CHAPTER IV

RESULT AND DISCUSSION

1. Inclusion Complex of Chloramphenicol : β-CD in Solution and Solid Complex

Phase Solubility data between Chloramphenicol and β-CD were shown in Table 1. Phase Solubility diagram (Figure 4) was constructed by plotting the molarity of chloramphenicol found in solution against the molarity of β-CD added. The plot showed typical B_s-type (47-51). In the absence of β-CD, the concentration of chloramphenicol found in solution was 1.68×10^{-2} M which is the solubility of chloramphenicol in water at room temperature. Any further addition of β-CD increases the total solubility of chloramphenicol. At point "a" in Figure 4, the system was saturated with respect to chloramphenicol:β-CD complex. The total solubility of chloramphenicol at the plateau region was found to be 3.93×10^{-2} M, an increase of 2.5 folds over free chloramphenicol.

After the solution is saturated with respect to complex and free chloramphenicol, further addition of β-CD into the system will result in the formation of chloramphenicol:β-CD complex which must then precipitate out of the solution. At the plateau region in Figure 4, the system still contains excess solid chloramphenicol and

therfore total solubility of chloramphenicol in solution remains constant.

At point "b" in Figure 4, we no longer have excess solid chloramphenicol in system. Further addition of β-CD will complex with free chloramphenicol and precipitate out of solution. Since the system no longer has excess solid chloramphenicol to keep the free chloramphenicol in solution at saturated level, the total amount of chloramphenicol in solution decreases as we add more β-CD into the system.

The stoichiometric ratio could be calculated from the rising region (52) and the plateau region (50) of the phase solubility diagram. The stoichiometric ratio of chloramphenicol:β-CD complex calculated from the rising portion and the plateau region were 0.980 and 0.967 respectively. Thus, we can conclude that the stoichiometric ratio of chloramphenicol:β-CD is 1:1, and the result is in good agreement with Aboutaleb, A.E., *et. al.*, (9).

The formation constant (stability constant) K_C was calculated from Equation 1 in the experimental section 3.1. The formation constant was found to be 9.63×10^{-2} M^{-1}.

The 1:1 chloramphenicol:β-CD complex could be prepared by stirring solution containing chloramphenicol and β-CD at room temperature for 5 days. Since the phase solubility diagram revealed the typical B_S-type, which meant insoluble complex was more thermodynamically stable

than free chloramphenicol. Therfore, once the system reached the equilibrium, the precipitate would be chloramphenicol:βCD complex not pure chloramphenicol. The solid complex can then be easily isolated by filtration process.

The IR spectroscopy was performed to verify the complex formation. The IR spectra of chloramphenicol, β-CD, physical mixture and the isolated complex were prepared, and the result were shown in Figure 5-8. The IR spectra in both physical mixture and isolated complex (Figure 7 and 8) showed major peaks of chloramphenicol and β-CD. However, the stretching vibration peak of carbonyl group (-C-) in the isolated complex was broader than that from the physical mixture, and the wave number were shifted from 1690 cm^{-1} in the physical mixture to 1700 cm^{-1} in the solid complex. In addition, the stretching vibration peak of hydroxyl group (ν-OH) from the isolated complex was broader than that from physical mixture sample. This indicated that chloramphenicol and β-CD were held together in the complex by hydrogen-bonding. The result supported the concept of the inclusion complex which formed by hydrogen-bonding (11).

From the differential thermal analysis (Figure 9), chloramphenicol (A) showed the endothermic melting peak at 152°C, and β-CD (B) showed melting peak at 73°C. In case of physical mixture (C) the thermogram showed an endothermic peak due to the melting peak of β-CD and

chloramphenicol at 73°C and 152°C respectively. In contrast, the differential thermogram of the complex showed no endothermic melting peak of chloramphenicol at 152°C and β-CD at 73°C, but showed the melting peak at 251°C. This confirmed that the isolated complound are not eutectic mixture but it is a pure compound. In addition, the strength of the crystal bonding of the complex is stronger than the strength of the crystal bonding of chloramphenicol.

The visual melting observations were also performed. Melting points of both chloramphenicol and chloramphenicol:β-CD were observed with the 1:1 physical mixture at about 73°C and 152° respectively. No melting process was seen with the 1:1 complex until temperature reached 251°C. All these evidences well correllated to the DTA analysis, and also confirm the complex formation.

Figure 10 compared the X-ray powder diffraction pattern of the complex (D) with that of the 1:1 physical mixture (C). The peaks of X-ray diffraction from 8-10 2θ and 30-32 2θ, which were found in case of the physical mixture 1:1, could not be seen in case of the complex. This indicated that the orientation in molecule of the complex is different from the physical mixture. In contrast, Figure 11 showed the peak patterns of physical mixture (C) which were actually resulted from the combined peak patterns of chloramphenicol (A) and β-CD (B).

The experimental results from phase solubility diagram, X-ray diffraction, IR spectra, and differential thermal analysis all indicated chloramphenicol formed inclusion complex with β-CD with the stoichiometric ratio of 1:1.

2. Kinetic Studies of β-CD Complex and Chloramphenicol

The HPLC method used in this experiment was modified from the published method (8). The retention times for both chloramphenicol and chloramphenicol:βCD complex were the same at 3.90 minutes, and internal standard (propyl paraben) was 5.52 minutes (see the details of procedures in the experimental section 3.4.1). The chromatogram of complex (Figure 13) was similar to the chromatogram of chloramphenicol (Figure 12) with the exception that the complex showed a broader peak than free chloramphenicol. However, the β-CD has no chromophore in its structure and therfore would not interfere with UV absorbance of chloramphenicol at 254 nm.

Data in Table 2 and 3 the ln A/A_o from the freezed method and non-freezed method, which were described in experimental section 3.4.3, were evaluated with paired t-test method. The result showed no statistically different at 95% probability. Thus, the freezed (F) method will be employed in all the experiments.

The accelerated thermodegradation process were performed at 70°, 60°, 50°, 40°C and at apparent room temperature. The order of kinetic for all temperatures were best fit as pseudo first order kinetic (see Table 5,7 in appendices).

Table 4 and 5 showed the data of chloramphenicol content remained ($\ln A/A_o$) at 70°, 6o°, 50°, 40°, and room temperature (33°C). The specific rate constant (k) which were calculated from slope of each linear line in Figure 16 (according to the first order rate expression) at 70°, 60°, 50°, 40°C, and room temperature, were 1.02×10^{-1}, 3.38×10^{-2}, 1.37×10^{-2}, 5.28×10^{-3}, and 2.60×10^{-3} day^{-1}, with the coefficient of determination (r^2) were 0.980, 0.993, 0.999, 0.993, and 0.984 respectively.

Similarily, the kinetic studies of chloramphenicol : β-CD complex were performed. Table 6 and 7 showed the data for chloramphenicol degradation from the complex at 70°, 60°, 50°, 40°C, and room temperature. The apparent specific rate constant (Figure 18) at 70°, 60°, 50°, 40°C, and room temperature were 4.13×10^{-2}, 1.77×10^{-2}, 3.73×10^{-3}, 1.45×10^{-3}, and 7.10×10^{-4} day^{-1}, and also the coefficient of determination were 0.996, 0.997, 0.992, 0.996 and 0.929 respectively.

Activation energy was calculated from the Arrhenius equation :

$$\ln k = \ln A - \frac{Ea}{R} \cdot \frac{1}{T}$$

where:

A = The constant which is termed the frequency factor.

Ea = The energy of activation which molecules must possess before they will interact.

R = The gas constant.

T = The absolute temperature.

By plotting ln k vs 1/T, Ea can be obtained from the slope. The extrapolated value of ln k at room temperature was also calculated from the linear regression line. The results of chloramphenical were shown in Table 8 of and Figure 16 in the appendices. Ea of chloramphenical was 20.8 kcal/mol and the coefficient of determination was 0.996.

Similarily, the Arrhenius plot for the 1:1 complex was constructed. The data of the complex were shown in Table 9 and Figure 18. The result of Ea and r^2 were 24.7 kcal/mol and 0.987 respectively.

The higher activation energy (Ea) of the complex indicated that higher energy was needed for the complex to be activated to the activated state.

The extrapolated specific rate constants, were calculated from the Arrhenius equation, were 2.32 x 10^{-3} and 5.43 x 10^{-4} day^{-1} for chloramphenicol and the complex respectively, and also the specific rate constants for apparent room temperature of chloramphenicol and the complex were 2.60 x 10^{-3} and 7.10 x 10^{-1} day^{-1} respectively.

The following equation was employed for the calculation of shelf-life which followed B.P.C. 1973 method.

$$t(110.0\% - 90.0\%\ L.A.) = \frac{\ln 110.0 - \ln 90.0}{k(roomtemp)}$$

The calculated shelf-life of chloramphenicol and the complex along with the comparison between the rate constant for the room temperature, 33°C (extrapolated values from Arrhenius plot) and the apparent room temperature were shown in Table 10. The shelf-life of extrapolated and the apparent room temperature of chloramphenicol were 2.63-3.14 and 2.35-2.80 months respectively. The shelf-life of the complex for extrapolated values and the apparent room temperature were 10.8-14.0 months and 8.04-11.2 months respectively.

3. The Microbiological Activity Test Between Chloramphenicol and the Complex

Agar diffussion method was used (details of the method were described in the experimental section 4), according to the C.F.R. method (50, 51). Each of the concentrations was compared with the reference standard of 50 µg/ml of chloramphenicol. The inhibition zone diameters (mm) of reference stardard, complex, and the chloramphenicol were listed in Table 11.

The inhibition zone diameters of free chloramphenicol and complex were used to obtain the equivalence concentrations of reference chloramphenicol standard by utilization of the standard curve of reference chloramphenicol standard (Figure 20). The free chloramphenicol was found to be equivalenct to 52.0 µg/ml of reference chloramphenicol standard whereas the complex was found to be equivalent to 50.0 µg/ml. Evaluation using paired t-test indicated that the results were not statistically different. Thus, Inclusion complex formation do not change the antimicrobial activity, and it may be better suited in the formulation of eye-drop preparation, especially, in reconstituted dosage form. However, clinical study is needed.