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Effects of Suspended Solids, pH and Salinity on the Chemical Fate of Oxolinic Acid in the Aquatic Environment

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해양환경에서 부유물질, 염분 및 pH의 옥소린산 화학적 거동에 미치는 영향

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ABSTRACT: The fate of chemical pollutants in the aquatic environment is generally considered to be strongly influenced by environmental factors such as pH, salinity and electrostatic charges on the surface of particles as well as by the characteristic of chemicals. Oxolinic acid was measured by chemical analysis using HPLC to determine the effect of salinity, pH and suspended solids on chemical binding and by bioassay for measuring bioactivity. The higher concentration of suspended solids in the medium, the lower concentration of oxolinic acid was detected in measurements from by both HPLC and bioassay analysis. This indicates particle may have a stronger binding or absorption effect on oxolinic acid. Bioassay analysis showed weaker bioacivity at higher salinity and pH 7.0, but this result of bioassay analysis was different from the result of HPLC.

KEY WORDS: Oxolinic acid, HPLC, Bioassay, Suspended solids, Salinity

요약: 해양환경에서 오염물질의 거동은 화학적 특성뿐 아니라 부유물질 표면의 정전하, pH 및 염분과 같은 환경 인자에 크게 영향을 받을 것이다. 이 연구는 해산어 양식장에서 빈번히 사용하고 있는 항생제인 옥소린산의 거동이 여러 종류의 부유물질, 염분 및 pH에 따라 어떻게 변동하는가를 HPLC에 의한 화학적 분석과 bioassay분석으로 검토하였다. 부유물질의 농도가 증가할수록 옥소린산은 HPLC분석에 의해서도 저농도로 검출되었고, 생물활성에 의한 bioassay분석으로도 현저히 감소하는 것으로 나타났다. 이 결과는 부유물질이 옥소린산을 흡착하여 수서환경에서 제거하는 역할을 한다는 것을 알 수 있다. 그리고 옥소린산은 염분 40‰과 pH 7에서 bioassay 분석에 의해 생물활성이 약간 저해되지만 HPLC분석은 조금 다른 양상을 보였다.

핵심용어: 옥소린산, HPLC, bioassay, 부유물질, 염분

1. Introduction

Most antibacterial agents are administrated as feed additives(oral treatment) and a major route of antibacterial release into the aquatic environment is from uneaten feed particles, as large portion of feed provided is uneaten by the fish. Although the quantity of uneaten feed is dependent upon management practices and the type of feed used, it ranges from 10 to 50%(Ackefors and Enell, 1994; Beveridge *et al.*, 1994, 1997). The uneaten feed settles to the nearby sediment whereas the dissolved form of antibacterial agent binds to suspended particles in water column. Wild animals around the fish farm are another destination for antibacterial associated with feed and oxolinic acid residues from the wild animal in the vicinity of fish cages have been detected (Samuelsen, 1990; Björklund *et al.*, 1992; Pouliquen *et al.*, 1992; LeBris *et al.*, 1995). The average of oxolinic acid detected in the muscle of wild fish was 3.8 ppm and residues were detected even in fish caught 400 m away from culture cages (Björklund *et al.*, 1991; Samuelsen *et al.*, 1992).

Faecal particles or urine released from the fish are another pathway of drug input to the aquatic environment. This is

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dependent upon the pharmacokinetics of the drug in the animals. According to Cravedi *et al.* (1987), faeces are an important source of oxolinic acid released into the aquatic system since 62–86% of the ingested antibacterial is discharged in the faeces of rainbow trout, and Roed(1992) claimed that 93% of oxytetracycline and 86% of oxolinic acid eaten by fish were excreted from the gut.

Whatever the routes of release may be, antibacterial residues eventually accumulate in the sediment and may affect the surrounding microbial community(Nygaard *et al.*, 1992; Smith *et al*, 1994; Le *et al.*, 2005). Some investigators have found little or no loss of bioactivity of flumequine and oxolinic acid residues in sediments after 6 months cease of antibiotic treatment (Kupka–Hansen, *et al.*, 1992; Samuelsen *et al.*, 1994) whereas Bj□rklund *et al.*(1991) found loss of antibacterial activity in oxolinic acid in the sediment after 10 days. These contradictory results indicate that chemical fate in aquatic environment is variable and likely to be influenced by environmental parameters such as levels of suspended solids, salinity, temperature and water currents. Therefore, understanding the ultimate fate of chemicals entering the aquatic environment is rarely achieved. In this study the fate of oxolinic acid was investigated in relation to major environmental parameters such as salinity, pH and different sources. The paired experiments were designed to investigate the main effect of parameters and interaction. These experiments were carried out for investigating each combination of two factors; 1) investigating the binding effects of clay and feed as small size particles 2) the binding effects of larger particles using sand and humus in peat moss and 3) the effects of salinity and pH variation. Bioactivity of oxolinic acid measured by inhibition bioassay was compared with chemical concentration measured by HPLC.

2. Material and Method

2.1 Effects of main factors on persistence of oxolinic acid

The effect test of main factors on oxolinic acid persistence were carried out using following parameters; pH(5, 7, 9), salinity(0, 20‰, 40‰), clay(0, 0.5w/v, 1.5w/v), feed particles(0, 0.5w/v, 1.5w/v), sand(0, 1g, 2g) and peat moss particles(0, 0.5g, 1.5g). Shrimp pellets formulated by the Prawn unit, IOA were used. They were broken up and passed through 0.1mm mesh of pore size. Both commercially available acid washed sand (0.1–0.3 mm) and peat moss were used. Peat moss was sieved through a 1 mm pore size mesh to remove the debris of plant root.

Trials were carried out in universal bottles each containing 20 ml liquid medium with five replicates for each sample. Oxolinic acid was introduced to give a final concentration of 1 ㎍/ml and the sample set was shaken on an orbital shaker (Model S1 50, Stuart Scientific, UK). 2 ml samples were taken after one day. Salinity was fixed at 20‰ with artificial seawater and pH was set at 7.8±0.2, except salinity and pH effect tests. Room temperature was maintained at 26℃±1.4℃.

The main effects of given parameters were determined by HPLC and bioactivity assay.

2.2 Effects of paired factors on persistence of oxolinic acid

Three tests were carried out using paired factors for oxolinic acid persistence such that pH and salinity were varied, mixed with clay and feed particles, mixed with sand and peat moss particles. Three salinities (0, 20, 40‰) and pH (5, 7 and 9) were paired, giving a total of 9 combinations. Three mixtures of clay(0, 0.5 %, 1.5 % of w/v) and mashed shrimp feeds(0, 0.5 %, 1.5 %, w/v) were added to giving 9 combinations. Oxolinic acid solutions containing 1g or 2 g sand or 0.5g or 1.5 g peat moss were compared with control solutions without additives.

The paired effects of given parameters were determined by HPLC and bioactivity assay.

2.3 HPLC analysis

The overlaying water sample was taken by 1 ml pipette and mixed with the same volume of 0.5 N NaOH using a vortex mixer. After centrifugation at 3,000 rpm for 10 minutes, 1 ml of supernatant was collected and mixed with the same volume of mobile phase before injection into the HPLC system. The sediment slurry sample was taken by pushing a glass tube into the sediment and suspending in 4 ml of 0.5 N NaOH. The mixture was homogenized on vortex mixer for at least one minute and then centrifuged at 3500 rpm for 25 minutes. The precipitant was washed twice with 2 ml of 0.5 N NaOH. 1 ml of supernatant from suspended sample was taken and the extracts were combined. After centrifuging at 3500 rpm for 15 minutes, 1 ml of supernatant was taken and injected into the HPLC system. Concentration was calculated by standard curve with peak height obtained.

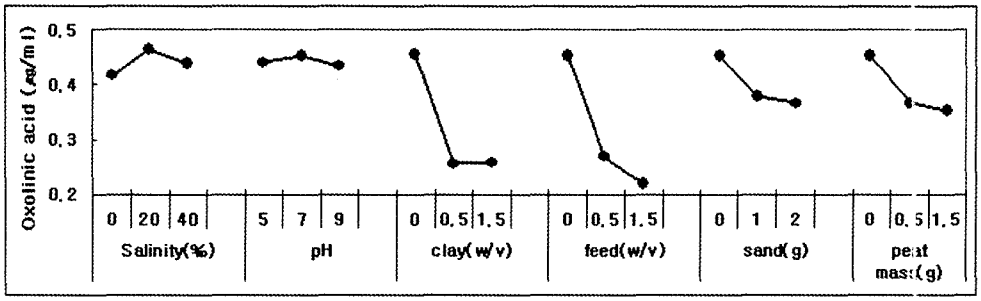

Fig. 1. The effect of salinity(‰), pH, clay, feed particles, sand and peat moss on persistence of oxolinic acid measured by HPLC.

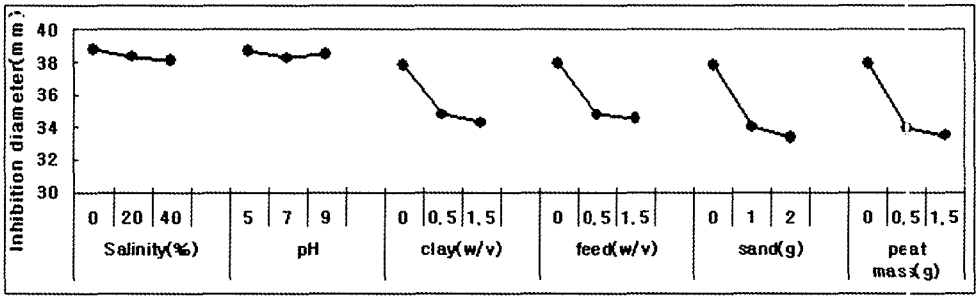

Fig. 2. The effect of salinity(‰), pH, clay, feed particles, sand and peat moss on persistence of oxolinic acid measured by bioassay.

2.4 Bioassay analysis

The bioactivity of the discs was determined after one day storage using the *Yersinia ruckeri*Italy strain as an indicator species on Iso Sensitest Agar (ISA)plates. Plates were made with exactly 20 ml ISA media to ensure consistency between treatments, therefore increasing the accuracy of the results. OA discs(2μg) were placed on the centre of prepared ISA plate and incubated at 28℃ for 24 hours. The inhibition zones were measured by taking five replicates.

3. Result

2.1 Effects of main factors on persistence of oxolinic acid

Oxolinic acid was measured by HPLC to determine the effects of salinity, pH, clay, feed, sand and peat moss on chemical binding(Fig. 1).

Higher concentration of oxolinic acid was detected in salinity 20‰ and pH 7 medium. However the range of changes in measurement was not very large, 0.046 μg/ml in salinity group and 0.02 μg/ml in pH. Lower concentration of oxolinic acid was found in higher contents of particles comparing with non-particle group designed as a control. However the differences of the concentrations between control and samples of small size particles(clay and feed) were much higher than that of the differences between control and samples of bigger size particles(sand and peat moss), relatively. This indicates feed particles may have a stronger binding or absorption effect than sand particles.

Bioassay results are given in Fig. 2. Weaker bioacivity was shown at higher salinity and at neutral pH value, which was opposite to the HPLC result. Higher bioacivity was shown at higher content of substrates, which was similar to the HPLC result. On the other hand, the differences among different sizes of particles were not significant.

2.2 Effects of paired factors on persistence of oxolinic acid

Paired effects of salinity and pH

The interaction effects of salinity and pH on the oxolinic acid measured by HPLC and bioassay showed different patterns(Fig. 3, Fig. 4). The effect of different levels of salinity does not depend on the level of pH, and thus there is no statistical significance between salinity and pH ($P>0.05$).

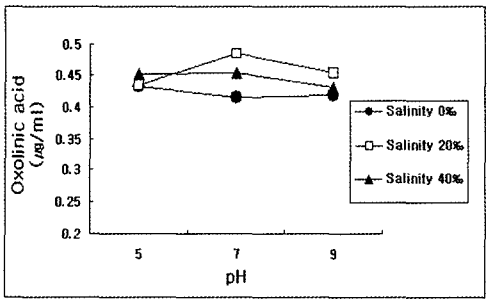

Fig. 3. Paired effect of salinity(‰) and pH on persistence of oxolinic acid measured by HPLC.

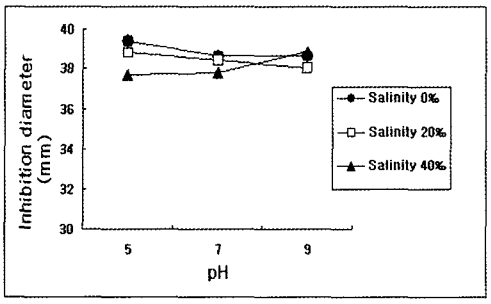

Fig. 4. Paired effect of salinity(‰) and pH on persistence of oxolinic acid measured by bioassay.

Paired effects of clay and feed

Higher concentration of particles in the medium and lower concentration of oxolinic acid were detected in measurements by both HPLC and bioassay analysis. As shown in Fig. 5, there was clear reduction of oxolinic acid in the groups having higher amounts of clay-feed comparing with non-particle group designed as a control. By the bioassay method, effect of particles on the bioactivity was shown in Fig. 6. The reduction of bioactivity was shown in higher content of substrates compared with the control group, except sample of 1.5(w/v)clay-1.5(w/v)feed pair.

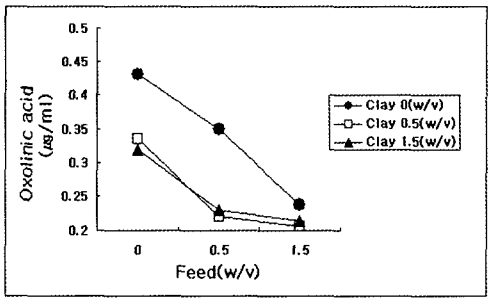

Fig. 5. Paired effect of clay and feed particles on persistence of oxolinic acid measured by HPLC.

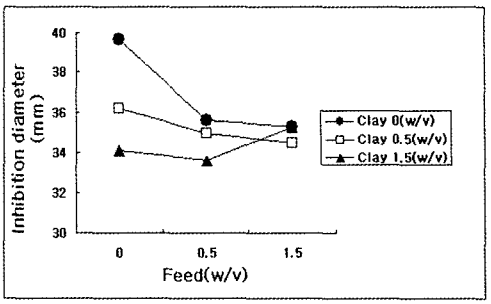

Fig. 6. Paired effect of clay and feed particles on persistence of oxolinic acid measured by bioassay.

Paired effects of sand and peat moss

HPLC results are given in Fig. 7. As the clay-feed paired group, the particles containing groups showed clearly lower concentrations, compared with the control group where no particles were added. But the effects of sand-peat moss paired group on the chemical binding of oxolinic acid was lower than that of clay-feed paired group.

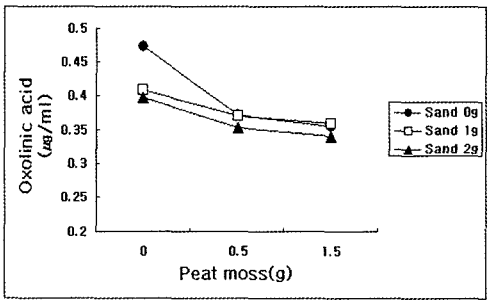

Fig. 7. Paired effect of sand and peat moss on persistence of oxolinic acid measured by HPLC.

The bioassay results of sand-peat moss paired group was similar in pattern to the HPLC results(Fig. 8). And the differences of bioactivity between sand-peat moss paired group and clay-feed paired group were not significant.

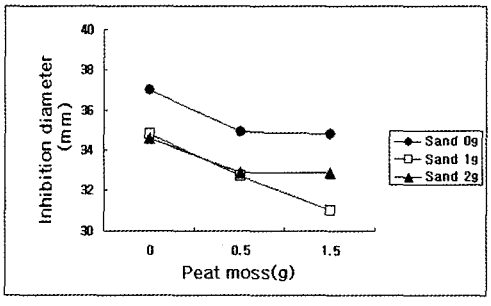

Fig. 8. Paired effect of sand and peat moss on persistence of oxolinic acid measured by bioassay.

4. Discussion

The fate of chemical pollutants in the aquatic environment is generally considered to be strongly influenced by environmental parameters such as pH, salinity and electrostatic charges on the surface of particles as well as by the characteristic of chemicals. As a chemical group introduced into aquatic environment from aqua-farming activities, the fate and impact of antibacterial agents has raised concerns and led to considerable research on their effects on the marine environment (Jacobsen and Berglind, 1988; Samuelsen, 1989, 1991; Bj□rklund *et al.*, 1991, 1992; Nygaard, 1992; Kupka-Hansen, *et al.*, 1992; Lunestad, 1991; Lunestad *et al.*, 1995, 2001; Le and Munekage, 2004) and freshwater fish farms sites (MaCracken *et al.*, 1976; Bernorth, 1991; Del□p□e and Pouliquen, 2003). Although there are considerable researches on controlling disease outbreaks in aquaculture, only few data are available on the chemical and physical characteristics of antibacterial agents in seawater or in sediment (Sithole and Guy, 1987; Pouliquen *et al.*, 1993; Lunestad *et al.*, 1995; Pouliquen and LeBris, 1996). Salinity and pH are known to have a major affection on fate of many chemicals. The rate of absorption of flumequine during bath treatment was reported to be dependent on the concentration of the agents, temperature, and pH as well as hardness of the water (O'Grady and Smith, 1992). The possible effect of pH on binding force between antibiotic compound and particle surface was also suggested by Sithole and Guy (1987). Increase in pH will increase the anionic charge on both the chemical and substrate, and thus diminish the binding force with tetracycline. This will decrease the number of protonated sites on the organic substrate available for hydrogen bonding. However, in this study there was no significance between binding effect of oxolinic acid with variation in pH or salinity. It is difficult to explain this result since there is no data available on binding effects of oxolinic acid with salts existing in seawater.

Cation effects on oxolinic acid concentration analysed by HPLC or bioactivity in this study were not significant unlike other results obtained from particle effects. Poliquen *et al.* (1994) suggested clay particles tended to bind more oxolinic acid. Even though the disc diffusion method gives a good indication of susceptibility against particular strain with particular antibacterials, it does not reflect what is happening in the natural environment. Nevertheless it has been suggested that susceptibility test of marine fish pathogens may be more accurately undertaken on medium prepared with natural or synthetic seawater (Samuelsen *et al.*, 1994). Also Lunestad and Goksoyr (1990) pointed out that the media prepared with 70% seawater gave considerable underestimation of the levels of resistance to quinolones and oxytetracycline compared with the media made without seawater. Further work is needed to unravel these conflicting reports. Polliquen and LeEris (1996) and Lunestad *et al.* (1990) studied the fate of oxolinic acid in the sediment and absorption efficiency of oxolinic acid on its substrate in seawater. They suggested that absorption of oxolinic acid increased with the sediment content of mineral and organic matter on particles of less than 63 ㎛ diameter. The same pattern was shown in this study that clay-feed paired group showed greater binding effect than sand-peat paired group. Polliquen *et al.* (1994) had also reported that sorption coefficient of oxolinic acid is lower in sand than in mud indicating the low sorption of oxolinic acid to sand. Sithole and Guy (1987) have studied the adsorption of tetracycline by the organic matter and found that the humic component in both peat and humic acid was the main binding agent to tetracycline. However, clay particles where the predominant source of suspended particles in overlying water as well as in sediment showed less binding effect than feed particles in this study. It is presumed as kaoline employed in this study has less binding capability, as CEC (Cation Exchange Capacity) of kaolinite ranges 1.59-2.53 whereas CEC of montmorillonite, a major clay component in marine sediment is 120. Also feed composed of a variety of organic materials may have more binding sites or a higher CEC than clay, so more interaction between oxolinic acid and outside hydrophilic surface of the organic particles would be possible. In fact, Booij (1993) and Wershaw *et al.* (1986) suggested that organic matter surrounding a reactive mineral surface constitutes an amphiphilic model. Endo *et al.* (1987) recommended that using ultrasize granule less than 1.0 ㎛ of oxolinic acid increased bioavailibility of oxolinic acid in red seabream and yellow tail compared with the ordinary granuals of oxolinic acid (6.4 ㎛ in diameter). Adsorption of tetracycline onto the humic acid and peat particles was reported to attain equillibrium in about 18 hours. Very

detailed chemical studies would be required to understand mechanism of binding reaction of oxolinic acid with organic components as humic acid and peat moss in this study, has complex binding reaction (Gestoettner and Fisher, 1997). Reduction of bioactivity of flumequine against *Aeromonas salmonicida* in the presence of the concentration of magnesium and calcium was reported (Pursell *et al*, 1995). The results obtained in this study also showed a significant reduction of bioactivity in overlaying water sample compared with nano purewater group, however there was no significant relationship of bioactivity between different concentrations of treatment. It is assumed that binding equilibrium would be lower than applied particle concentrations. When inhibition zones from bioassay were converted into the concentration units by standard calibration, concentration obtained from bioassay were lower than that of HPLC, whereas Barker (1994) reported the detection limit of oxolinic acid from rainbow trout muscle ranging 0.03-0.06 g/ml. Also the correlation of coefficient of the bioassay result was slightly lower than HPLC analysis. This is the indication of higher analytical precision of HPLC. However, it is argued that HPLC analysis tends to overestimate the concentration of biologically active antibacterials in the marine environment (Smith *et al.*, 1995). Also analytical or standardised methods to measure antibacterial agents from marine environment should be considered. Many protocols for measuring antibacterial residue in human are available for pharmacokinetic studies, however there is still no guide line for measuring antibacterials in the marine environment. For instance, 15 different methods to measure the residue of oxolinic acid in marine environment was reported(Ikai *et al.*, 1989; Samuelsen, 1989, 1990; Bjorklund, 1990; McGill and Hardy, 1991; Pouliquen *et al.*, 1994).

As HPLC analysis and bioassay are used for measuring different parameters, for instance, the total amount and bioavailability of antibacterial agents respectively, both should be utilized and standard methods for each parameter should be established.

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