Chapter 4

Discussion and Conclusion

1. Pharmacokinetic study

In the present study, lyophilization was usefully applied in concentrating tetracycline, extracted from the tissues (muscle and liver) by 0.1 M phosphate buffer, pH 4.5. By the process of extraction, the extracted drug solution before being lyophilized was diluted, 10 folds of that in the tissue. This would cause the undetectable result from the tissue having the drug concentration less than 1 mcg/gm (the sensitivily of the method is about 0.1 mcg/ml) by using the microbiological assay method as the drug quantitative analysis. The advantage of this technique is the evaporation of water from a sample while the sample temperature is below $0^{o}C$. Thus, the drug loss by heat during the concentrating procedure was avoided

The recoveries of tetracycline in tissues (about 70-80%) were obtained in the present study (Table 5). Such result was also reported by Poiger and Schlatter (1976).[14] They used Fluorometry in determining tetracyclines (tetracycline and chlortetracycline), extracted from the biological materials (serum, muscle, kidney, liver and milk powder). The recoveries obtained were between 30 and 45%. The bindings of tetracyclines with proteins were thought to be the main reason for the low recoveries. In addition, another

work reported by Hermansson (1982)[15] showed that the recoveries of tetracycline in plasma were 68.7 ± 2.3 and 72.8 ± 1.1% after adding two different concentrations 0.12 and 3.50 mcg/ml, into it, respectively. He used the High Performance Liquid Chromatography as the assay method. But it can be noticed that Poiger and Schlatter (1976) and Hermansson (1982) did not use the microbilogical assay in determining tetracycline in biological materials, however they did show the low recoveries of tetracycline.

The possible explanation for under 80% recoveries of tetracycline in tissues obtained in the present study may be the bindings of tetracycline with proteins[11,48] and/or the drug loss during the extraction process.[11] According to the previous works concerning the binding of the drug with proteins, by Powis (1974)[48] and by Kunin (1967)[11], 53% and 24 to 64% of tetracycline was bound in the body to serum proteins, respectively. Whilst the works of Carlin and Perkin supporting the view of the drug loss during the extraction revealed that epimerization at carbon atom 4 of tetracycline occurred at pH 2 to 6 (the extracting solvent pH of the present study was 4.5), producing a ratio of epimer to tetracycline of 0.6 : 1 in 24 hours. The epi-isomers were less active therapeutically.[11]

It could be noted that the drug levels in serum and muscle from 24-48 h after the 3 routes of administration were decreased. By contraries, the drug levels in liver from the same period after intramuscular and intraperitoneal administration were increased, however, after oral administration were decreased, but very slowly. Such phenomenon may be due to the effect of the reabsorption of tetracycline released from bile. The phenomenon, called "the entero-

hepatic circulation of tetracycline" was also found in humans,[11] rats[49] and dogs.[50] Previously, another work showed that oxytetracycline (MW = 460.44) which is closely related in chemical structure with tetracycline (MW = 444.1) was found in the bile and liver of rainbow trout after a single dose (15 mg oxytetracycline/kg) of the oral administration.[51] Such previous findings and present findings would partly support the view that the enterohepatic circulation of tetracycline occurs in catfish. However, more investigation should be done to confirm this assumption eg. the determination of tetracycline in bile and the hepatic portal vein after the intravenous or intramuscular administration.

The result from pharmacokinetic study after the three routes of administration showed that the individual variation in oral absorption was larger than in intraperitoneal and intramuscular absorption. It was probable that some amount of drug might be released from fish stomach in some fish by their natural rejection activity which was the uncontrollable factor. Another reason might be due to the much variation of each fish oral absorbability. The latter assumption seems consistent with the previous reports revealing the large individual variation in oral absorption of oxytetracycline (a tetracycline derivative) in dogs[50] and humans.[11]

The present study demonstrated that the biological half-life of tetracycline (in serum) in catfish (33.10 h) was quite longer than the biological half lives of the drug in humans (8.5 h)[11], dogs (3-4 h)[13] and cows (5.73 h)[24]. The plausible explanation for this discrepancy is the species variation and/or the environments (eg. water temperature).

Concerning to the species variation, Baggot et al (1977)[25] stated that among species, variation in extent of distribution of the drug in body fluids and tissues, and to a much lesser degree, in binding to serum proteins may also influence the rate of elimination. Whilst, Mccracken et al(1976)[32] showed that the increase in water temperature of fish would increase the rate of elimination of active antibiotic residues. Their findings compared favorably with those of Ljungburg et al (1969).[52] So the water temperature change would affect the biological half lives of drugs in fish,for fish are the cold-blooded animals whose body temperature is changible to the environment temperature, by contraries, mammals are warm-blooded animals whose temperature are fixed to about 37°C. In the present study, the water temperature (about 25°-30°C) was lower than the body temperature of mammals (about 37°C). Perhaps, the lower water temperature might be a factor causing the longer half-life in the catfish.

From AUC_o^{α} interpretation, it was found that the drug absorption after intraperitoneal administration was distinctly better than after the intramuscular administration and oral administration respectively. However, it was not fully confirmed because the administrations were not conducted at the concurrent time and the water temperature was not properly controlled. Previous works revealed that water temperature affects drug elimination in fish.[32] The absorption after oral dosing was the least despite the largest dose (10 times more than the intraperitoneal dose). This finding suggests incomplete absorption after oral dosing in catfish which agrees with the previous findings in humans.[11]

The absorption after intramuscular administration was worse than after the intraperitoneal in spite of their equivalent doses: This may partly result from the leakage of the medication back out of the injection site.[6] The skin of a fish apparently does not leave a great deal of contractibility, and once a needle hole is punched into it, it does not seal it self as well as animal tissues.

Previous works [32,51] showed that after administrations of oxytetracycline, a derivative of tetracycline, in rainbow trout, the drug was excreted very slowly. Maccracken et al (1976)[32] reported that after 5 mg oxytetracycline/kg fish intraperitoneal injection and 750 mg oxytetracycline (in medicated feed)/kg fish/day for 14 days in 5°C water temperature, the drug could be detected within 17 days and 14 days respectively. Additional report of Silven et al (1968)[51] who found that after 75 mg/kg oxytetracycline fish oral administration in 12.2-19.8°C water temperature, the drug could be detected in muscle within 17 days and in liver and bile at least within 24 days. In the present study, after 5 mg tetracycline/kg catfish intraperitoneal administration and 50 mg tetracycline/kg catfish oral administration, in 25-30°C water temperature, the drug could be found at least 6 days and 4 days respectively. Such previous findings are somewhat reasonable in comparing with the present finding because there are some differences eg. the detection method, the detection microorganisms, the extracting solvents and the water temperature, however, both of them demonstrated the slow elimination rate of the drugs. In the determination of the drug by Mccracken et al (1976), Silven et al (1968) and the present study, the results were reported as the diameters of the inhibition zone of the drug, positive or negative and mcg/ml or gm,

respectively. In experimenting, the water temperature of the present study (25^{o}-30^{o}C) was higher than of Mccracken et al (5^{o}C) and Silven et al (16.2-19.8^{o}C). As to the test organisms, both Maccracken et al and the present study used Bacillus cereus ATCC 11778, but Silven et al used 2 microganisms (Staphylococcus aureus and Sarcina lutea). For the extraction of drugs from the tissues, methanol/HCl (98:2) and 0.1 M phosphate buffer, pH 4.5 were used by Mccracken et al and the present study respectively; but Silven et al did not describe the solvent being used.

Another point of interest is that the drug levels (in serum, muscle and liver) were sustained at the high levels (about 1 mcg/ml or gm) until 24 h. Such result suggests that the administration of tetracycline in catfish may be done once a day, for the purpose of the prophylaxis or therapy of the infection of the tetracycline susceptible microorganisms(MIC < 1 mcg/ml),effectively by the large dose first and the small dose at the next consecutive days. Such comment is different from the recommended drug administration in mammals (usually 2-4 times a day).[11,26]

Another interesting observations are some patterns of the drug levels (in serum, muscle and liver) after each administration. After the intraperitoneal and oral administration, from 0.5-2h, the drug levels in liver were distinctly higher than in serum and in muscle, but after the intramuscular administration, the drug levels in serum were slightly higher than in muscle and in serum. Such observations suggested that after the intraperitoneal and oral administration, most of the drug being absorbed might be transferred to the liver, before being distributed into the blood circulation, and on the

other hand, after the intramuscular administration, most of the drug was expected to be absorbed into the blood circulation, and then being distributed into tissues.

The biological half-lives of tetracycline in serum and in muscle after each administration, obtained from the present study were slightly different. Such result indicated that the drug elimination rates in serum and in muscle might be almost equal.

The present study showed that the biological half-life (in serum or in muscle) of tetracycline obtained after the intraperitoneal administration was longer than after the intramuscular and the oral administration, respectively. This finding should be more investigated for the statistical significance because,even though, about 50-60 fish were used in each administration, only one half-life ($t_{\frac{1}{2}}$) was obtained, since, after drawing the blood from the heart of each fish, the fish was killed immediately for the drug analysis in muscle and liver at the concurrent time. The plausible explanation for the discrepancies may be due to the uncontrolled water temperature, the different lots of the experimental fish and/or the variation of absorbability of each fish especially after the oral administration, during the different periods of each administration. These are only expectations. However, the real reason is still obscure.

Remarkably,none of the biological half-lives of tetracycline in liver after the three routes of the administration was determined in the present study, for, the irregularity of the drug levels in liver (Figure 5,9,13 and Result Conclusion, p. 72.) which was expected to be the effect of the enterohepatic circulation of tetracycline in the catfish (see pp. 83-84).

2. Determination of minimal inhibitory concentration (MIC) of tetracycline to inhibit growth of Aeromonas hydrophila strains

Agar dilution techniques were chosen for the determination of minimal inhibitory concentration (MIC) of tetracycline to A. hydrophila strains, in the present study, for they have four major advantages over broth dilution methods.[53]

1. By using an inoculum replicating apparatus, a fairly large number of strains may be tested at the same time.

2. Microbial heterogeneity or contamination can be detected readily by observing the nature of bacterial growth on the surface of the agar plates as compared to that in a broth medium.

3. The medium may be supplemented with whole blood or blood products to permit testing of some of the nutritionally fastidious microorganisms that cannot be tested satisfactorily in a clear broth medium.

4. The standard procedure may be modified in a number of ways in order to permit testings of a particular type of microorganism and this is quite acceptable as long as appropriate controls are included to demonstrate that the modification does not affect the end result.

The major disadvantage to the agar dilution procedure for susceptibility testing is the fact that test plates cannot be sub-cultured easily in order to determine the bactericidal activity of the antimicrobic. For that purpose, broth dilution procedure are more ideally suited. Broth dilution MICs are not always identical to those determined with an agar dilution procedure.

The result from the MIC study shows that A. hydrophila isolated not only from catfish (50%) and shake-head fish (46.42%) but also from enviromental sources (37.5%) (water and soil in culturing ponds) can resist to tetracycline, on the other hand, the microorganisms isolated from humans and recieved from JCM do not possess this ability. The resistant strains found in environmental sources may be due to the effect of the drug contaminated in the fish culturing ponds and/or the migration of the resistant microorganisms from the fish. The resistance may result from the widely use of the antimicrobial agents in the fish culturing. Such result supports the previous works of Aoki and Egusa (1971)[54] and Aoki (1975).[5]

It is interesting to notice that the microorganisms isolated from humans and recieved from JCM are susceptible to tetracycline. The possible explanation may be that they had never been exposed to tetracycline, before.

The resistant strains found in this study probably carry R-factors. This assumption is supported by the previous evidence[23] showing that the Aeromonas hydrophila carry R-factors having markers of resistance to sulfonamide, tetracycline, streptomycin and chloramphenicol However, the origin of R-factors related to fish culturing is not yet known.[4]

3. Prophylaxis testing of tetracycline against A. hydrophila infection in catfish

In the present study, all of the catfish in the control groups developed ulcer except 1 catfish in the first challenged group (6h), Table 11). This catfish might have its own individual natural resistance. The catfish was then repeatedly challenged with about 10^9 viable cells of the bacterial suspension after the first challenge 24 h and no ulcer was developed. The plausible explanation might not only be from its individual resistance, but also from the immunity to A. hydrophila F181 after the first challenge. Concerning the latter expectation Saitanu et al [40,41] found that the immunity preventing the A. hydrophila infection could be produced after the injection of the vaccines prepared from heat killed or formalinized A. hydrophila. However, it was shown that there was a fish having natural immunity to A. hydrophila.

The prophylaxis testing result obtained in the present study demonstrated that tetracycline could prevent A. hydrophila F181 infection 100% within 3 days after the intraperitoneal administration. Then, the protection was decreased to 80%, 0% and 0% after the administration 4, 6 and 7 days respectively (Table 12). Such result closely corresponded with the theoretical tetracycline levels in muscle (studied in this thesis) (Figure 15). It could be noticed that the protection was usually 100% or 0% whereas the theoretical drug levels were higher or lower than the MIC of tetracycline to A. hydrophila F181 (0.50 IU/ml or 0.51 mcg/ml), respectively. Therefore, undoubtedly, the protection was 100% within 3 day after the administration because of the theoretical drug levels at that time, above the MIC. But the

actual protection at d 4 was only 80%, even though, the mean of the theoretical drug level at d 4 was higher than the MIC. However, the lower limit of the theoretical drug levels at d 4 was lower than the MIC because of the absorbability variation of each catfish. Thus, it could be explained that at d 4, there might be some catfish having the theoretical drug levels below the MIC, causing some catfish infected. In addition, it may be due to the fact that the gradually decrease of the drug levels in the infected site caused some catfish being infected for at this time the theoretical drug levels were almost equal to the MIC. Perhaps, the drug levels might not be maintained at the high levels long enough to eradicate the microorganisms. It could also be explained why no protection at d 6 and d 7 was found since at that time the theoretical drug levels were below the MIC.

It was worthnoting that at the same day of each testing (at d 4, d 6 and d 7), the ulcer severity of the treated groups was less than of the control groups. The less severe ulcer in the treated groups may result from the effect of the subinhibitory concentrations of tetracycline. This finding corresponds with the previous works.[55,56,57] Lorian et al (1977)[55] pointed out that even sub-inhibitory concentration of antibiotics might enhance phagocytosis and tip the balance in favor of the host. Scanning electron-microscope studies of bacteria also demonstrate that the effects of drugs on bacteria can be detected at much lower concentrations than those required to inhibit growth in broth.[55,56,57]