CHAPTER III

EXPERIMENTAL

3.1 Apparatus and chemical substances.

3.1.1 Apparatus

1. BRUKER ACF 200 pulsed NMR spectrometer, 200 MHz., Switzerland
2. CELL-DYN 610 Hematology Analyzer, Sequoia-Turner Corporation,USA.
3. Duo-Dilutor model 125 Hematology Dilutor, Sequoia-Turner Corporation, USA.

3.1.2 Chemical substances

1. Deuterium oxide (D_2O), Fluka chemika AG.
2. Sodium chloride (NaCl), Aldrich Chemical Co, Ltd.
3. Potassium chloride (KCl), Aldrich Chemical Co, Ltd.
4. Di-sodiumhydrogen phosphate-2-hydrate ($Na_2HPO_4 \cdot 2H_2O$), Aldrich Chemical Co, Ltd.
5. Potassium-dihydrogen phosphate (KH_2PO_4), Aldrich Chemical Co, Ltd.
6. Distilled deionized water
7. Ethylenediamine tetraacetic acid (EDTA), Aldrich Chemical Co, Ltd.
8. Nitrogen gases (N_2), Thai Industial Gas (TIG) Co, Ltd.

9. Calcium chloride (CaCl), Aldrich Chemical Co, Ltd.

10. Concentrated sulphuric acid (H_2SO_4), Aldrich Chemical Co, Ltd.

11. Silica gel (drying agent)

12. Isotonic Detergent, L&R Co, Ltd.

13. Isotonic Diluent, L&R Co, Ltd.

3.2 Sample preparation

3.2.1 Preparation of Phosphate buffer saline solution (PBS)

Sodium chloride (8.0 g.), potassium chloride (0.2 g.), disodium hydrogen phosphate (1.44 g.) and potassium dihydrogen phosphate (0.24 g.) were weighed accurately and put into a beaker. This mixture was dissolved in distilled, deionized water and then poured into the volummetric flask (1000 ml). When the mixtures completely dissolved, adjusted the volume to 1000 ml by distilled, deionized water and made it homogeneous by inversion technique.

3.2.2 Preparation of red blood cells solutions

1. About 5 ml of the fresh blood was added into a test-tube containing the ethylenediamine tetraacetic acid (EDTA) (1mg/1ml of blood). The tube was closed then mixed by inversion technique.

2. The hematocrit values (HCT,%) of the whole blood was measured by using the Hematology analyzer.

3. The remaining of the blood was centrifuged at 13,000 cycle/min. for 3-4 minutes. The red blood cells were packed at the bottom of the test-tube, while the layer above the red blood cells was blood platelets, white blood cells and plasma.

4. Plasma, blood plateletes and white blood cells were removed by mean of a dropper out of red blood cells. The red blood cells were washed with phosphate buffer saline solution (PBS) and centrifuged again for further 3-4 minutes and then separated the PBS from the red blood cells. (repeated this step for 2-3 times to make sure that no blood plateletes, white blood cells and plasma remained in the red blood cells).

5. The concentrations of the red blood cells were adjusted to 50%, 30%, 15% and 7.5% by adding PBS solution to the red blood cells and mixed them by inversion method. These solutions of red blood cells were measured by Hematology analyzer and NMR spectrometer.

3.3 Test method

3.3.1 Mean corpuscular volume (MCV), Hematocrit (HCT) determination.

Before running the samples on CELL-DYN 610, background count for RBC and PLT was measured by placing the cup with 10 ml of fresh diluent under the trabsducer of CELL-DYN 610. Acceptable values were:

RBC less than or equal to 0.05

PLT less than or equal to 10

If the RBC background was greater than 0.05 or platelet background was greater than 10, then the background was measured again with a fresh cup of diluent.

If the RBC and PLT background were acceptable, the CELL-DYN 610 automatically switched to and measured the WBC and HGB background. Acceptable values were:

WBC less than or equal to 0.5

HGB less than or equal to 0.2

If all values were acceptable, cleaned the transducer on CELL-DYN 610 was closed with 20 ml of fresh diluent. After that, the CELL-DYN 610 was now ready to operate.

Before measuring the blood to measure, mixed the room temperature specimen (whole blood) was mixed by gentle inversion (20 times). Then whole blood (40 microliters) was externally diluted in a ratio of 1:250 with diluent using Duo-Dilutor and dispensed into an unused sample vial and swirl to mix. A 1:25,000 dilution was serially prepared from this initial 1:250 dilution and dispensed into another unused sample vial and swirled to mix. During each RBC, HCT and MCV measured cycle, the vial with the 1:25,000 was placed under the transducer of CELL-DYN 610 hematology analyzer. The size and number of red blood cells (RBC), hematocrit (HCT) and mean cell volume (MCV) presented in the diluted whole blood were measured and results were printed and recorded.

3.3.2 Measurement of relaxation times

Each concentration of the red blood cells' solutions (30%, 15% and 7.5%) was added into NMR tubes and then puts the capillary tube containing dueterium oxide into them. The tubes were sealed with paraffin films to protect oxygen intering into the NMR tubes. After that, the proton relaxation times of each concentration of red blood cells were measured.

All NMR measurements were carried out using a BRUKER ACF 200 pulsed NMR spectrometer at 200 MHz frequencies with the probe temperature at 37 ^{o}C. The T1 measurements were made every 20 minute and stoped at 120 minutes. The chemical shift was measured relative to external dueterium oxide containing was contained in a separated capillary tube placed in NMR tubes.

In this experiment of work, the spin-lattice relaxation time (T1) was measured using the inversion-recovery pulse sequence and spin-pain relaxation time (T2) was measured using the CPMG pulse sequence.

3.3.2.1 Spin-lattice relaxation time measurements

In this study, the standard inversion-recovery method, employing a $[180^{o}\text{-}\tau\text{-}90^{o}\text{-Aq-T}]_{n}$ pulse sequence, was used to determine the spin-lattice relaxation time (T1). An interface microcomputer with software program provided rapid data acquisition and analysis. The T1 decay curve was the product of the resultant analysis of 10 free induction decay (FID) peak heights with a sequence of increasing interpulse delay times. The important parameters in this method were:

τ was the variable time between an applied 180^{o} pulse that completely inverted the spins, and a 90^{o} observing pulse that determined the extent of the remaining magnetization after time t.

T (or D1) was the time between acquisition time (Aq) and the next pulse sequence, in which the system was assumed to come to equilibrium after time T. In all measurements, T was greater than 5xT1.

VD was the variable delay times list which in all measurements, usually starts with the shortest delay time and the last delay time was the time which relaxation is complete.

NE was the number of intensity-time data sets that used for determination of each relaxation time. It was equal to the number of Tau (τ)values in the VD list (the number of FID stored).

Following Fourier transform of the free induction decay, the remaining magnetization was determined by measuring the intensity of peaks.

The 180° pulse width was determined by minimization of the free induction decay of the spectrum and was checked again after the first pulse to get the minimum intensity of the peaks.

The 90° pulse width was set at half of the 180° pulse width.

At least 10 intensity-time data sets were used for the determination of each relaxation time.

The experiments started with the determination of optimized parameters for the T1 measurements that were described above. All parameters can be obtained by using the approximated T1 value that was approached to the real T1 value of sample.

In determination of approximated T1 value, the inversion-recovery method for determining T1 can be used for a rough-and-ready measurement. From the relaxation theory, since the z magnetization at the end of the τ interval was given by:

$$Mz = Mo(1-2e^{-\tau/T1}) \qquad (3\text{-}1)$$

then, the detected signal passed through a null point when

$$e^{-\tau/T1} = 1/2 \qquad (3\text{-}2)$$

Therefore, in variable delay time, started the experiments were started with very small τ and then values were varied. In this case, the τ values that were used in these experiments were 0.05, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0, 3.0, 5.0, and 10.0 seconds so that the numbers of intensity-time data was 10. Because of the proton spin-lattice relaxation time for the medium molecular weight molecules in the presence of

oxygen were often only a second or two and plenty of exceptions occur both to longer and shorter values, therefore T1 was supposed to be 1 or 2 and D1 in this experiment were 5 or 10 (generally D1 is greater than 5xT1).

The sample that used to determine optimized parameters of T1 measurements was PBS solution because all samples that used for this study were the solution of red blood cells in PBS solution.

After the accumulation of FID signals, 10 spectrums were obtained by Fourier transformation of FID signals. The value was determined by observing the null point from the stacked plots (the value that the peak was inverted at this stage) (Fig. 2-4) and after that the approximated T1 value was calculated by:

$$T1 = \tau/\ln 2 \qquad (3\text{-}3)$$

This approximated T1 value was used in the aid to design all τ values in these experiments.

From approximated T1 value, all designed τ values were 0.05, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, and 5.0 and appropriated D1 was 5. At this point the number of loops through the VD list was reduced to be 1 cycle and NS (number of scan of FID accumulation) was reduced to be 2 (from the multiple of 2^n, where n=0, 1, 2,...). The times for T1 measurements was not long because the life-times of red blood cells were short otherwise some red blood cells break. If this case happened, the T1 value might be incorrect.

Before starting T1 measurements, the NMR tubes with suspensions of red blood cells were leaved in the NMR probe temperature at 37°C for 5-10 minutes to make sure that the temperatures of all red blood cells suspensions were constant and

stable. The procedure made the red blood cells to sink to the bottom of NMR tube to avoid the effect from red blood cells to signal accumulation.

All parameters for T1 experiments were shown below:

VCLIST.001

number of experiments = 10

number of loops = 1

D1 = 5

VDLIST.001 : the delay times; 0.05, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, and 5.0

number of scans = 2

After the peak picking operation was completely, the display showed the relative intensities of the peak picking through a series of plus sign with the last point being displayed as a circle and after the T1 calculation was completely, the result was printed and the exponential line fitting curve was displayed with a standard deviation of points in this curve as shown in figure 3-1.

3.3.2.2 Spin-spin relaxation time measurement

The T2 values were measured by using CPMG pulse sequence and all parameters for measurement were the same as the ones that used in the measurement of T1. After the peak picking operation was completely, the display showed the relative intensities of the peak picking through a series of plus sign with the last point being displayed as a circle and after the T2 calculation was completely, the result was printed and the exponential line fitting curve was displayed with a standard deviation of points in this curve as shown in figure 3-2.

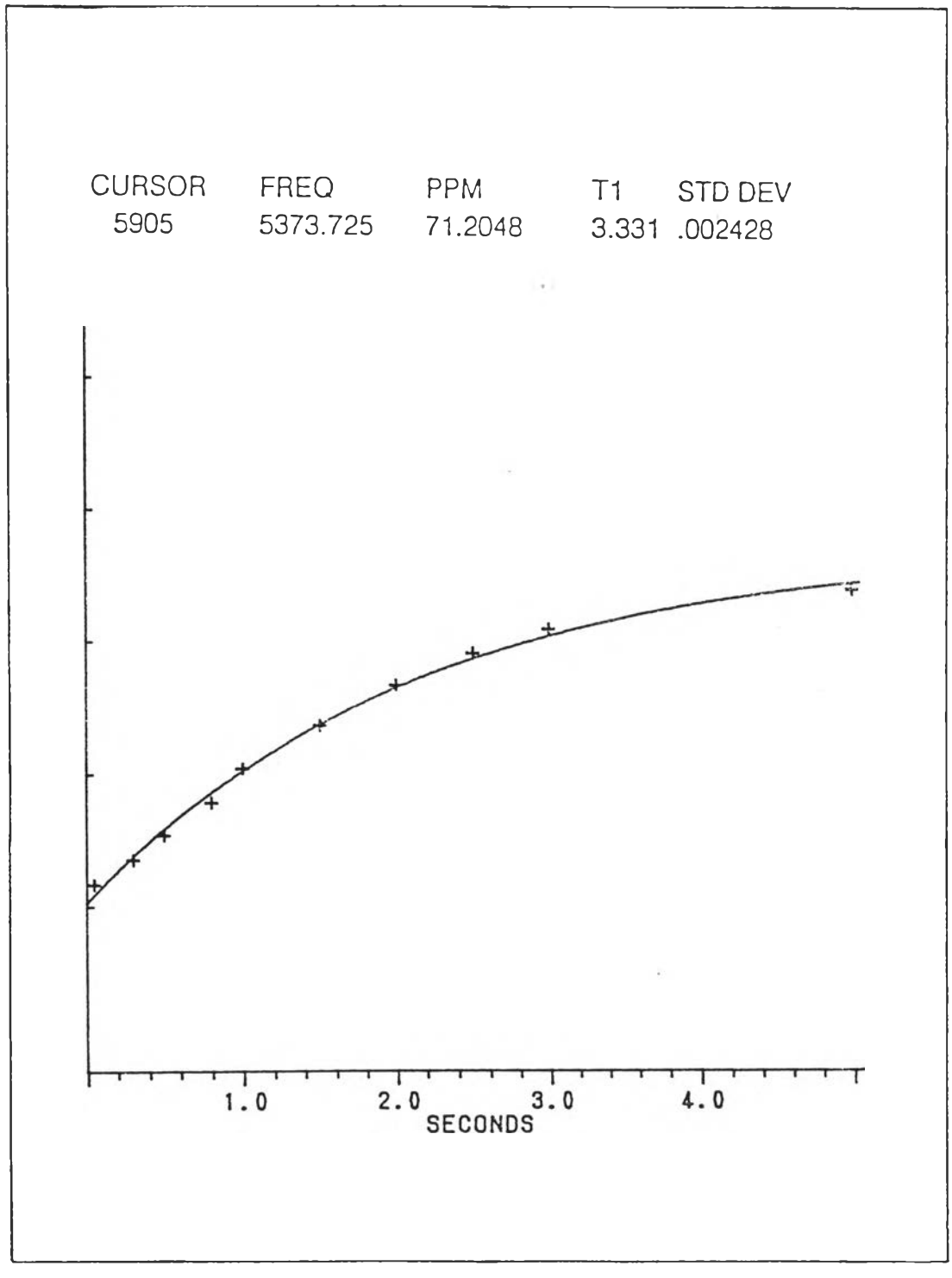

Figure 3-1 Exponential curve plot from the relative intensities of the peak picking through a series of plus sign with delay times displayed as a circle : inversion-recovery pulse sequence

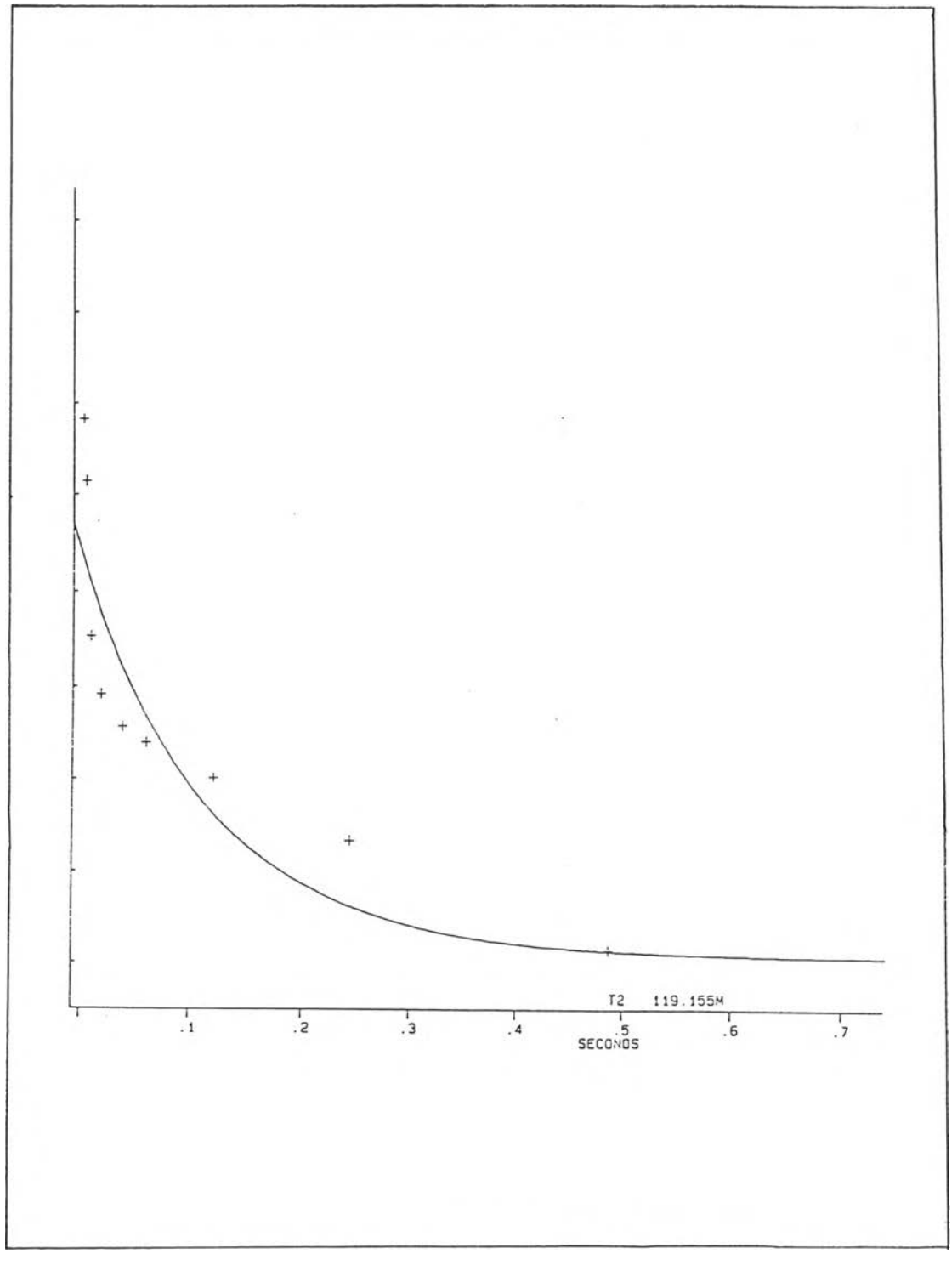

Figure 3-2 Exponential curve plot from the relative intensities of the peak picking through a series of plus sign with delay times displayed as a circle : CPMG pulse sequence