# CHAPTER IV

## DISCUSSION

### Inclusion Complexation of Chloramphenicol : 2-HP-β-CD in Solution and Solid State

Figure 11 showed the phase solubility diagrams obtained for chloramphenicol with 2-HP-β-CD. According to Higuchi and Connors (1965), the A type curve indicated the formation of soluble complex between chloramphenicol and 2-HP-β-CD. There was a 5.4 fold increse in solubility of chloramphenicol in 0.1 M 2-HP-β-CD. This is greater than the maximum solubility using β-cyclodextrin as a solubilizer. The increase of the solubility of chloramphenicol with the addition of 2-HP-β-CD was considered due F 2-HP-β-CD could be obtained in the same way as with natural cyclodextrin (Duchene and Wovessidjewel, 1990). β-cyclodextrin molecules have been shown to have a toroidal, hollow, truncated cone structure with cavities of specific size. The polar sugar hydroxyl groups are oriented to the cone exterior. This provides for aqueous solubility for β-cyclodextrin. By contrast, the core interior is highly non-polar and provides a lipophilic microenvironment which can solubilize various materials. The result of this property, 2-HP-β-CD can form soluble, reversible inclusion complex with water insoluble compound resulting in a soluble molecular inclusion complex. In this process the size of drug to be solubilized must be considered, it must fit at least partially into the 2-HP-β-CD cavity. The wider edge of the 2-HP-β-CD cavity has a diameter of 6-7.8 A° (Szejtli,1982).

The slope of the phase solubility curve gives a value of 0.62 indicating a highly efficient chloramphenicol : 2-HP-β-CD interaction. In addition, the $k_C$ of the complex was found to be 118 M⁻¹, indicating that a low stable complex is formed.

Uekama et al., (1987) have put forward a scheme which emphasis the factors that are influential in the absorption of drug administered as inclusion complexes with cyclodextrins. There are a series of successive stages in the absorption process (Figure 30) which are important when the drug

Solid drug $\xrightarrow{\text{G.I. Tract}}$ Dissolved drug ——————→ Circulatory system

Solid D-CyD ——————→ Dissolved
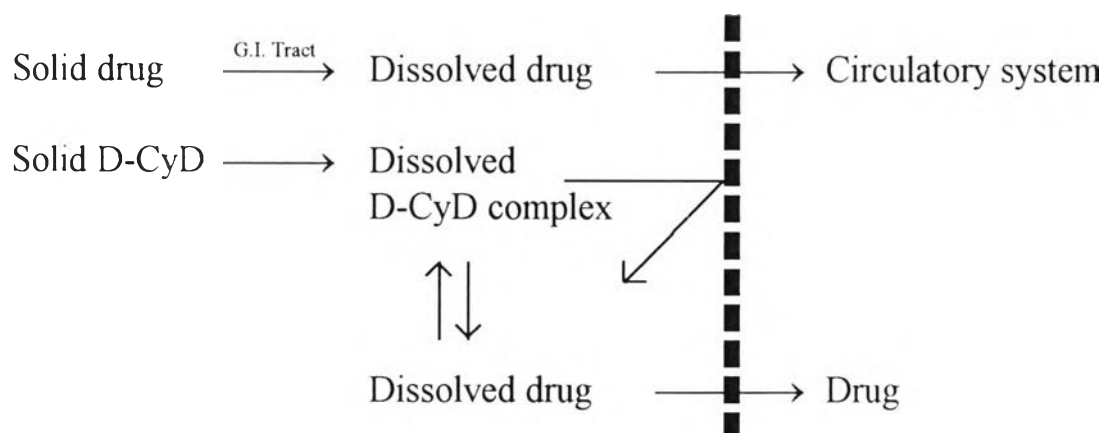D-CyD complex

↑↓

Dissolved drug ——————→ Drug

Figure 30 Schematic representation of dissolution-dissociation-absorption
process of a drug-cyclodextrin (D-CyD) complex.

passes into the circulatory system. If the rate of absorption of drugs is limited
by the rate of dissolution, it is advisable to increase the dissolution rate by
forming inclusion complexs with the cyclodextrins. Thus, it should be aware
of the stability constant of the complex. When administered the complexes
with low stability constants, they rapidly release the drug, possibly reducing
the effect that complexation has on the bioavailability of the drug. If the
stability constant of the complex is high, the drug release rate will be slow
and the quantity of the free drug around the area where it is absorbed will be
low. Szejtli (1988) has established that inclusion complexes with stability
constants between 200 and 5000 $M^{-1}$ can be used to improve the bioavailability
of hydrophobic drugs. For the chloramphenicol : 2-HP-$\beta$-CD complex, the
concentration of free chloramphenicol around the area where it is applied
will be high because of rapid release but it can not be concluded that it
enhances the bioavailability of chloramphenicol.

Solution of chloramphenicol and 2-HP-$\beta$-CD was able to be
conveniently lyophilized to generate a white powder which was stable and
easily reconstituted. Upon reconstitution, the chloramphenicol did not
precipitate. Lammers et al., (1975) indicated that the structure of cyclodextrin
inclusion compound in solution and solid state differ significantly. In solution,
the guest molecule occupies the cavity of the cyclodextrin and the complex
is surrounded and solvated by water molecules. In the solid state, the guest
may be enclosed in a void space of a lattice and not necessarily by individual
cyclodextrin molecules. This arrangement may result in the formation of
nonstoichiometric inclusion compounds. Exception to this behavior are certain
amorphous cyclodextrin derivatives such as 2-HP-$\beta$-CD that do not form

lattice structured inclusion compounds. However, the inclusion complex in solid state are more stable than in solution that the dissociation of the complex takes place due to the presence of water and the inclusion complexation is a reversible process.

## Solid Chloramphenicol : 2-HP-β-CD Complex Characteristics

It was important to identify the physicochemical properties of chloramphenicol when it was incorporated with 2-HP-β-CD because upon inclusion within the 2-HP-β-CD cavity, chloramphenicol might change its 2-HP-β-CD was examined by IR spectroscopy, X-ray diffractometry and differential thermal analysis measurement, and compared with the corresponding physical mixture in the same molar ratio.

The IR spectra of chloramphenicol, 2-HP-β-CD, the physical mixture and the inclusion complex were compared in the Figure 12. The IR spectrum of the physical mixture did not show any significant differences. The characteristic carbonyl stretching band at 1686 cm$^{-1}$ appeared in both the chloramphenicol and physical mixture. In the case of chloramphenicol : 2-HP-β-CD complex, N-H bending of amide II at 1564 cm$^{-1}$ was absent. Moreover, the 1686 cm$^{-1}$ band of solid complex shifted to 1692 cm$^{-1}$ and the intensity of carbonyl stretching band was reduced.

This spectral change might have resulted from the inclusion of chloramphenicol within the cavity of 2-HP-β-CD and the dissociation of the intermolecular hydrogen bonds of chloramphenicol through inclusion complexation. The observed decrease in intensity of carbonyl stretching band might have resulted from its restriction within the 2-HP-β-CD cavity.

Supporting evidence for the complex formation was obtained from thermal analysis studies. The DTA thermogram of chloramphenicol, 2-HP-β-CD, the physical mixture and the inclusion complex were presented in Figure 13. The DTA thermogram of chloramphenicol showed one endothermic peak at 150 °C. While the DTA thermogram of 2-HP-β-CD showed two broad endothermic peaks at 67°C and 226 °C. On the other hand, the DTA thermogram of the physical mixture showed three endothermic peaks characteristic of chloramphenicol and 2-HP-β-CD at 66°, 145° and 240°C. However, the DTA thermogram of inclusion complex revealed the absence of the endothermic peak characteristic of chloramphenicol and 2-HP-β-CD but showed the melting peak at 164°C.

Further evidence of complex formation was obtained from X-ray powder diffraction studies. The X-ray powder diffractograms of the chloramphenicol, 2-HP-β-CD, the physical mixture and the inclusion complex were shown in Figure 14. From X-ray diffraction pattern of the chloramphenicol and physical mixture, it is observed that chloramphenicol and physical mixture exhibited crystalline characteristics. The peak pattern of the physical mixture was resulted from the combined peak patterns of chloramphenicol and 2-HP-β-CD. On the other hand, the diffraction pattern of the complex shows an amorphous characteristic. This indicated that the orientation in molecules of the complex is different from the chloramphenicol and physical mixture. The absence of crystalline characteristic of chloramphenicol might be caused by inclusion complex of chloramphenicol in 2-HP-β-CD cavities.

## Determination of Physical Properties

### 1. Viscosity

In general requirement, the maximum viscosity of eye drops preparations was not limited. (Alfonso and Gennaro, 1985). Schoenwald et al., (1979) indicated that increasing viscosity increased the contact time to the eye, reduced the drainage rate and enhanced ocular bioavailability.

In this study the viscosity data were determined at 25°C for 4 months. The viscosity values of all preparations were between 2-5 cps, they were very low and rather constant throughout 4 months. Thus, all preparations should containe viscosity-increasing agent to enhance clinical efficacy. The most common viscosity-increasing agents used in aqueous ophthalmic formulations are hydroxypropyl methylcellulose, carbomers, hydroxyethyl cellulose, methylcellulose and polyvinyl alcohol. However, the selection of the viscosity-increasing agents should be considered their compatibility with other components of the formulations, e.g., the buffer system (boric-borate buffer) caused polyvinyl alcohol to precipitate from aqueous solution. The concentration of the viscosity-increasing agents should be also considered. The potential disadvantage for using viscosity-increasing agents are the formation of a crust around the eyelids and cause blurring of vision, especially if used in high concentrations.

## 2. pH

In general, chemical stability and ocular comfort of aqueous ophthalmic preparations depend on the pH of the preparations. Ocular comfort is the absence of stinging or burning upon instillation in the eye. Ocular comfort also depend on type and concentration of buffer and the nature of the drug substance. The buffer system should provide adequate buffer capacity to maintain the pH of the preparation during storage but low enough to allow the tear fluid to adjust the preparation to the physiological range upon instillation in the eye (Conner, 1981).

The pH of aqueous ophthalmic preparations should be $7.4 \pm 0.1$, equal to the pH of the natural tears, to minimize discomfort and disruption of the natural buffer system of the tear fluid. An acceptable range for eye drops which will not irritate epithelium is pH 5.0 to pH 9.0 (Conner, 1981). It will not irritate epithelium because the instillation of the solution to the eyes stimulate the flow of tears and the rapid neutralization of excess hydrogen or hydroxyl ions with the buffer capacity of the tears (Alfonso, 1985).

Higuchi and Marcus (1954) reported that the optimum pH of chloramphenicol eye drops is between 2-7 and maximum stability being at pH 6. James and Leach (1970) suggested that the complexation between borate ion and chloramphenicol (1 : 2) was responsible for increased stability of chloramphenicol in the boric-borate buffer system. And Kenneth et al., (1989) recommanded that a boric-borate buffer pH 6 should be used for dispensing chloramphenicol in eye drops solutions. Then, Siriwan Ruengsawad (1989) studied effect of a boric-borate buffer pH 6 to stability of chloramphenicol eye drops and reported that the stability of the formulation in boric-borate buffer pH 6 was not greater than at pH 7 due to less concentration of borax in the formulations of pH 6 than those of pH 7 (0.3% at pH 7, 0.05% at pH 6). Thus, the concentration of borate ion at pH 6 was too low to form complexation completely. In this study, the shelf-life of the preparation containing 2-HP-$\beta$-CD was longer than that of BPC 1973. It could be concluded that 2-HP-$\beta$-CD could improve stability of chloramphenicol eye drops. The pH values of all preparations were around pH 7.6 and rather constant at room temperature (25°C) for 4 months. Thus, 2-HP-$\beta$-CD did not affect buffer system of the preparations and ocular irritation did not occur. However, futher study was suggested.

### 3.Tonicity

Tonicity refers to the osmotic pressure exerted by a solution from the solutes or dissolved solids present. It is important to adjust the tonicity of aqueous ophthalmic preparations to the physiological range of 290-310 milliosmole/kg for optimum comfort upon instillation in the eye. Sodium chloride is most commonly used to adjust the tonicity of ophthalmic solution. Tear fluid and other body fluids exert an osmotic pressure corresponding to that of 0.9% sodium chloride solution. A solution that contains a greater amount of solutes than the tear fluid has a greater osmotic pressure and is called "hypertonic". By contrast, a solution with less solute has a lower osmotic pressure and is "hypotonic" (Salvatorn et al., 1987).

In this study, the tonicity of the preparations (absence of sodium chloride) was slightly increase when 2-HP-β-CD was added. The complex solution produced slightly higher tonicity than upper tolerance limit. This formulation may irritate the eyes unless the flow rate of tears was rapid enough to neutralize any excess tonicity. Conversely, chloramphenicol eye drops BPC 1973 produced slightly lower tonicity than lower tolerance limit whereas the tonicity of Formula I and II were isotonic.

## Degradation Studies of Chloramphenicol : 2-HP-β-CD Complex and Chloramphenicol

The contents of chloramphenicol were assayed by HPLC. The degradation products and the other ingredients in the formulations were reported not to interfere the assay of chloramphenicol (Doungsamorn, 1983; Suwanna, 1985). In this experiment, the peak of 2-HP-β-CD, boric acid, borax and phenylmercuric acetate were not appeared. Thus, all additives also did not interfere the analysis of chloramphenicol.

### 1. Order of reaction rate

The accelerated thermodegradation process were performed at 65°, 55°, 45°, 37°. In this study common method to determine the order of reaction is linear regression analysis. The coefficient of determination ($r^2$) and other statistical parameters were determined. The order of reaction rate of all preparations were best fit to first order kinetics, which is similar to previous report (Higuchi et al., 1954).

## 2. Comparison of rate constant

The rate constant (k) of the four preparation at five temperatures were compared in Table 19. The k values were orderly ranked from the minimum to maximum. It was found that, the stability of preparations at five temperatures were assumed orderly rank as complex solution, reconstituted powder (Formula II) for eye drops, reconstituted powder (Formula I) for eye drops and chloramphenicol eye drops BPC 1973. Thus, 2-HP-β-CD could be used to improved stabilityof chloramphenicol eye drops. However, chloramphenicol complex solution that did not be freeze-dried, its rate constant was lower than that of reconstituted powder.

## 3. Heat of activation (Ea)

Heat of activations were in the range of 18-25 kcal/mol, which were in agreement with the reports by Suwanna Laungchonlatan (1985); 20-22 kcal/mol, K.A.Connors (1979); 20 kcal/mol and T.Higuchi (1954); 24 kcal/mol.

The heat of activation (Ea) represents the influence of temperature on the reaction rate. If heat of activation is in the range of 10 to 30 kcal/mol, the reaction rate depends on temperature. Thus, the advantage is obtained by accelerated temperature studies in predicting the reaction rate at low temperature.

Heat of activation of each reaction was different, it was reported that Ea of the halogenation of chloramphenicol was 30 kcal/mol (Higuchi and Bias., 1953) but amide hydrolysis of chloramphenicol is 23 kcal/mol (Higuchi et al., 1954). Thus, the major degradation of chloramphenicol in this study might be an amide hydrolysis.

The different activation energy of four preparations were observed. The activation energy of reconstituted powder (Formula II) for eye drops was the highest (24.886 kcal/mol) and the activation energy of chloramphenicol eye drops BPC 1973 was the lowest (18.886 kcal/mol). It was due to slightly different slopes of Arrhenius plot that affected the estimation of extrapolated degradation rate at 25°C, 8°C and hence calculated shelf-life.

The higher heat of activation of the complex indicated that higher energy was needed for the complex to be activated to the exciting state. Thus, the degradation of preparation containing 2-HP-β-CD occured more difficult than that of chloramphenicol eye drops BPC 1973.

## 4. Calculated rate constant and shelf-life at room temperature (25°C) and 8°C

The confidence of calculation of rate constant at room temperature depended on the small standard deviation, the better linearity and the greater number of measurement. The coefficient of determination ($r^2$) of the four preparations were in the range of 0.9621-0.9971(Table 20-23), thus, the accuracy of the data and linearity were rather good. The number of data point collected were 4 points of 4 temperatures (65°, 55°, 45°, and 37°C) that obtained resonable accuracy in applying the Arrhenius treatment and making an extrapolation (Leon et al., 1986). The extrapolated degradation rate constants which calculated from the Arrhenius equation and apparent degradation rate constant at room temperature were compared in Table 28. It was found that the extrapolated degradation rate constant values were higher than the apparent degradation rate constant, except reconstituted powder for eye drops (Formula II).

At 25°C, the extrapolated shelf-life according to the 90-100 % LA was orderly ranked in order from the longest to the shortest, as following; reconstituted powder (Formula II) for eye drops, complex solution, reconstituted powder (Formula I) for eye drops and chloramphenicol eye drops BPC 1973. The preparation containing 2-HP-β-CD showed the longest shelf-life. According to the 90-100 % LA, the extrapolated shelf-life of reconstituted owder (Formula II) for eye drops was 7.74 months whereas the extrapolated shelf-life of chloramphenicol eye drops BPC 1973 was 1.86 months. Therefore the extrapolated shelf-life of the preparation containing 2-HP-β-CD was four times longer than that of BPC 1973, similar to the result calculated according to the standard of BP 1993. Moreover, it was observed that the shelf-life of chloramphenicol eye drops BPC 1973 according to BP 1993 specification was shorter than the limitation. (the limitation of BPC 1973 at 25°C is 4 months).

At 8°C, the extrapolated shelf-life orderly ranked in order from the longest to the shortest was similar to the result of the extrapolated shelf-life at 25°C. The shelf-life of all preparations at 8°C were more than at 25°C. Thus chloramphenicol eye drops should be kept at refigerated temperature.

The intervals of the extrapolated shelf-life of reconstituted powder (Formula I, II) for eye drops and complex solution according to the 90-100 % LA and BP 1993 were rather widen. Nevertheless, it was observed that the extrapolated shelf-life of all preparations differed from normal room condition storage.

## Stability of Reconstituted Powder for Eye Drops of Chloramphenicol

In the case of solid state, reconstituted powder (Formula I and II) for eye drops were investigated when kept at 45°C and 75% RH for 4 months and at room temperature for 7 months. The percent remained of two preparations are more than 95 %. It could be concluded that the two preparations appeared to be stabilized at 45°C and 75%RH without lossing of the chloramphenicol. When the two preparations were kept at room temperature (25°C), they produced negligible degradation. Thus, the 2-HP-$\beta$-CD complexation could improve the stability of sensitive active substance such as chloramphenicol against surroundings. So the tentative shelf-lives for two years of reconstituted powder could be presumed.

Although the stability effect of cyclodextrin complexation appeared both in solution and solid state, these effects were more pronounced in solid state than in solution which the dissociation of the complex occured due to the presence of water. (inclusion complexation was a reversible process).

## Antimicrobial Activity Test of the Chloramphenicol and Complex

In this study, the agar diffusion method was used according to the U.S. Code of Federal Regulations (C.F.R.) and The United State Pharmacopoeia. The agar diffusion method or cylinder plate depends upon diffusion of the antibiotic. When a dilute aqueous solution of chloramphenicol or the complex is placed in contact with a solid agar gel, the chloramphenicol or complex will diffuse from the solution through the interface into the gel until the equilibrium is attained. The agar gel which contains nutrient to support the growth of microorganism is inoculated with a suspension of a *Micrococcus leuteus* (ATCC 9341) that is sensitive to the chloramphenicol.

Upon incubation, the growth of the micro-organism was prevented entirely in a circular area around the cylinder containing a solution of chloramphenicol or complex.

The inhibition zone diameters of chloramphenicol and complex were used to obtain the equivalence concentration of reference chloramphenicol standard by utilization of the standard curve of reference standard. The chloramphenicol and complex were found to be equivalent to 51 $\mu$g/ml and 52 $\mu$g/ml, respectively. The results were not statistically different by evaluation using paired t-test. Thus, inclusion complex formation did not change the antimicrobial activity of chloramphenicol.

## Eye Irritation Test of Chloramphenicol and Chloramphenicol : 2-HP-β-CD Eye Drops in Rabbit

The use of animal eyes as test objects for pharmacological and toxicological examination has been established procedure for a long time. Draize et al., (1944) described method for testing unknown compounds of the formulations as eye irritants, that the material was instilled directly into the conjunctival sac of the albino rabbits. The two reasons of using the albino rabbit as a laboratory animal were

1. Its eye has a rather large surface of exposed globe for observation.
2. The lack of pigmented iris eases the interpretation of iristitis.

The results of this study were presented in Table 33. It was found that an approximate volume of 0.05 ml (2 drops) of three solutions (0.9% sodium chloride, chloramphenicol : 2-HP-β-CD eye drops, chloramphenicol eye drops BPC 1973) did not cause corneal cloudiness (opacity) or damage, did not produce conjunctivitis, did not cause swelling (edema), congestion, hemorrhage of the iris, did not alter pupillary light reflex. Thus, it was concluded that chloramphenicol : 2-HP-β-CD eye drops did not produce eye irritation in albino rabbit in this study.

Although the result of this study showed no eye irritation of the complex in the rabbits, the anatomy and physiology between the eyes of rabbit and human were different. The obvious anatomical and physiological difference between the rabbit eye and the human eye is the presence of the nictitating membrane (third eyelid) in the rabbit. This muscular membrane sweeps the corneal surface and acts as a protective device of the eyes. A function of the nictitating membrane is removing irritating materials from the corneal surface. At the same time, it could act as a reservior to entrap irritating materials, in spite of tearing, which could prolong the effect of the irritating materials.

Another factor that would seem to be a major significance is the difference in pH of aqueous humors. Best and Taylor (1961) have reported a pH of 7.1-7.3 for human whereas the aqueous humor of rabbit was reported to be 8.2. (Carpenter and Symth, 1946) In addition, it has been reported that the blinking reflex of the rabbit was slower than the human and could be delayed for as long as 20 minutes. (Mann and Pullinger, 1942).

Moreover, Carpenter and Smyth (1946) reported that the corneal epithelium of human measured 45.4 μm in histologic section compared to a corresponding 33.6 μm in rabbit. It could be concluded that the rabbit eye was more sensitive than the human eye because of the difference in thickness.

Thus, it has not been possible to use the results of the rabbit study to predict accurately the actual eye irritation in human that might occur after instillation.