DETERMINATION OF RESIDUAL OXYTETRACYCLINE IN FISHES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

[Determinasi residu oxytetracycline pada ikan dengan menggunakan HPLC]

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ABSTRACT

A high performance liquid chromatography method (HPLC) was developed for the determination of oxytetracycline (OTC) residues in fishes. OTC was extracted from fish tissues with 5% trichloroacetic acid containing 5% disodium ethylenediaminetetraacetate (Na_2EDTA). The extract was centrifuged and concentrated. The concentrated solution was washed with n-hexane and passed through a Sep-pak C_{18} cartridge. The cartridge was washed with distilled water, before use. Elute OTC with methanol and evaporate to dryness. The residue was dissolved in acetonitrile-distilled water (3:7), and determined by HPLC (with absorbance measurement at 360 nm) on a TOSOH TSK-GEL ODS 80 Tm Column (250 mm x 4,6 mm) with methanol-acetonitrile-0.2 M oxalic acid (1 : 1 : 4.4; pH 2,0) as a mobil phase. The average recoveries of OTC from blood, liver, muscle and kidney of rainbow trout, *Oncorhynchus mykiss* were 90, 72, 84, and 73%, respectively. The detection limits for muscle was 0.05 ppm, and those for liver and kidney were 0.1 ppm.

Key words: determination, fishes, HPLC, oxytetracycline, residue.

INTRODUCTION

Oxytetracycline (OTC) is a commonly used antibiotic in commercial aquaculture of freshwater and marine fish species. OTC is a bacteriostatic compound with a broad antibacterial activity against both Gram-positive and Gram-negative microorganisms, both aerobic and anaerobic species (Grondel *et al.*, 1987).

For the purpose of prevention and/or treatment of infection fish diseases, various antimicrobial agent have been widely used in aquaculture. In consequence, many drug-resistant strains of fish pathogens have appeared in fish frams (Watanabe *et al.*, 1971; Aoki, 1975). The drug may be used for prophylaxis or therapy of bacterial infections. Several diseases are describe, i.e. carp erythrodermatitis, columnaris disease, edwardsiellosis, enteric redmouth diseases and furunculosis, on which occasion OTC are recommended drugs for treatment (Austin, 1984).

The quantitative determination of drug in fish is usually done by bioassay, but is well known that this assay is lacking in sensitivity and specifity to drug (Sporns *et al.*, 1986). A simple and sensitive method for determination of OTC in fish tissue by high performance liquid chromatography (HPLC) has been reported (Ueno *et al*., 1989), however could not enough to find the high recoveries of OTC, because of the influence of the interfering substances. Therefore, it is necessary to develop a HPLC method for the determination of OTC residues in fishes. The present study was performed on the determination of OTC residues in various tissues of yellowtail, red sea bream, ayu, carp and rainbow trout by means of HPLC.

MATERIALS AND METHODS

Special chemicals

Oxytetracyline hydrochloride 4-[dimethyamino]-1, 4, 4x, 5, 5, 6, 11, 12a-octahydro-3, 5, 6, 10, 12, 12a-hexahydroxy-6-methyl-1,11-dioxo -2-napthacenecarboxamide, OTC used in this experiment was obtained from Kyowa Hakko Kogyo., Ltd. Japan, and a Sep-pak C_{18} cartridge was from Water Assoc. (Milford, MA., USA).

Other chemicals were of analytical grade or of the grade for HPLC. The chemical structure of OTC is shown in Fig. 1.

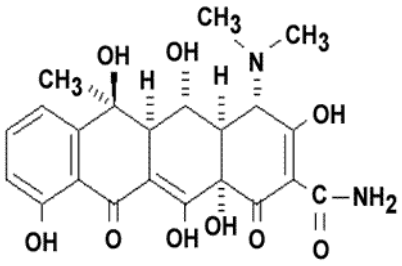


Fig. 1. The chemical structure of oxytetracycline

Fish

Five species, namely yellowtail, *Seriola quingueradiata*; red sea bream, *Pagrus major*; ayu, *Plecoglossus altivelis*; carp, *Cyprinus carpio*; and rainbow trout, *Oncorhynchus mykiss* employed in this experiment were obtained from fishfarms and aclimatized in aquarium al least for one week prior to use for the experiment. The averages body length and weight of fishes and water temperature of aquarium are shown in Table 1.

Tabel 1. The averages body length and weight of fishes, and water temperature

Species	Body length (mm)	Body weight (g)	Temperature (°C)
Yellowtail	290	461	24
Red sea bream	169	168	21
Ayu	177	82	22
Carp	148	86	21
Rainbow trout	202	135	16

Administration of OTC

All the test fishes were starved for one day before oral administration of OTC. Ninety fish of each species were acclimatized in separated aquarium (1,000 liters capacity) equipped with flowing water system. After one week acclimatization, the fishes were transfered to 50 ppm MS-222 water and anaesthetized for 10 min. Then, the diets containing adequate amount of OTC was orally administrated to fish at one dose of 50 mg in 3 g diet kg^{-1}-body weight, using Eppendorf Combitips. Then, the ninety fish of each species were immediately transfered into each 150 liters aquarium, dividing into three groups, each two aquarium in duplicate including control.

At 2, 4, 8, 12, 26, 50, 74, 98, 146, 194, and 242 hr after OTC administration, the three fish of each group were taken out from aquarium and cut at the hind brain with scissors. The blood was drawn out from cardiac puncture with a heparinized syringe. The blood, liver, kidney, muscle, and intestine collected from each three fish at each interval were pooled, frozen in liquid nitrogen and then stored in a freezer at -20°C.

Determination of OTC in fish

The extraction of OTC from fish tissues was carried out by a modified method of Ueno *et al*. (1989) (Fig. 2). The HPLC analysis of OTC extracted from tissues was performed under the conditions of instrument, TOSOH HPLC 8010 system; Column, TOSOH TKS-GEL ODL 80 Tm (250 x 4.6 mm^2); Mobil phase, methanol-acetonitrile-0.2 M oxalic acid (1:1:4,4; pH 2,0); Flow rate, 0.5 ml min^{-1}; Temperature, 35°C; Detector, UV 360 nm (TOSOH UV 8010); Sensitivity, 0.1 AUFS; Sample volume, 20 µl; Data analysis. TOSOH Super System Controller.

RESULTS AND DISCUSSIONS

It is known that OTC antibiotic has a tendency to combine with proteins and to form chelate with metal ions (Oka *et al*., 1985; Spons *et*

al., 1986). Therefore it is difficult to extract the OTC from fish tissues. Ueno *et al*., (1989) reported analytical procedure for OTC in rainbow trout tissues and was used 5% tricholoro acetic acid containing 5% Na_2EDTA to the trichloro acetic acid solvent only effective to gain the 81.70% recovery of OTC in serum, compared with those tissues. The recovery of OTC from muscle, kidney, and liver 74.2; 72.2; and 75.3% (Ueno *et al*., 1989).

In order to increase the accuracy of OTC recovery, in this experiment, an attemps were made a more convenient method. Disodium ethylenediaminetertaacetic acid has also been known as one of typical chelating agents and prevents OTC from forming complex with metal ions. Oxytetracycline is subject to chelation and therefore the recovery of this compound from fish tissues is difficult. Therefore, in this experiment the 5% Na_2EDTA was added 3 times to sample and homogenized, compared to the extraction procedure reported by Ueno *et al.* (1989) utilising 5% Na_2EDTA only for once.

A mixture of methanol-acetonitrile-0.2 M oxalic acid (aqueous, 1:1:4.4, pH 2.0) was used for the two purposes: (1) to elute the OTC from the Sep-pak C_{18} column, and (2) as a OTC mobile phase. This solvent system was modified from a method developed by Ueno *et al*. (1989) using methanol-acetonitrile-0.2 M oxalic acid (aqueous, 1:1:3.5, pH 2.0). The modification of the extraction systems from twice (Ueno *et al*., 1989) to three times and addition of 5% Na_2 EDTA to solvens, following the utilisation of methanol-aecetonitrile-0.2 M oxalic acid (1:1:4.4 pH 2.0) as a mobil phase gave the best average recovery (79.8%) (Table 2) higher than the recovery of OTC from various tissues of rainbow

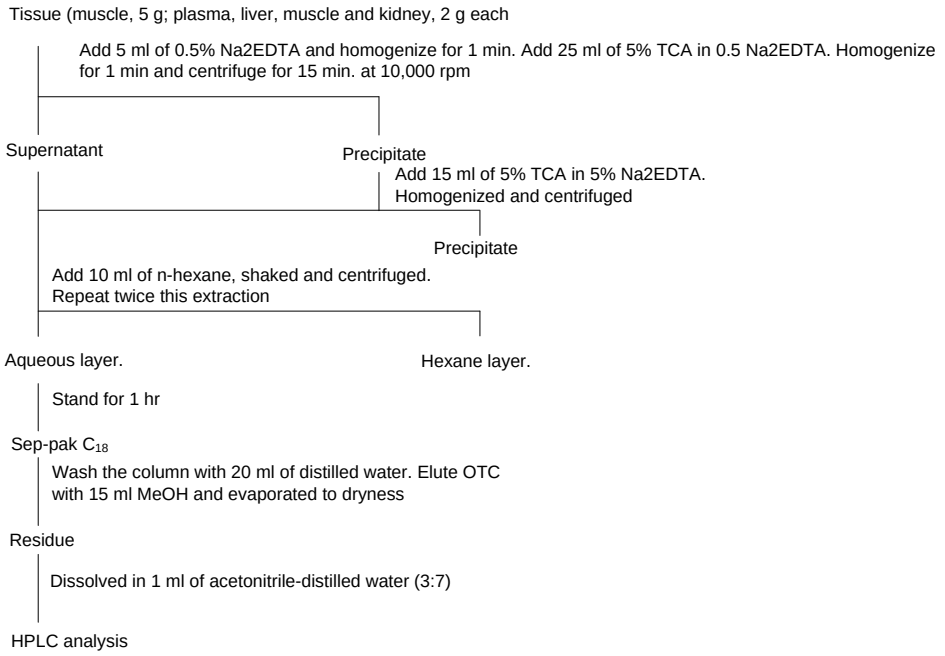
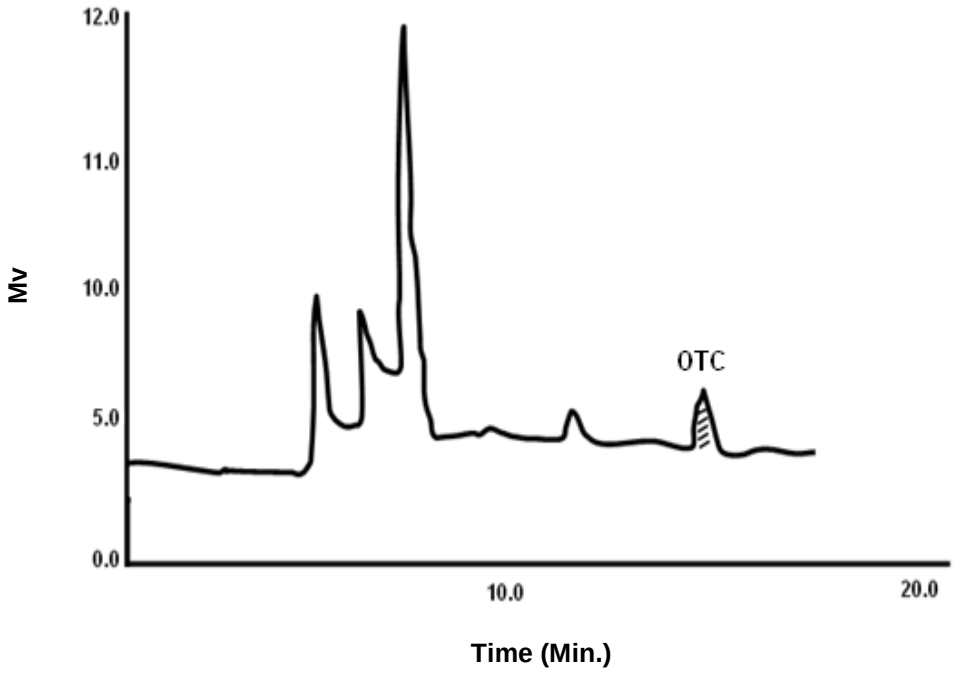


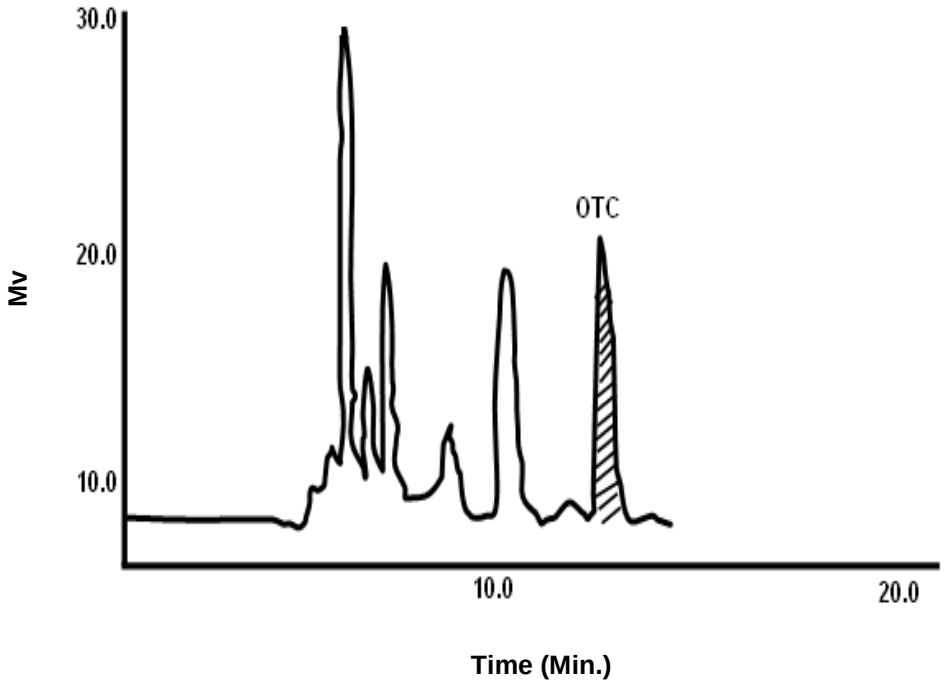
Fig. 2. Analytical procedure for oxytetracycline in fish tissues

trout reported by Ueno *et al*. (1989) reached an average recovery at 73.5%. It seems this method prevents OTC from forming complex with metal ions, then the hexane treatment and a method using Sep-pak C_{18} cartridge were a suitable cleanup procedure not only made easy for sample

preparation but also improved effectively the elimination of lipids and interfering substances from the fish tissues. Morever, the suitable condition of mobile phases was effective to find the high recoveries of OTC compared to the previous by Ueno *et al*, (1989).

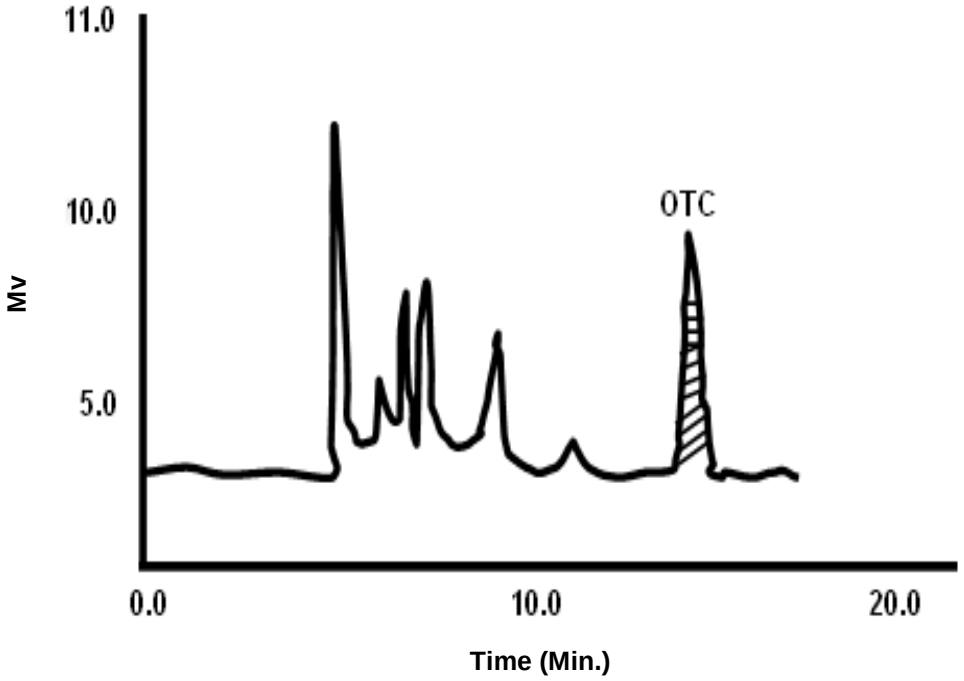


(a)

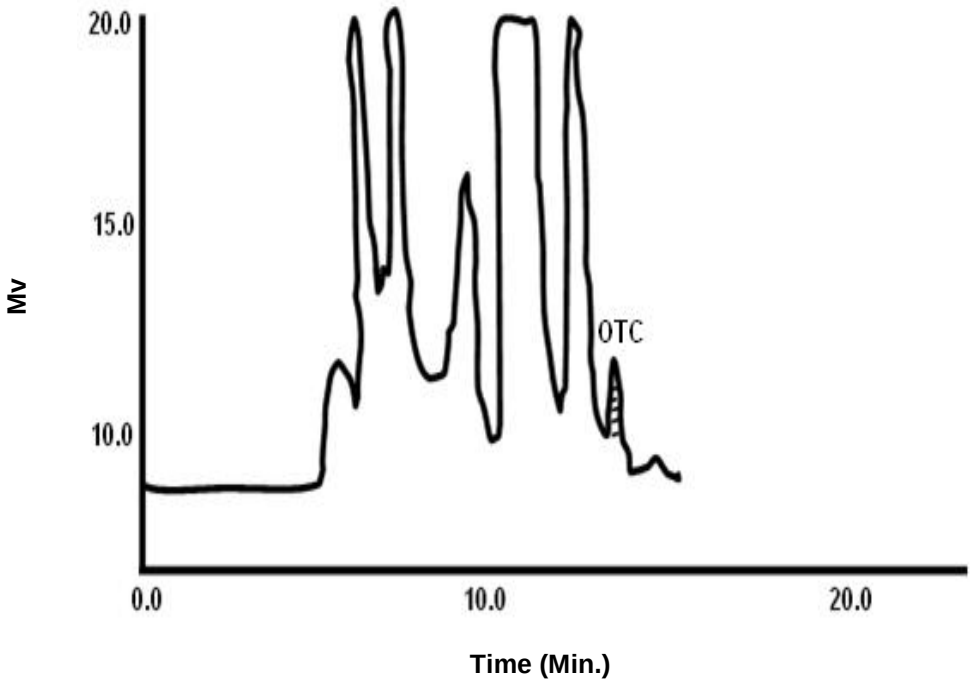


(b)

Fig. 3. Typical chromatograms of oxytetracycline from tissues (a) plasma and (b) liver of rainbow trout on HPLC



(a)



(b)

Fig. 4. Typical chromatograms of oxytetracycline from tissues (a) plasma and (b) liver of rainbow trout on HPLC

The recoveries of OTC from plasma, liver, muscle, and kidney were 90, 72, 84, and 73%, respectively (Table 2), was higher that of reported by µeno *et al*., (1989) corresponding to the average recovery at 73.5%. The detection limits of OTC for muscle was 0.05 ppm, and those for liver and kidney were 0.1 ppm. Fifteen percent loss of recovery might be cause by absorption of OTC with the gel Sep-pak C_{18} catridge, chemical degradation and the irreversible binding to protein during the storage period as reported by Murray *et al*., (1987). This values were in agreement with the standard values provided for the settlement of Residue Analysis (recovery > 70%) (Norin Suisan Sho, 1980). High recovery of OTC was observed in the order plasma (90%), muscle (84%), kidney (73%) and liver (72%). The results of this experiment show marked differences in the absorption levels of OTC tested in various tissues of rainbow trout. This low recovery value in liver may be explained that the OTC administrated to fish might be biotransformed toward the respective extractable forms by the various reactions such as hydroxylation, epoxidation, S-oxidation, dealkylation, reduction, hydrolysis and conjugation (Lumban Batu, 2001a). Our data also show that the OTC quickly released from the kidney, resulting in the low recovery of OTC in kidney compared with those tissues.

Oxytetracycline orally administrated was easily absorbed and quickly distributed in issues of tested fishes. Table 3 shows the maximum concentration and its required time, and also the biological half-life of OTC in the liver and kidney of the tested fishes. The maximum concentration of OTC were observed higher in the liver of tested fishes compared with those in kidney (Table 3). The maximum OTC concetrations in the tissues of yellowtail, red sea bream, ayu, carp and rainbow trout were attained respectively at 4-2 hr, 12 hr, 12 hr, 12 hr, and 26-50 hr post dosing. The time required to attain to the respective maximum levels and biological half-life of OTC in the tested fishes were in the same order with those in oxolinic acid and thiamphenicol (Lum-ban Batu, 2001a; 2002b). The highest maximum concentration of OTC was found in the liver of ayu (10.8 ppm), and the lowest in the red sea bream (2.3 ppm). The highest maximum concen-tration of OTC was found in the kidney of ayu (1.8 ppm) and the lowest in carp and rainbow trout (0.6 ppm) (Table 3). The duration of OTC in fishes shows in Table 3 by both the biological half-life values.

Table 3. Maximum concentration, required time, and biological half-life of oxytetracycline in the liver and kidney of fishes after oral administration

Fish	Maximum concentration (ppm)	Required time (hr)	Biological Half-life (hr)
Yellowtail			
Liver	2,2	4	8-10
Kidney	1,6	2	6
Red sea bream			
Liver	2,3	12	16
Kidney	1,1	12	12
Ayu			
Liver	10,8	12	12
Kidney	1,8	12	14
Carp			
Liver	2,8	12	12
Kidney	0,6	12	12
Rainbow trout			
Liver	7,4	26	70
Kidney	0,6	50	50

Figs. 4-9 shows the changes in the tissues level of OTC after oral administration at dose of 50 mg/kg-body weight to yelowtail, red sea bream, ayu, carp and rainbow trout. The remaining OTC was observed in blood, liver, muscle and kidney of yellowtail, red sea bream, ayu and carp 98 hr after oral administration (Figs. 4-7). Figure 8. shows the changes in the OTC concentration at a dose of 50 $mgkg^{-1}$ -body weight. The intestine of rainbow trout showed the longest duration of OTC among those of fishes, i.e. the OTC in the intestines of tested fishes except rainbow trout disappeared after 24 hr post dosing, in shorter periods than in their other tissues. However, the OTC in the intestine of rainbow trout still remained at 242 hr post dosing (Fig. 8.). In conclusion, this experiment indicate that the digestive absorption of OTC administrated to the tested fish differs from another. In our previous paper was reported that oxolinic acid and thiamphenicol also remained for a long time in the intestine of rainbow trout compared with other tested fishes (Lumban Batu, 2002a; 2002b). Thus, the long duration of OTC observed in the tissues of rainbow trout might due to the slow digestive process of the rainbow trout.

With the exception of rainbow trout which showed a digestive singularity as mentioned above, a good relationship was observed between the activities of drug-metabolizing enzymes, especially aryl hydrocarbon hydroxylase and glucuronyl transferase in fishes, and the duration of OTC orally administrated to the fishes (Lumban Batu, 2002c).

By using this developed HPLC method has enabled to demonstrate the presence of OTC residues in the various tissues of fish, and allows for higher recoveries compared with the previous paper reported by Murray *et al*., (1987) and Ueno *et al*., (1989).

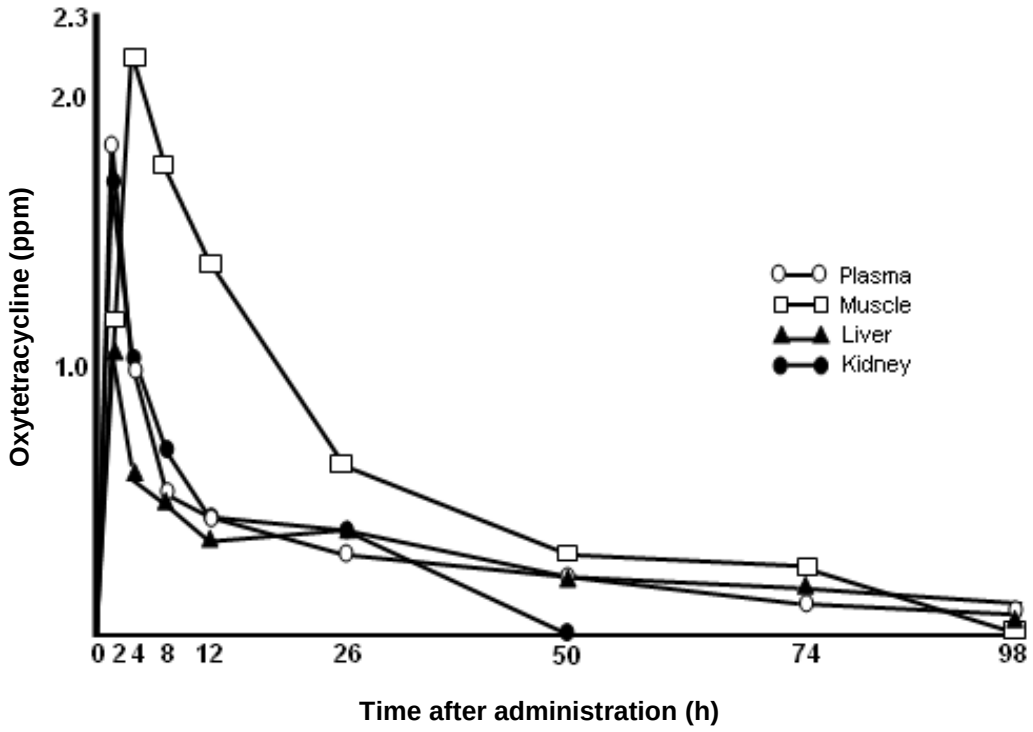


Fig. 4. Channges in the concentration of oxytetracycline in the tissues of yellowtail after oral administration at a dose of 50 mg/kg-body weight

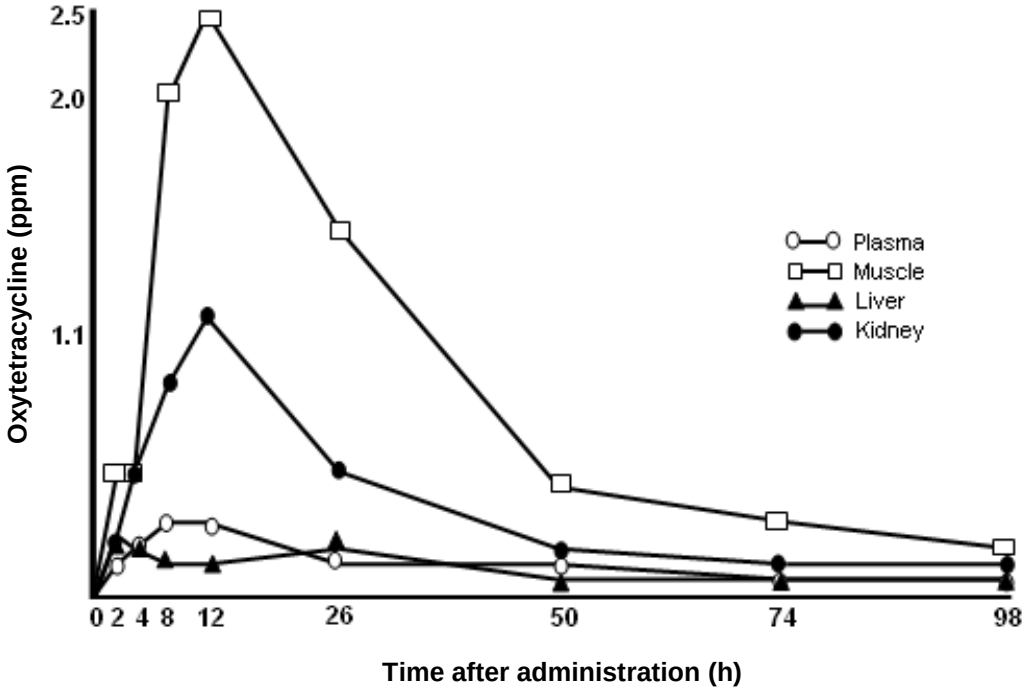


Fig. 5. Channges in the concentration of oxytetracycline in the tissues of red dea bream after oral administration at a dose of 50 mg/kg-body weight

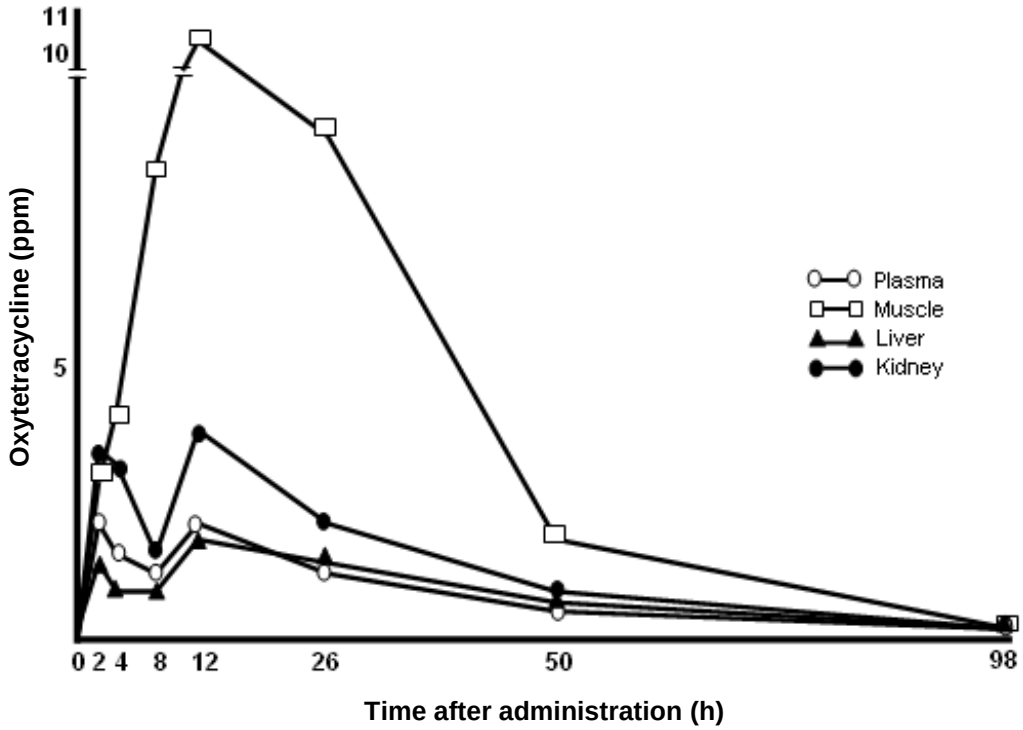


Fig. 6. Channges in the concentration of oxytetracycline in the tissues of ayu after oral administration at a dose of 50 mg/kg-body weight

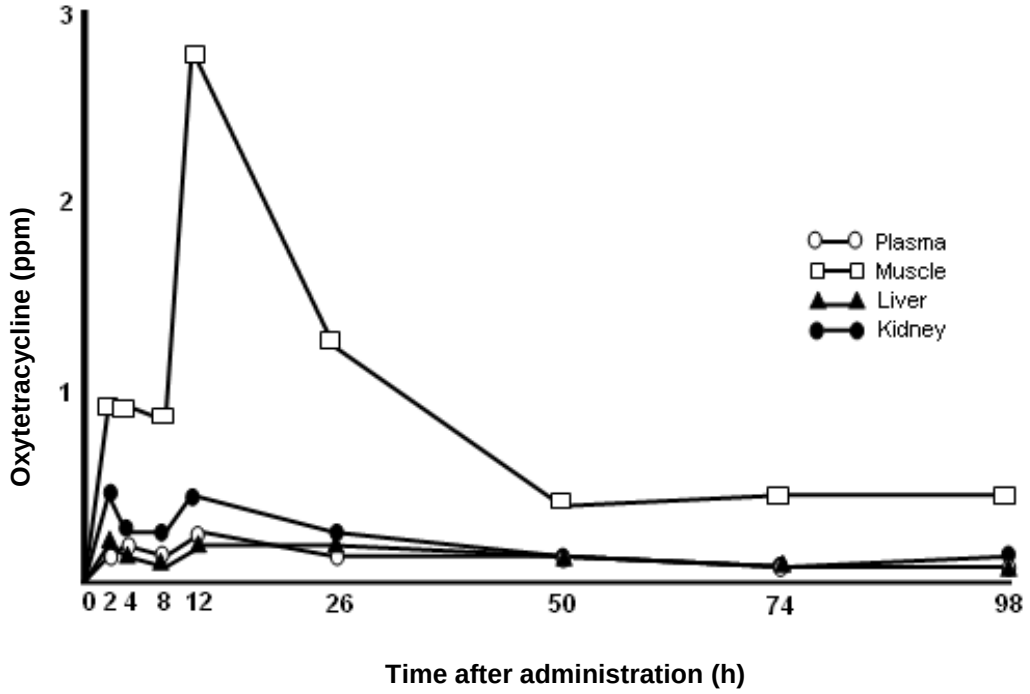


Fig. 7. Channges in the concentration of oxytetracycline in the tissues of carp after oral administration at a dose of 50 mg/kg-body weight

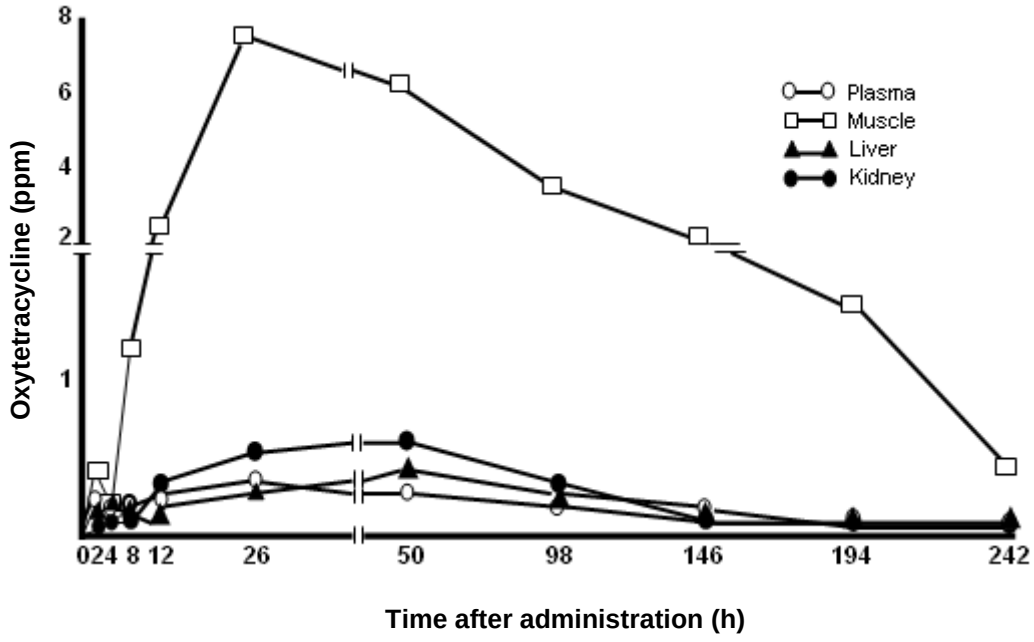


Fig. 8. Channges in the concentration of oxytetracycline in the tissues of rainbow trout after oral administration at a dose of 50 mg/kg-body weight

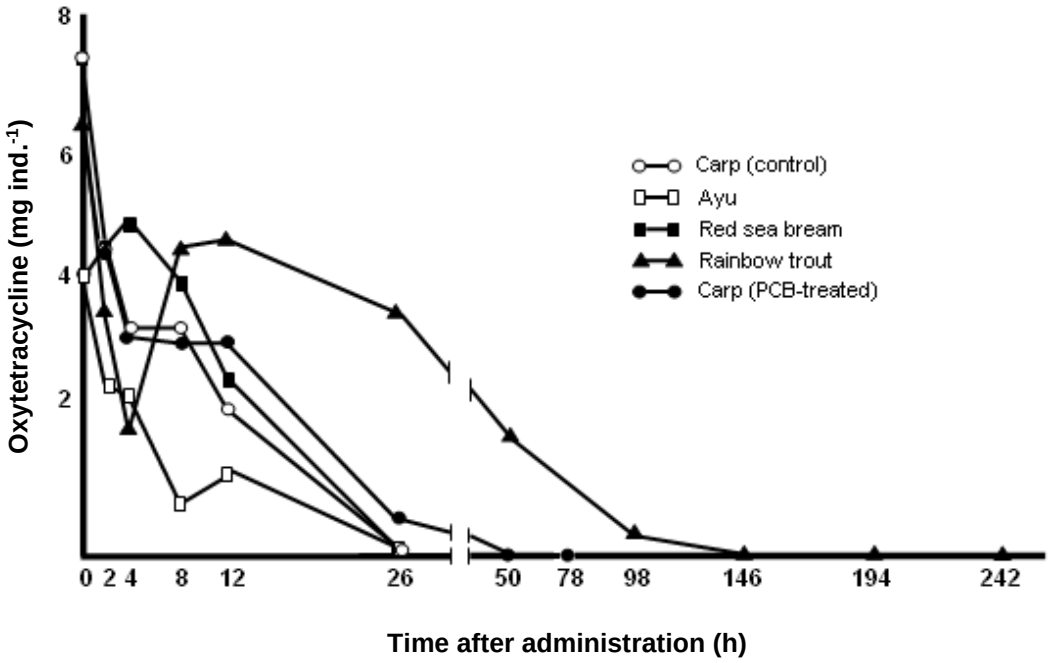


Fig. 9. Channges in the concentration of oxytetracycline in the intestine of fishes after oral administration at a dose of 50 mg/kg-body weight

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