CHAPTER III

EXPERIMENTS

1. EQUIPMENTS

1.1 Spectrophotometer : Shimadza Double Beam Balance, UV-180.

1.2 Differential Thermal Analysis : Shimadzu DT-30 thermal analyser.

1.3 Infared Spectrophotometer : Shimadza IR-440.

1.4 Top to Bottom Rotating Shaker, thermostatically controlled at 37°C ± 0.5°C.

1.5 X-ray Diffractometer : Philips, Type PW 1730/10, 220 V, 7250 UA, 50/60 HZ, NC9430 017 30101, No. Dy 1023.

1.6 Perkin Elmer 240C Eleniental Analyser.

1.7 Incubater

1.7.1 Precision Theles Model 4, 120 Volts, 160 Watts, and Serial No. 12-V-10, Cat. No. 31483, Chicago, U.S.A.

1.7.2 Laboratory Thermal Equipment Ltd., Type 8 CI XI.

1.7.3 Laboratory Thermal Equipment Ltd., Green-field NR oldham, 220 V, 500 Watts, Serial No. 39384.

1.7.4 Heraus., type 140y, 220 Volts.

1.8 Analytical Balance : Sartorius Type 2443 Fabv. Nr. 159299.

1.9 pH meter : Radiometer Type RHM 62.

1.10 HPLC : Water Associates.

Pump : Water Model 510.

Detector : Water Associates Model 440.

Intregrator : Waters 740 Data Model.

1.11 Melting Apparatus : Buchi Cappiltary Melting point Apparatus.

2. MATERIALS

2.1 Chloramphenicol base : Siam-Chemi-Pharm Lot 434945, Chemical Assay 1005.2 ug/mg and microbiological assay 986.32 ug/mg.

2.2 β-cyclodextrin (β-CD) (Sigma Chemical Company, Lot # 76E-3462, No. C-4767).

2.3 Chloroform ($CHCl_3$) (E. Merck. Lot # 703 K3647245, AR grade).

2.4 Methanol (CH_3OH) (AR grade, E. Merck Lot # 708 K4184709).

2.5 Boric acid (H_3BO_3) (AR grade, BDH Chemical Ltd., Poole England, Lot # 1724850).

2.6 Borax (AR grade, E. Merck, Lot # 310 K 2147303) ($Na_2B_4O_7 : 10H_2O$).

3. METHODS

3.1 Phase Solobility Analysis

Solubility measurements were carried out according to Higuchi and Lach (45). 200 mg chloramphenicol was added into a series of screw caped tubes, then 10.0 ml of water was added to each tube. Known amount of β-cyclodextrin were then added to each tube. These tubes were immediately sealed and rotated in the Top to Bottom Rotator at room temperature for 12 hours. After solubility equilibrium was ascertained, an aliquot volume was centrifuged and filtered through Whatman No. 1 filtered paper. A 1 ml of aliquot of this clear solution was diluted with distilled water to make up a suitable concentration and analysed with spectrophotometrically at 278 nm against the blank (distilled water).

The concentrations of soluble chloramphenicol were determined from standard curve and the molarity of soluble chloramphenicol in each solution was calculated in moles/L The results were shown in table 1, and the phase solubility diagram was constructed as shown in Figure 4. The apparent formation constant (K_C) was calculated from the initial linear portion of the phase solubility diagram according to the following equation (46).

$$K_C = \frac{\text{slope}}{\text{intercept (1-slope)}} \quad \ldots\ldots (1)$$

And the stoichiometric ratio of the complex may be estimated by the following equation (46).

$$\text{Stoichiometric Ratio} = \frac{L_b - L_a}{S_t - S_p} \qquad \ldots\ldots (2)$$

(see figure 4 in the experimental section)

Where :- S_t = total amount of solid substrate in each tube expressed in term of concentration.

S_p = The amount of soluble substrates at plateau region.

L_a, L_b = The molarity of ligands at point a and b on the plateau region.

3.2 Preparation of β-CD-chloramphenicol solid complexes

Solid complex of chloramphenicol with β-CD was prepared by using conditions derived from the descending part of the solubility diagram (Figure 4). A 2.27 g β-CD (0.1 m) and 0.645 g chloramphenicol were dissolved in about 25 ml of distilled water in a round bottom flask. The mixture was stirred at room temperature for 5 days. The complex which precipitated as a white microcrystalline powder was separated, and washed with chloroform to remove excess chloramphenicol. The microcrystalline powder obtained was dried under vacuum at 60°C for 2 days, and was subjected to further investigations.

3.3 Investigation of the Chloramphenicol : β-CD Solid Complex.

3.3.1 Infrared Spectrophotometry

The IR spectra of the complex, β-CD, Physical mixture and chloramphenical was performed utilizing potassium bromide disc technique. The resulting spectra were shown in Figure 5-8.

3.3.2 Differential Thermal Analysis (DTA)

The Shimadzu DT-30 Thermal Analyser was used. Operating conditions selected for the instrument were the follows : sample size 2-10 mg, heating rate 10°C/min, range-100 μ, and a nitrogen effluent flow rate of 50 ml/min. The thermogram were shown in Figure 9.

3.3.3 X-ray Diffraction Studies

The X-ray diffraction patterns were performed with this operating condition :

Gain (G)	120/min
Range (R)	4×10^3
Lower level (LL)	3.50
Window (W)	4.0
(time constant)	1.0
mA	30
kV	40
Start at 4 (2θ)	

Samples for X-ray diffraction studies were prepared as follows : A thin rectangular plate with a cavity was placed over a glass slide. Fine powder of the sample was packed fully and firmly into the cavity. The sample was then covered firmly with another glass slide, which was fastened to the metal plate with adhesive tape. The whole set was reversed, the glass slide base was reversed, the flat surface of the sample was exposed for the diffraction study, ultilizing a phillips X-ray Diffractometer. All diffraction patterns were run at 1 min^{-1} from 4 to 46 in terms of the 2θ angle. All X-ray diffraction patterns were shown in Figure 10 and 11.

3.3.4 Melting Point Determination

The melting point of chloramphenicol, β-CD, 1:1 physical mixture and the solid complex were determined in cappillary tubes.

3.4 Stability Studies

3.4.1 Condition of HPLC Analysis

The HPLC method for chloramphenicol was modified from the published method of Suwanna Laungchonlatan (8). The HPLC was performed using an equipment of Water Associated as described before. The eluent was monitored spectrophotometry at 254 nm. The separation ulitilized a Water Associated reverse phase column (μ-bondapak C_{18}, 10μ, 300 x 39 mm i.d.). Operating

at a flow rate of 1.0 ml/min with using methanol : H_2O (60:40) as the mobile phase. And propyl paraben was used as an internal standard, and the injection volume was 20 µl.

3.4.2 Standard Curve of Chloramphenicol and Complex Solution

Chloramphenicol and complex solution were prepared at exactly four concentrations (1.0, 1.5, 2.0 and 3.0 mg/ml) in boric-borate buffer pH 6.85. The known solution were chromatographed under the condition described in 3.4.1. Then, peak area of chloramphenicol, complex and internal standard from each chromatogram were recorded. The standard curve of chloramphenicol and its complex were constructed by plotting between the concentration (mg/ml) and the ratio of peak area which were shown in Figure 14 and 15.

3.4.3 Study of the Difference Degradation Rate Resulted from Freezed Samples and Non-Freezed Samples

The objective of this study was to test the difference of degradation rate of chloramphenicol and its complex, calculated when the samples were in freezed condition in a period of time before analysis (Freezed Method), as compared to the samples which were analysis immediately after sampling (Non-Freezed Method).

0.5% w/v chloramphenicol and its complex solution were prepared in boric-borate buffer pH 6.85. The solutions were packed and sealed in 5 ml glass-resistance vials, and then stored in incubator at $70^{o} \pm 0.5^{o}C$. After 2 days of incubation, two vials were sampling each day. The remained chloramphenicol in each vial was immediately determined using condition which described in 3.4.1, and the other vial was kept in the freezer for a period of time. The content remained of chloramphenicol in the freezed samples would then determined by the condition discussed in 3.4.1. Similar procedures, were also used for chloramphenicol : β-CD complex.

The ln A/A_{o} of chloramphenicol and its complex from both freezed method and non-freezed method were calculated, and the results were shown in Table 2 and 3. Then, the degradation rate constants (k) which were calculated from data in Table 2 and 3, were evaluated statistically by paired T-Test.

3.4.4 Investigation Method for the Rate Constant at Various Temperature

0.5% w/v chloramphenicol and its complex were prepared in boric-borate buffer pH 6.85. The solutions were packed and sealed in the series of 5 ml vials, and then stored in incubator set at 40^{o}, 50^{o}, 60^{o}, $70^{o} \pm 0.5^{o}C$ and at ambient temperature (room temperature was from 30^{o} - $35^{o}C$ and the average room temperature $33^{o}C$

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was used). Sample were taken at the appropiated time, and then kept in the freezer. The content remained of chloramphenicol and its complex were determined by the conditions described in section 3.4.1. The resulting data were shown in Table 4-7. Then, the curve of chloramphenicol and its complex were constructed by plotting between the values of ln A/A_o and time (day). The degradation curves were shown in Figure 16 and 17. From the degradation curves, calculated the specific rate constants from slope of each linear line.

3.4.5 Arrhenius Relationship

The Arrhenius plot of chloramphenicol and its complex were constructed by plotting between the logarithm of specific rate constants (lnk) and the reciprocal absolute temperature ($^oK^{-1}$). The Arrhenius plot were shown in Figure 18 and 19.

The activation energy (E_a) of chloramphenicol and its complex were then calculated, and the results were shown in Table 8 and 9.

3.4.6 Compared the Extrapolated Shelf-life Values with the Apparent Shelf-life Values of Chloramphenicol and its Complex at Apparent Room Temperature.

Extrapolated the specific rate constant (lnk) from Arrhenius plot (Figure 18,19) by using the absolute room temperature (306°K). The extrapolated specific rate constants of chloramphenicol and its complex were shown in Table 8 and 9 respectively. The shelf-life values of chloramphenicol and its complex were then calculated by substituted the values of extrapolated specific rate constant in the following equation :

$$t\ (110.0\% - 90.0\%\ \text{L.A.}) = \frac{\ln 110.0 - \ln 90.0}{* k_{rt}}$$

(k_{rt} = k at room temperature)

In similar way, the apparent shelf-life values were calculated by using the specific rate constants which were determined from the experiments at ambient temperature. The resulting data were shown in Table 10.

4. The Quantitative Agar Diffusion Assay Used for Testing Activity of Chloramphenicol vs Complex

4.1 Materials

4.1.1 Assay medium used antibiotic medium 1. (Difco) (Difco Laboratories, Detroit Michigan U.S.A. Lot 0263-01-1)

Peptone	6.0 g
Pancreatic digest of casiene (Trypticane)	4.0 g
Yeast Extract	3.0 g
Beef Extractives	1.5 g
Dextrose (glucuse)	1.0 g
Agar	15.0 g
Distilled water q.s.	1000.0 g

Final pH 6.5 - 6.6 after sterilization

4.1.2 Test Organisms

Micrococcus leuteus (ATCC 9341) is maintained by culture on slant of the assay medium No. 1 at 37°C for 24 hours.

4.1.3 Chloramphenicol Standard : A standard chloramphenicol was supplied by the Asean Reference Substances, Control No. S183003

4.1.4 Solution 1 (1% Potassium Phosphate buffer, pH 6.0)

Dibasic Potassium Phosphate	2.0 g
Monobasic Potassium Phosphate	8.0 g
Distilled Water q.s.	1000.o ml

Adjust with 18 N H_3PO_4 or 10 N KOH to yield a pH 5.95 to 6.05 before sterilization.

4.1.5 Steriled 0.9% Normal Saline Test Solution

These mentioned solutions should be prepared from AR grade Reagents and steriled by autoclave at 15 lbs/inch^2 for 15 minutes before.

4.2 Method of Testing

4.2.1 Preparation of Reference Standard Chloramphenicol Solution

Prepared the Asean Reference Standard chloramphenicol (do not dried) with concentration 10 mg/ml, stored at 4-7°C. For each assay, diluted the stock solution to the required concentrations in steriled phosphate buffer pH 6.0. At five lelvels of concentration (32, 40, 50, 62.5 and 78.1 µg/ml) were prepared; it was essential to prepare these solutions each day as required.

4.2.2 Preparation of Chloramphenicol Sample Solution and Complex Solutions

The powder of chloramphenicol and complex should be prepared at levels 50 ug/ml (the mid-value in steriled phosphate buffer pH 6.0. These solutions should be prepared freshly for the day of assay.

4.2.3 Preparation the Optimum Inoculum Size

To obtain reasonable sensitivity from the microbiological assay, the size of inoculum used is important. A typical overnight (incubated at 37°C for 24 hours) culture of *Micrococcus leuteus* in Antibiotic Medium I were investigated in the test system by diluting the inoculum size with steriled NSS. 0.9% until the result of % transmittance (%T) of 1:10 of stock agar slant were obtained 10-12%.

(Determined % Transmittance at 580 nm)

4.2.4 Preparation of the Assay Plates

The inoculum of the test organism is prepared by emulsifying the growth from a slant culture medium with 3.0 ml of steriled NSS. The optical extinction of the suspension of microorganisms was adjusted to give a transmission of 12%T in a 1 cm cuvette at 580 nm. After mixing the melted and tempered (50 $\pm$ 2°C) assay medium throughly, pipetted this medium 4.0 ml on the base layer, gentle swirled until seed agar covered over the base layer.

After 15 minutes, Placed six cups by using steriled forceps on the seed layer (Allowing exactly 60 θ between adjacent cups).

The dilution of standard and sample preparation prepared as described above, were placed into cups on the agar. The plates were incubated over night at 37°C, and measured the diameter of inhibition zones with vernier callipers.

Then, plotted curve for standard response curve, and from the standard curve, calculated the % relative potency of chloramphenical and complex.

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