spectrophotometer	Stained with Ponceau S. destain with 5% acetic acid, clear in acetic alcoholsol., scan with densitometer.

Table 2 Comparison of the amount of abnormal hemoglobin components obtained from three methods

Types of Hb	Disease	n	DEAE Sephadex chromatography mean ± S.D, range%	Cellulose acetate electrophoresis mean ± S.D., range%	Cellogel micro electrophoresis mean ± S.D, range%
A_2	normal	150	2.63 ± 0 45	3 30 ± 0.72	4.11 ± 0.82
			1.9 – 3.8	1.6 – 4.7	2.3 – 5.8
	B–thal. traits	50	5.77 ± 1.12	6.98 ± 1.81	8.43 ± 1.72
			3.9 – 7.9	4.8 – 12.6	6.0 – 12.6
E	Hb E trait	30	29.68 ± 5.74	29.45 ± 5.32	33.89 ± 5 52
			16 0 – 40.0	14.2 – 45.6	20.5 – 45.9
	thal. Hb E disease	15	50.92 ± 14.27	48.65 ± 17.44	50.64 ± 15.84
			36.0 – 88.0	27.6 – 91.8	35.9 – 89.4
F	thal. Hb E diaease	15	49 08 ± 14.27	51.35 ± 17.44	49.36 ± 15.84
			12.0 – 64.0	8.2 – 72.4	10.6 – 64.1
H	Hb H disease	26	–	11.6 ± 5.19	10.08 ± 6.13
				2.0 – 22.0	2.0 – 22.6
Bart's	thal. Hb Bart's	9	–	7.05 ± 3.77	8.44 ± 4.02
				1.1 – 9.30	2.3 – 12.8

Types 3 The t tests and correlation coeffient of DEAE sephadex chromatography, cellulose acetate electrophoresis and Cellogel microelectrophoresis

Types of Hb	Disease	n	t test and correlation coefficient		Interpretation	Probability
			DSC v s. CGME	CAE v.s. CGME		
A_2	normal	150	19.22	9.04	S.D.*	$P < 0.01$
			0.33	0.30		
	B-thal trait	50	9.17	4.11	S.D.	$P < 0.01$
			0.63	0.64		
E	Hb E trait	30	3.14	3.40	N.S.**	$P > 0.05$
			0.60	0.82		
	B-thal Hb E disease	15	0.05	3.40	N.S.	$P > 0.50$
			0.94	0.85		
F	B-thal Hb E disease	15	0.05	3.40	N.S.	$P > 0.05$
H	Hb H disease	26	—	1.14	N.S.	$P > 0.05$
Bart's	Hb Bart's disease	9	—	0.77	N.S.	$P > 0.05$
				0.76		

*S.D. = Significantly different

**N.S. = Not significantly different

Table 4 Hemoglobin A_2 values in normal controls and in patients with B-thalassemia traits by various technics.

Authors	Medium	No. of test	Normal person mean ± 1 S.D. range %	No. of test	B-thal. traits mean ± 1 S.D. range %
Hilgartner[8]	paper electrophoresis	22	9.88 ± 3.4	27	17.3 ± 4.2
			5.0 – 14.3		13.2 - 23.4
Hoffman[9]	paper	10	1.52 ± 0.51	7	5.47 ± 2.0
			0.50 – 2.54		1.47 - 9.47
Hilgartner[8]	starch block	22	2.60 ± 0.40	37	5.27 ± 0.35
			1.8 – 3.2		3.3 - 8.4
Sunderman[10]	starch block	25	2.27 ± 0.41	10	4.90
			1.5 – 3,1		3.2 - 8.2
Rozman[17]	cellulose acetate	42	3.30 ± 0.40	12	6.3 ± 0,7
			2.50 – 4.10		4.90 - 7.7
Biere[12]	cellulose acetate	30	2.46 ± 0.47	7	5.17 ± 0.64
			1.52 – 3.4		4.40 - 6.30
Pabis[8]	" Cellogel "	30	2.50 ± 0.60	33	6.3 ± 1.5
			1.40 – 3.8		4.9 - 10.4
Penalver[9]	" Cellogel "	50	2.37 ± 0.23	30	4.39 ± 5.2
			1.90 -- 2.8		
Huisman[7]	DEAE-Cellulose	124	2.80 ± 0.28	45	4.97 ± 0.35
			1.5 - 3.0		3.5 - 6.3
Huisman[7]	DEAE-sephadex	40	2.65 ± 0.37	15	5.6 ± 0.94
			1.9 - 3.2		3.9 - 6.7

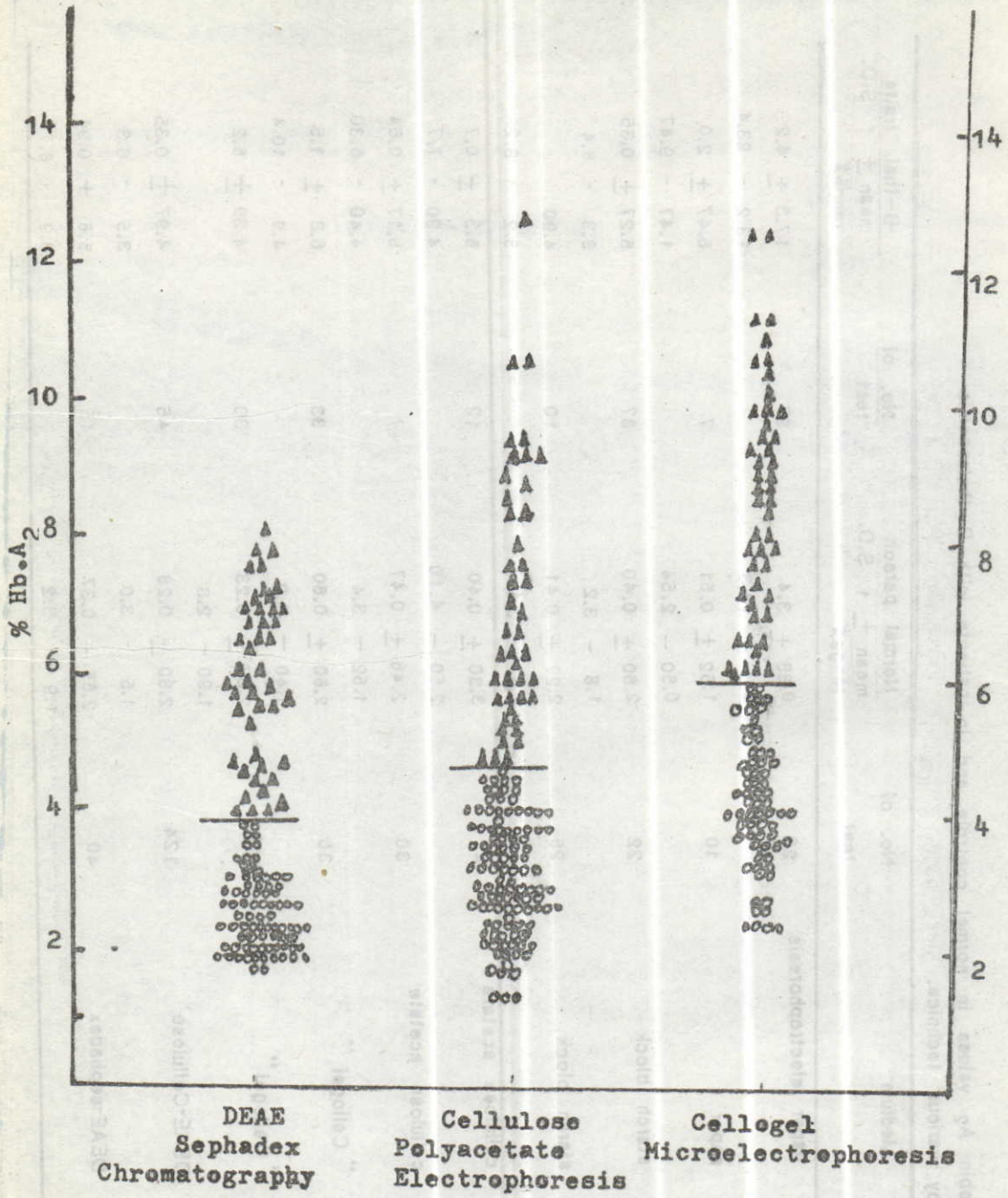


Fig. 1. Comparison of hemoglobin A_2 values as derived by three different methods.
Hemolysates from the control (Normal A_2 levels) are designated as O, and from beta-thalassemia as ▲

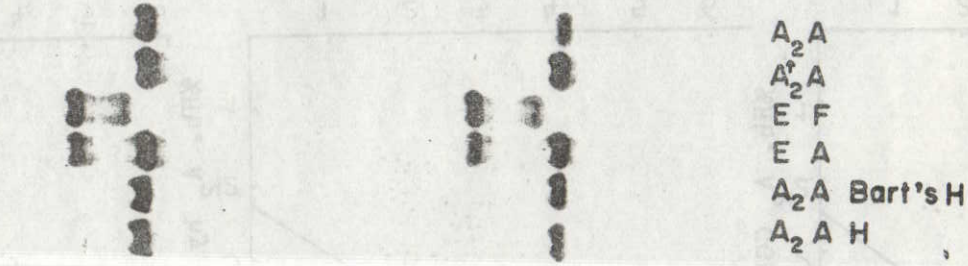


Figure 4. Hemoglobin electrophoresis in microelectrophoretic system using Sepraphore III and Cellogel as supporting membrane.

A
Y = 2.58+0.27Y
r = 0.33
%Hb. A2 obtained from CAE
%Hb.A2 obtained from DSC
Y = 2.59+0.58X
r = 0.32
%Hb. A2 by CGME
%Hb. A2 obtained from DSC
Y = 2.53+0.60X
r = 0.33
%Hb. A2 CGME
%Hb. A2 obtained from CAE

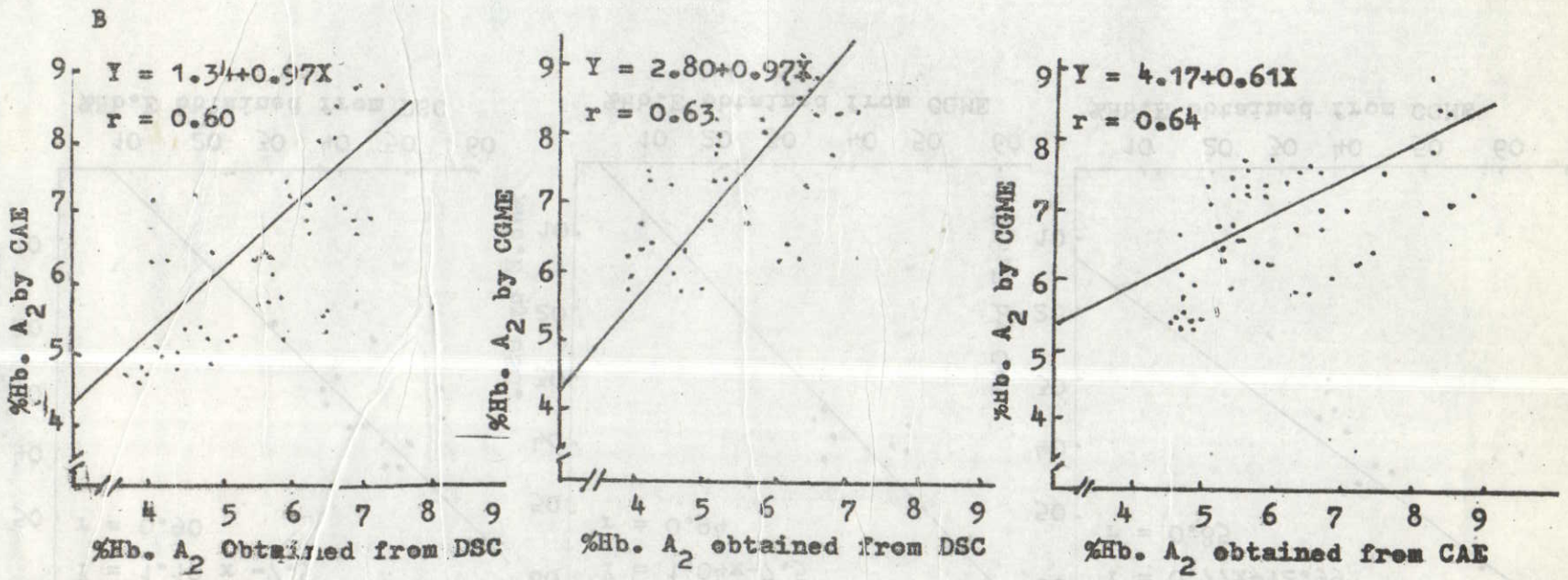


Figure 3 Correlation of hemoglobin A_2 obtained from DSC, CAE, and CGME
A. hemoglobin A_2 from control n=150 B. hemoglobin A_2 from B-thalassemia trait n=50

A
Y = 1.10 x -7.5
r = 0.90
%Hb. E by CAE
%Hb.E obtained from DSC
Y = 1.04x-7.5
r = 0.94
%Hb.E by DSC
%Hb.E obtained from CGME
Y = 0.77x+12.99
r = 0.85
%Hb.E by CAE
%Hb.E obtained from CGME

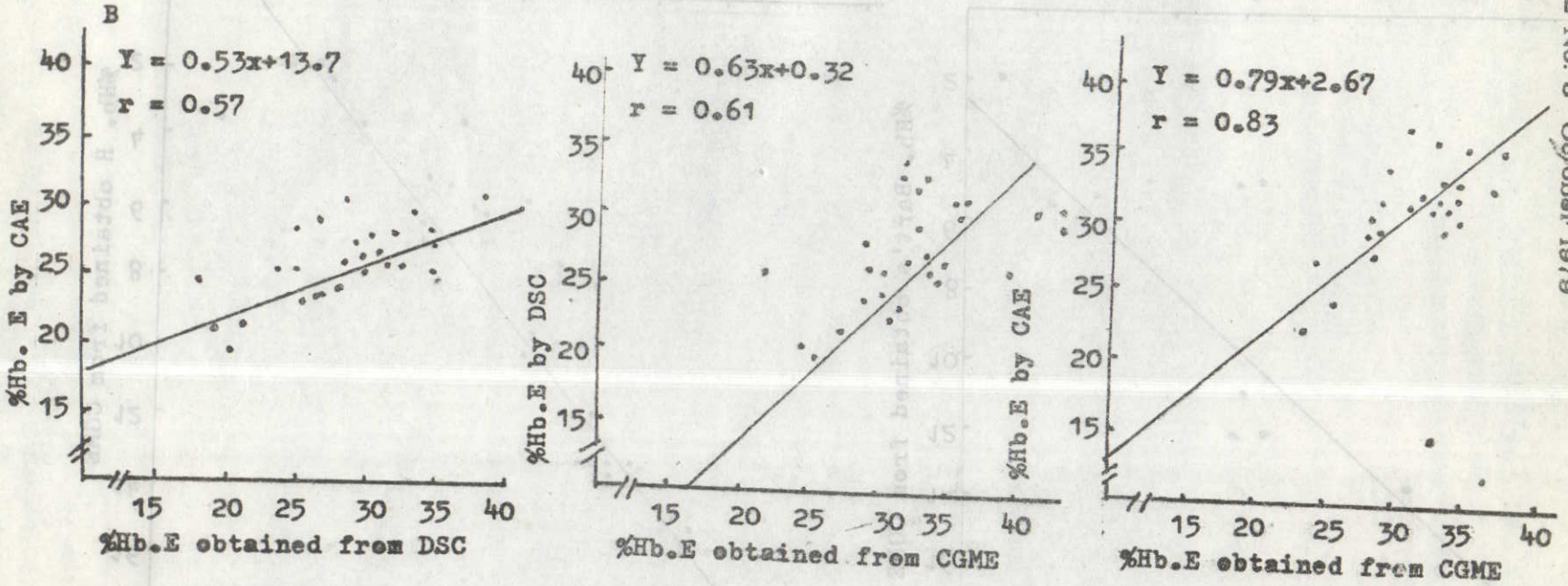
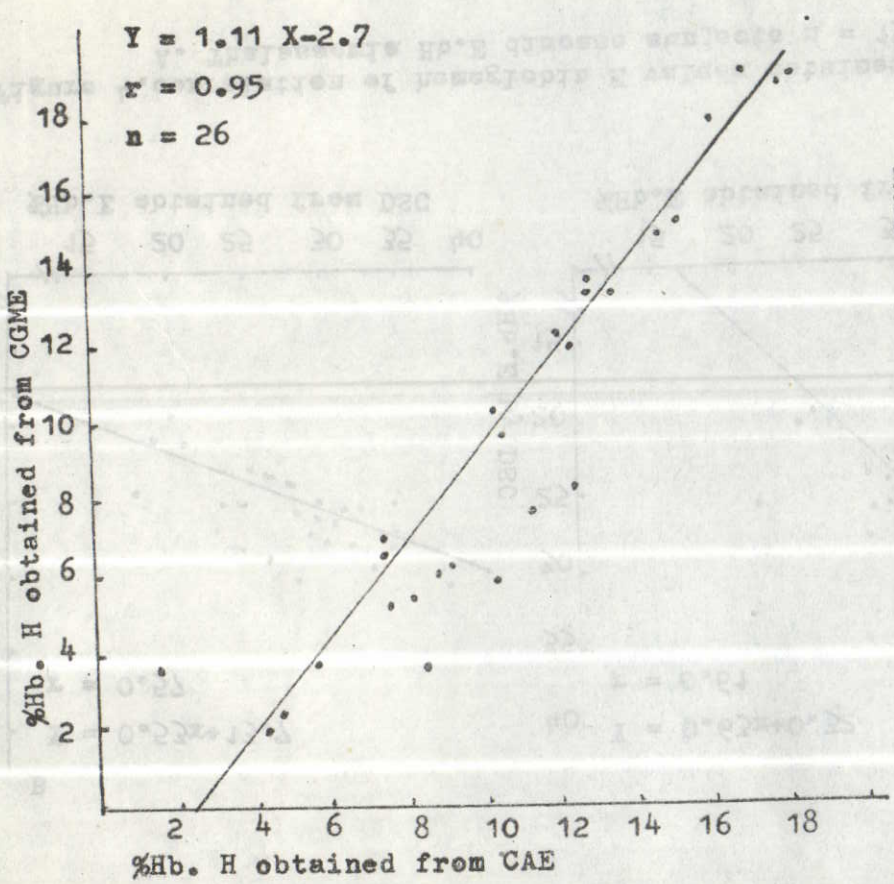


Figure 4.correlation of hemoglobin E values obtained from DSC, DGME and CAE
A. Thalassemia Hb.E disease sunjects n = 15 B. Hemoglobin E traits subject n = 30



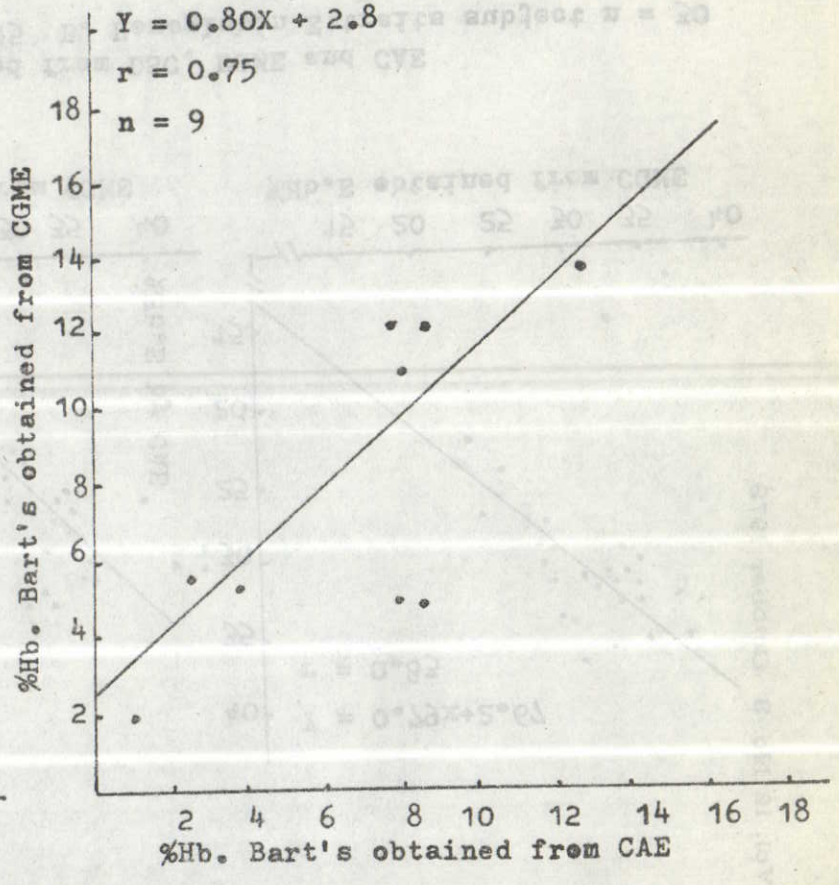


Figure 5. Correlation of hemoglobin H and Bart's obtained from CAE and CGME

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การประเมินผลวิธีการหาปริมาณของฮีโมโกลบิน (เปรียบเทียบ 3 วิธี)

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ย่อเรื่อง

ผู้ทำการทดลองได้รายงานเปรียบเทียบค่าของฮีโมโกลบินที่ปกติและผิดปกติ โดยวิธี Cellogel microelectrophoresis, cellulose acetate electrophoresis และ DEAE sephadex column chromatography ในการตรวจหาฮีโมโกลบินเอสองในคนปกติและในคนใช้ทาลาสซีเมีย ชนิด เบต้าไมเนอร์ พบว่ามีสูงเมื่อหาโดยวิธี cellogel microelectr phoresis ทั้งนี้ อาจเป็นเพราะมีผลกระทบกระเทือนจากโปรตีนชนิดอื่น นอกเหนือไปจากฮีโมโกลบินเมื่อย้อมสี และหาค่าโดยใช้เครื่องวัดความเข้มของสี

สำหรับค่าของฮีโมโกลบินอี, เอฟ, เอช และบาร์ท ซึ่งหาโดยวิธีทั้งสามจะไม่แตกต่างกันทางสถิติ

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