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Residue of Clindamycin in the Muscles of Eel and Flounder Infected by *Streptococcus* sp. by HPLC

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ABSTRACT *: Residue and recoveries of clindamycin were investigated by reversed-phase high performance liquid chromatography (RP-HPLC) which was infected for the control of streptcoccal infection in Anguilla japonica and Paralichthys olivaceusis. Detection limit was 0.1 ppm. Recoveries of clindamycin in muscles of flounder and eel were 80.4 and 78.8%, respectively. The clindamycin in eel and flounder was detected up to 13 and 15 days after dosing, respectively.*

Key Words *: Clindamycin, RP-HPLC, Residue, Anguilla Japonica, Paralichthys Olivaceusis*

I. INTRODUCTION

Clindamycin is a semisynthetic lincosamidal antibiotics which substituted Cl for OH at seventh carbon of lincomycin (Fig. 1). Including staphylococcus and streptococci, most of gram positive cocci are susceptible to it. A complete cross-resistance occurs in lincomycin and a partial cross-resistance in erythromycin. Because of a good absorption from alimentary canal and an excellent permeability in cell membrane, the application of clindamycin tends to increase more than that of lincomycin in clinical medicine. The antibacterial action of clindamycin which binds to the susceptible 50S ribosomal subunits of bacteria inhibits a protein synthesis. As the inhibition of bacterial protein synthesis, in terms of antibiotics, the minimum inhibitory and sterile concentration is all much the same and has a good antibacterial activity. As the drug has the antibacterial activity in *Aeromonas salmonicida* besides the Streptococcus sp. which cause a bacterial streptococcal infection, it is expected an excellent clinical effect in another fish disease (Austin, 1985; Austin and Austin, 1987).

Most of studies on clindamycin have been focused on rat, dog, and human (Gray, 1983; Sun, 1973). No studies have attempted in fish, as far as we are aware of, especially to consider the residual determination of clindamycin by reversed-phase high performance liquid chromatography (RP-HPLC). In Korea and Japan, macrolide antibiotics such as lincomycin and josamycin (Takemaru and Kusuda 1988) was used to treat the streptococcal infection which occurred every year frequently in fish farm of eel and flounder. The effect was, however, weekened as the fish got the resistance to these antibiotics. Therefore, the finding of a proper antibiotics and its application to infected fish became the main issue of the aquaculture.

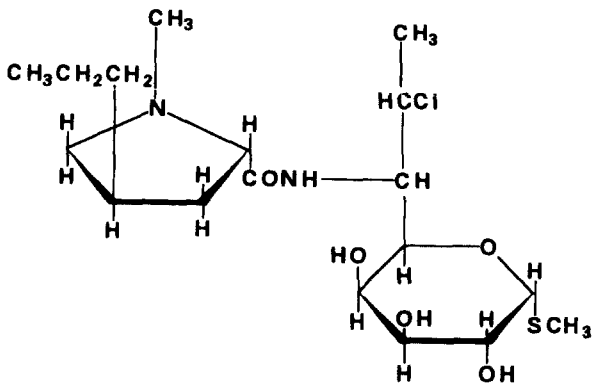

Fig. 1. Structure of the clindamycin

In this study, for the first time, the residual pro-

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perties of clindamycin was characterized in the muscles of eel and flounder using RP-HPLC with ultraviolet detection.

II. MATERIALS AND METHDOS

1. Test drug and reagents

A commercial form of 10% clindamycin hydrochloride (Kwang-jin Pharm. Co. Ltd., Seoul, Korea) was used in a challenge test.

Acetonitrile was HPLC grade (E. Merck, Darmstardt, Germany) and all other reagents were of analytical grade including phosphoric acid and sodium phosphate. The water was deionized with NANO pure II (Barnstead, Iowa, USA).

2. Experimental fish

Healthy eel (Anguilla japonica) measuring 35.0-42.0 cm and weighing 155-172 g and flounder (Paralichthys olivaceus) measuring 6.1-7.3 cm and weighing 6.5-7.6 g were randomly collected from 4 aquaria of fish farm.

For the laboratory challenge test, 500 fish were stocked into each of 4 of fish farm with aeration, continuous light, and water recirculation. Water temperature was maintained at $25\pm2^{\circ}C$ for eel and $21\pm2^{\circ}C$ (salinity, 30.5-32.0%) for flounder. Fish were fed twice a day during the experiment period.

3. Bacteria

Two bacterial strains of Streptococcus sp. isolated from clinilcally diseased fish in Korea were used in this study (Table 1).

4. Laboratory challenge test

Two bacterial strains of Streptococcus sp. (HEO 0734, HEO958) were cultured in trypticase soy broth (TSB) at $23\pm2^{\circ}C$ for 24 h. Three hundred mililiter of the culture (3.6×10^{6} CFU/ml) was inoculated into 100 *l* tanks (HEO0734 to eel, HEO958 to flounder). The bath challenge was conducted for 5 h before the fish were returned to their original aquaria.

After the outbreak of clinical disease in challenged fish, infected eel and flounder were orally administered clindamycin at dosage of 100 mg/kg body weight per day. Clindamycin was mixed with vegetable oil coated onto feed and fed daily for 3 d.

Infected fish were collected and cultured bacteriologiocally. Colonies were tested by general bacterial procedures for Streptococcus sp. confirmation.

5. Sampling

Among infected treated groups and infected non-treated groups, ten fish from each group were collected randomly every day right after administration of test drug for a week and every second day for another two weeks. Collected fish were labelled and stored in $-76^{\circ}C$ without venesection until their usage. The samples were thawed in room temperature before they were applicated to the experiment.

6. Standard calibration curve of clindamycin

A stock solution of clindamycin was diluted into the given concentrations (0.1, 1, 1.25, 2.5, 5, 8, 10 ppm) and injected onto the HPLC. In order to determine the concentration of clindamycin in fish, a standard calibration curve of clindamycin concentration (ppm) vs. peak height (cm) was determined.

7. Recoveries and reproducibility of clindamycin from the spiked tissues of flounder and eel

To investigate the concentration of clindamycin in the testing fish, muscle was homogenized. Varying the concentrations of the spiked tissues (1, 10,

Table 1. Bacterial strains used in this study.

Species	strains	year	Source fish	Country
Streptococcus sp.	HEO0734	1995	Eel	Korea
Streptococcus sp	HEO958	1995	Flounder	Korea

50, 100 ppm), the recoveries of clindamycin were carried out using HPLC after the 30 min incubation.

After the peak height was obtained, the percent recovery was determined using the standard calibration curve.

8. Apparatus and HPLC conditions

Methanol and 0.05 M NaH_2PO_4 buffer adjusted to pH 2.5 with phosphoric acid, (20:80, v/v) was used as a mobile phase. It was filtered through 0.45 μ nylon filter.

GuardPak Nova-Pak C_{18} (Waters Assoc.) as a guard column and Ultracarb 5 ODS 20 (300×4.6 mm i.d., Phenomenex, Torrance, CA, USA) as an analytical column were used.

The HPLC system consists of SHIMADZU Class LC10 pump, a Rheodyne 7125 injector (SHIMADZU, Japan), and SPD-10A UV/VIS detector (SHIMADZU, Japan). The detector wavelength was 214 nm.

9. Preparation of samples

Muscles of eel and flounder after 3 days successive exposures to 100 mg/kg oral administration of clindamycin with the given intervals were provided from College of Veterinary Medicine, Chungbuk National University. Three grams of tissue was taken and put into the 10 m*l* of phosphate buffer solution and then homogenized. 2 m*l* of homogenate (0.6 g tissue) was incubated for 30 min at 37°C and 2 m*l* of methanol was added and then vortexed to precipitate protein. The mixture was centrifuged for 5 min. at 12,000 rpm and 2 m*l* of the supernatant was collected and evaporated with N_2 gas. The residue was then dissolved in methylene chloride and centrifuged for 5 min at 12,000 rpm and then the methylene chloride layer was evaporated. Eventually, the last residue was reconstituted with 50 μ*l* 0.05 M phosphate buffer (pH 2.5) and filtered, and the filtrate (20 μ*l*) was injected onto the HPLC.

III. RESULTS AND DISCUSSION

1. Standard calibration curve of the clindamycin

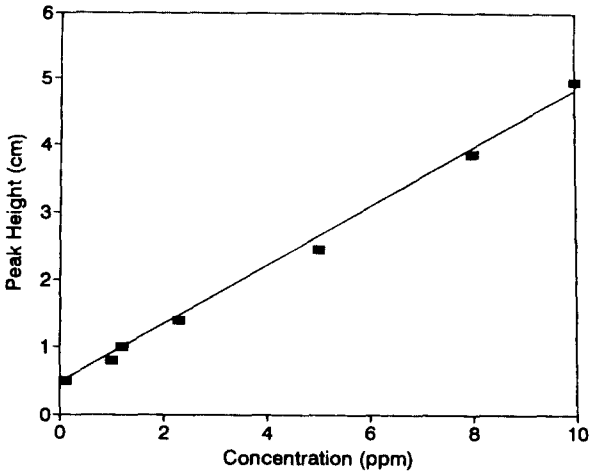

Fig. 2. Standard curve of clindamycin using RP-HPLC

Standard calibration curve was generated by least-square regression of the clindamycin peak area vs the concentration. The regression analysis showed linearity for clindamycin over the 0.1-10 μg/μ*l* range with correlation coefficient ≥0.995 (n= 5) (Fig. 2).

2. Detection limit

The developed RP-HPLC method showed an excellent precision with high sensitivity and speed, and low detection limit. In this study, the detection limit of clindamycin in two different fishes was determined. The detection limit using the method of this study was 0.1 ppm. The result can be regarded as the standard method because most of countries authorize the less than 10% of standard deviation. The typical chromatograms of blank and spiked clindamycin in tissues were shown in Fig. 3(A, B, and E).

3. Recoveries and reproducibilities of clindamycin in muscles of eel and flounder

Recoveries of clindamycin in muscles of eel and flounder were found with the mean value of 78.8± 2.36% and 80.4±3.11%, respectively. Precision (defined as the coefficient of variation of replicate analysis) of the assay for clindamycin in each muscle was evaluated over the concentration range studied (Table 2 and 3). The coefficient of variation for intra- and inter- day assay were less than 3.5%.

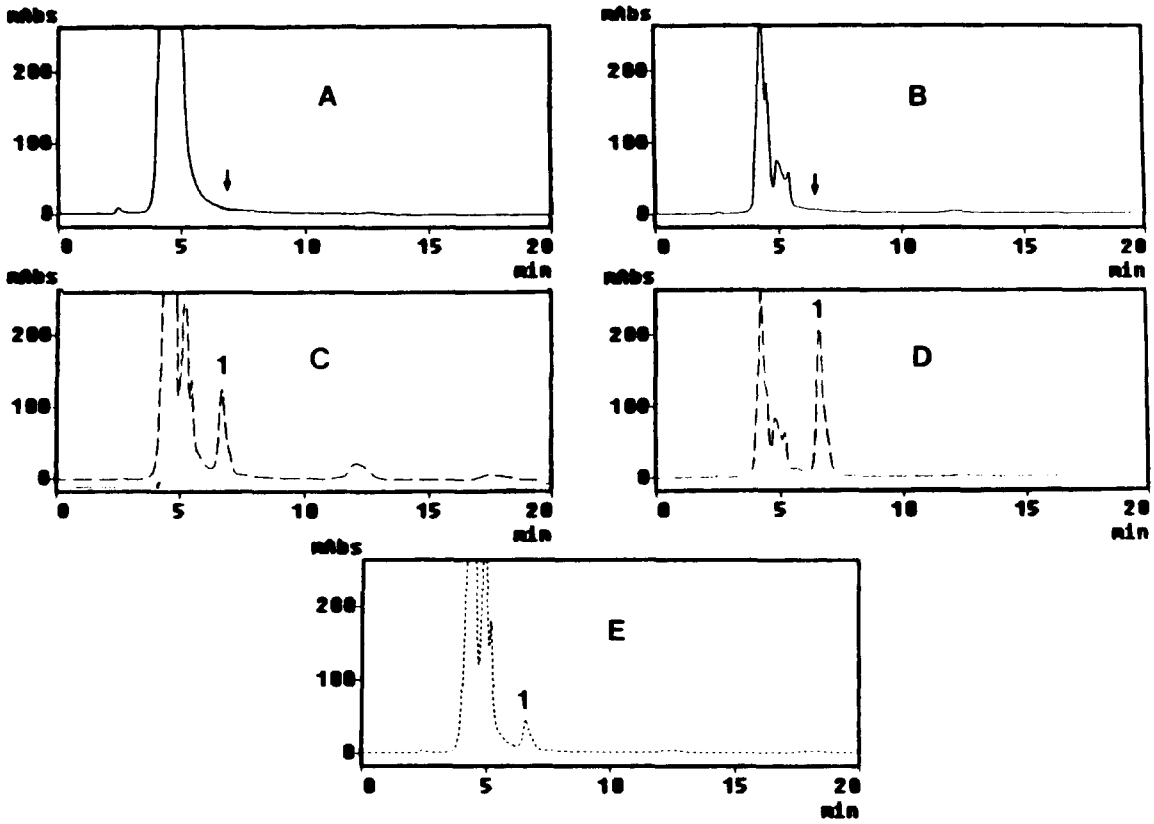

Fig. 3. Chromatograms of (A) muscle of flounder as blank, (B) muscle of eel as blank, (C) muscle of flounder 3days after clindamycin p.o., (D) muscle of eel 3days after clindamycin p.o., and (E) muscle of flounder spiked with 0.1 ppm clindamycin. Peak 1 : Clindamycin.

Table 2. Intra-and inter-day assay validation of clindamycin in flounder tissues (n=5)

Amount Added (ppm)	Amount Found (ppm)		Coefficient of Variation (%)	
	Intra	Inter	Intra	Inter
1	0.760	0.803	1.05	1.02
10	8.033	8.317	1.56	1.02
50	39.13	41.75	0.84	3.64
100	78.37	82.03	1.73	1.45

Table 3. Intra-and inter-day assay validation of clindamycin in eel tissues (n=5)

Amount Added (ppm)	Amount Found (ppm)		Coefficient of Variation (%)	
	Intra	Inter	Intra	Inter
1	0.753	0.787	2.79	2.52
10	7.900	8.168	1.54	1.75
50	38.53	40.40	1.17	2.05
100	78.83	79.23	2.94	3.12

4. Pharmacokinetics of clindamycin in muscles of eel and flounder

The extent and time of accumulated drug control the chemical and biological properties of drug. Especially, compared with mammalians as the fish has a low capacity of metabolism, the indiscriminate administration of drug can give rise to the residual toxicity (Austin, 1985 and Sun, 1973). A major deficiency in our present state of knowledge regarding use of clindamycin employed in flounder and eel is that we know little regarding the fate of the drug once it administered. Therefore, it is necessary to know pharmacokinetic data such as absorption, distribution, excretion, and metabolism for the public health. The lack of such information is particularly critical because of our meager knowledge concerning aquatic toxicology in general and the behaviour of clindamycin in nonvertebrate tissues in particular. In this study, the concentration of clindamycin in the edible portion of tissues was determined. Changes of concentration

Table 4. Changes of concentration of clindamycin in flounder and eel muscles after 3 exposures to 100 mg/kg oral administration of clindamycin (n=5)

Days after treatment	Concentration of clindamycin(μg/g tissue)	
	Flounder	Eel
1	13.43±4.1	17.75±4.2
2	11.25±3.5	15.43±2.9
3	10.20±3.1	13.75±3.5
4	7.53±3.4	12.16±2.7
5	6.50±2.1	11.15±3.0
6	2.58±1.7	9.53±2.9
7	1.35±0.7	8.64±2.3
9	0.41±0.1	3.89±1.2
11	0.25±0.1	1.35±0.5
13	0.10±0.02	0.24±0.1
15	*	0.10±0.05
17	*	*
19	*	*
21	*	*
$t_{1/2}$	38.67	47.52
K_e(day^{-1})	0.43	0.35
C_o(μg/g)	22.38	29.58

* below 0.1 ppm or not detected
$t_{1/2}$: Time for concentration of clindamycin in muscle to decrease by one-half.
K_e : Elimination rate constant
C_o : Concentration of clindamycin at time=0

of clindamycin in flounder and eel muscles after 3 exposures to 100 mg/kg oral administration with the given intervals were observed using HPLC (Table. 4), and its typical chromatograms were shown in Fig. 3(C and D).

In table 4, the half life of clindamycin in flounder and eel shows a little difference. It assumed that the longer residual time in flounder to compare with that of eel may be resulted from the habitual slow moving. As observed the above, the difference of half life in fish presents the necessity of determination of the dose and withdrawal time. This result was similar to the study reported by Park (1991) which showed the pharmacokinetic differences in a carp, catfish in Africa, and rainbow trout. A policy on the permission of a standard of residual drug and the establishment of withdrawal time can be effective but the determination of adequate concentration needs to take a prudent attitude. The result of this study shows the withdrawal time of clindamycin which arrives at the below 0.1ppm was 13 days in eel and at least 15 days in flounder, respectively.

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