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질산화 활성슬러지 내에서의 클린다마이신 항생제 생분해

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The investigation of clindamycin biodegradation in nitrifying activated sludge

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요약 : 본 연구의 목적은 미량오염물질인 클린다마이신(Clindamycin) 항생제의 생분해성을 질산화 슬러지내 에서 평가하는 것이다. 우선 단기간 배치 실험(Batch)을 통한 10ppb의 클린다마이신 생분해 실험결과, 클린다마이신이 반으로 줄어드는 시간 ($t_{0.5}$)은 질산화 슬러지내에서는 9.1시간으로 측정되었으나, 질산화가 저해된 슬러지내에서는 $t_{0.5}$ 시간이 26.1시간으로 증가하였다. 본 실험을 통해, 클린다마이신 분해산물이 질산화 슬러지내에서 발견되었고 이는 clindamycin-sulfoxide (m/z 441)인 것으로 추정되었다. 이 분해산물은 항생능력이 있는 것으로 판단되었다. 이 클린다마이신 분해산물은 장기간 배치실험을 통해서도 줄어들지 않는 것으로 관찰 되었다. 따라서, 활성슬러지를 통한 클린다마이신의 완전 생분해는 쉽지 않은 것으로 판단되었다.

핵심용어 : 미량오염물질, 클린다마이신, 분해산물, 하수, 질산화, 활성슬러지

Abstract : The aim of this study is to evaluate the biodegradability of the micro-contaminant, clindamycin antibiotic, under nitrifying activated sludge (AS) condition. Based on the short-term clindamycin degradation batch test at an environmentally relevant concentration (10 ppb), clindamycin disappearance half-life ($t_{0.5}$) was estimated to be 9.1hrs under nitrification condition. However, biodegradation was slower (26.1 hrs) when nitrification was inhibited. Also, one clindamycin metabolite was detected under nitrification condition, but not under inhibited nitrification condition. Based on the mass spectra, the metabolite is suspected to be clindamycin-sulfoxide (m/z 441), which is known to have antimicrobial activity. The metabolite was not degraded during the long term batch study, suggesting that under the conditions tested, biodegradation of clindamycin in activated sludge systems is ineffective.

Keywords : *Microcontaminant, Clindamycin, Metabolites, Wastewater, Nitrification, Activated sludge*

1. Introduction

Providing the sufficient clean water to the public becomes a challenging issue around the world since reliable water resource is getting diminished (Saeijs et al., 1995; World Health Organization and UNICEF., 2005). Wastewater is an alternate and attractive water resource if appropriate treatment technology can be achieved (Papaiacovo, 2001). This is one of the reasons why the removal of micro-contaminants in wastewater, such as pharmaceuticals, is a very important issue (Sedlak et al., 2000). In general, these compounds are present at parts per billion (ppb) levels or less in wastewater (Hirsch et al., 1999; Kim et al., 2005 Batt et al., 2008). Although these concentrations are relatively

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lower than other wastewater pollutants, such as carbon or ammonia, the significance of these compounds to human and wildlife is not negligible. For example, even ppt level of estrogen can disrupt the hormone system of aquatic species (de Mes et al., 2005).

In addition, many of these pharmaceutical compounds are not regulated in wastewater. Accordingly, there is an increasing concern about toxicological effects towards both humans and environment at trace concentrations (ppb-ppt) for long durations. Recent studies reported that biological wastewater treatment processes with higher solids retention time (SRT) (> 10 days) tends to have better removal efficiencies for these compounds compared to lower SRT processes (Ternes et al., 2004 Reif et al., 2008). The enhanced nitrification activity under long SRT could be one of possible answers for this observation. It is noted that ammonia oxidation bacteria (AOB) can co-metabolize various polyhalogenated ethanes(Rasche et al., 1991) and monocyclic aromatic compounds (Keener et al., 1994). Several researchers showed that trimethoprim antibiotic was removed more in nitrifying activated sludge (high SRT) compared to that in activated sludge (short SRT) (Eichhorn et al., 2005; Perez et al., 2005). Although a number of biodegradation studies have been conducted for evaluating the fate of pharmaceutical compounds in activated sludge (Kalsch, 1999; Ingerslev, 2001; Haib and Kummerer, 2005) several pharmaceutical compounds have not been examined in nitrifying activated sludge.

Clindamycin is known as highly effective antibiotic against gram-negative anaerobes and aerobes as well as gram-positive anaerobes. This antibiotic is used widely for human treating respiratory, skin, genital tracts and bone infections (Martens-Lobenhoffer et al., 2001). Clindamycin is ranked at 94th among the top 300 most prescript drugs in U.S according to 2005 survey (Rxlist, 2009). Although the frequency of detection of clindamycin in the environment is relatively lower than other antibiotics (Alexy et al., 2006 Batt et al., 2006), significance of its presence in the environment might not be negligible. Based on the ratio of expected introductory concentrations (EIC)/probable no effect concentrations (PNEC) in previous study (Bisceglia et al., 2005), clindamycin was the second highest potentially-risky compound for aquatic organism (Daphina sp.) among the top 200 prescription drugs in the United States.

Clindamycin is considered a non-biodegradable substance under OECD guideline biodegradable test (Zahn-Wellens test) (Gartiser et al., 2007). A recent study indicated that sorption is not a major elimination process of clindamycin in biosolids (Wu et al., 2009). Accordingly, it is important to know if clindamycin and its metabolites can be biodegradable under nitrifying activated sludge condition to judge the fate of potentially toxic substance clindamycin in environment.

2. Materials and methods

2.1. Chemicals and reagents

Clindamycin (CLD) was purchased from MP Biomedicals (Aurora, OH, USA). Standard solution at a concentration of 1 mg/mL was prepared in methanol. This standard solution was stored at -40 ℃ for a maximum of 3 months. Using this stock standard solution,

standard curve was generated (Figure 1). Working standard solution was prepared fresh on the day of analysis by diluting stock solution with water. An isotopically labeled sulfonamide, $^{13}C_6$-sulfamethazine, was used for internal standard and was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

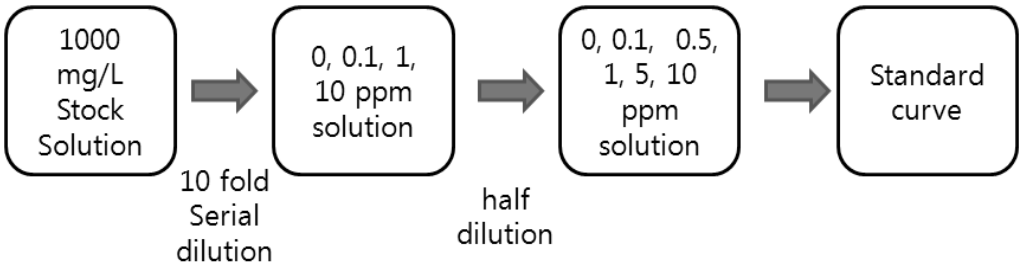


Fig. 1. Standard curve preparation procedure

2.2 Short term batch test

Three 6 litter batch reactors, consisting of glass flasks wrapped in aluminum foil, were set-up to contain 5-L of biomass in each reactor. The biomass of each batch reactor was adopted from the stage-2 activated sludge of Amherst WWTP, NY. This stage-2 activated sludge is a suspended growth system for enhanced nitrification following by stage 1, which is designed to remove mainly organics. The collected biomass was aerated for one day to reduce dissolved organic matter content. Aeration was achieved by the air stone which is connected to the atmospheric air blower. The MLSS (mixed liquor suspended solid) concentrations of biomass was 3,300 mg/L. At time 0, these three reactors (Batch-1, 2, 3) were treated with CLD 10 μg/L and ammonia 50 mg/L as a final concentration of each compound. Also, one of these two batch reactors (Batch-2) was treated with allythiourea (5 mg/L final concentration) (Batt et al., 2006) to inhibit nitrification by AOB in activated sludge but not in Batch-1 and 3. In Batch-3, 1% (w/w) sodium azide (NaN_3) was added at time 0 to inhibit the any microbial activity in activated to estimate the other removal mechanism such as sorption except biodegradation. These reactors were mixed using magnetic stirrers at 300 rpm. Duplicate 500-mL aliquot samples (total 1 L) were withdrawn from each reactor at 0.1, 6, and 24 hr after treatment. Samples were centrifuged at 2000 g for 10 min. After centrifuge, samples were prepared by solid phase extraction (SPE). After SPE, these samples were stored at -40 ºC freezer before the analysis. All samples were analyzed within 24 hrs after the end of batch study. The final concentrations of each CLD and its metabolites in the dissolved phase from these samples were determined by directly injecting 20 μL aliquots into the LC/MS/MS. Ammonium (NH_4-N), nitrate (NO_3-N), and pH were monitored to track the nitrification activity in each batch reactor (0.1, 2, 4, 6, 24 hrs). Concentrations of ammonium (NH_4-N) and nitrate (NO_3-N) were determined using the

colorimetric tests following the protocol of HACH® Ammonia Nitrogen Test Kit (Model NI-SA, Cat. No. 24287-00) and HACH® Nitrtate Test Kit (Model NI-11, Cat. No. 1468-03), respectively.

2.3 Long term batch test

Another clindamycin biodegradation test was designed and conducted with same manner as described above but longer test periods (12 days) under nitrifying activated sludge reactor. One 6-liter batch reactor, consisting of glass flasks wrapped in aluminum foil, was set-up to contain 2 L of biomass in each reactor. The biomass was adopted from the stage-2 activate sludge (AS). Same MLSS concentration (3,300 mg/L) was used in the initial test. Samples were sampled at 0, 1, 4, 6 and 12 days after start. Duplicate 5 mL of samples were centrifuged at 2000 g for 10 min and analysis was conducted instantly after centrifuge. The pH of the reactors was measured daily, and if the pH dropped below 6.5, the pH was adjusted to around 7.5 with sodium hydroxide. The water level of bioreactor was checked every day and add tap water if any loss of water was found.

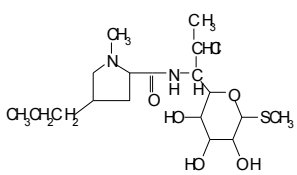
2.4 Solid phase extraction

Five-hundred mg Oasis HLB cartridges were conditioned with 5 mL acetonitrile followed by 5 mL water. Samples were loaded at a rate of 3 to 5 mL per minute, and the cartridges were eluted twice with 4 mL acetonitrile into a glass tube containing 50 µL of the internal standard (corresponding to 50 µg of $^{13}C_6$-sulfamethazine). The volume of the acetonitrile in the extracts was reduced to 0.2 mL under a stream of air, water was added to a final volume of 1.0 mL. More details were delineated in a reference (Batt et al., 2005).

2.5 LC/MS/MS

Samples were analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS) using a LCQ Advantage™ ion trap mass spectrometer (IT- MS) equipped with an electrospray ionization source (ESI) operated in positive ion mode (Thermo Finnigan, San Jose, CA, USA). The structure of clindamycin and LC/MS/MS analysis information are summarized in (Table 1).

Table 1. A summary of the target analytes, MS/MS parameters, and method limits of detection(LOD)

Compound (Abbreviation) Class	Structure	Precursorion (MH^+)	Productions 1, 2	Collision energy (%)	Isolation width	LOD (µg/L)
Clindamycin (CLD) Lincosamide		425	377,126	40	1.0	0.060

The column used was a BetaBasic-18 C_{18} column (100 X 2.1 mm internal diameter with 3 μm particle size) equipped with a UNIPHASE™ guard cartridge (10 X 2.1 mm internal diameter with 3 μm particle size), both purchased from Thermo Hypersil-Keystone (Bellefonte, PA, USA). The flow rate was 200 μL per minute, the column oven temperature was 30℃, and the full loop injection volume was 20 μL. The separation was performed using a gradient mobile phase consisting of acetonitrile (A), methanol (B) and water with 0.3% formic acid (C). The details are explained in a reference (Batt et al., 2005).

3. Results and duscussion

3.1 Nitrification effect to clindamycin degradation

Only the Batch-1 reactor generated nitrate (nitrification) after ammonium injection during the short-term batch study (data is not shown here). During the study, higher clindamycin disappearance in Batch-1 (mass to charge ratio, m/z 425) was observed compared to other batch reactors (Figure 2). When the simple first-order degradation equation was applied, clindamycin disappearance half-life ($t_{0.5}$) was estimated as 9.1 hrs under nitrification condition (Batch-1). However, under inhibited nitrification condition (Batch-2) the half-life was 26.1 hrs As shown in (Figure 2), there is no significant disappearance of clindamycin in Batch-3, which was designed for inhibiting the biological activity of activated sludge (AS). The results indicate that biosorption or hydrolysis of clindamycin is negligible within the 24-hr time period, which is consistent with earlier an study reporting negligible clindamycin biosorption (K_d = 3.9) (Jarnheimer et al., 2004).

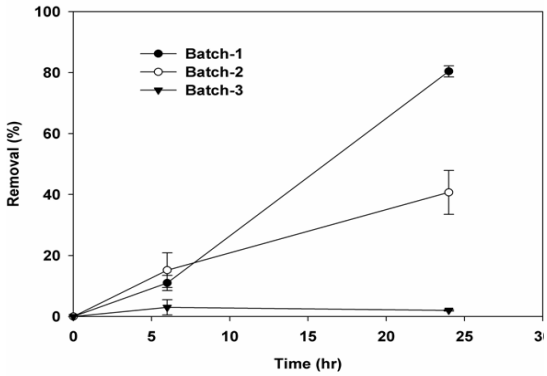


Fig. 2. The disappearnce of clindamycin under three different batch reactor conditions [Batch-1:Biomass treated with clindamycin and ammonium; Batch-2: Biomass treated with clindamycin, ammonia and allythiourea; Batch-3: Biomass treated with clindamycin, ammonia and sodium azide] (error bar: standard deviation).

3.2 Nitrification sludge effect to metabolites

One of the objectives in this study is to characterize the clindamycin metabolites under nitrifying activated sludge. After analyzing the samples under full scan LC/MS from each batch reactor, a new compound (m/z 441) was observed which appeared to increase over time in Batch-1 reactor (Figure 3, 4). Upon further examination of this new peak in the LC/MS chromatograms, it was concluded that this peak is a biodegradation product of clindamycin. The same metabolite has been reported in a previous study (Wynalda et al., 2003) using human liver microsomal incubations. This metabolite is a clindamycin-sulfoxide (Figure 3) and has been reported to have some bioactive property. The long-term batch test (12 days)

was followed to trace the fate of clindamycin and its metabolite. Although the disappearance of clindamycin and the generation of the suspected clindamycin-sulfoxide metabolite were observed, no other metabolites produced. Interestingly, the metabolite was generated only in the presence of high nitrate but it was not decreased during the study period (12 days) (data is not shown here). Therefore, this metabolite might be generated by ammonia oxidation bacteria (AOB) which can co-metabolize various polyhalogenated ethanes(Rasche et al., 1991) and monocyclic aromatic compounds (Keener et al., 1994). However, we can not exclude the possibility that other monooxygenase using bacteria can be responsible for generating this metabolite since we could not identifying the metabolite generating bacteria.

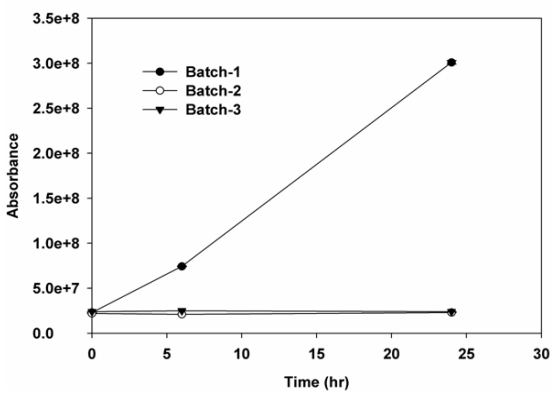


Fig. 4. The m/z=441 generation profile under three different batch reactor conditions [Batch-1:Biomass treated with clindamycin and ammonia; Batch-2: Batch-1 process added with allythiourea; Batch-3: Batch-1 process added with sodium azide] (error bar: standard deviation).

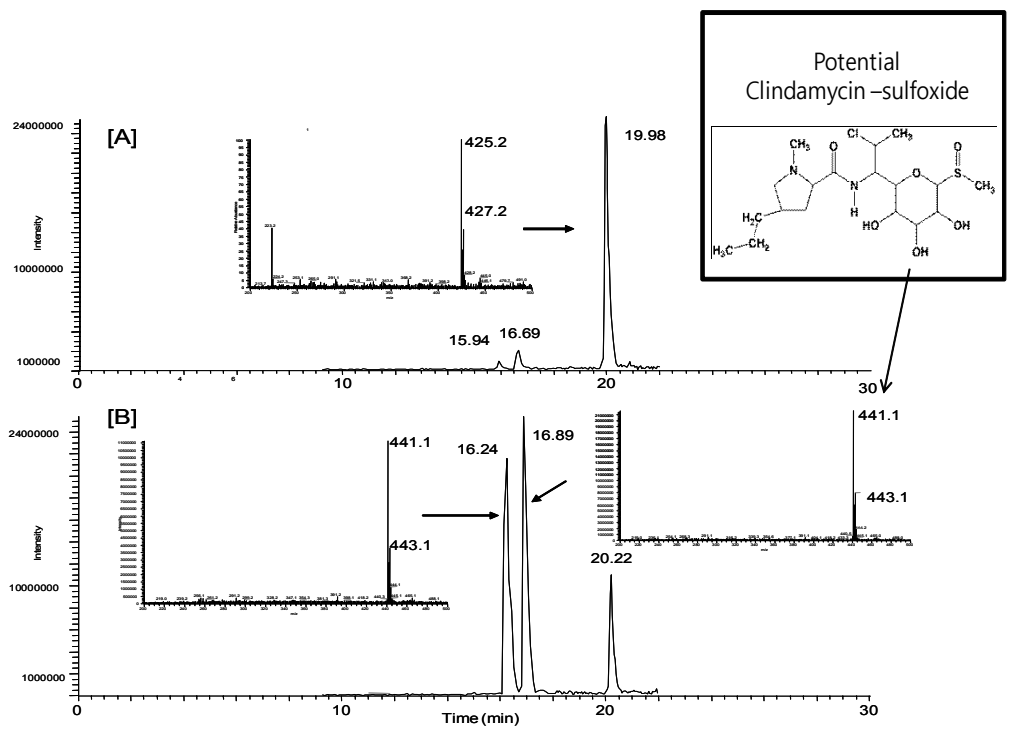


Fig. 3. The clindamycin metabolite generation under nitrifying activated sludge during 24hr incubation [clindamycin (m/z, 425), potential clindamycin sulfoxide (m/z, 441)] (A: Time 0 hr; B: Time 24hrs).

4. Conclusions

This study has been conducted to monitor the fate of clindamycin antibiotic in nitrifying activated sludge process. Enhanced clindamycin disappearance was observed in nitrifying activated sludge relative to nitrifying-inhibited activated sludge. The generation of clindamycin-sulfoxide (m/z 441) metabolite was observed in nitrifying activated sludge with nitrate generation although it is not clear that metabolite was generated by nitrifying bacteria but other bacteria such as other monooxygenase using bacteria. Since metabolite was not degraded in both batch reactors, it is important to emphasize that monitoring only for the presence of parent pharmaceutical compounds in environment might lead to underestimation of the potential impacts of micro-contaminants. This study indicated that the disappearance of the parent compound in biological wastewater treatment plant does not guarantee of the complete removal of the bioactivity of micro contaminants from wastewater.

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