CHAPTER 3

RESULTS AND DISCUSSION

3.1. Elution profiles of hemoglobin

DEAE-Sephadex bead has diethylaminoethyl ($-OCH_2CH_2N^+H(CH_2CH_3)_2$) functional groups that interact with anionic groups on hemoglobin and thus can retain all types of hemoglobin. Different types of hemoglobin contain different amounts of net negative charge and therefore can be separated with the pH gradient elution. Upon elution using the more acidic buffer, containing more HCl, hemoglobin becomes less negative and thus anionic groups of hemoglobin captured by DEAE-bead can be exchanged with Cl^-. The order of hemoglobin eluted from a DEAE-Sephadex column is HbA_2 co-eluted with HbE, then HbA and finally HbF [55].

Examples of elution profiles of normal blood sample and that of thalassemia patient obtained from the proposed system are illustrated in **Figures 3.1 (a) and (b)**, respectively. It should be noted that the Y-axis is in voltage that relates to transmittance and therefore peak height decreases with increasing of absorbance. The patterns of both elution profiles are similar but different in ratio of peak areas. Abnormal blood has higher amount of HbA_2 and HbE as compared to that of normal blood sample.

Composition of hemoglobin types in blood can vary depending on age. Normal adult blood has been known to contain approximately 95-98% HbA, 2-3% HbA_2 + HbE, and 0.8-2% HbF [56]. An individual with β-thalassemia trait usually has an elevated level of HbA_2, HbE and maybe HbF along with the evidence of microcytosis and distinctive abnormal facial feature [53, 57]. Patients with α-thalassemia, on the other hand, usually have HbA_2 and HbE the same level as normal people have. Therefore ratio of HbA_2 and HbE to total hemoglobin cannot pinpoint what type of thalassemia the patient has without further examination.

From the elution profiles of blood samples, areas under each peak were integrated and the sum of those areas was counted as total hemoglobin. The percentage of HbA_2 + HbE was calculated from the ratio of area under HbA_2 + HbE peak to the total area. Examples of calculation are also summarized in **Figures 3.1 (a) and (b)**.

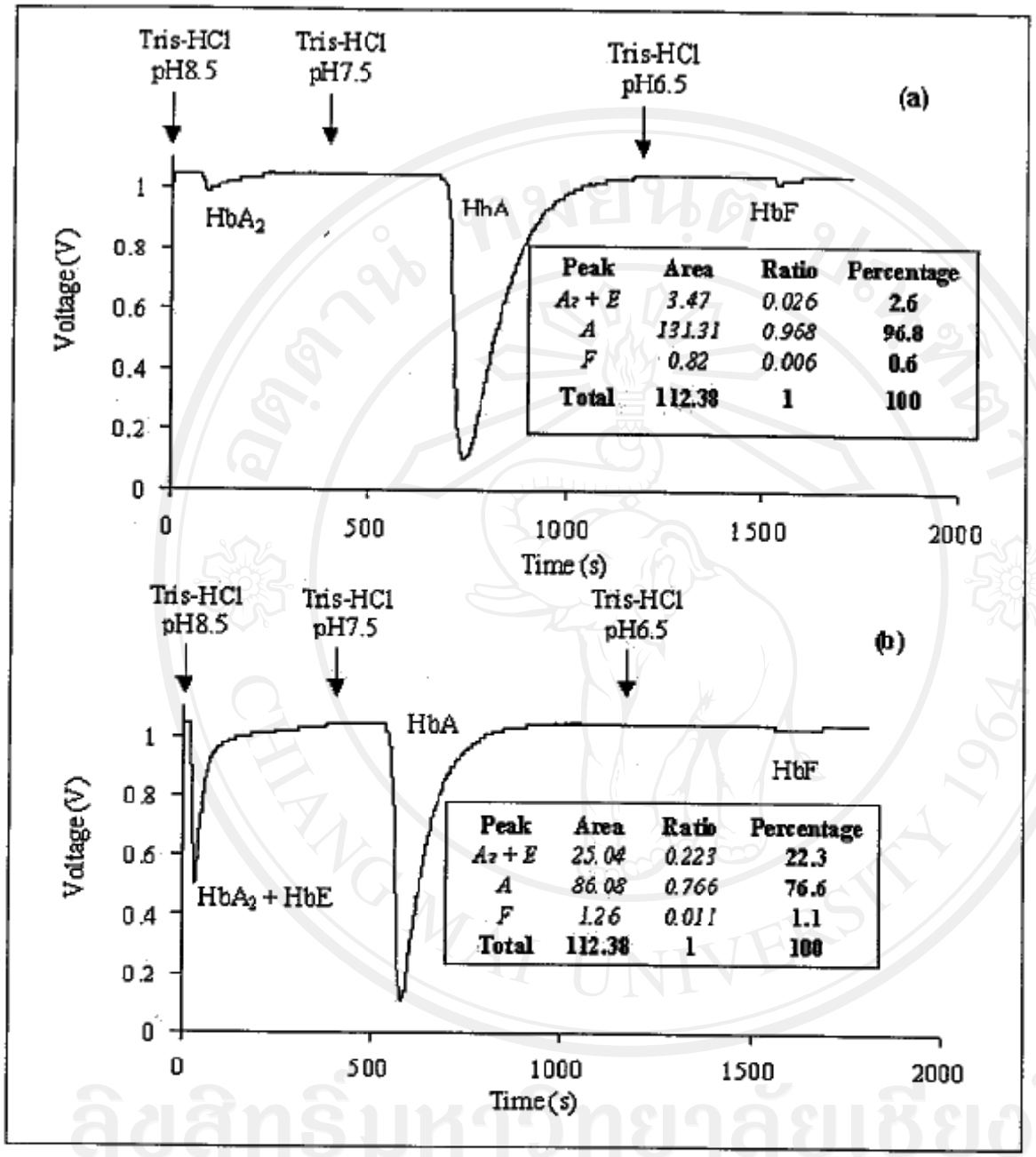

Figure 3.1 (a) elution profiles of normal blood sample obtained from the proposed system
(b) elution profiles of thalassemia patient blood sample obtained from the proposed system

3.2 Optimization

3.2.1 pH of buffer solutions

In this optimization, the normal blood sample was used to select the suitable pH for elution of Hbs. According to the conventional microcolumn method, the percentage of Hb A_2 of this normal blood sample is 3.6%. Along with the fact that normal adult blood contains approximately 95-98% of Hb A with small amount of Hb A_2 about 3.5% and Hb F has less than 1%. These values were used as reference values to evaluate different pH gradient. **Table 3.1** show that the 4th condition of pH 8.5, 7.5 and 6.5 is the most suitable pHs condition for the FI reduced volume column system. These buffer solutions could completely elute Hb A_2 (3.7%), Hb A (95.5%) and Hb F (0.8%) respectively, which were closed to the reference values of the normal adult blood.

Table 3.1 Optimization of the pH of buffer solutions.

Condition of pH gradient	pH peak	Hb Type	Peak area	Area ratio	Relative Percentage
1	8.2		13.95	0.2770	27.7
	6.5		36.41	0.7230	72.3
	Total		50.36	1	100
2	8.5	A_2	2.38	0.0362	3.6
	8	A	54.2	0.8243	82.4
	6.5	F	9.17	0.1395	13.9
	Total		65.75	1	100
3	8.5	A_2	3.22	0.0369	3.7
	7.6	A	78.8	0.9023	90.2
	6.5	F	5.31	0.0608	6.1
	Total		87.33	1	100
4	8.5	A_2	2.45	0.0369	3.7
	7.5	A	63.71	0.9547	95.5
	6.5	F	0.56	0.0084	0.8
	Total		66.42	1	100

3.2.2 Flow rate

Another factor that affects the purposed system is the flow rate of the system. The range of variation of the flow rate was varied from 2.0 to 0.5 ml/min. Again, a normal blood sample (2.6% with Hb A_2 by conventional method) was used in this study. The flow rate of 2.0 ml/min was too high and it produced a high backpressure that caused the tubing connections to leak. At the low flow rate of 0.5 ml/min, the separation time was too long and the small chromatographic column was clogged from some matrices in blood sample that settled down before separation could be achieved. Again this situation generated the pressure to the system and the leakage of the connection point occurred.

The flow rate at 1.5 ml/min did not generate the high backpressure but it was too fast to accurately separate the types of Hb. It resulted high percentage of Hb A_2 of the normal blood level as compared to the result obtained from the reference conventional system. Moreover, only 2 peaks of Hb types (Hb A_2 and Hb A) were obtained as shown in **Table 3.2**. The flow rate of 0.8 ml/min was found to be the most appropriate for the proposed system because it did not cause the leak and the amounts of Hbs were reasonable as compared to reference values obtained from the conventional method.

Table 3.2 Optimization of the elution flow rate.

Flow rate (ml/min)	FI-reduced volume column system				Percentag of HbA_2 (Conventional system)
	Peak	Peak Area	Area ratio	Percentage	
1.5	HbA_2	18.86	0.1847	18.47	2.6
	HbA	83.27	0.8153	81.53	
	HbF	-	-	-	
	Total	102.13	1	100	
0.8	HbA_2	4.55	0.0262	2.6	2.6
	HbA	168.49	0.9707	97.1	
	HbF	0.54	0.0031	0.3	
	Total	173.58	1	100	

3.2.3 The amount of resin

A reduced volume micro-column was made of acrylic piece into the dimension of 3 mm i.d. and 2 cm long.The amount of resin was packed in the column varied from 140 to 70 µl. The same sample of a normal level Hb A_2 (2.6% by conventional method) was used in this study. The results in Table 3.3 showed that the suitable amount of resin was 140 µl, as it gave the same normal level of Hb A2 as compared to the conventional method. Lower amount of resin (e.g. 80 µl) was not sufficient to separate all Hb types. Co-elutions of Hb A-Hb A_2, and Hb A-Hb F were observed. Higher amount of resin (e.g. > 140µl) can be produced the high back pressure to the system that can cause the connection leakage.

Table 3.3 The optimization of the amount of resin.

Amount of resin (µl)	FI-reduced volume column system				Percentage of HbA_2 (conventional system)
	Peak	Peak Area	Area Ratio	Percentage	
140	HbA_2	2.54	0.0254	2.5	2.6
	HbA	97.04	0.9688	96.8	
	HbF	0.59	0.0059	0.6	
	Total	100.17	1	100	
70	HbA_2	11.00	0.1605	16.0	2.6
	HbA	57.53	0.8395	84.0	
	HbF	-	-	-	
	Total	68.53	1	100	

In regions where routine thalassemia testing is most needed due to its prevalence in the populations, such as in Southeast Asia and Africa, it is often also the case that economic restrictions on the medical systems prevent the use of the latest technologies that exist. In the laboratories that are not well equipped with technically advanced instrumentation such as HPLC [58-59], diagnosis of Hb type is normally accomplished by conducting several different tests in combination, namely Osmotic Fragility Test (OFT), DEAE-column separation, HbE screening test (E-screen) and Polymerase Chain Reaction (PCR) with gel electrophoresis. The significant of each test already explained briefly in the introduction chapter. The relationship between results of each test and the diagnosis of Hb type is summarized as shown in **Table 3.4**.

In this work 40 adult blood samples (packed cells) with positive OFT were examined. To summarize and compare all the results **Table 3.5** shows calculated percentages of HbA_2+HbE obtained from the proposed FI- reduced volume column system along with the results from a conventional column, E-screen, PCR and Hb types diagnosis were done at the hospital. Similar to a conventional column, results from the FI-reduced volume column could predict the existence of thalassemia (i.e. β, E trait, EE homozygous) from the patients who have more than 3 % HbA_2 + HbE.

Based on the hospital diagnostic results, the correlation plot between the two systems, R=0.9271, was obtained as shown in **Figure 3.2**. The ranges of HbA_2+HbE percentages estimated in each case using the proposed and the conventional systems that were compared as shown in **Table 3.6**. Two samples (one β-trait (18.4%) and one α-trait

(17%) were statistically eliminated from **Table 3.6** because they gave results much higher than their standard ranges (β-trait 4-8%, α-thal-1-trait 2.5-3.5%) that are normally obtained even when using the conventional column. Because of the differences in the system characteristics and column size, the average amounts of HbA_2+HbE obtained from the proposed system are lower than those obtained from the conventional system, especially for the case of E-trait and EE homozygous. The cause of deviation needs further investigation. However, the proposed system can differentiate normal and α-thal-1-trait sample (HbA_2+HbE <3%) from abnormal samples (>3%) and was still able to yield accurate positive test results. The level of HbA_2+HbE in EE homozygous samples was higher than in E-trait and β-trait samples respectively, the same trends as found when using the conventional column.

For other types of Hb such as HbS, no testing could be conducted, as there were no samples available in Thailand. However, according to a previous study by Dozy et al, the DEAE column can separate Hb by descending pH gradient between 8.5-7.0. The order of Hb eluted from the column were HbA_2, Hbs, HbA and finally HbF, respectively, in that study. Similarly, in this proposed study, HbA_2, HbA and HbF were eluted in the same order based on a similar pH range. Therefore, this proposed method should be able to screen for HbS if it is contained within the sample.

Reproducibility of the system was tested by running 7 replicates of 3 samples (normal, β-trait and E-trait) each of which represented different levels of HbE. RSD of

the method was found to be 3.4% for normal sample and 4.6% for both β-trait and E-trait samples as shown in **Table 3.7-3.9**.

Table 3.4 Summarization of the test results with the diagnosis of Hb type

OFT	HbA_2 (conventional column)	E-screen	PCR	Hb type
+	-	-	-	A_2 normal
+	+ (>3%)	-	-	β-trait
+	+ (>60%)	+ (70-90%)	-	EE homozygous
+	+ (>3%)	+ (25-30%)	-	E-trait
+	-	-	+	α

Table 3.5 Comparison of calculated percentages of HbA_2 + HbE peak areas from the proposed FI-reduced volume column system and from the conventional column system. The results from E-screen, PCR and Hb Type diagnosis were done by the Thalassemia Research Laboratories, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University (+ is positive result and - is negative result)

sample	Percentages HbA_2 + HbE			E-screen	PCR	Hb Type
	FI-red. V column	Conventional column	Ratio FI-red.V : convention			
A4	2.6	2.9	1 : 1.1	-	-	A_2- Normal
B6	1.3	2.2	1 :1.7	-	-	A_2- Normal
B11	2.2	2.9	1 : 1.3	-	-	A_2- Normal
B13	1.9	2.5	1 : 1.3	-	-	A_2- Normal
B15	2.6	3.4	1: 1.3	-	-	A_2- Normal
B16	2.7	3.4	1 : 1.3	-	-	A_2- Normal
C3	1.9	2.5	1 : 1.3	-	-	A_2- Normal
C25	2.8	2.8	1 : 1.0	-	-	A_2- Normal
D17	2	2.3	1 : 1.2	-	-	A_2- Normal
E6	2.6	2.4	1 : 0.9	-	-	A_2- Normal
E25	2.3	2.6	1 : 1.1	-	-	A_2- Normal
C8	2.9	2.7	1 : 0.9	-	-	A_2- Normal
F18	2.4	2.5	1 : 1.0	-	-	A_2- Normal
C5	3.4	4.5	1 : 1.3	-	-	β-Thal-trait
D3	5.4	4.8	1 : 0.9	-	-	β-Thal-trait
D9	4.1	4.7	1 : 1.1	-	-	β-Thal-trait

D18	7.3	18.4	1 :2.5	-	-	β-Thal-trait
E24	4.8	4.6	1 : 1.0	-	-	β-Thal-trait
A9	1.8	2.9	1 :1.6	-	+	α-Thal -1- trait
A8	3.1	5.5	1 : 1.8	-	+	α-Thal -1- trait
A28	2	2.5	1 : 1.3	-	+	α-Thal -1- trait
B2	2.2	2.5	1 : 1.1	-	+	α-Thal -1- trait
C2	1.7	1.1	1.06	-	+	α-Thal -1- trait
C11	1.4	2	1 : 1.4	-	+	α-Thal -1- trait
C12	1.2	2.2	1 : 1.8	-	+	α-Thal -1- trait
C16	2.7	2.4	1 : 0.9	-	+	α-Thal -1- trait
D2	1.3	2.2	1 : 1.7	-	+	α-Thal -1- trait
E7	1.9	2.1	1 : 1.1	-	+	α-Thal -1- trait
E10	2	1.9	1 : 1.0	-	+	α-Thal -1- trait
E20	8.5	17	1 : 2.0	-	+	α-Thal -1- trait
A1	5.5	21.3	1 : 3.9	+	-	E-trait
A23	8	21.1	1 : 2.6	+	-	E-trait
B4	8.4	18.7	1 : 2.2	+	-	E-trait
C13	6	21.4	1 : 3.6	+	-	E-trait
D20	11.1	25	1 : 2.3	+	-	E-trait
E19	8.5	26.1	1 : 3.1	+	-	E-trait
C7	7.8	21.7	1 : 2.8	+	-	E-trait
A39	12.5	81.2	1 : 6.5	+	-	EE-homo
C24	11.7	78.2	1 : 6.7	+	-	EE-homo
A37	20.7	93.8	1 : 4.5	+	-	EE-homo

Table 3.6 Comparison of HbA_2 +HbE percentages obtained from the FI-reduced volume column and from the conventional larger column. The Hb type diagnosis were done by the Thalassemia Research Laboratories, Maraj Nakorn Chiang Mai Hospital, Chiang Mai University

[a] number of samples for each Hb Type

* Independently run

Note: there were no SD data for the results obtained from the conventional column

Hb type	Hb A_2 + Hb E percentage				Standard range (HPLC)*
	FI-reduced volume column*		Conventional larger column*		
	range	Mean±SD	range	Mean	
A_2-Normal (13)[a]	1.3-2.9	2.3±0.1	2.2-3.4	2.7	2.5-3.5
β-Thal- trait (4)[a]	1.2-3.1	1.8±0.1	1.1-2.9	2.5	2.5-3.5
α-Thal-1-trait (11)[a]	3.4-5.4	4.1±0.1	4.5-4.8	4.6	4.0-8.0
E-trait (7)[a]	5.5-11.1	8.0±0.1	18.7-26.1	22.2	>10
EE-Homo (3)[a]	11.7-20.7	14.3±0.1	78.2-93.8	84.5	>60

Table 3.7 Reproducibility of the system that was tested by running 7 replicates of normal sample

No. of Run	Area				percentage		
	A2	A	F	Total	A2	A	F
1	4.55	168.49	0.54	173.58	2.6	97.1	0.3
2	7.92	288.21	0.88	297.01	2.7	97.0	0.3
3	5.08	194.08	1.18	200.34	2.5	96.9	0.6
4	8.52	304.99	0.60	314.11	2.7	97.1	0.2
5	9.43	332.47	0.85	342.75	2.8	97.0	0.2
6	5.98	224.04	0.91	230.93	2.6	97.0	0.4
7	5.50	214.57	0.57	220.64	2.5	97.2	0.3

Average percentage of HbA_2	**2.6**
SD	**0.1**
%RSD	**3.4**

Table 3.8 Reproducibility of the system that was tested by running 7 replicates of β-trait sample

No. of Run	Area				percentage		
	A2	A	F	Total	A2	A	F
1	18.06	302.33	0.76	321.15	5.6	94.1	0.2
2	18.82	314.37	0.44	333.63	5.6	94.2	0.1
3	11.11	208.81	0.84	220.76	5.0	94.6	0.4
4	10.99	209.56	0.94	221.49	5.0	94.6	0.4
5	9.74	176.63	0.91	187.28	5.2	94.3	0.5
6	11.41	207.58	0.37	219.36	5.2	94.6	0.2
7	12.74	232.16	1.17	246.07	5.2	94.3	0.5
Average percentage of HbA_2					**5.3**		
SD					**0.2**		
%RSD					**4.6**		

Table 3.9 Reproducibility of the system that was tested by running 7 replicates of E-trait sample

No. of Run	Area				percentage		
	A2	A	F	Total	A2	A	F
1	9.29	112.68	0.52	122.49	7.6	92.0	0.4
2	12.07	131.05	0.68	143.80	8.4	91.1	0.5
3	11.25	132.80	0.30	144.35	7.8	92.0	0.2
4	13.79	152.20	0.51	166.50	8.3	91.4	0.3
5	9.47	102.81	0.15	112.43	8.4	91.4	0.1
6	8.78	96.47	0.49	105.74	8.3	91.2	0.5
7	8.18	102.85	1.17	112.20	7.3	91.7	1.0

Average percentage of HbA_2	**8.0**
SD	**0.4**
%RSD	**4.6**

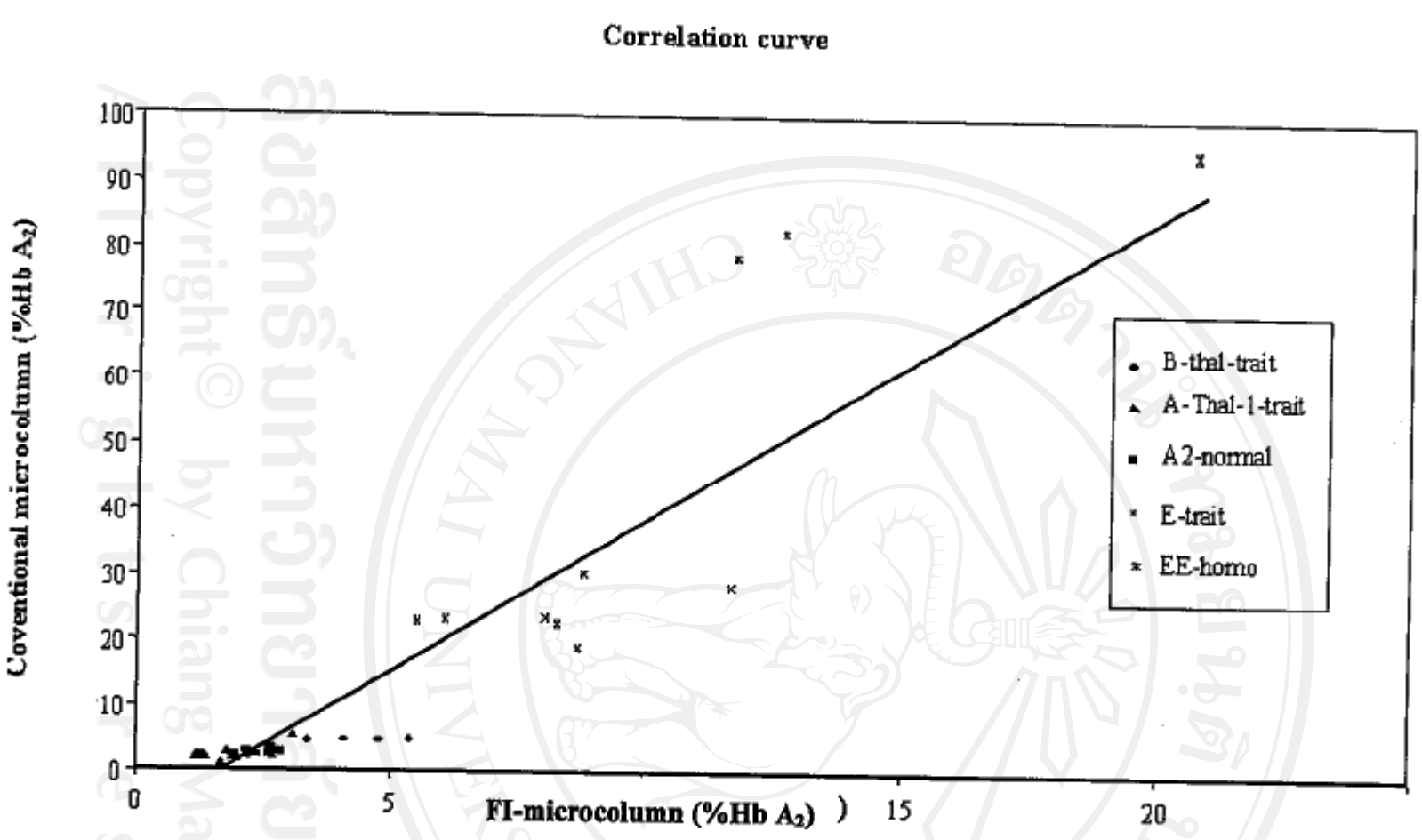

Figure 3.2 The correlation plot between the results obtained from the FI-microcolumn system and those obtained from the conventional microcolumn, y=2.8728x-3.5247, R =0.9271

3.4 Advantages and disadvantages

Both the proposed and the conventional column systems cannot point out patients with α-Thalassemia due to non-differentiated level of HbA_2+HbE from normal people (<3%). PCR or HPLC result is needed in this case.

The proposed reduced volume column, just like the conventional one, has to be repacked after a couple of runs due to constriction of the flow caused by coagulation of some matrices in blood. However, the amount of beads used and the time it takes for column packing in the proposed system are minimal as compared to those of the conventional column. The limitation is also compensated by other benefits offered using the proposed system such as ease of operation, low cost, small amount of sample, and fast analysis time. The analysis time using the FI-reduced volume column is about 35 min per sample as compared to 4 hours using a conventional column. Amount of sample for each run was as little as 80 µl of 50 times diluted sample, a major improvement as compared to the 2 ml of undiluted packed cell that is needed for a conventional column technique.