CHAPTER 2

EXPERIMENTAL

2.1 Materials and apparatus

1. Peristaltic Pump (FIA Lab, USA)
2. Tygon Tubing (Saint-Gobain Performance Plastics, USA)
3. Reduced Volume Micro-column (acrylic piece; 3 mm i.d. and 2 cm long)
4. Six-port Injection Valve (Upchurch Scientific, USA)
5. PTFE Tubings (Cole Palmer, USA) i.d. 1/32 inches
6. Syringe size 1 ml
7. Eppendorf tube size 1 ml
8. Flow through cell (HELLMA, Germany)
9. Spectronic 21 (Spectronic Instrument, USA)
10. Computer Software (Metex Corp., USA)
11. Computer (Compaq Presario 425)

2.2 Reagents

1. Tris (hydroxymethyl) aminomethane: Tris-HCl (UBS, USA)
2. Potassium Cyanide: KCN (Riedel-De Haen Ag Seelze-Hannover, Germany) (see **APPENDIX D**)
3. Hydrochloric acid: 37% HCl (Merck, Germany)

4. Diethylaminoethyl(DEAE)-Sephadex A-50, 65-120 μm (Phamacia Biotech, Sweden)

2.3 Preparation of buffer solutions

Tris-HCl 0.05 M buffer solutions of different pHs were prepared by dissolving 6.057 g Tris (hydroxymethyl) aminomethane and 0.1 g KCN in 1000 ml distilled water and adjusted to the desired pH with HCl to create the pH gradient between 8.5 to 6.5. Tris-HCl buffer solution has to be degased before use to avoid bubble.

2.4 Preparation of blood samples

Total of 40 blood samples (packed red cells) were obtained from the Thalassemia Research Laboratories, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University where all subjects were routinely checked up. Each blood sample was centrifuged to separate the pack red cell and the serum then the packed red cell was collected by a micropipette. The packed red cell was hemolysated by 50 times dilution with Tris buffer pH 8.5 prior to use.

2.5 Packing of DEAE-Sephadex A-50 beads

Before column packing, DEAE-Sephadex A-50 beads 10 g were swollen in 900 ml of Tris-HCl 0.05 M buffer solutions pH 8.5 at least 12 hours at room temperature. Then DEAE-Sephadex A-50 beads were degased get rid of air to bubble. Then the beads were packed into a reduced volume micro-column shown in **Figure 2.1**. It was made of acrylic piece into the dimension of 3 mm i.d. and 2 cm long. This

dimension is much smaller than conventional column, which is about 1 cm i.d. and 5 cm long. One end of the reduced volume micro-column was closed and sealed with cotton wool before packing the beads. Then 140 µl of 40-120 µm DEAE-Sephadex A-50 beads was introduced into the reduced volume micro-column by using a pasteur pipette to aid the packing. The amount of beads used in the reduced volume micro-column was about 20 times less than the amount of beads needed for packing a larger conventional column. After that another side of the column was closed and again sealed with cotton wool.

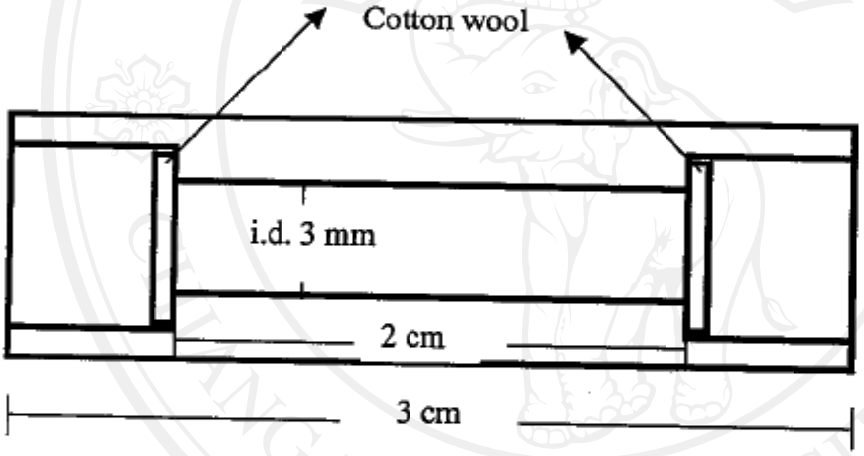

Figure 2.1 A reduced volume micro-column

2.6 Manifold and operation steps

All pump tubings were tygon. The rest of the flow lines were assembled from PTFE tubings. A peristaltic pump was used to deliver buffer solutions. Samples were introduced by means of a six port injection valve with an 80 µl sample loop. Then passed into the flow through cell with 1 cm path length was which in the Spectronic 21. The detection of hemoglobin was done at 415 nm. The absorbance data were

converted to voltage and recorded with a computer software (Metex Corp., USA) installed in a personal computer (Compaq Presario 425). These data were transferred and integrated with Microsoft Excel (Microsoft Corp., USA).

The diagram of a simple manifold used is shown in **Figure 2.2.** Buffer solution pH 8.5 was pumped through the unloaded column to condition the column at a flow rate of 0.8 ml min^{-1}. Blood sample (80 µl) was injected into the system through a valve V_2. After the first peak of co-eluted HbA_2 and HbE appeared, the pH 8.5 buffer solution was replaced with pH 7.5 buffer solution by switching the valve V_1. In between the gradient of pH 8.5 to 7.5, the HbA was eluted. Via the valve V_1 then the buffer solution was again changed to pH 6.5 to elute out HbF. As a precaution the valve V_3 can be used to drive off any observed air bubbles before they can get into the column.

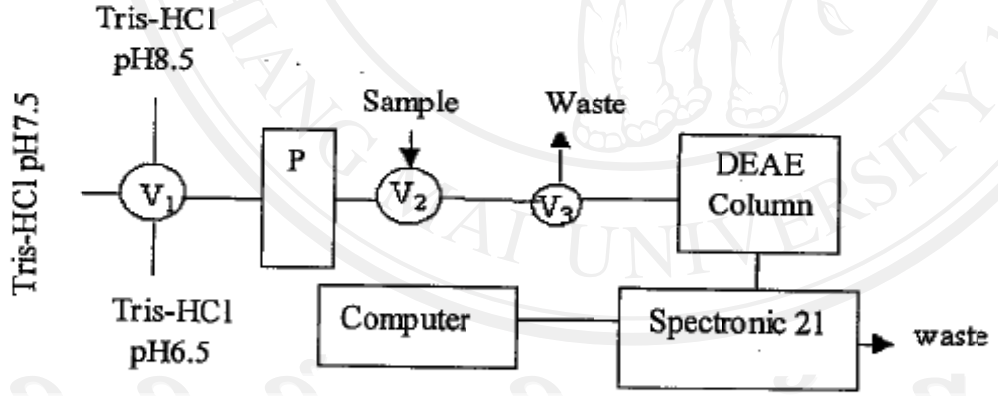

Figure 2.2 FI-reduce volume column manifold; V_1= 3-way valve, P= peristaltic pump, V_2 = 6-port injection valve, V_3 = 3-way valve

2.7 Optimization

In this work, the suitable pHs of Tris-HCl buffer solution that used as the carrier buffer for elution of each Hb type was studied. The flow rate, which can affect the separation, was varied from 2.0-0.5 ml/min. In addition, the amount of resin that was packed in the column was also optimized from 70-140 µl. The normal adult blood samples were used in this optimization study.

2.8 Evalutation of FI-reduce volume column method

All the results obtained from the proposed system were compared to those obtained from the conventional column system and the correlation plot between the 2 systems was also done. The reproducibility of the proposed system was tested by running 7 replicates of 3 samples (normal, β-trait and E-trait) each of which represented different levels of Hb E. The sample through put, the amount of reagent used per sample per run and the limitation of the proposed system will be discussed in Chapter 3.