PHOTOLYSIS OF OXYTETRACYCLINE-DIVALENT CATION COMPLEXES IN BUFFER SOLUTION

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โฟโตไลซิสของสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลิน-ไดวาเลนท์แคตไอออนในสารละลายบัฟเฟอร์

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ออกซีเตตตร้าซัยคลินเป็นยาต้านเชื้อแบคทีเรีย มีการใช้กันอย่างแพร่หลายในการเพาะเลี้ยงสัตว์น้ำ ทำให้มีปัญหาการตกค้างของยาในแหล่งน้ำและดิน กระบวนการโฟโตไลซิสจัดเป็นกระบวนการหลักของการสลายตัวของออกซีเตตตร้าซัยคลินในธรรมชาติ เป็นที่ทราบกันว่าออกซีเตตตร้าซัยคลินเกิดสารประกอบเชิงซ้อนกับไดวาเลนท์แคตไอออนหลังเกิดสารประกอบเชิงซ้อนโครงสร้างของออกซีเตตตร้าซัยคลินจะเปลี่ยนจากโครงสร้างบิดโค้งงอเป็นแนวระนาบทำให้ไวต่อการสลายตัวโดยแสง ดังนั้นการเกิดสารประกอบเชิงซ้อนกับไดวาเลนท์แคตไอออนนี้จึงเป็นวิธีการใหม่ในการช่วยเร่งการสลายตัวของออกซีเตตตร้าซัยคลินในสิ่งแวดล้อม

การศึกษานี้ศึกษาการเกิดสารประกอบเชิงซ้อนของออกซีเตตตร้าซัยคลินกับไดวาเลนท์แคตไอออน 3 ชนิด ได้แก่ Mg^{2+}, Ca^{2+}, Cu^{2+} ติดตามโดยใช้วิธีทางสเปกโตรฟลูโอโรเมตรีและคำนวณหาค่าคงที่การจับ (binding constant, K) ของสารประกอบเชิงซ้อนในสารละลาย ทริส-ไฮโดรคลอไรด์บัฟเฟอร์ พีเอช 7.80 หรือสารละลายฟอสเฟตบัฟเฟอร์ พีเอช 7.80 โดยทริส-ไฮโดรคลอไรด์บัฟเฟอร์ถูกเลือกใช้เป็นสารละลายในการศึกษา เพราะมีความสามารถต้านการเปลี่ยนแปลงพีเอชของสารละลายสูง ค่าคงที่การจับของสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลินกับ Mg^{2+}, Ca^{2+}, Cu^{2+}ในสารละลายทริส-ไฮโดรคลอไรด์บัฟเฟอร์ เท่ากับ 3,921.8 , 4,386.8 , 1,149.9 โมลาร์$^{-1}$ ตามลำดับ และค่าคงที่การจับของสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลินกับ Mg^{2+}, Ca^{2+} ในสารละลายฟอสเฟตบัฟเฟอร์ เท่ากับ 2,704.6 , 1,791.2 โมลาร์$^{-1}$ ตามลำดับโดยค่าคงที่การจับจะมีค่าแตกต่างกันเมื่อใช้ระบบบัฟเฟอร์ต่างชนิดกัน

การสลายตัวโดยโฟโตไลซิสของสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลิน-Cu^{2+} หรือ ออกซีเตตตร้าซัยคลิน-Ca^{2+} เป็นปฏิกิริยาการเสื่อมสลายอันดับหนึ่ง ซึ่งอัตราเร็วของปฏิกิริยาเป็นสัดส่วนโดยตรงกับความเข้มข้นของออกซีเตตตร้าซัยคลินในช่วงความเข้มข้น 0.101-0.402 มิลลิโมลาร์ ในขณะที่การสลายตัวของสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลิน-Mg^{2+} เป็นปฏิกิริยาการเสื่อมสลายอันดับศูนย์ ซึ่งอัตราเร็วของปฏิกิริยาไม่ขึ้นกับความเข้มข้นของออกซีเตตตร้าซัยคลิน

ความเข้มข้นของไอออนมีผลต่อการสลายตัวโดยโฟโตไลซิสของสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลิน-Cu^{2+} โดยมีการสลายตัวมากขึ้นเมื่อความเข้มข้นของเกลือในรูปไอออน ของสารละลายมีค่าระหว่าง 0.07-0.1 โมลาร์ แต่เมื่อความเข้มข้นของเกลือในรูปไอออนของสารละลายสูงกว่า 0.1 โมลาร์ อัตราการสลายตัวของสารประกอบเชิงซ้อนจะลดลง ในขณะที่การสลายตัวของสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลิน-Ca^{2+}และ ออกซีเตตตร้าซัยคลิน-Mg^{2+} อัตราการสลายตัวมีค่าเพิ่มมากขึ้นเมื่อความเข้มข้นของเกลือในรูปไอออนของสารละลายมีค่าระหว่าง 0.04-0.6 โมลาร์ แสดงว่าการเสื่อมสลายโดยโฟโตไลซิสได้รับผลกระทบจากความเข้มข้นของเกลือในรูปอิออน จากการศึกษานี้จึงเชื่อว่ากระบวนการโฟโตไลซิสช่วยเร่งการสลายตัวของสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลิน-ไดวาเลนท์แคตไอออน ดังนั้นสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลิน-ไดวาเลนท์แคตไอออน โดยเฉพาะอย่างยิ่งสารประกอบเชิงซ้อนออกซีเตตตร้าซัยคลิน-Cu^{2+} สามารถใช้เป็นวิธีการใหม่ในการช่วยเร่งการสลายตัวของออกซีเตตตร้าซัยคลินในสิ่งแวดล้อม

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PIPAT SITTISAK :PHOTOLYSIS OF OXYTETRACYCLINE-DIVALENT CATION COMPLEXES IN BUFFER SOLUTION. ADVISOR: ASST. PROF. WALAISIRI MUANGSIRI, Ph.D., CO-ADVISOR: ASST. PROF. PORNPEN WERAWATGANONE, Ph.D., PROF. LEE E. KIRSCH, Ph.D., 100 pp.

The antibiotic oxytetracycline is commonly used in aquaculture and can eventually lead to contamination of the surrounding environment such as surface water and soil. In nature, a major degradation mechanism of oxytetracycline is photolysis. Oxytetracycline is known to form complexes with cations. After complex formation, oxytetracycline changes from a folded to a planar conformation which is more sensitive to light. Thus, the oxytetracycline-cation complex can be used as a new means of accelerating oxytetracycline degradation in the environment.

In this study, the complex formation of oxytetracycline with three different types of divalent cations, i.e. Mg^{2+},Ca^{2+}, and Cu^{2+}, was studied using a spectrofluorometer and the binding constants (K) of oxytetracycline with cations in either Tris-hydrochloride buffer pH 7.80 or phosphate buffer pH 7.80 were calculated. Tris-hydrochloride buffer was chosen for use as a solvent for further studies because of its higher buffer capacity. The binding constants of oxytetracycline with Mg^{2+}, Ca^{2+}, and Cu^{2+} ions in Tris-hydrochloride buffer pH 7.80 were 3,921.8 , 4,386.8 , and 1,149.9 M^{-1} respectively and the binding constants of oxytetracycline with Mg^{2+}, Ca^{2+} ions in phosphate buffer pH 7.80 were 2,704.6 , 1,791.2 M^{-1} respectively. The binding constants were different in other buffer system.

Photolysis degradation of oxytetracycline-Cu^{2+}complex or of oxytetracycline-Ca^{2+}complex followed first order reaction. Direct photolysis rate was found to be dependent on the initial oxytetracycline concentration, in the oxytetracycline concentration from 0.101 to 0.402 mM. While degradation of oxytetracycline-Mg^{2+}complex was a zero order reaction, photolysis rate was not dependent on the initial oxytetracycline concentration.

Ionic strength had influence on photolysis degradation of oxytetracycline-Cu^{2+}complex. Increasing ionic strength from 0.07 to 0.1 M led to the increase of the degradation rate constant. But ionic stregnth above 0.1 M, the degradation rate constant decreased. While, photolysis degradation of oxytetracycline-Ca^{2+}complex and oxytetracycline-Mg^{2+}complex were increased in ionic strength concentration range 0.04 to 0.6 M, an indicative of ionic strength on photolysis. This study showed that photolysis could be accelerated by oxytetracycline-divalent cation complex. Thus, the oxytetracycline-divalent cation complex especially oxytetracycline-Cu^{2+} can be used as a new means of accelerating oxytetracycline degradation in the environment.

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LIST OF ABBREVATIONS

%	percentage
˚C	degree Celcius
μ	micro (10^{-6})
μg	microgram
μL	microlitre
μm	micrometre
1st	first
2nd	second
Ca^{2+}	calcium ion
Cu^{2+}	copper ion
et al.	*et alii*, and other
etc.	*et cetera* (and other similar things)
g	gram
h	Planck' constant
HCl	hydrochloric acid/ hydrochloride
HPLC	high performance liquid chromatography
hr	hour (s)
K	binding constant
k	reaction rate constant
L	litre (s)
m	milli (10^{-3})
M	molar (s)

Mg^{2+}	magnesium ion
mg	milligram
min	minute (s)
mL	milliliter
mm	milimetre
NaOH	sodium hydroxide
NaCl	sodium chloride
nm	nanometre
no.	number
pH	potential of hydrogen
rf	relative fluorescence
RT	room temperature
S.D.	standard deviation
UV-Vis	ultraviolet-visible
t	time
$t_{0.75}$	time of remain 75% concentration
v/v	volume by volume
w/w	weight by weight
[*]	activated species
[C]	concentration
π	transition state

CHAPTER I

INTRODUCTION

1. Background and significance of the study

Oxytetracycline, an antibiotic in the tetracycline group, is widely used in the treatment of bacterial infection in humans as well as in aquaculture, especially in fish and shrimp farming. The use of oxytetracycline in aquaculture eventually results in the surrounding water and soil being contaminated by the antibiotic (Austin, 1985; Aoki et al.,1987; Shaojun et al., 2008) leading to the emergence of microbial resistance. The core chemical structure of oxytetracycline is the naphthacene structure, rings A to D (Figure 1) (Buckley and Smyth, 1986; Hong-Xia et al., 2004; Novak-Pekli et al., 1996). Oxytetracycline is an amphoteric substance with the 3 pKa values of 3.57, 7.49, and 9.88 due to the deprotonation of the hydroxyl groups at the C3 and C12 positions and the tertiary amine of the 4-α-dimethyl amino group (Shaojun et al., 2008).

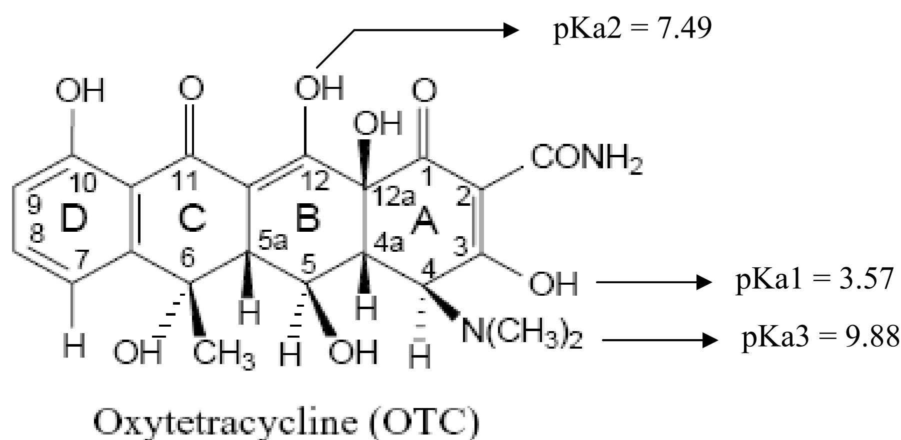


Figure 1 Chemical structure of oxytetracycline, ionizable groups, and its pKa values.

Oxytetracycline is prone to several degradation mechanisms due to the presence of active phenolic hydroxyl groups and amino group. In acidic solution, oxytetracycline undergoes epimerization at C-4 position giving rise to the formation of 4-epi-oxytetracycline (EOTC) which is reduced in antibacterial activity (Buckley and Smyth, 1986). Oxytetracycline also undergoes dehydration resulting in the formation of 2 isomers, α-apo-oxytetracycline (α-AOTC) and β-apo-oxytetracycline (β-AOTC), with low antibacterial activity (Guindy et al., 1985; Khan et al., 1996; Lykkeberg et al., 2004). However, hydrolysis and photolysis are two major degradation pathways in the environment (Xuan et al., 2010). The degradation rate of oxytetracycline under sunlight is influenced by the pH of the solution; that is, it increases in an alkali solution, but decreases in an acidic one (Doi and Stoskopf, 2000).

Oxytetracycline in the oxytetracycline-cation complex has been reported to undergo photolysis at a faster rate than the oxytetracycline free molecule (Xuan et al., 2010). It was reported that cations form a complex with oxytetracycline at 12-OH (hydroxyl group) and 11-CO (carbonyl group) positions (Anderson et al.,2005). Complex formation changes the folded conformation of the napthacene ring in oxytetracycline to that of a planar conformation (Jin et al., 2007). As a result, the complex has a higher quantum yield and is prone to undergoing photolysis at a higher degradation rate (Yuan et al., 2010). Thus, a strategy of using the oxytetracycline-cation complex to accelerate oxytetracycline degradation can be employed in order to reduce the contamination of oxytetracycline in surface water.

Extensive studies on formation of divalent cation-tetracycline complex had been done (Martin, 1979). The results from those studies can be used to roughly forecast formation of divalent cation-oxytetracycline complex due to chemical structure similarity of these two compounds. Previous studies on employing divalent cation-oxytetracycline complex to accelerate oxytetracycline degradation were also performed under specific conditions such as in a particular solvent system (Xuan et al., 2010). Therefore, there are gaps between scientific researches on this subject and its applications. Systematic approach to the issues is needed. This study reveals that solvent system is one of the key factors on complex formation. This finding may directly affect efficacy of using divalent cation-oxytetracycline complex in enhancing oxytetracycline degradation.

2. Objectives of the study

2.1 Complex formation of oxytetracycline with three different types of divalent cation, i.e. Mg^{2+}, Ca^{2+}, and Cu^{2+}, was studied and compared the binding constant (K) of oxytetracycline with divalent cation were calculated.

2.2 Photolysis of oxytetracycline-divalent cation complexes in buffer solution and influence of oxytetracycline initial concentration, ionic strength on the reaction were studied. The degradation rate constant (k) of oxytetracycline complex was compared to that of free oxytetracycline.

3. Hypothesis

Photolysis of oxytetracycline-divalent cation complexes can be used to accelerate oxytetracycline degradation.

CHAPTER II

LITERATURE REVIEW

Oxytetracycline, an antibiotic in the tetracycline group, is widely used in the treatment of bacterial infection in humans as well as in aquaculture, especially in fish and shrimp farming. The mode of action of the compound involves inhibition of protein synthesis by blocking the attachment of transfer RNA to ribosomes. The chemical structure of this compound is shown in Figure 1 (Buckley and Smyth, 1986). In literature reviews, degradation mechanism of oxytetracycline and oxytetracycline-divalent cation complex are different. Difference in type and concentration of divalent cations affects degradation rate of oxytetracycline-divalent cation complex. However, this information is unclear for being used as a new means of accelerating oxytetracycline degradation in the environment.

1. Physicochemical properties of oxytetracycline

Oxytetracycline structure

Oxytetracycline (Figure 1), has a molecular weight of 496.9. The core chemical structure of oxytetracycline is a naphthacene structure, ring A to D (Hong-Xia et al., 2004; Novak-Pekli et al., 1996). Any alterations to the general oxytetracycline ring skeleton such as, ring breaking, aromaticity disruption of ring D, and aromatization of ring A or C and consequently lead to destroy oxytetracycline antimicrobial activity. The ketones at C1 and C11 and the hydroxyls at C3, C10, C12, and C12a are essential since they are involved in an extensive network of RNA binding interactions composed of hydrogen bonds. Neither the hydroxyl nor the methyl at C6 is essential for the activity. No direct ribosome interaction is observed

for the C6 substituents. Removal of the dimethylamino group at C4 diminishes in vitro activity and loses in vivo activity (Abraham, 2003).

pKa of oxytetracycline

Oxytetracycline is an amphoteric substance with the 3 pKa values of 3.57, 7.49 and 9.88 due to the deprotonation of the hydroxyl groups at the C3 and C12 positions and the tertiary amine of the 4-α-dimethyl amino group. The four ionization states are represented by H_3OTC^+, H_2OTC, $HOTC^-$, and OTC^{2-}. Figure 2 shows the structure, the four ionization states and the ionization equilibrium of oxytetracycline (Shaojun et al., 2008).

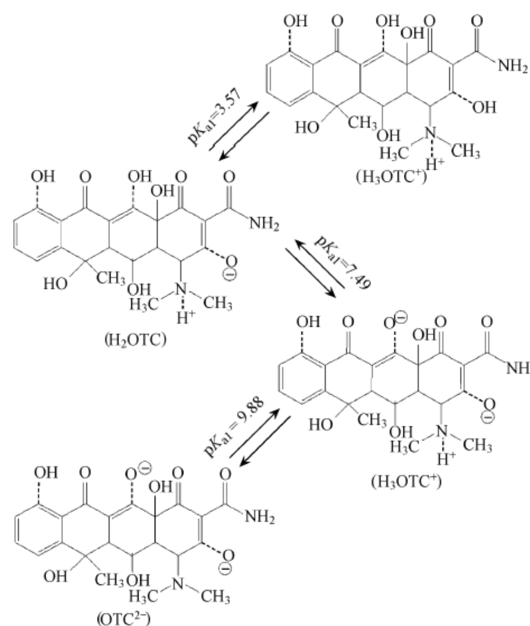


Figure 2 Structure, ionization states, and dissociation constant (pKa) of oxytetracycline.

Chromophore of oxytetracycline

Oxytetracycline chemical structure contains chromophores that can absorb UV light. Oxytetracycline consists of 3 chromophores (I, II, and III) as shown in Figure 3 with UV maxima at 245 or 270, 310 and 350-380 nm, respectively (Hong-Xia et al., 2004). Aromatic structure or conjugated double bond of oxytetracycline results in low energy level of the transition from π to π^* state.

Oxytetracycline in excited state can be reduce energy level to ground state by collisional vibration through exothermic or fluorescence. Fluorescence maximum of oxytetracycline is varied according to solution's pH value. Fluorescence maximum of oxytetracycline was reported to be at 450, 510-520 and 500-505 nm when pH values of solutions were <8, 8-12 and >12, respectively (Hong-Xia et al., 2004). Chelation complexes dramatically enhances the fluorescence of tetracycline, a weak fluorescent compound. Molecular conformation has a great impact on fluorescence intensity, especially, when the conformation of the BCD rings is a planar structure through rigid binding of the ligand at the diketone functional group (Anderson et al., 2005).

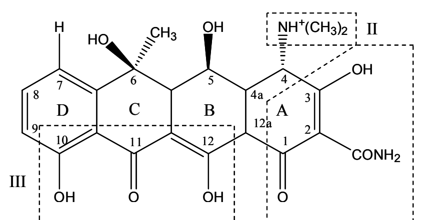


Figure 3 Chemical structure of oxytetracycline.

2. Degradation of oxytetracycline

2.1 Epimerization and dehydration

Factors affecting oxytetracycline degradation are pH, temperature and light intensity. Oxytetracycline degradation depends greatly on solution pH. Acidic conditions increase oxytetracycline stability but alkaline conditions aid oxytetracycline degradation. The phenolic hydroxyl and amine groups in oxytetracycline result in an existence of different ionic species at different pH values. Certain ionic species are more labile than others and thus are proned to degradation (Xuan et al.,2010). In acidic conditions, oxytetracycline undergoes epimerization at C-4 position giving rise to the formation of 4-epi-oxytetracycline (EOTC). This EOTC is essentially inactive as antibiotics. Thus, this feature is carefully controlled in products in the market. The optimum pH range for the epimerization is about 2-6 (Abraham and Rotella, 2010). Under acidic conditions (pH around 2-6), anhydro-oxytetracycline (AOTC) is also formed due to the dehydration involving the hydrogen at position C5a and the hydroxyl group at position C6. AOTC is very unstable in acidic solution resulting in scission of ring B to be more stable aromatic isomers, α-apo oxytetracycline (α-AOTC) and β-apo oxytetracycline (β-AOTC) (Lykkeberg et al., 2004) (Figure 4). Mechanism of oxytetracycline degradation under acid-base conditions is similar to mechanism of tetracycline degradation. In addition, the degradation rate is increased when temperature is increased.

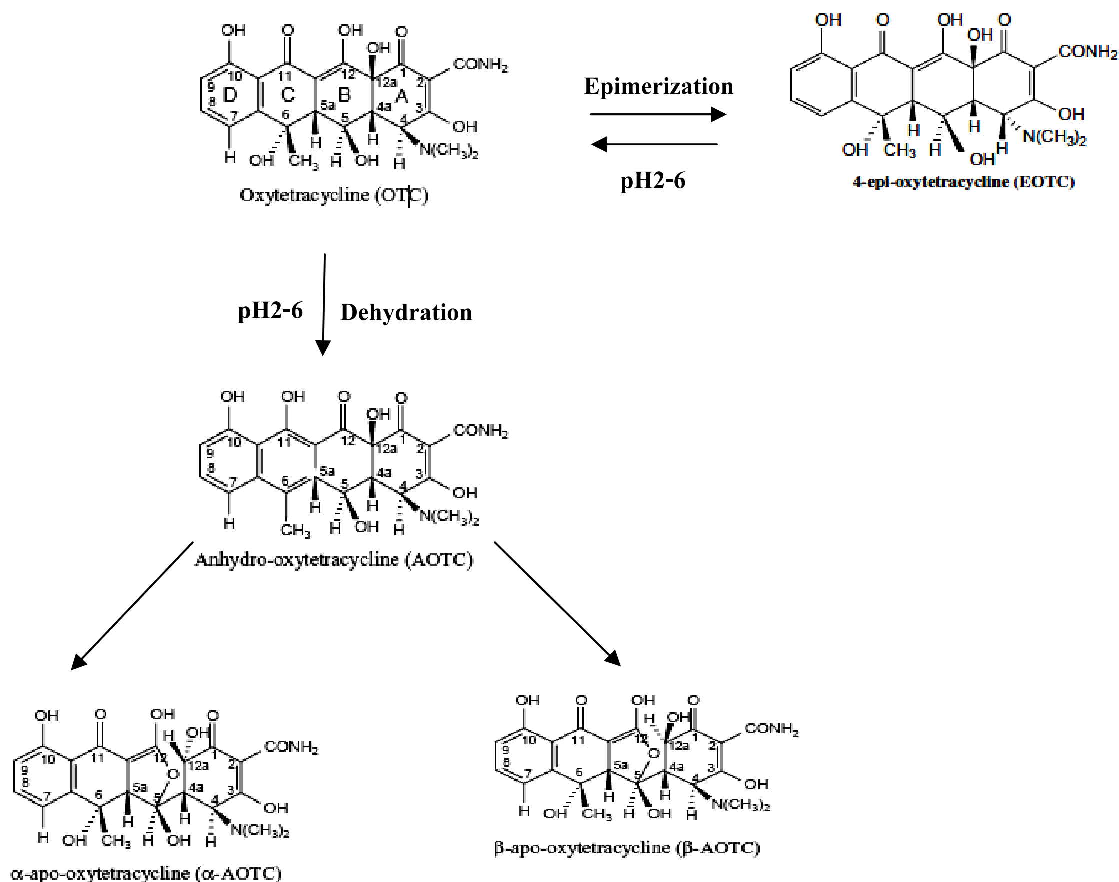


Figure 4 Degradation scheme of oxytetracycline (Khan et al., 1987).

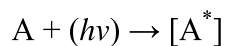
2.2 Photolysis

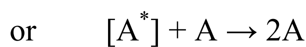
2.2.1 Mechanism of photolysis

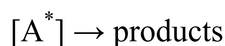
In photolysis, a quantum of light is represented by *hv* activates a species, where *h* is Planck's constant ($6.626x10^{-27}$ erg s) and *v* is the frequency of the light (in unit of s^{-1}). After light activation, the activated species denoted by [*] returns to ground state by either.

1. Emits light with different frequency to that of the incident beam. This is known as fluorescence or phosphorescence.

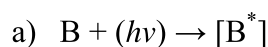
2. Decompose, photolysis. The simplest sequences in photolysis is


$A + (h\nu) \rightarrow [A^*]$


or $[A^*] + A \rightarrow 2A$

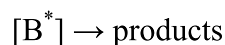

$[A^*] \rightarrow$ products

The second component of the system, B or a photosensitizer, absorbs light and undergoes:

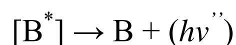

a) $B + (h\nu) \rightarrow [B^*]$

b) $[B^*] + A \rightarrow B + [A^*]$

c) If only (a) predominates, it may be followed by


$[B^*] \rightarrow$ products

or


$[B^*] \rightarrow B + (h\nu'')$

Then B is called a screening agent where ν'' means a different frequency. In the last case, concentration of B is not degrade over time and thus protects other photosensitive compounds in the preparation (Carstensen, 2000).

2.2.2 Reaction order of photolysis

In photolysis of aqueous oxytetracycline, the degradation rate constants depends on the oxytetracycline initial concentration. The increase of oxytetracycline

initial concentration leads to the decrease of the rate constants (Shaojun et al., 2008). The oxytetracycline photolysis kinetics is a first order reaction.

$$C = C_0e^{-kt}$$

Where, C_0 is the initial oxytetracycline concentration, C is the remaining oxytetracycline concentration at irradiation time t, and k is the rate constant.

In pharmaceutical systems, most reported photolysis are first order reactions. An example of this is cefotaxime photolysis (Lerner et al., 1988). However, non first order can be observed and is the zero order reaction is a rare case (Carstensen, 2000). For example, photolysis of nimodipine is a first order reaction in methanol, but it is a zero order reaction in a mixed solvent of acetonitrile and water (Riekes et al., 2012).

2.2.3 Photolysis of oxytetracycline

Oxytetracycline has strong UV absorbance in a range of 200-400 nm making oxytetracycline susceptible to sunlight irradiation. In addition, photolysis rate depends on sunlight intensity and pH value of the solution (Xuan et al., 2010). First order rate constant of oxytetracycline photolysis is increased with an increase of solution's pH.

OTC^- is prone to photolysis while OTC^+ does not undergo photolysis (Shaojun et al., 2008). The oxytetracycline molecules are in a neutral and positively charged forms under acidic condition; in contrast, OTC^- are dominant in a alkaline solution. Thus, increasing solution pH leads to the increase in the photolysis rate constant indicating low stability of oxytetracycline at high pH. It is attributed to the formation oxytetracycline molecules from OTC to OTC^-, concomitant with the transition of π to

π^* state of oxytetracycline chromophore. Additionally, negatively charged oxytetracycline molecules, tend to attract reactive species, which accelerate oxytetracycline photolysis. Figure 5 depicts photolysis of oxytetracycline which is more complicated than oxytetracycline degradation under acid-base condition (Shaojun et al., 2008).

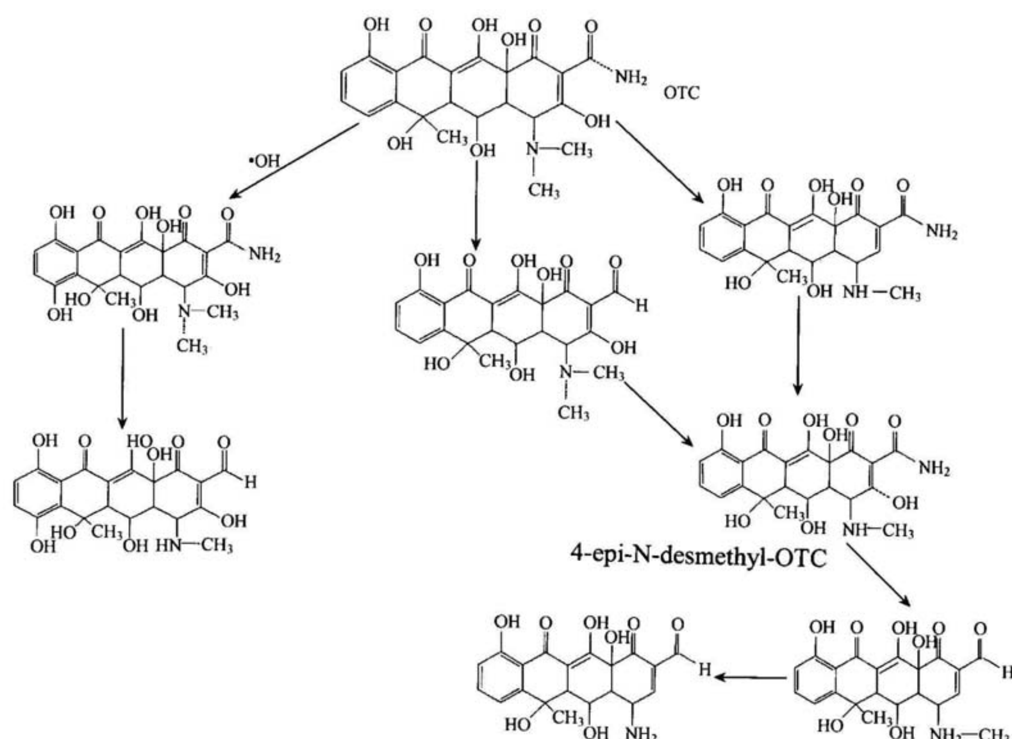


Figure 5 Photodegradation pathway of oxytetracycline (Shaojun et al., 2008).

2.2.4 Photolysis of oxytetracycline in environment

Oxytetracycline quickly undergoes photolysis. After 14 days of light exposure at 8°C, in which 70% of oxytetracycline was photodecomposed in freshwater (Poliquen et al., 2007). Photolysis of oxytetracycline in freshwater was reported to be a first order reaction with no initial lag phase.

Photolysis of oxytetracycline in seawater by sunlight irradiation was a first-order reaction but the degradation rate was much slower when compare with oxytetracycline photolysis in freshwater (Lunestad et al., 1995). In quartz containers, oxytetracycline concentration is reduced from 50 to 2 mg/L in seawater after 9 days of exposure at sea level.

Photolysis, product of oxytetracycline present in soil surface water was studied using LC-MS-MS. The degraded products included α-apo-oxytetracycline, β-apo-oxytetracycline, 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC), N-desmethyl-oxytetracycline (N-DM-OTC, m/z 447), 4-epi-N-desmethyl-oxytetracycline (E-N-DM-OTC), N-didesmethyl-oxytetracycline (N-DDM-OTC, m/z 433), and 4-epi-N-didesmethyl-oxytetracycline (E-N-DDM-OTC) (Halling-Sorensen et al., 2003).

However, photolysis of oxytetracycline may not major degradation mechanism in turbid water, solid manure, and soil because of low accessibility of sunlight in those media. Seawater contains high amount of suspended particles, cations and organic matter, which can scatter, form complex and adsorb oxytetracycline resulting in preventing of oxytetracycline from photolysis. In addition, the photolysis rate depends on light intensity, which varies with season and latitude.

3. Complexation

3.1 Tetracycline complexation

Most studies on tetracycline-metal ion complexes have been focused on Ca^{2+} and Mg^{2+} ions because they are physiologically relevant divalent cations. Binding constants, stoichiometry, and binding sites are determined using a variety of spectroscopic and electrochemical methods.

Tetracycline have four pKa values of 3.2, 7.6, 9.6, and 12. Thus, 5 ionization states of tetracycline can be written as H_4Tc^+, H_3Tc, H_2Tc^-, HTc^{2-} and Tc^{3-}. Tetracycline assumes several conformations depending on the environment. The kinetic and equilibrium behavior of each conformations is independent of the nature and concentration of the buffer used. Studies on tetracycline-metal ion complex formation limited by low solubility of metal ions in a high pH range and low binding affinity of tetracycline to the metal ions at a low pH range (Jin et al., 2007). In addition, the formation of tetracycline-metal ion complexes laters overall ionization state of the complex and pKa values of tetracycline.

The A ring and the BCD moiety of tetracycline are reported to be binding sites for metal cations. More specifically, (a) the C10-C11 ketophenol moiety, (b) the C11-C12 β-diketone moiety, (c) the C4 dimethylamine and the C3 or C12a hydroxyl moieties, (d) the C1-C3 tricarbonyl methane moiety, and (e) the C11-C12 β-diketone and C1-C3 tricarbonyl methane moiety have been reported to be binding sites where the β-diketone moiety is the major binding site (Buckley and Smyth., 1986; Conover, 1956).

The second and the third ionizable protons of tetracycline at the C12 (the β-diketone moiety of the BC rings) and at the C4 (dimethylammonium of the A ring), respectively, directly involve in complex formation with Mg^{2+} and Ca^{2+} ions. Thus, pKa values of tetracycline are altered upon Mg^{2+} and Ca^{2+} binding. A change in a folded conformation of free tetracycline to an extended form is closely linked with the deprotonation of the dimethylammonium group. This conformational change is a result of formation of a hydrogen bond between positively charged dimethylammonium and alkoxide ion at C12a. Therefore, in a pH range of 7.5-8.5 where preferable conformation of tetracycline is the extended form, tetracycline-Ca^{2+} complex is in the extended form. Unlike Ca^{2+}, tetracycline-Mg^{2+} complex is reported to process the folded conformation (Jin et al., 2007).

Chelation of tetracycline with Mg^{2+}, Ca^{2+}, and Zn^{2+} has been shown to depend on ionic radius (Anderson et al., 2005). Mg^{2+}, and Zn^{2+} with comparable ionic radius of 86 and 74 pm, respectively, show higher binding constants to tetracycline than the binding constants of tetracycline to Ca^{2+} with ionic radius of 114 pm. In another study, binding constant of tetracycline with Mg^{2+}or Ca^{2+} in 0.01 M Tris-HNO_3 buffer pH 7.80 (with an ionic strength of 0.15 M) are reported to be 1,515 and 875 M^{-1}, respectively using a UV spectrophotometry method (Martin, 1979).

3.2 Oxytetracycline complexation

The result from divalent cation-tetracycline complex studies can be used to roughly forcast formation of divalent cation-oxytetracycline complex due to chemical structure similarity of these two compounds. The oxytetracycline forms complex with many metal cations. The proposed sites of complexation are at (a) the C_{10}-C_{11}

ketophenol moiety, (b) the C_{11}-C_{12} β-diketone moiety, (C) the C_4 dimethylamine and the C_3 or C_{12a} hydroxyl moieties (Buckley and Smyth.,1986). The site of complexation can be linked with the deprotonation sequence, which changed with the solution's pH value. The deprotonation sequence is as following : (1) the C3 hydroxy moiety, (2) the C10 phenol moiety, (3) the C4 dimethylamine, (4) the C12 hydroxy moiety, and (5) the C12a hydroxyl moiety (Hong-Xia et al., 2004). Binding constants of oxytetracycline with Mg^{2+}or Ca^{2+} in 0.01 M Tris-HNO_3 buffer pH 7.80 (with an ionic strength of 0.15 M) are reported to be 2,625 and 1,150 M^{-1}, respectively using a UV spectrophotometry method (Martin, 1979).

The presence of Ca^{2+} plays an important role in oxytetracycline hydrolysis. A non linear correlation of first order plot of oxytetracycline in the presence of Ca^{2+} at pH 5.88 (phosphate buffer) and pH 9.78 (NaOH solution) shows that oxytetracycline hydrolysis deviates from the first order reaction and markedly slow down the hydrolysis (Xuan et al., 2010).

Rapid photolysis of oxytetracycline-Ca^{2+} complex is observed under sunlight irradiation (Xuan et al., 2010). The photolysis of oxytetracycline-Ca^{2+} complex is a first order reaction. The photolysis rate constant of oxytetracycline is comparable to that of hydrolysis at 60°C and markedly increases in the presence of Ca^{2+} (Xuan et al., 2010). This implying that oxytetracycline becomes vulnerable to sunlight irradiation after chelating with Ca^{2+}. However, the effect of the multi-covalent cations such as Mg^{2+}, Cu^{2+} on the photolysis of oxytetracycline has not been well documented.

4. Divalent cation in aquaculture

Calcium ion

In shrimp farm, Ca^{2+} is generally used to reduce soil acidity (Wurts and Masser, 2004), therefore Ca^{2+} is employed during pond preparation and adjusting water pH during cultivation period. Ca^{2+} is not only used for adjusting pH of soil and water, which directly affects shrimp survival, but also used for stabilizing pH of water in order to promote biological productivity. The optimum water pH range for shrimp cultivation is 7.4-8.5 (Rakbankerd, 2012:online). In addition, in order to ensure survival of shrimps, pH value of water should not fluctuate more than o.5 pH unit. Generally, one of parameter determining water quality is alkalinity.

Total alkalinity is the total concentration of titrable bases in the water. The base species in water are HCO_3^- and CO_3^- ions. Thus, the total alkalinity has a unit of milligrams per liter (ppm) of equivalent calcium carbonate. The alkalinity of seawater is normally higher than 100 ppm, but the alkalinity of surface water is often low, particularly during the rainy season (Wurts and Masser, 2004). Ca^{2+} is added to adjust the alkalinity to its optimum value of 70-90 ppm (Neospark, 2013:online).

Copper ion

Copper has been used as an effective algicide and antiparasite in aquaculture. (Chen and Lin, 2001). Copper is used to control algae in ponds, including filamentous and higher algae such as *Chara* (“Stink weed”). Copper also controls most external parasites of fish, including “Ich”. However, the difference between therapeutic concentration and toxic level of copper is very narrow. The toxicity of copper to fish increases as the total alkalinity decreases.

Copper has several salt forms which can be used in water. Copper sulfate is the cheapest and most commonly used in aquaculture because it is water soluble. A proper concentration of copper sulfate in ppm to be added in fish farm is calculated by dividing the total alkalinity (in parts per million) by 100.

Total alkalinity (ppm)/100 = Copper sulfate need (ppm)

When the total alkalinity is lower than 50 ppm, a water treatment with copper should be avoid because of high toxicity of copper under such conditions. When the total alkalinity is higher than 250 ppm, copper sulfate at a concentration not more than 2.5 ppm should be added (University of Florida, 2013:online).

Magnesium ion

Seawater with a salinity of 35% contains 53 mM Mg^{2+} (Table 1) (Lunestad and Goksoyr, 1990). Mg^{2+} is a major divalent cations present in seawater.

Table 1 Chemical composition of seawater (Wikipedia, 2013:online)

Symbol	Name	% of total	mmoles
Cl^-	Chloride	55.29	546
Na^+	Sodium	30.74	469
Mg^{2+}	Magnesium	3.69	53
SO_4^{2-}	Sulphate	7.75	28
Ca^{2+}	Calcium	1.18	10.3
K^+	Potassium	1.14	10.2

Oxytetracycline has been known to form complex with divalent cations. After complex formation, conformation of oxytetracycline changes form the folded to the planar conformation which is more sensitive to light. Thus, the purpose of this study to investigate a new means of accelerating oxytetracycline degradation in the environment using oxytetracycline-divalent cation complexes. Divalent cations chosen in this studied included Mg^{2+}, Ca^{2+}, and Cu^{2+} because Mg^{2+} is prevalence in seawater; Ca^{2+} is used preparation pit in fish and shrimp farming; and Cu^{2+} is used as algicide and antiparasite in aquaculture.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Raw material

1.Oxytetracycline hydrochloride (Lot.1425721 Sigma-Aldrich, USA)

2.Oxytetracycline hydrochloride (Lot.10060441 Dafeng Huashu Pharmaceutical, China)

1.2 Equipment and instruments

1. Spectrofluorometer (Jasco Model FP-777, Japan)
2. Shimadzu Liquid Chromatography (HPLC Class-LC 10, BARA Scientific Co., Ltd))
3. Symmetry Column C18, 3.5µ, 4.6x150 mm. (WAT200632, Water Corporation, USA)
4. pH meter (Orion Model 420A, USA)
5. Analytical balances (Mettler Toledo No.MT-035, Switzerland)
6. Ultrapure water (Model Elgastat Maxima UF, ELGA Ltd., England)
7. Micropipettes (50-200 µL), (100-1000 µL), (1-5 mL) (BIOHIT)
8. Micropipettes tips (BIOHIT)
9. Disposable syringe 3 mL without needle (NIPRO)
10. Membrane filter (13 mm, 0.45 Nylon, LUBITECH)
11. Membrane filter (13 mm, 0.22 Nylon, LUBITECH)

12. Centrifuge (CENTRIFUGETTE 4206)

1.3 Chemicals

1.Methanol (HPLC grade, Lot.11060197 RCI Labscan Limited)

2.Acetronitrile (HPLC grade, Lot.11060107 RCI Labscan Limited)

3.Calcium chloride dehydrate (Lot.1059031 Fluka chemika, Switzerland)

4.Magnesium chloride hexahydrate (Lot.6E417306F Carlo Erba Reagent, Italy)

5.Copper sulphate-5-hydrate (Lot.1E40334 Riedel-De Haen Ag Seelze-Hannover)

6.Tris-Hydroxymethyl-Methylamine (Lot.1005118 Asia Pacific Specialty Chemicals Limited, Australia)

7. Triethylamine (Lot.4E230094G Carlo Erba Reagent, Italy)

8. Hydrochloric acid (Lot.7647-01-0 J.T Baker, Thailand)

9. Formic acid (Lot.8B746158E Carlo Erba Reagent, Italy)

10.Standard Buffer pH 7.0 (Lot.Orion 910107 Thermo Fisher Scientific, USA)

11.Standard Buffer pH 4.01 (Lot.Orion 910104 Thermo Fisher Scientific, USA)

12.Standard Buffer pH 10.01 (Lot.Orion 910110 Thermo Fisher Scientific, USA)

13.Sodium phosphate monobasic (Lot.G3794 8D5560A Carlo Erba Reagent, Italy)

14.Di-Sodium Hydrogen Phosphate Anhydrous (Lot.8E121148G Carlo Erba Reagent, Italy)

2. Methods

2.1 Complex formation of oxytetracycline with divalent cations

The complex formation of oxytetracycline with divalent cations was studies using a fluorescence spectroscopy technique. Influence of buffer, difference types and concentrations of divalent cations on complex formation were evaluated.

2.1.1 Determination of excitation and emission wavelengths of oxytetracycline

Oxytetracycline solutions in 0.005 M phosphate buffer pH 7.80 were prepared in a concentration range of 10^{-6} to 10^{-3}M. Excitation wavelength was primarily determined from UV absorption maxima of oxytetracycline using a spectrophotometer. Then, excitation and emission wavelengths of oxytetracycline were determined by a Jasco spectrofluorometer model FP-777.

2.1.2 Influence of buffer on oxytetracycline-divalent cation complex formation

Stock solutions of oxytetracycline in 0.005 M phosphate buffer pH 7.80 or in 0.1 M Tris-hydrochloride buffer pH 7.80 were prepared at concentrations of 10^{-3} and 5×10^{-4} M, respectively. Stock solutions of $MgCl_2$, $CaCl_2$ or $CuSO_4$ were also prepared at concentrations of 0.04, 0.02, or 0.05 M, respectively, in 0.005 M phosphate buffer pH 7.80 or 0.1 M Tris-hydrochloride buffer pH 7.80. Oxytetracycline- divalent cation complexes were formed by an addition of divalent cation stock solution in to

oxytetracycline stock solution to make various ratios of oxytetracycline:divalent cation in the range of 1:0 to 1:300. The mixtures were then adjust to the volume and oxytetracycline initial concentration around $6x10^{-5}$ M. Fluorescence intensity of each solution were measured within 30 minutes after mixing using an excitation and emission wavelengths obtained from 2.1.1

2.1.3 Determination binding constant (K) of oxytetracycline-divalent cations complex

A binding profile of each complex was constructed by plotting a graph between fluorescence intensities (I) and divalent cation concentrations [C^{2+}]. The binding constant (K) was determined by fitting the binding profile with equation (1) (Arias et al., 2007).

$$I = \frac{I_f + I_b K_{OTC}[C^{2+}]}{1 + K_{OTC}[C^{2+}]} \qquad \text{Equation (1)}$$

Where,

I_f = fluorescence intensity of oxytetracycline in the absence of cation.

I_b = fluorescence intensity of oxytetracycline in the presence of cation.

pH values of each solutions were determined before and after the fluorescence intensity measurements. The obtained binding constants were used to calculate concentrations of divalent cations needed to ensure that 90% of oxytetracycline formed oxytetracycline-divalent cation complexes.

2.2 Development of HPLC method for an analysis of oxytetracycline (High Performance Liquid Chromatography method, HPLC)

HPLC method have been widely used for the analysis of active ingradient in pharmaceutical product. Therefore, HPLC method was selected for quantitative analysis of oxytetracycline and degrade products. In this study, the HPLC method was adopted a previous method reported by Lykkeberg et al (2004). Briefly, the separation was carried on an Luna C18 chromatographic column (150 mm x 4.6 mm i.d., 5μm) operated at 35°C using a gradient elution method. Mobile phase A was methanol:0.08 M formic acid in water (5:95, v/v). Mobile phase B was methanol:0.08 M formic acid in water (95:5, v/v). The gradient elution procedure was as follows. The linear gradient elution procedure was 0-6 minutes: 89%A ; 6-11 minutes: 89%A→50%A ; 11-20 minutes: 50%A ; 20-25 minutes: 50%A→89%A ; 25-30 minutes: 89%A. The injection volumn was 20 μl. The flow rate 1 ml/min and the analytical wavelength of the detector was set at 254 nm. Solution of standard oxytetracycline solution (0.402 mM) was prepared by dissolving standard oxytetracycline with ultrapure water (USP35). The hydrolysis sample of oxytetracycline was prepared by heating the standard oxytetracycline solution on hot plate for 20 min.

The HPLC analytical method was further modified by changing elution techniques from gradient to isocratic elution technique, modifying aqueous composition of the mobile phase among 0.08 M formic acid in water pH 3.2, 0.1% formic acid pH 3.0 and phosphate buffer pH 3.1, changing methanol composition of the mobile phase in a range of 14-20%, and reducing the flow rate 1 to 0.8 ml/minute.

Finally, the modified HPLC method was perform using a symmetry C18 column (150 mm x 4.6 mm i.d., 3.5 µm). The mobile phase composed of 0.1% formic acid in water:methanol (85:15 v/v) employing isocratic elution technique with a flow rate 0.8 ml/minute. The injection volumn was 10 µl. The analytical wavelength was set at 254 nm and the run time was 30 minutes.

The modified HPLC analytical method was proven to be a stability indicating assay using 2 techniques, determination of peak purity using a peak purity index from diode array detector and determination of peak purity from mass using LC/MS technique. The standard oxytetracycline sample and the hydrolysis sample of oxytetracycline were prepared as mentioned above. Photodegraded samples of oxytetracycline-divalent cation complexes were prepared by an addition of 2.475 mM Mg^{2+}, 2.233 mM Ca^{2+} or 8.006 mM Cu^{2+} in to 0.201 mM oxytetracycline in Tris-hydrochloride buffer pH 7.80 prior to expose the samples to 1,680 Lux.hours of UV light. Then the samples were kept in the dark at $4^{o}C$ until assay. In determination of peak purity, UV spectrum of oxytetracycline peak obtained from HPLC analysis of the standard oxytetracycline sample, the hydrolysis sample, and the photodegrade samples of oxytetracycline-divalent cation complex were compared.

In LC/MS method, the LC conditions was the same conditions as mentioned above. The separation was done on a Hypersil Gold aQ 2.1 150 mm 3 micron, with a flow rate of 0.25 ml/minute. LC/MS experiment was performed on Bruker Daltonics equipped with a ESI source. The mass spectrometry data were collected in a positive scan mode in a mass scan range 200-700 m/z. The spray voltage was set at 4.2 kV.

Thirty possed per square inch of nitrogen gas was used as nebulizer gas at a rate of 10 L/minute.

2.3 Kinetic studies of photolysis of oxytetracycline-divalent cation complex in buffer solution

2.3.1 Selection of quenching technique

In order to stop or delay photolysis reaction of oxytetracycline-divalent cation complexes, 2 quenching techniques were investigated; pH 7.80 at 4°C in the dark or pH 2.0 at 4°C in the dark. A oxytetracycline-Cu^{2+} complex was chosen in this quenching study because of it underwent photolysis at the fastest rate. A oxytetracycline-Cu^{2+} complex solution was prepared by an addition of $CuSO_4$ stock solution in 0.1 M Tris-hydrochloride buffer in to oxytetracycline stock solution in 0.1 M Tris-hydrochloride buffer. The mixture was then diluted with 0.1 M Tris-hydrochloride buffer. Initial concentrations of oxytetracycline and Cu^{2+} were 0.201 and 8.006 mM, respectively. In this study, the mixture was exposed to 1,680 Lux.hours of UV light. The sample were then kept in the darkness at 4°C or adjust pH by preparing the solution in 0.1 N HCl to pH value about 2 and kept in the dark at 4°C. At appropriate time points, the sample were analyzed for remaining oxytetracycline concentration using HPLC technique as mentioned in 2.2. Suitable quenching technique was selected based on capability of the quenching technique, that allowed further degradation of oxytetracycline less than 10% for at least 6 hours.

2.3.2 Determination of reaction order

Oxytetracycline-divalent cation complexes were prepared by an addition of divalent cations stock solution; i.e. Mg^{2+}, Ca^{2+} or Cu^{2+} to in oxytetracycline stock solution and adjust to the volume with 0.1 M Tris-hydrochloride buffer pH 7.80. The final concentration of oxytetracycline in each solutions was about 0.201 mM and the final concentrations of Mg^{2+}, Ca^{2+}, or Cu^{2+} were about 2.475, 2.233, or 8.006 mM, respectively. Ionic strength of each solution was adjust to 0.07 M by an addition of sodium chloride. The samples were kept in tightly sealed borosilicate vials and subjected to 280 Lux of UV light in a stability chamber at 30°C. At appropriate time points, samples were withdrawn. The reaction was quenched prior to sample analysis for the remaining oxytetracycline concentration using HPLC technique as mentioned in 2.2. pH values of each reaction solutions were measured by a pH meter at initial time point and at end of the experiment to ensure that pH values of the reaction mixtures were constant throughout the experiment course. Photolysis of free oxytetracycline under the same conditions was studied as a control study. Concentration time profiles and first order plots were constructed along with other appropriate data analysis techniques in order to evaluate reaction order and estimate rate constant (k).

Initial rate and fractional time technique was employed in order to determine reaction order (Conners, 1990). Oxytetracycline-divalent cation complexes were prepared by an addition of divalent cations stock solution in to oxytetracycline stock solution. Initial concentration of oxytetracycine was varied in a range of 0.101-0.402 mM, while the final concentration of Mg^{2+},

Ca^{2+}, or Cu^{2+} were 2.653, 2.410, or 8.176 mM, respectively. The samples were exposed to UV light and analyzed for the remaining oxytetracycline concentration as mentioned above. Concentration time profiles of oxytetracycline from each reaction were plotted.

In initial rate technique, initial degradation rates were estimated from slopes of each concentration time profile where the degradation was less than 10%. Then, initial rate value (k) were plotted against initial concentration of oxytetracyclin. In fractional time technique, $t_{0.75}$ or time required for 25% loss was directly estimated from corresponding concentration time profiles. Then, $t_{0.75}$ value were plotted against initial concentration of oxytetracycline.

2.3.3 Ionic strength effects

In this study, the oxytetracycline-divalent cation complex solutions were prepared as mentioned in 2.3.2. The ionic strength of each solution was varied from 0.04-0.6 M using sodium chloride. The samples were exposed to UV light and analyzed for the remaining oxytetracycline concentration as mentioned 2.3.2.

CHAPTER IV

RESULTS AND DISCUSSION

1. Complex formation of oxytetracycline with divalent cations

1.1 Determination of excitation and emission wavelengths of oxytetracycline by fluorescence spectroscopy

UV spectrum of 5×10^{-5}M oxytetracycline in phosphate buffer pH 7.80 showed UV absorption maxima wavelength at 272 and 362 nm corresponding to UV absorption maxima of chromophore I and III, respectively (Figure 6). This observation was consistent with results previously reported by Hong-Xia et al (2004). In this study, excitation wavelength was chosen to be 390 nm because chromophore III of oxytetracycline was expected to be a binding site with divalent cation (ลาวัลย์ ศรีพงษ์, 2554).

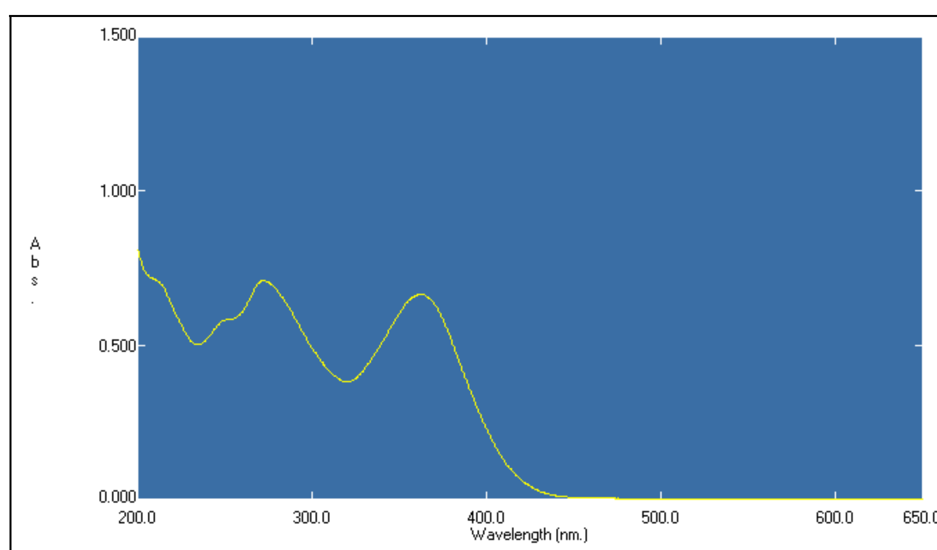


Figure 6 UV spectrum of $5x10^{-5}$M oxytetracycline in phosphate buffer pH 7.80.

Figure 7 illustrated emission spectrum of oxytetracycline using the excitation wavelength of 390 nm with an emission maximum at 510 nm. Therefore, fluorescence intensities of oxytetracycline at various concentrations were determined by setting excitation and emission wavelengths at 390 and 510 nm, respectively. Figure 8 showed that, in a concentration range of 10^{-6} M to 1×10^{-4} M, fluorescence intensities of oxytetracycline increased as oxytetracycline concentration increased. However, at concentration above 10^{-4} M, fluorescence intensities were reduced because of quenching phenomena at high concentration of oxytetracycline. Since the instrument gives precise and accurate measurement when relative fluorescence intensity is higher than 300 rf, appropriate oxytetracycline concentration for further studies is in a range of $5\text{x}10^{-5}$ M to 10^{-4} M employing excitation and emission wavelengths at 390 and 510 nm, respectively.

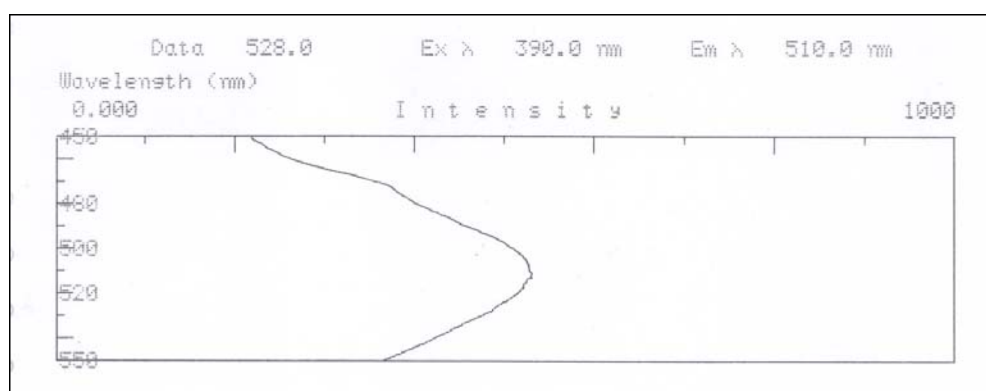


Figure 7 Fluorecence spectrum of 10^{-4} M oxytetracycline in phosphate buffer pH 7.80.

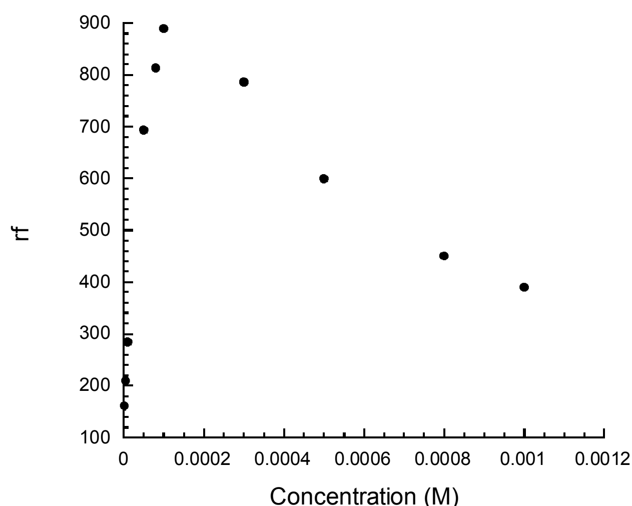


Figure 8 Fluorescence intensities of oxytetracycline at various concentrations in phosphate buffer pH 7.80.

1.2 Influence of buffer to complex formation of oxytetracycline with divalent cation

pH value of water in fish and shrimp farming in Nakhorn Pathom, Thailand was 7.80 (n=3). Thus, complex formation between oxytetracycline with divalent cations was studied in buffer pH 7.80 in order to mimic the pH value of water in real condition.

1.2.1 Complex formation of oxytetracycline with divalent cations in phosphate buffer solution

Complex formation of oxytetracycline with divalent cations was studied in phosphate buffer pH 7.80 in presences of $6x10^{-5}$ M oxytetracycline and 0 to $1.8x10^{-2}$ M Mg^{2+}, 0 to $6x10^{-3}$ M Ca^{2+}, 0 to $1.8x10^{-3}$ M Cu^{2+}. In the presence of Mg^{2+}, oxytetracycline fluorescence intensity was enhanced and redshifed to 515 nm

(Figure 9) when the excitation wavelength was set at 390 nm. The results indicated that the chromophore III of oxytetracycline directly involved in complex formation.

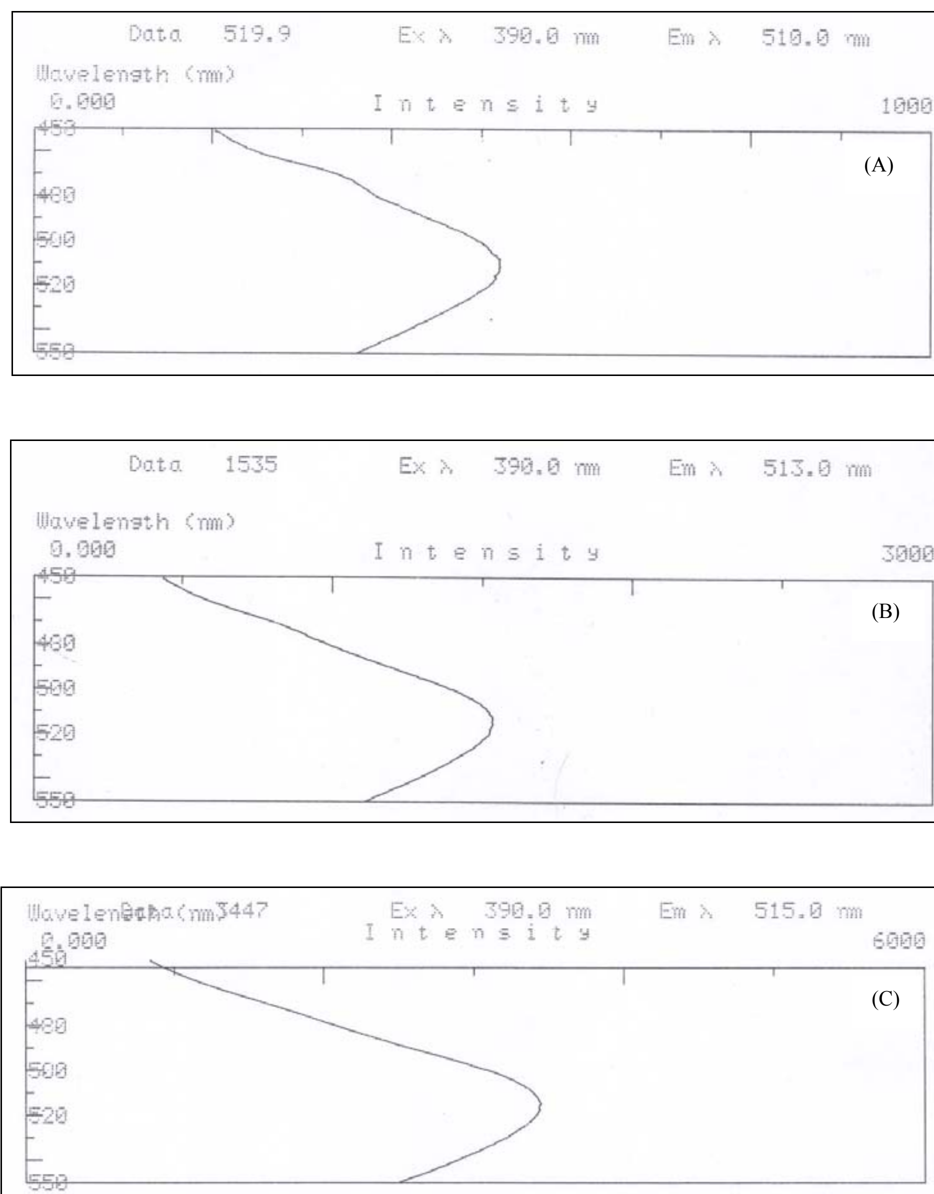


Figure 9 Fluorescence spectra of 6×10^{-5} M oxytetracycline with Mg^{+2} concentration ratio OTC: Mg^{+2} 1:0 (A), 1:1 (B), 1:10 (C)

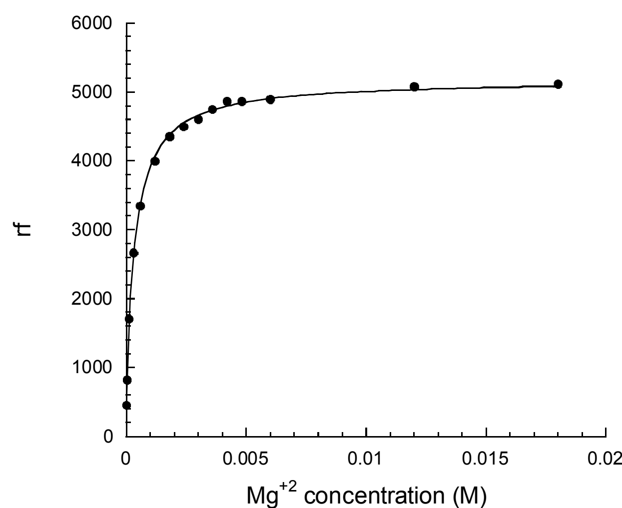


Figure 10 Fluorescence of oxytetracycline-Mg^{2+} complex at various Mg^{2+}concentrations in phosphate buffer pH 7.80. Excitation and emission wavelengths of 390 and 510 nm., respectively.

Figure 10 showed that, in phosphate buffer pH 7.80, fluorescence intensities of oxytetracycline was increased as concentration of Mg^{2+} was increased and reached a maximum around 5,000 rf. When Mg^{+2} concentration was higher than 0.012 M or oxytetracycline : cation ratio was more than 1:200, enhancing in fluorescence intensity leveled off. Enhancing in fluorescence intensities of oxytetracycline in the presence of Mg^{2+} was also indicative of complex formation. The maximum fluorescence enhancement was observed because most of oxytetracycline molecules already formed complex with the magnesium cation. Therefore, increasing Mg^{2+} concentration could not result additioned formation of oxytetracycline-Mg^{2+} complex. Figure 10 also showed a typical characteristic of 1:1 complex formation as reported by Arias et al (2007). In addition, after the complex solutions were kept at room

temperature for 10 days. The complex solutions were clear but the pH value was decreased from 7.80 to 7.34. The result indicated that buffer capacity of the phosphate buffer (5×10^{-3} M) was not high enough to maintain constant pH which was one of the critical parameters in kinetics studies. However, attempts to increase concentration of phosphate buffer led to precipitation of $MgCl_2$ in stock solution.

Fluorescence spectrum of oxytetracycline in the presence of various Ca^{2+} concentrations also showed fluorescence enhancement with redshifted to 515 nm as they were observed in the presence of Mg^{2+} (Figure 9). The observed fluorescence enhancement was due to complex formation of oxytetracycline-Ca^{2+} involving chromophore III of oxytetracycline when excitation wavelength of 390 nm was employed. However, investigation of complex formation in the presence of Ca^{2+} at a concentration higher than $6x10^{-3}$ M could not be performed due to solubility limit of $CaCl_2$ in the solvent. This observation was in accordance with the statement mentioned earlier that the complex formation took place at carbonyl carbon at C11 and hydroxyl group at C12 resulting in conformation change of the naphthacene ring from the folded conformation to the planar conformation (Jin et al., 2007). Fluorescence enhancement of oxytetracycline after complex formation with Mg^{+2} or Ca^{+2} was consistent with the results previous reported by Arias et al (2007).

$CuSO_4$ does not dissolve in phosphate buffer pH 7.80. Attempts to investigate complex formation of oxytetracycline with Cu^{2+} by changing order of phosphate buffer introducting were failed.

The above results indicated that the phosphate buffer system was not suitable for studying the formation of the oxytetracycline-divalent cation complex due to 3

reasons. Firstly, the phosphate buffer caused salting out effect of $CaCl_2$. Secondly, the phosphate buffer was unable to control the solution's pH value. And thirdly, $CuSO_4$ did not dissolve in phosphate buffer.

1.2.2 Complex formation of oxytetracycline with divalent cation in Tris-hydrochloride buffer pH 7.80

In this study, Tris-hydrochloride buffer pH 7.80 was employed as a solvent. Oxytetracycline solutions in the presence of Mg^{2+}, Ca^{2+}, or Cu^{2+} gave rise to clear solution with constant pH value for at least 10 day at room temperature. This result indicated that the buffer system had a direct impact on complex formation. The phosphate buffer system was not suitable for the formation of the oxytetracycline-cation complex because the phosphate buffer caused precipitation at high concentration of divalent cation and unable to control the pH of solution, while the Tris-hydrochloride buffer could control the pH of solution for a longer times. Therefore, Tris-hydrochloride buffer was selected for further studies on degradation of the oxytetracycline-divalent cation complex.

In Tris-hydrochloride buffer pH 7.80, fluorescence enhancement of oxytetracycline in the presence of Ca^{2+} or Mg^{2+} indicated the complex formation (Figure 11). At high divalent cation concentrations, the fluorescence enhancement of the complex between oxytetracycline with Mg^{2+} or Ca^{2+} was reached a maximum and then leveled off. The plataue of fluorescence intensity at high Ca^{2+} or Mg^{2+}concentration was due to depletion of free oxytetracycline to form complex with divalent cation. Thus, further increase in fluorescence intensities of oxytetracycline was not observed. Fluorescence enhancement of oxytetracycline-Mg^{2+} or

Ca^{2+}complex implied that both Mg^{2+} and Ca^{2+} formed complex with chromophore part III of oxytetracycline (Figure 3). The observed fluorescence enhancement was consistent with the previous results, indication of complex formation (Arias et al., 2007). Arias et al (2007) also reported that oxytetracycline-Mg^{2+}complex has a higher quantum yield than that of oxytetracycline-Ca^{2+} complex. As a result, the fluorescence intensity of oxytetracycline- Mg^{2+} complex was higher than that of oxytetracycline-Ca^{2+} complex (ลาวัลย์ ศรีพงษ์, 2554).

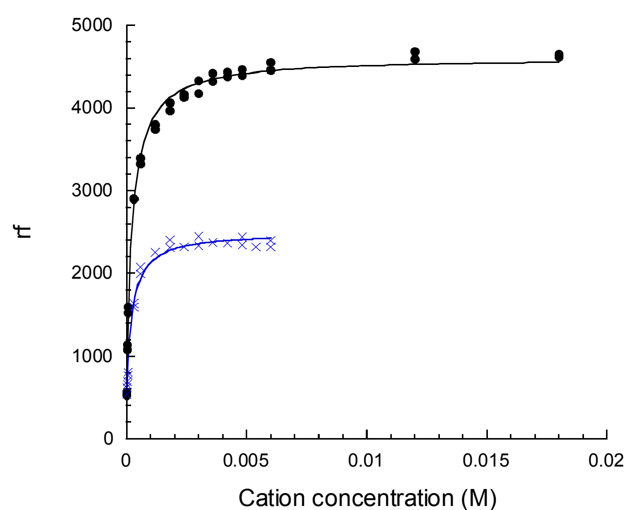


Figure 11 Fluorescence intensity of oxytetracycline in the presence of Mg^{2+} (·) and Ca^{+2} (×) in Tris-hydrochloride buffer pH 7.80 at the excitation and emission wavelengths of 390 and 510 nm., respectively (n=2). The solid lines represented a fitting curve according to equation (1).

On the contary, fluorescence intensity of oxytetracycline in the presence of Cu^{2+} was reduced as Cu^{2+} concentration was increased (Figure 12). The quenching of

oxytetracycline fluorescence intensities reached a certain value and then leveled off at a high Cu^{2+} ion concentration. Causes of the observed quenching effect are unclear but probably due to the difference in positions at which the oxytetracycline formed the complex with the divalent cation. Therefore, the planar conformation of naphthacene could not be formed. Fluorescence characteristic of oxytetracycline-Cu^{2+} complex has never been reported. Chen et al (2011) reported that UV spectrum of oxytetracycline-Cu^{2+} complex showed a UV absorption maxima around 374-386 nm with a UV absorbance value 2 times higher than that of free oxytetracycline. The fluorescence quenching was observed, when the excitation wavelength of 390 nm was employed, and the result reported by Chen et al (2011) implied that chromophore III of oxytetracycline involved in complex formation. We suggested that Cu^{2+} cation might form a complex at hydroxyl group at C10 and carbonyl oxygen at C11 of oxytetracycline. The oxytetracycline-Cu^{2+} complex formation did not create ring constrain so that the folded conformation of napthacene ring would not be changed to the planar conformation. However, further investigation on oxytetracycline-Cu^{2+} complex should be determined the conformation in order to prove the above speculation.

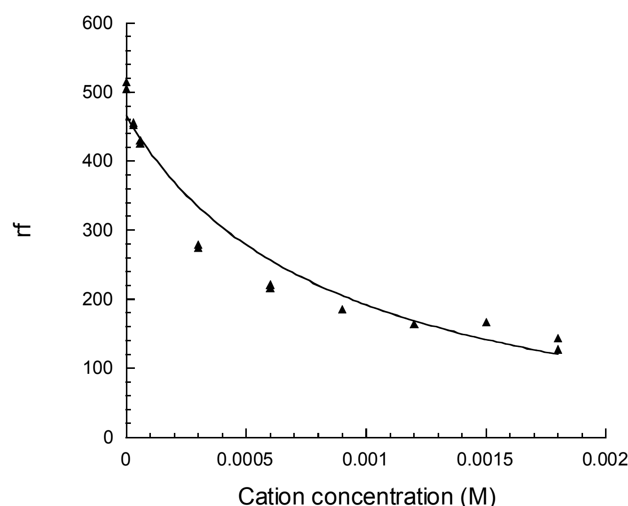


Figure 12 Fluorescence intensity of oxytetracycline in the presence of Cu^{2+}in Tris-hydrochloride buffer pH 7.80 at the excitation and emission wavelength of 390 and 510 nm., respectively (n=2). The solid lines represented a fitting curve according to equation (1).

1.3 Binding constant (K) of oxytetracycline-divalent cation complex

Arias et al (2007) developed a mathematic equation for determination of a binding constant (K) using a relationship between fluorescence intensities of complex solution and divalent cation concentrations. The equation was derived and based on assumption that a stoichiometric of the oxytetracycline-cation complex was 1:1 and complex possesses different UV and fluorescence characteristic from that of free molecule. The observed fluorescence intensity was a result of fluorescence intensities of free oxytetracycline and oxytetracycline complex. In other words,

$$\varepsilon_{app} l[OTC]_t = \varepsilon_f l[OTC]_f + \varepsilon_b l[OTC]_b \qquad \text{Equation (2)}$$

Where, l = light pathway (in a standard cell is equal to 1 cm)

ε = Extinction coefficient

$[OTC]_t$ = Total oxytetracycline concentration

$[OTC]_f$ = Respective concentrations of free oxytetracycline

$[OTC]_b$ = Oxytetracycline-divalent cation complex concentration

Mass balance equation is $[OTC]_t = [OTC]_f + [OTC]_b$

Where $[OTC]_t$, $[OTC]_f$ and $[OTC]_b$ are total concentration of oxytetracycline in the solution, concentration of free oxytetracycline and concentration of oxytetracycline complex, respectively. $[OTC]_t$, $[OTC]_f$ and $[OTC]_b$ are related to complex formation constant, K_{OTC}, defined as

$$K_{OTC} = \frac{[OTC]_b}{[OTC]_f\,[C^{2+}]} \qquad \text{Equation (3)}$$

Where, $[C^{2+}]$ is divalent cation concentration

Finally, Arias et al (2007) developed the following mathematic.

$$I = \frac{I_f + I_b K_{OTC}[C^{2+}]}{1 + K_{OTC}\,[C^{2+}]} \qquad \text{Equation (1)}$$

Where, I_f is oxytetracycline intensity in the absence of cation and I_b is oxytetracycline intensity in the presence of cation.

According to binding profiles of oxytetracycline with Mg^{+2}, Ca^{+2} or Cu^{+2} shown in Figure 11 and 12, a curve fitting program was employed in order to determine binding constant employing equation (1). Good fitting of the solid lines in

Figure 11 and 12 represent curve indicated 1:1 complex formation between oxytetracycline and divalent cations; i.e. Mg^{2+}, Ca^{2+} and Cu^{2+}. Based on equation (1), the binding constants of oxytetracycline with Mg^{+2}, Ca^{+2} and Cu^{+2} ions in Tris-hydrochloride buffer pH 7.80 were estimated to be 3,921.8 , 4,386.8 and 1,149.9 M^{-1} , respectively. Arias et al (2007) reported the binding constants of oxytetracycline-Mg^{2+} or oxytetracycline-Ca^{2+} complex at pH around 4-5 to be 66 and 55 M^{-1} , respectively. Differences in binding constants were mainly due to difference in ionization state of oxytetracycline. At pH 4-5, 50% of oxytetracycline were zwitterions and the rest were monocationic form. However at pH 7.80, 50% of oxytetracycline were zwitterions and the rest were monoanionic form, form that easily form complex with divalent cation. Thus, binding constants of oxytetracycline to divalent cations at pH 7.80 is higher than that of at pH 4-5. In another study, Martin (1979) reported binding constant of oxytetracycline with Mg^{2+} or Ca^{2+} in Tris-KNO_3 buffer pH 7.80 to be 2,625 and 1,150 M^{-1}, respectively using a UV spectrophotometry method. The difference of binding constants between these 2 studies were speculated to be due to differences in an analytical technique, buffer system and/or ionic strength of the system.

The estimated binding constants were utilized in order to design further kinetic studies. For example, if initial concentration of oxytetracycline free molecule was 0.201 mM, initial concentration of Mg^{2+}, Ca^{2+}, or Cu^{2+}needed for 90% complex formation was calculated to be 2.475, 2.233, or 8.006 mM, respectively.

2. Development of HPLC method for an analysis of oxytetracycline (High Performance Liquid Chromatography method, HPLC)

HPLC analytical methods of oxytetracycline and oxytetracycline hydrochloride were reported in The United States Pharmacopeia (USP35), in British Pharmacopeia (BP2010), and in other scientific articles (Khan et al., 1996; Lykkeberg et al., 2004; Papadoyannis et al., 2000; Smyrniotakis and Archontaki, 2007; Xuan et al., 2010). However, a suitable analytical method for oxytetracycline stability studies should be a stability indicating assay.

In the USP35 monographs, separation of oxytetracycline and oxytetracycline hydrochloride was carried on using a spherical styrene-divinylbenzene copolymer column. This column is quite expensive. The elution method is isocratic, but the monographs did not show that the assay method was a stability indicating assay.

In the BP2010 monographs, separation of oxytetracycline and oxytetracycline hydrochloride was also performed on a spherical styrene-divinylbenzene copolymer column using a gredient elution method. The method is a stability indicating assay because the method could separate oxytetracycline from its degrade products, 4-epi-oxytetracycline ,α- and β-apo oxytetracycline.

Other analytical techniques of oxytetracycline proven to be a stability indicating assay include spectrophotometry, chemiluminescence, HPLC, and capillary electrophoresis (Yuwono and Indrayanto, 2005). HPLC method has been widely used for the analysis of active ingredient in pharmaceutical product. Lykkeberg et al (2004) reported a HPLC analytical method which was a stability indicating assay using C18 column. The separation was performed using gradient elution with a flow rate of 0.25

ml/min. Retention times of oxytetracycline and it's impurities; 4-epi-oxytetracycline, oxytetracycline, α- apo oxytetracycline and β-apo oxytetracycline, were around 4, 7, 14 and 21 min, respectively (Figure 13).

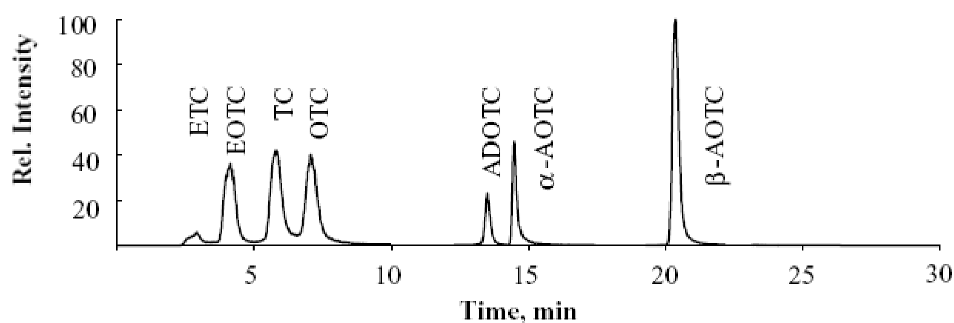


Figure 13 A typical chromatogram of oxytetracycline and it's degrade products reported by Lykkeberg et al (2004) a C18 column was used as a stationary phase. The separation was performed using gradient elution with a flow rate 0.25 ml/min. The chromatogram shows separation of epi-tetracycline (ETC), 4-epi-oxytetracycline (EOTC), tetracycline (TC), oxytetracycline (OTC), 2- acetyl-2-decarboxamido-oxytetracycline (ADOTC), α- apo oxytetracycline (α-AOTC) and β-apo oxytetracycline (β-AOTC).

This study was firstly adapted the HPLC method reported by Lykkeberg et al (2004). A typical chromatogram of oxytetracycline hydrochloride in ultrapure water shows one broad peak with a retention time of around 7 min (Figure 14) consistent with the retention time of oxytetracycline previously reported.

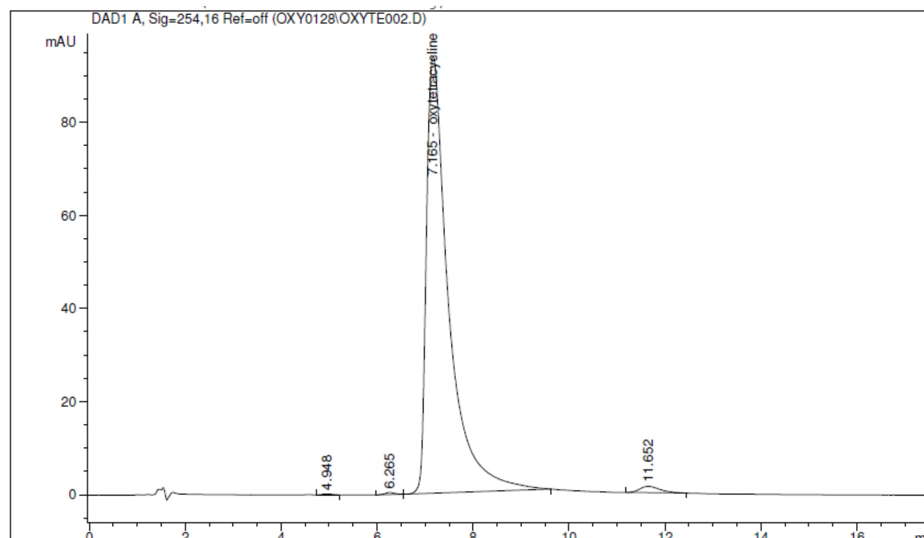


Figure 14 A typical chromatogram of oxytetracycline hydrochloride in ultrapure water. A C18 column was used as a stationary phase. The separation was performed using gradient elution with a flow rate of 1 ml/min.

Oxytetracycline hydrochloride in ultrapure water underwent forced degradation by heating on a hot plate for 20 min. Chromatogram of the degraded sample consisted of 8 peaks (Figure 15). According to the peak pattern, a peak with retention time of 4 min was predicted to be 4-epi-oxytetracycline (EOTC). Peak hight and peak area of oxytetracycline peak at the retention time of 7 min decreased, an indication of oxytetracycline degradation. The peak at a retention time of 11.5 min was not expected to be a peak of 2- acetyl-2-decarboxamido-oxytetracycline (ADOTC) because ADOTC is an impurity from oxytetracycline synthesis process (Lykkeberg et al., 2004). Therefore, peak area of the ADOTC peak was expected to be constant or decreasing if it could degrade to other compounds. In this study, peak area at the retention time about 11.5 min increased over time indicating that it was an unknown degrade product of oxytetracycline. Therefore, 2 peaks at retention times of 4 and 7 min were expected to be 4-epi-oxytetracycline and oxytetracycline,

respectively, while peaks at around 6, 11.5, 15.5 min were unknown degrade products. The HPLC conditions reported by Lykkeberg et al (2004) gave rise to a broad and asymmetrical oxytetracycline peak. The HPLC method was further modified using an isocratic elution method.

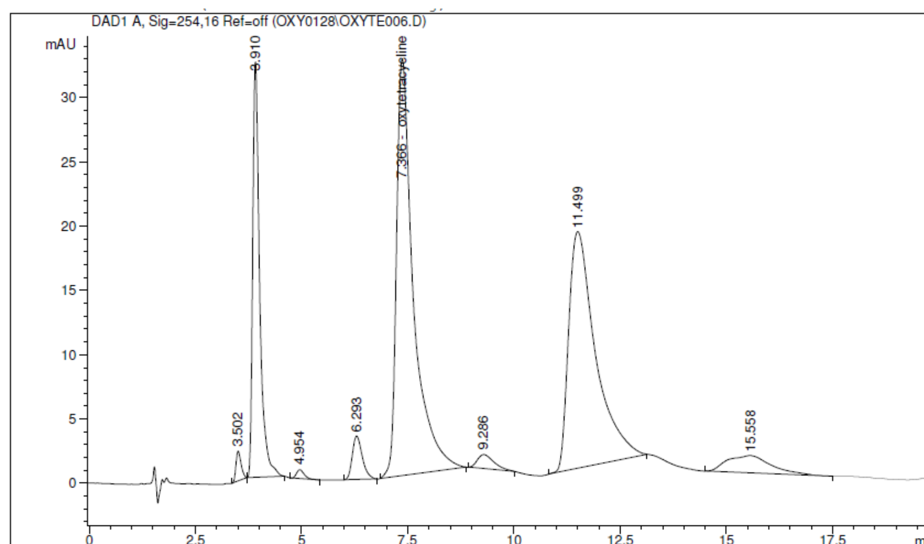


Figure 15 Chromatogram of oxytetracycline hydrochloride in ultrapure water after heat exposure. The separation was carried out on a C18 column with a gradient elution method. The flow rate was set at 1 ml/min.

By modifying aqueous composition of the mobile phase from 0.08 M formic acid in water pH 3.2 to phosphate buffer pH 3.1. Chromatogram of oxytetracycline hydrochloride in ultrapure water showed a symmetrical peak with a retention time of 22.6 min (Figure 16). Chromatogram of oxytetracycline degraded under heating on a hot plate for 20 min showed 5 major peaks (Figure 17). Although all peaks were well separated under these HPLC conditions, the run time was 40 min. The mobile phase composition was further modified in order to shorten the run time and apply the LC conditions with mass spectrometry detector.

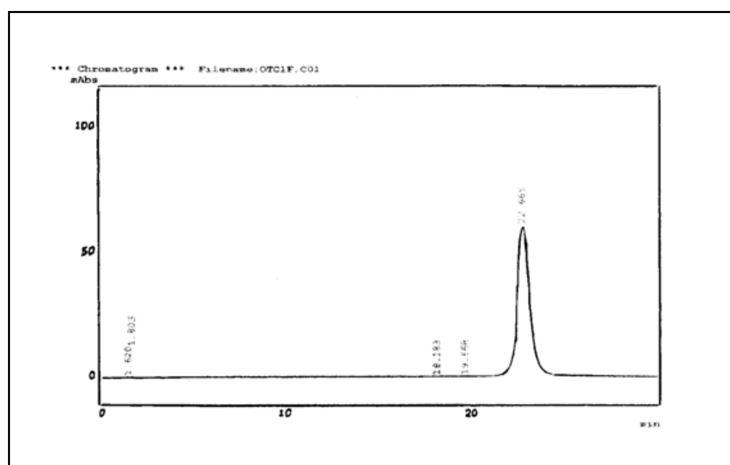


Figure 16 Chromatogram of oxytetracycline hydrochloride in ultrapure water. The separation was carried out on a C18 column with an isocratic elution method. The mobile phase was buffer : methanol (86 : 14 v/v) with a flow rate of 1 ml/min.

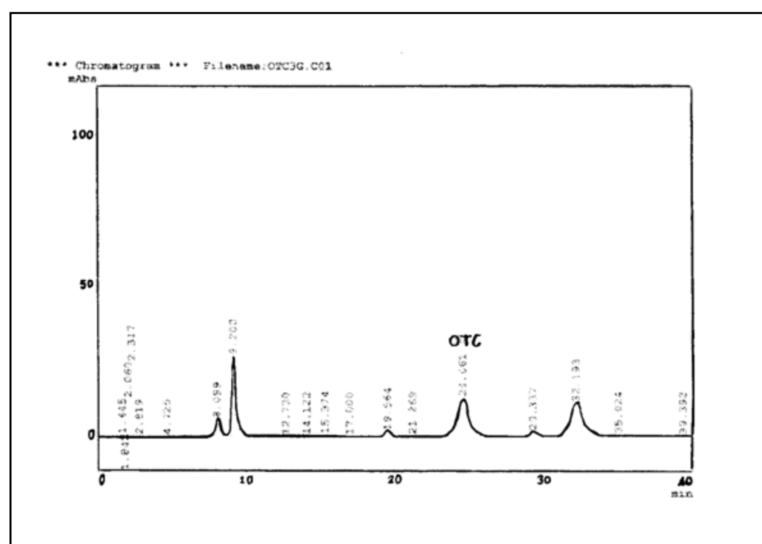


Figure 17 Chromatogram of oxytetracycline hydrochloride after heat exposure. The separation was carried out on a C18 column with an isocratic elution method. The mobile phase was buffer : methanol (86 : 14 v/v) with a flow rate of 1 ml/min.

By increasing organic composition of the mobile phase from 14% to 20%, the retention time of oxytetracycline peak was charged from 22.6 to 12 min (Figure 18).

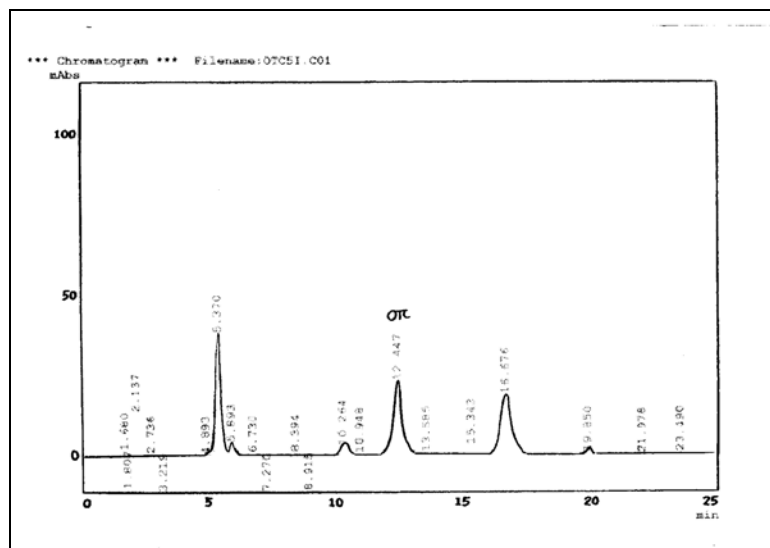


Figure 18 Chromatogram of oxytetracycline hydrochloride after heat exposure. The separation was carried out on a C18 column with an isocratic elution method. The mobile phase was buffer : methanol (80 : 20 v/v) with a flow rate of 1 ml/min.

The mobile phase composition was further modified by utilizing formic acid instead of using phosphate buffer and adjusting the methanol concentration from 20 to 15%. Under these conditions, oxytetracycline was eluted at 9.2 min (Figure 19). Chromatogram of oxytetracycline hydrochloride after heat exposure showed 5 major peak (Figure 20). All peaks were well separated and symmetrical. Thus, this HPLC condition was the analysis method employed for further studies on degradation of the oxytetracycline-divalent cation complex.

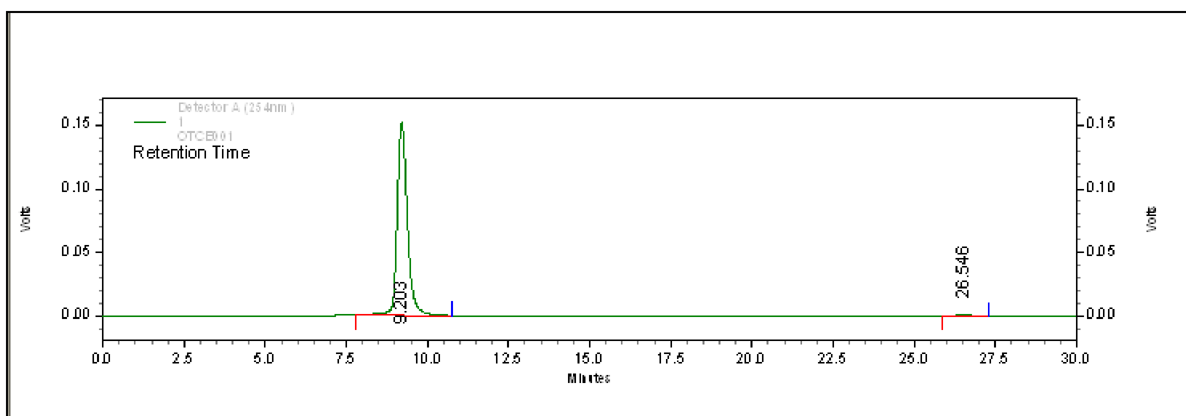


Figure 19 Chromatogram of oxytetracycline hydrochloride in ultrapure water. The separation was carried out on a C18 column with an isocratic elution method. The mobile phase was buffer : methanol (85 : 15 v/v) with a flow rate of 0.8 ml/min.

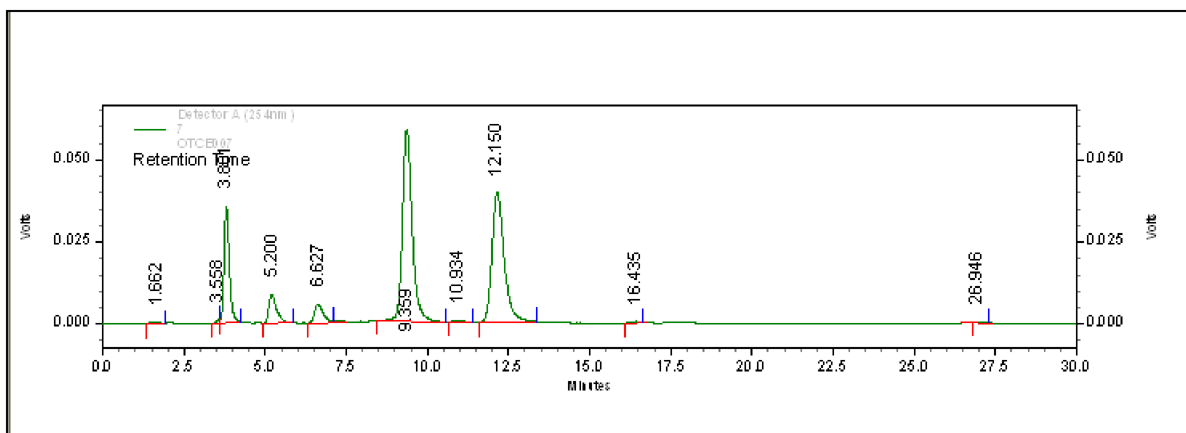


Figure 20 Chromatogram of oxytetracycline hydrochloride after heat exposure. The separation was carried out on a C18 column with an isocratic elution method. The mobile phase was buffer : methanol (85 : 15 v/v) with a flow rate of 0.8 ml/min.

In conclusion, the HPLC conditions used in this study employed a C18 column as a stationary phase. The mobile phase composed of buffer : methanol (85 :

15 v/v) with a flow rate of 0.8 ml/min. The elution method was isocratic elution method with a run time of 30 min. The analytical wavelength was set at 254 nm

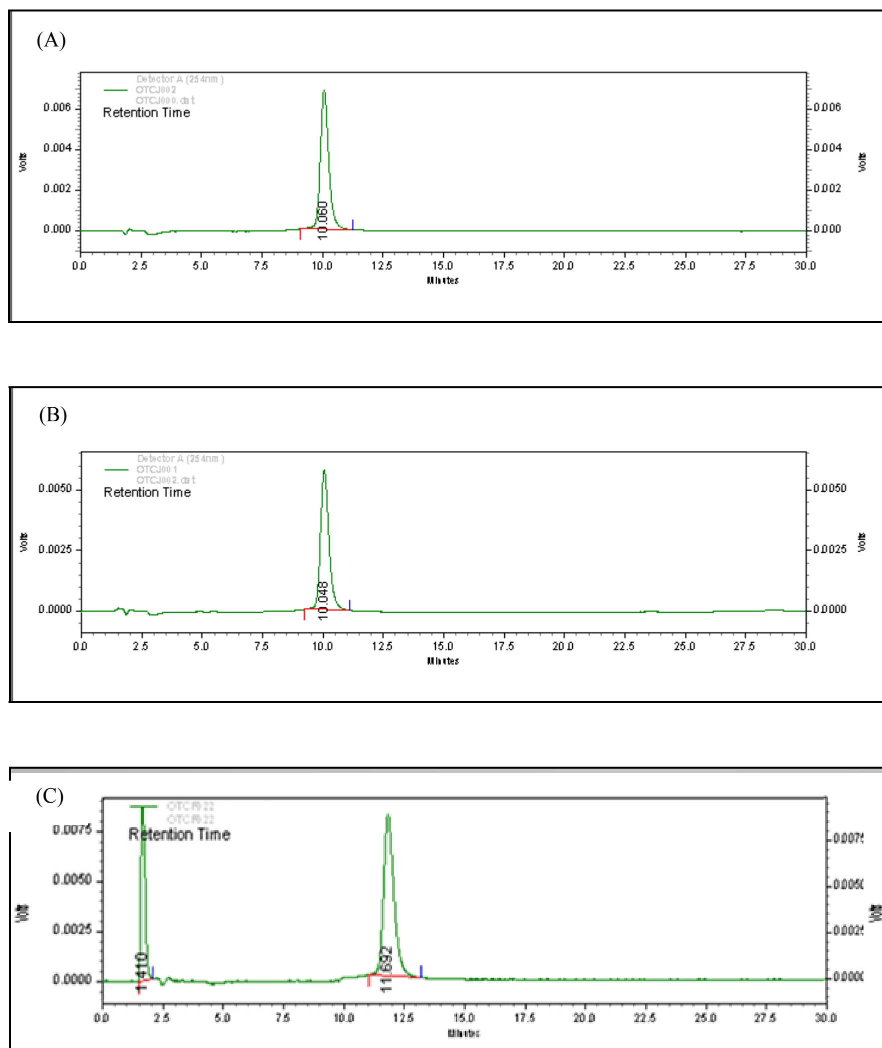


Figure 21 Chromatogram of oxytetracycline of freshly prepared oxytetracycline-Mg^{2+}complex (A), oxytetracycline-Ca^{2+}complex (B) and oxytetracycline-Cu^{2+}complex (C) was carried out on a C18 column with an isocratic elution method. The mobile phase was buffer : methanol (85 : 15 v/v) with a flow rate of 0.8 ml/min.

In the presence of divalent cations; i.e. Mg^{2+}, Ca^{2+} and Cu^{2+}, chromatograms of freshly prepared oxytetracycline-divalent cation complexes showed one major peak corresponding to free oxytetracycline (Figure 21 A, B and C). Peaks of the complexes were not seen due to 2 reasons. Firstly, the complex formation was a reversible process. Secondly, under the HPLC analytical conditions, pH value of the mobile phase was 3. At pH 3, cationic form of oxytetracycline was unfavorable for complex formation.

UV characteristics determining from the HPLC diode array detector and mass determining from a HPLC/MS technique were employed to show that the modified HPLC method was an stability indicating assay. In this study, oxytetracycline standard solution and the sample after UV stress were analyzed using the HPLC instrument equipped with a diode array detector. UV spectrum of oxytetracycline peak obtained from oxytetracycline standard solution was compare with that of heat-degraded oxytetracycline peak. Superimpossability of the two UV spectra, with a peak purity index of 0.9983, indicated that other compounds were not co-eluted with the oxytetracycline peak (Figure 22).

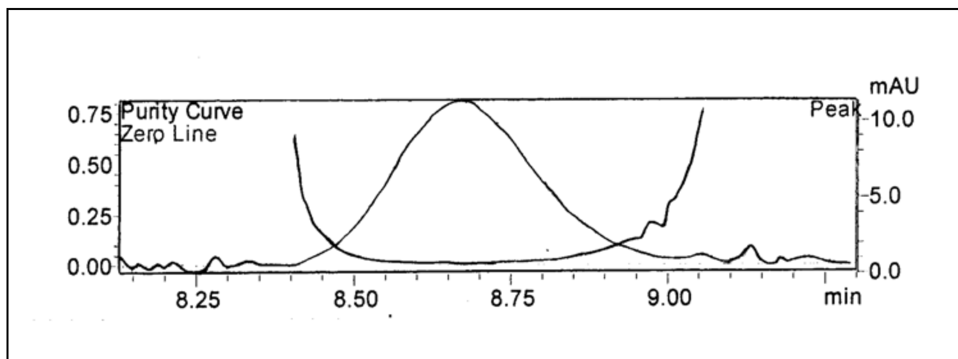


Figure 22 Peak purity of oxytetracycline peak at the retention time about 8.7 min. that comes from epimerization and dehydration. Not detect impurity . Peak purity index = 0.998320

In another study, the hydrolysis sample of free oxytetracycline in ultrapure water, the photodegraded samples of oxytetracycline-Mg^{2+} and oxytetracycline-Ca^{2+} complexes were analyzed using a HPLC instrument equipped with a mass spectrometry detector. The samples were prepared by an addition of 2.475 mM Mg^{2+} or 2.233 mM Ca^{2+} in to 0.201 mM oxytetracycline in Tris-hydrochloride buffer pH 7.80. The samples were then exposed to UV light providing 1,680 Lux.hours at 30°C. Then the samples were kept in the dark at 4°C prior to assay. The hydrolysis sample of oxytetracycline gave rise to a chromatogram as shown in Figure 23. A peak at retention time of 14.14 min was considered as oxytetracycline (m/z 461.1). The result indicated that this LC/MS analysis condition can separate peaks of oxytetracycline and degrade products and can be applied for further use in HPLC analysis method.

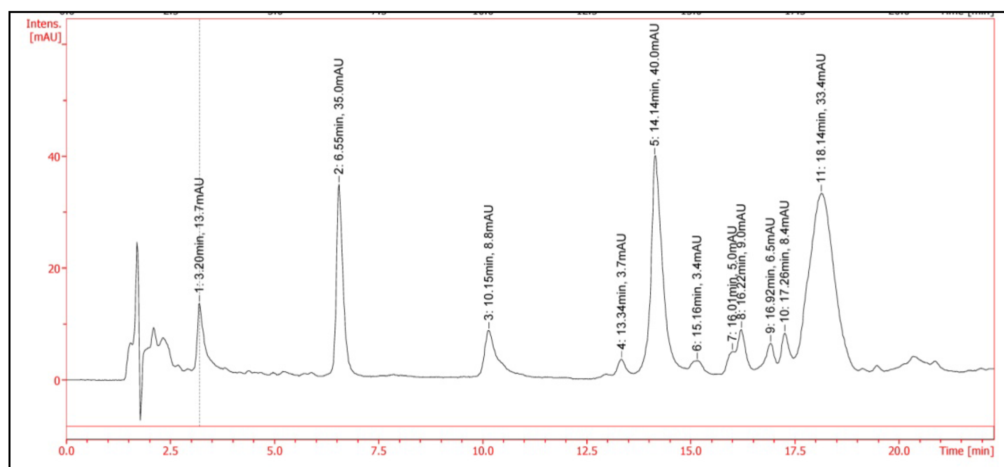


Figure 23 Chromatogram compound mass spectrum analysis by LC/MS method of oxytetracycline in ultrapure water that comes from epimerization and dehydration.

Figure 24(A) showed a typical chromatogram of oxytetracycline-Mg^{2+} complex or oxytetracycline-Ca^{2+} complex after photodegradation with 2 major peaks at around 13 and 14 min. Under the same conditions as mentioned earlier but on a different HPLC instrument. The mass analyser showed that a peak at the relative retention time around 14 min consisted of 1 mass, 461.2, corresponding to free oxytetracycline (Figure 24C). While a peak at the retention time around 13 min composed of 2 masses, 429.1 and 447.1 (Figure 24B). The degraded product with molecular mass of 447.1 was believed to be 4-epi-N-desmethyl-oxytetracycline (E-N-DM-OTC), a known photodegraded product of oxytetracycline found in soil (Halling-Sorensen et al., 2003). The degraded product with molecular mass of 429.1 was expected to be a degraded product of E-N-DM-OTC which underwent dehydration. However, further studies such as NMR should be done in order to confirm the formation of these 2 degraded products.

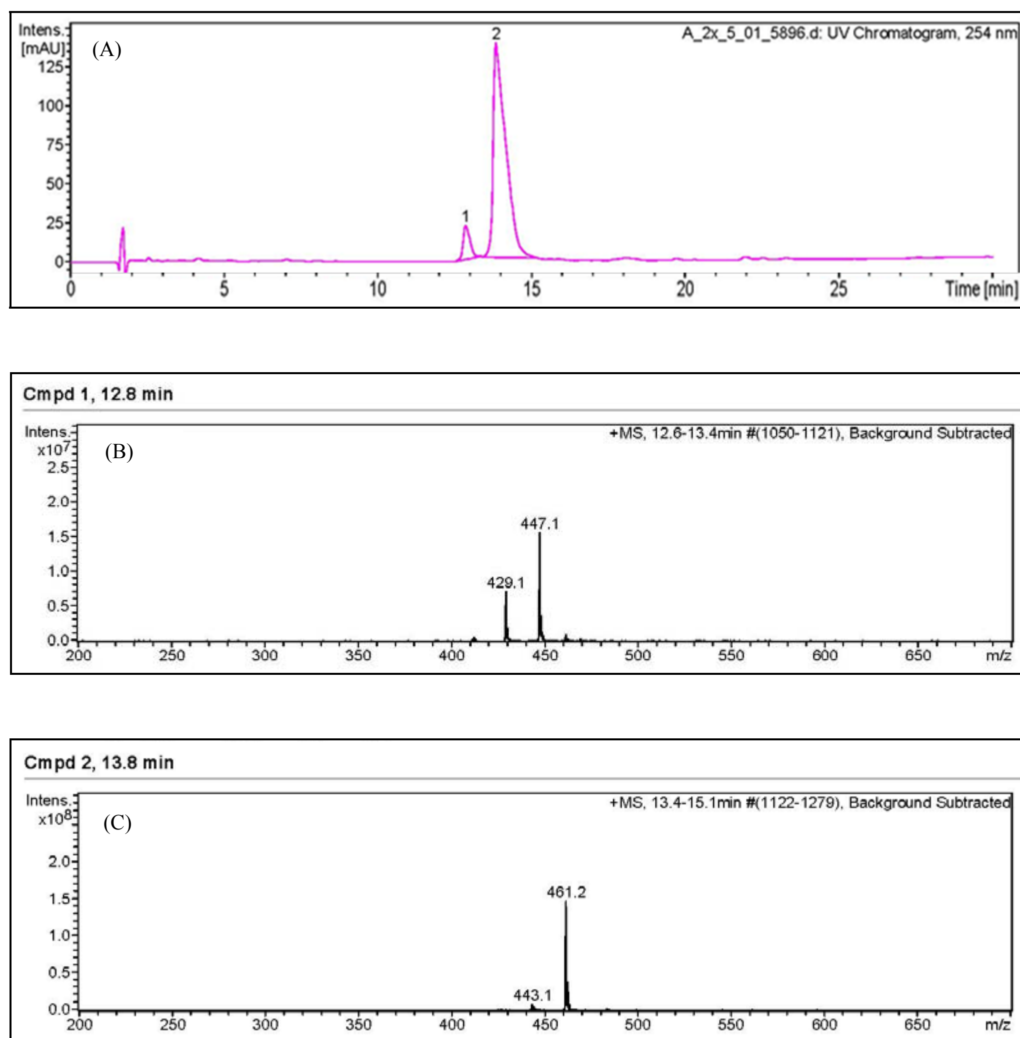


Figure 24 Chromatogram (A) and mass spectra of oxytetracycline-Mg^{2+} complex after photo-stress. Mass spectrum of peak at retention around 13 (B) and 14 min (C).

Chromatogram obtained from LC/MS analysis of oxytetracycline-Cu^{2+} complex after photodegradation was shown in Figure 25. Oxytetracycline-Cu^{2+} complex underwent photodegradation at a fast rate so that oxytetracycline was completely degradation after 1 hour of UV exposure.

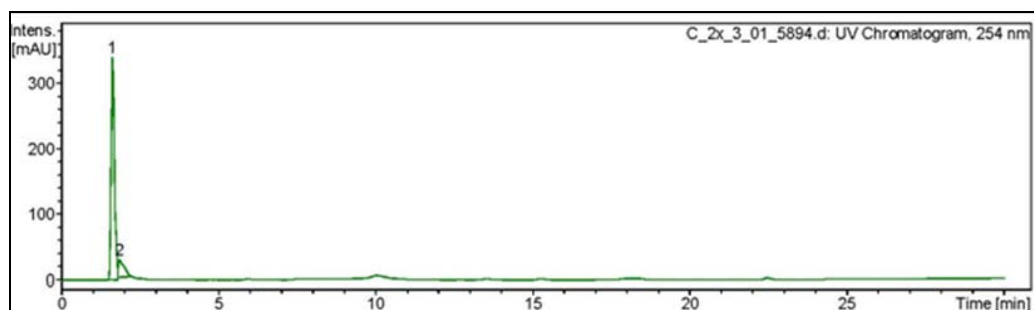


Figure 25 Chromatogram of oxytetracycline-Cu^{2+} complex after photodegradation.

3. Photolysis of oxytetracycline-divalent cation complexs in buffer solution

3.1 Selection of technique for quenching the photolysis of oxytetracycline-divalent cation complex

Preliminary result showed that photolysis of oxytetracycline-Cu^{2+} complex was very fast; therefore, proper quenching technique was necessary in order to obtain accurate remaining oxytetracycline concentration at each sampling time point. Two quenching techniques were proposed; i.e. pH 7.80 at 4°C in the dark, and pH 2.0 at 4°C in the dark. Employing the darkness, the oxytetracycline-Cu^{2+} complex were kept in the dark at 4°C. Figure 26 showed that, in the dark at low temperature, about 37% of oxytetracycline was further degraded over 6 hours. In other words, the photolysis rate of oxytetracycline-divalent cation complex was not slow down by keeping samples in the dark and at 4°C.

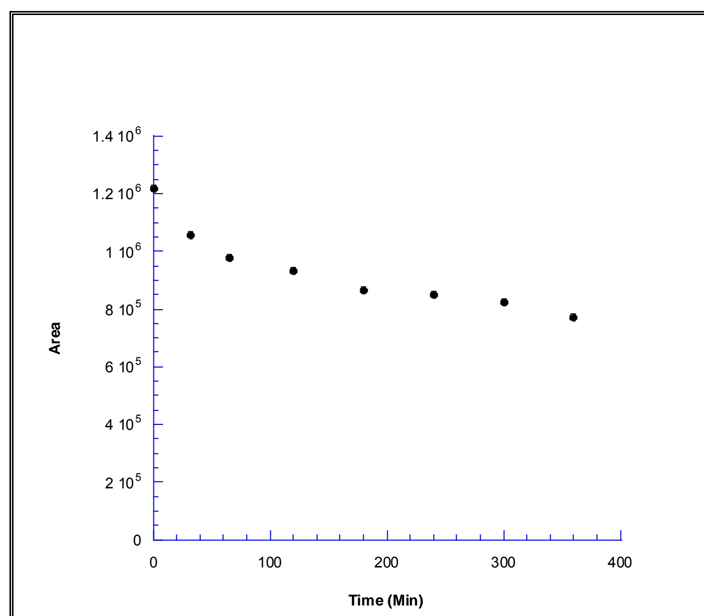


Figure 26 A peak area versus time profile of oxytetracycline in samples containing oxytetracycline -Cu^{2+} complex in 0.1 M Tris-hydrochloride buffer pH 6.5 after exposure to UV light for 10 min and storage in the dark at 4°C for 6 hours.

In the second technique, the reaction rate was expected to decrease by preparing the solution in 0.01 N HCl (pH 2). In acidic condition, oxytetracycline is protonated and possesses a positive charge which is an unfavorable from to form complex. In addition, free oxytetracycline was reported to be quite stable under acidic condition (Doi and Stoskopf, 2000). In this study, oxytetracycline -Cu^{2+} complex in Tris-hydrochloride buffer, after exposure to UV light, was adjusted to pH 2 and stored in dark at 4°C. Figure 27 showed that under such condition, about 4% of oxytetracycline was further degraded over 6 hours. In other words, the reaction was effectively quenched by adjusting sample pH value. Therefore, sample pH adjustment

to 2.0 and storage at 4°C in the dark was selected as the quenching technique in further kinetic studies.

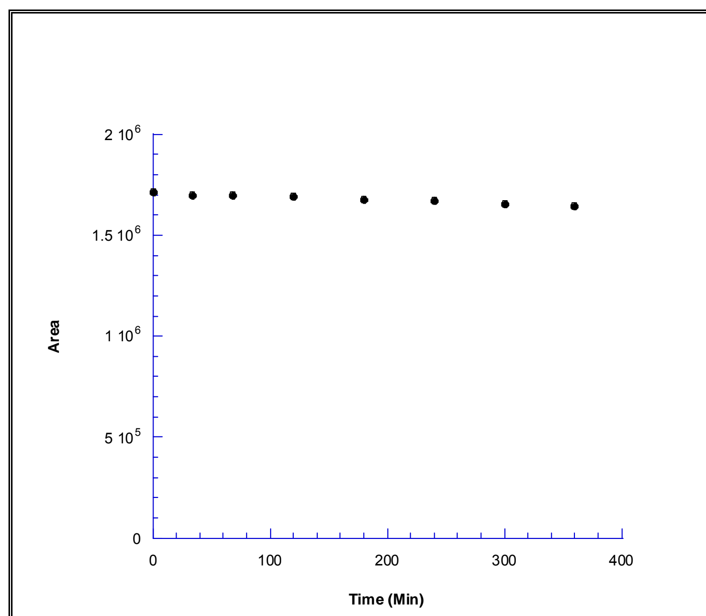


Figure 27 A peak area versus time profile of oxytetracycline in samples containing oxytetracycline -Cu^{2+} complex in 0.01 N HCl (pH 2) after exposure to UV light for 10 min and storage in dark at 4°C for 6 hours.

3.2 Reaction order determination of photolysis of oxytetracycline-divalent cation complex

This part of the studies was aimed to determine reaction order of photolysis of oxytetracycline-divalent cation complex. Reaction order was determined by 3 techniques; i.e., integrated rate equation technique, initial rate technique and fractional time technique.

3.2.1 Determination of photolysis reaction order of oxytetracycline-Mg^{2+} complex

Photolysis of oxytetracycline-Mg^{2+} complex was studied in 0.1 M Tris-hydrochloride buffer pH 7.8 under 5,880 Lux.hours of UV light in a stability chamber. At the beginning of reaction, concentrations of oxytetracycline and Mg^{2+} were 0.201 and 2.475 mM, respectively. Based on binding constant (K) of oxytetracycline and Mg^{2+}, it was precalculated that 90% of oxytetracycline was complex form while the rest was free molecule of oxytetracycline. The solution's pH values at the beginning and at the end of reaction were 7.80±0.02.

Concentration time profile of oxytetracycline showed that 92% of oxytetracycline degraded within 21 hours (Figure 28). The linear correlation of the concentration time profile indicated that degradation of oxytetracycline-Mg^{2+} complex was a zero order reaction. According to the integrated rate equation of a zero order reaction, $[C]_t = [C]_0 - kt$ (Conners, 1990), reaction rate constant (k) was estimated to be 8.93×10^{-3} mM/hour.

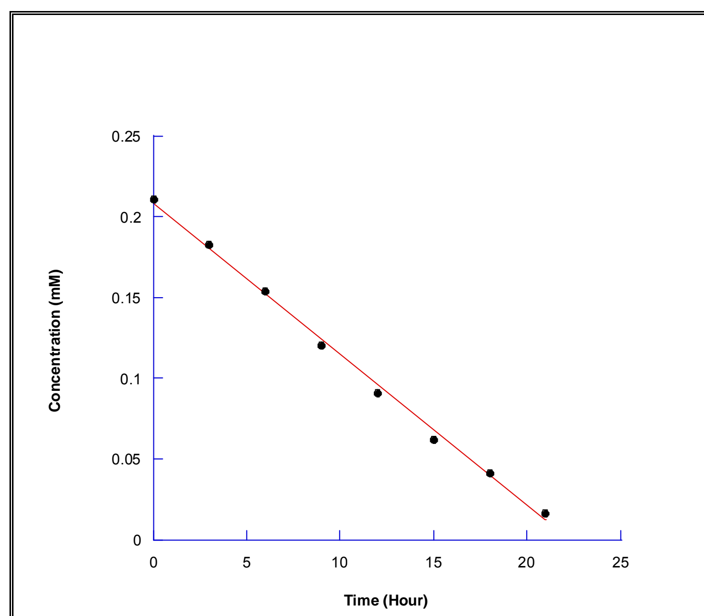


Figure 28 Concentration time profile of oxytetracycline in oxytetracycline-Mg^{2+} complex in Tris-hydrochloride buffer pH 7.80 under UV light.

Initial rate technique was employed in order to determination reaction order. Based on rate equation of a zero-order reaction,

$$\frac{d[C]}{dt} = -k$$

Initial loss rate of oxytetracycline should be constant and independent of oxytetracycline initial concentrations. In this study, initial concentration of oxytetracycline was varied in a range of 0.101-0.402 mM, while initial concentration of Mg^{2+} was 2.653 mM. Concentration time profiles of oxytetracycline from each reaction were shown in Figure 29. Initial degradation rates were estimated from slopes of each profiles where the degradation was less than 10% (Conners, 1990). A correlation between initial rates and initial concentrations with a slope around zero indicated that photolysis of oxytetracycline-Mg^{2+} complex followed zero order

reaction (Figure 30). In other words, the parallel concentration time profiles indicated that was zero order reaction.

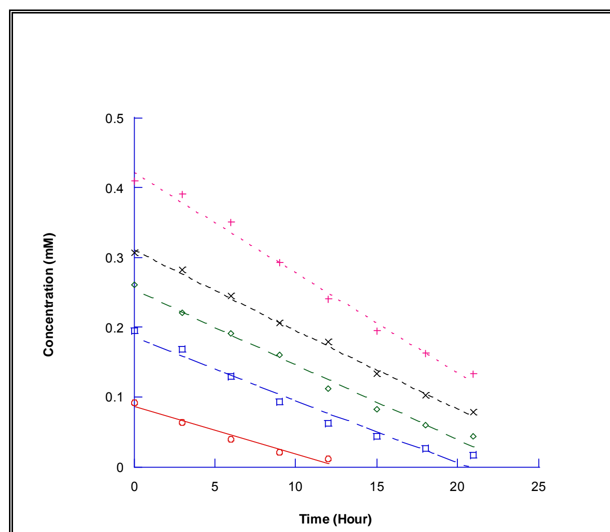


Figure 29 Concentration time profiles of oxytetracycline-Mg^{2+} complex under UV light 5,880 Lux.hours at different initial oxytetracycline concentrations; 0.101(ₒ), 0.201(□), 0.262(◊), 0.302(×), 0.402(+) mM

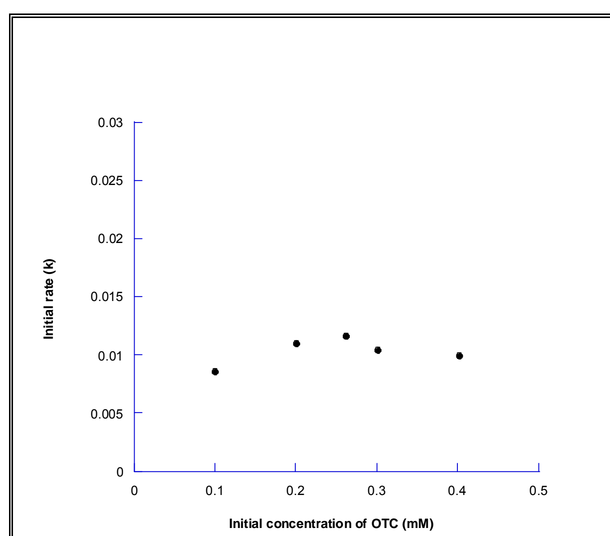


Figure 30 Correlation between initial rate of photolysis with initial concentration of oxytetracycline in oxytetracycline-Mg^{2+}complex solution

Fractional time technique was also employed in determination of reaction order. In this study, $t_{0.75}$ or time required for 25% loss was directly estimated from corresponding concentration time profiles. Then, $t_{0.75}$ value were plotted against initial concentration of oxytetracycline (Conners, 1990) (Figure 31). A linear correlation indicated that photolysis of oxytetracycline-Mg^{2+} complex followed the zero order reaction. In conclusion, photolysis reaction order of oxytetracycline-Mg^{2+} complex was determination using 3 techniques and was concluded to follow the zero order reaction.

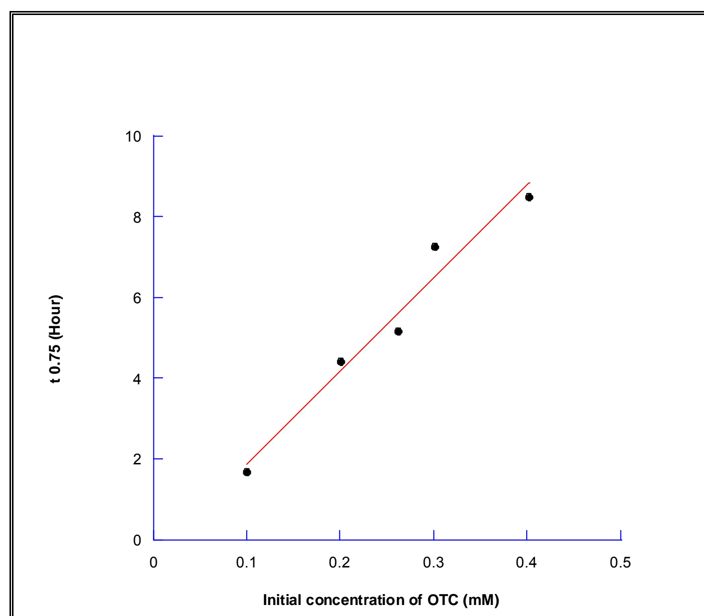


Figure 31 Correlation between initial oxytetracycline concentration (mM) with time of 75% oxytetracycline remaining ($t_{0.75}$) of oxytetracycline-Mg^{2+} complex photolysis in Tris-hydrochloride buffer pH 7.80 under UV light.

3.2.2 Determination of photolysis reaction order of oxytetracycline-Ca^{2+} complex

Photolysis of oxytetracycline-Ca^{2+} complex was studied in 0.1 M Tris-hydrochloride buffer pH 7.80 under 4,760 Lux.hours of UV light at 30°C. Initial concentrations of oxytetracycline and Ca^{2+} were 0.201 and 2.233 mM, respectively. Under such conditions, 90% of oxytetracycline was in complex form while the rest was free oxytetracycline. The solution pH's value at the beginning and at the end of reaction were constant at 7.80±0.01.

Concentration time profile of oxytetracycline showed that around 90% of oxytetracycline degraded within 17 hours (Figure 32). The observed non-linear correlation implied that photolysis of oxytetracycline-Ca^{2+} complex did not follow a zero order reaction. According to the integrated rate equation technique, the first reaction order was then assumed. The integrate rate equation for the first order reaction was derived based on the following rate equation

Rate equation : $d[C]/dt = -kC$

Integrated rate equation : $C_t = C_0e^{-kt}$ or $\ln C_t = \ln C_0 - kt$ (Conners, 1990)

The above integrate rate equation implied that a linear relationship should be observed when of remaining oxytetracycline concentration was logarithymic transformed and plotted against time. In other words, a linear relationship of first order plot should be observed from a first order reaction. In this study, first order plot of oxytetracycline-Ca^{2+} complex was shown in Figure 33. The linear correlation

indicated that the photolysis of oxytetracycline-Ca^{2+} complex followed a first order reaction with a rate constant of 0.14 $hour^{-1}$.

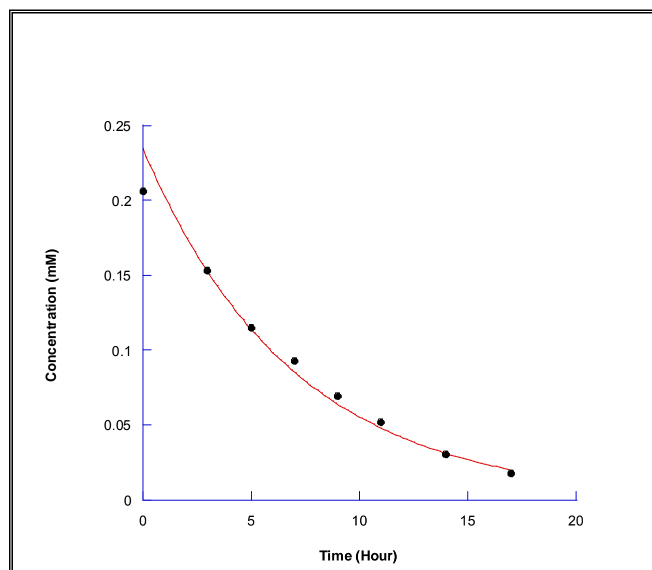


Figure 32 Concentration time profile of oxytetracycline in oxytetracycline-Ca^{2+} complex in Tris-hydrochloride buffer pH 7.80 under UV light.

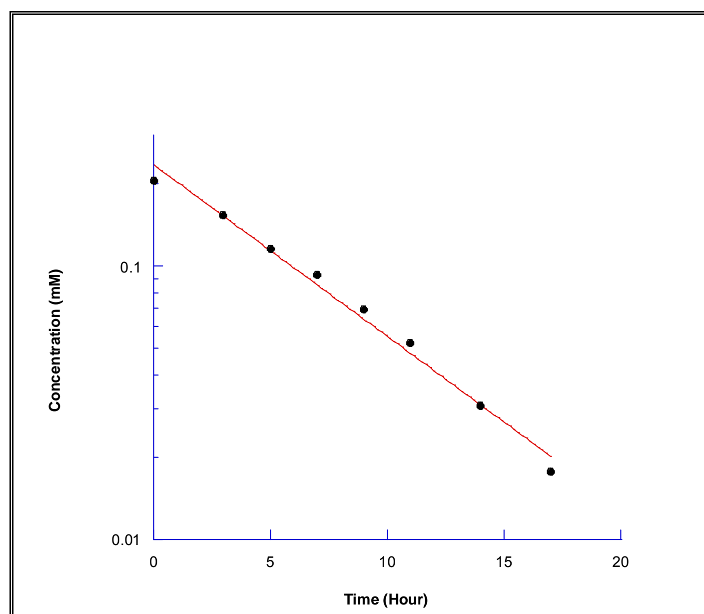


Figure 33 First order plot of oxytetracycline-Ca^{2+} conplex in Tris-hydrochloride buffer pH 7.80 under UV light.

Initial rate technique was also employed in determination of reaction order. The rate equation of a first order reaction is

$$d[C]/dt = -k[C]$$

Initial loss rate is estimated the degradation is less than 10% so that $[C]_t \approx [C]_0$. In other words, for a first order reaction, initial loss rate is linearly proportional to initial concentration and the proportional constant is the rate constant (Conners, 1990). In this study, initial concentration of oxytetracycline was varied in a range of 0.101-0.402 mM while initial concentration of Ca^{2+} was 2.410 mM. First order plots of oxytetracycline from each reaction were shown in Figure 34. Initial degradation rates were estimated from slopes of the profiles and plotted against its corresponding initial concentrations (Figure 35). A linear correlation between initial loss rate and initial concentration indicated that photolysis of oxytetracycline-Ca^{2+} complex followed first order reaction. The slope represent the first order rate constant of 0.04 $hour^{-1}$. In other words, the parallel first order plots indicated that was first order reaction.

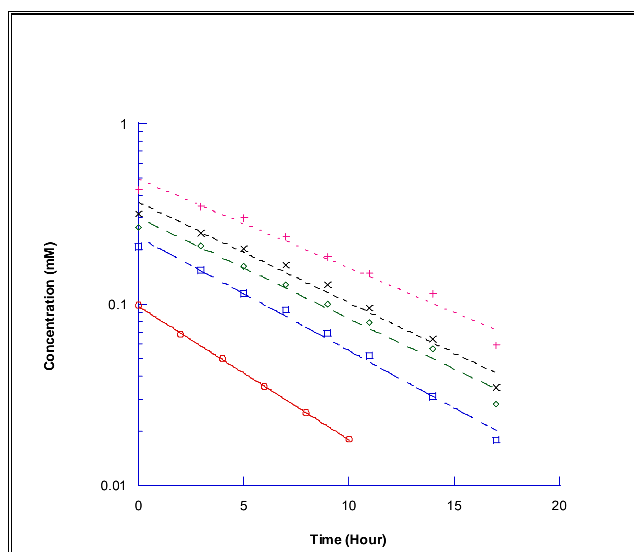


Figure 34 First order plots of oxytetracycline Ca^{2+} complex under UV light 4,760 Lux.hours at different initial oxytetracycline concentrations; 0.101(∘), 0.201(□), 0.262(◊), 0.302(×), 0.402(+) mM

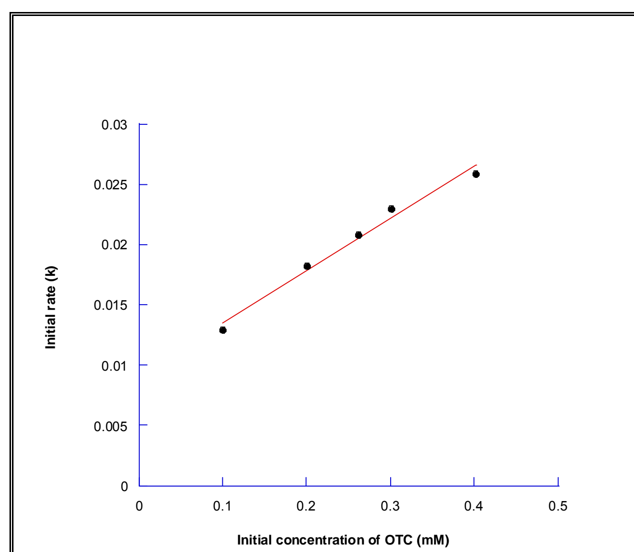


Figure 35 Correlation between initial rate of photolysis with initial concentration of oxytetracycline in oxytetracycline-Ca^{2+}complex solution.

Fractional time technique was also employed in determination of reaction order. In this study, $t_{0.75}$ or time required for 25% loss was directly estimated from corresponding concentration time profiles (Conners, 1990). Then, $t_{0.75}$ valus were plotted against initial concentrations of oxytetracycline with a slope around zero indicated that photolysis of oxytetracycline-Ca^{2+} complex followed the first order reaction (Figure 36). In conclusion, photolysis reaction order of oxytetracycline-Ca^{2+} complex was determined using 3 techniques and was concluded to follow the first order reaction.

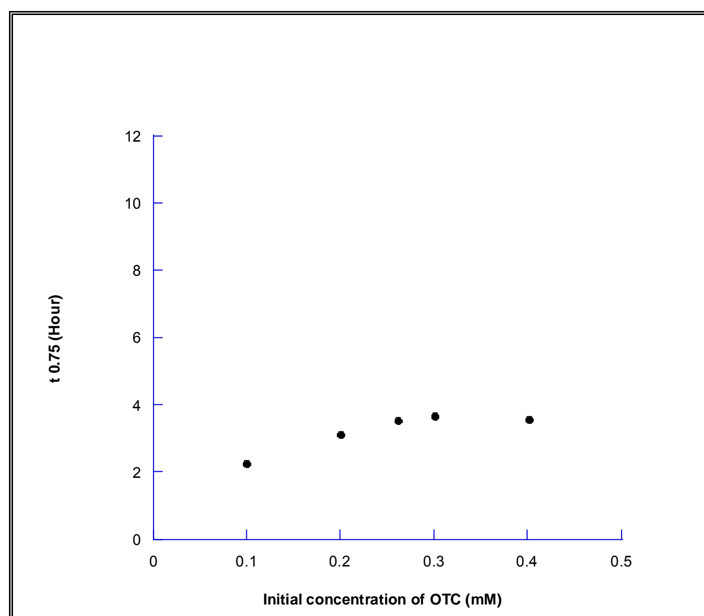


Figure 36 Correlation between initial oxytetracycline concentration (mM) with time of 75% oxytetracycline remaining ($t_{0.75}$) of oxytetracycline-Ca^{2+} complex photolysis in Tris-hydrochloride buffer pH 7.80 under UV light.

Employing integrate rate equation and initial rate techniques, first order rate constant values of oxytetracycline-Ca^{2+} complex photolysis were estimated to be 0.14 and 0.04 $hour^{-1}$, respectively. In general, rate constant obtained from initial rate

technique is more accurate than rate constant obtained from integrate rate equation technique because rate constant obtained from initial rate technique comes from more than 1 set of data. However, accurate estimation of initial rate is difficult especially when the reaction rate is very fast. In this study, photolysis of oxytetracycline-Ca^{2+} complex was quite fast and sampling time point was not frequent enough so that initial rate estimation was not accurate. Therefore, photolysis rate constant of oxytetracycline-Ca^{2+} complex was expected to be 0.14 $hour^{-1}$ rather than 0.04 $hour^{-1}$.

Photolysis of oxytetracycline-Ca^{2+} complex in phosphate buffer pH 5.85 under sunlight has been reported to follow a first order kinetic with a rate constant of 0.23 $hour^{-1}$ (Xuan et al.,2010). The rate constant estimated in this study was not consistent with the rate constant reported by Xuan et al (2010). Difference in both reported values were due to differences in buffer system, pH values, and intensity of light. This study previously showed that buffer systems had a great impact on solubility of oxytetracycline-divalent cation complexes and on its ability to maintain constant pH value which was a critical characteristic of pseudo first order conditions. In addition, ionization state of oxytetracycline was influenced by pH value. As a consequence, oxytetracycline-Ca^{2+} binding constant and effect of oxytetracycline conformational change on oxytetracycline degradation resulted in differences in observed rate constants. Xuan et al (2010) studied photolysis of oxytetracycline-Ca^{2+} complex under sunlight which mimic degradation of the complex in nature. However, light intensity of the sun is not constant throughout the day or year.

3.2.3 Determination of photolysis reaction order of oxytetracycline-Cu^{2+} complex

Photolysis of oxytetracycline-Cu^{2+} complex was studied in 0.1 M Tris-hydrochloride buffer pH 7.80 under 840 Lux.hours of UV light at 30°C. Initial concentration of oxytetracycline and Cu^{2+} were 0.201 and 8.006 mM, respectively. Under the above conditions, 90% of oxytetracycline was in complex form while the rest was free oxytetracycline. After Cu^{2+} was added in the solution, pH value of the solution was dropped. However, the solution pH value at the beginning and at the end of reaction were constant at 6.50±0.02.

Concentration time profile of oxytetracycline showed that more than 90% of oxytetracycline degraded within 3 hours (Figure 37). The observed non linear relationship of the concentration time profile indicated that photolysis was not a zero order reaction. According to the integrated rate equation of a first order reaction, first order plot was constructed (Figure 38). The observed linear relationship of the first order plot indicated that the photolysis of oxytetracycline-Cu^{2+} complex was a first order reaction with a rate constant of 0.61 hour^{-1}.

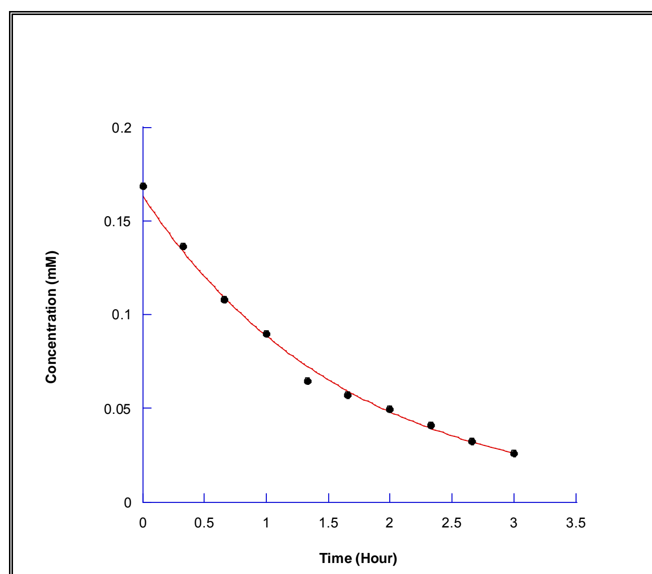


Figure 37 Concentration time profile of oxytetracycline in oxytetracycline-Cu^{2+} complex in Tris-hydrochloride buffer pH 6.50 under UV light.

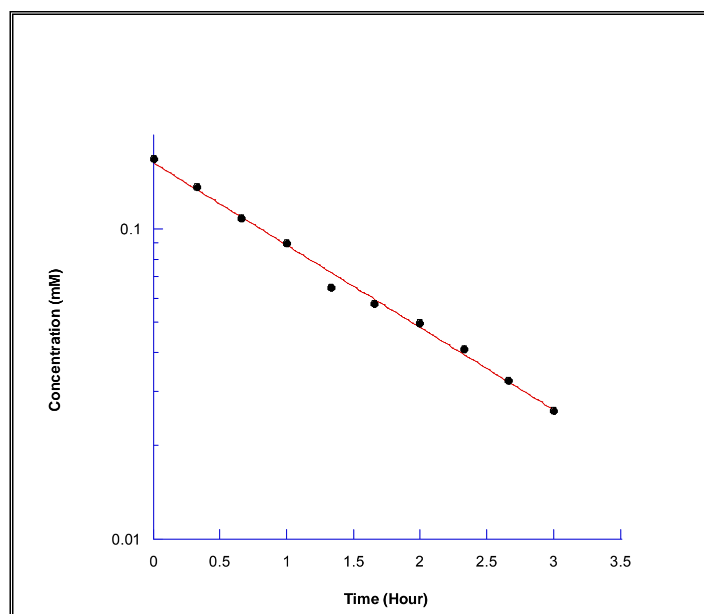


Figure 38 First order plot of oxytetracycline-Cu^{2+} complex in Tris- hydrochloride buffer pH 6.50 under UV light.

Employing initial rate technique, initial concentration of oxytetracycline was varied in a range of 0.101-0.402 mM while initial concentration of Cu^{2+} was 8.176 mM. First order plots of oxytetracycline from each reactions were shown in Figure 39. Initial degradation rates were estimated from slopes of the profiles and plotted against its corresponding initial concentrations (Figure 40). A linear relationship between initial loss rate and initial concentration indicated that photolysis of oxytetracycline-Cu^{2+} complex was a first order reaction. The slope of the plot represented the first order rate constant of 0.48 $hour^{-1}$. In other words, the parallel first order plots indicated that was first order reaction.

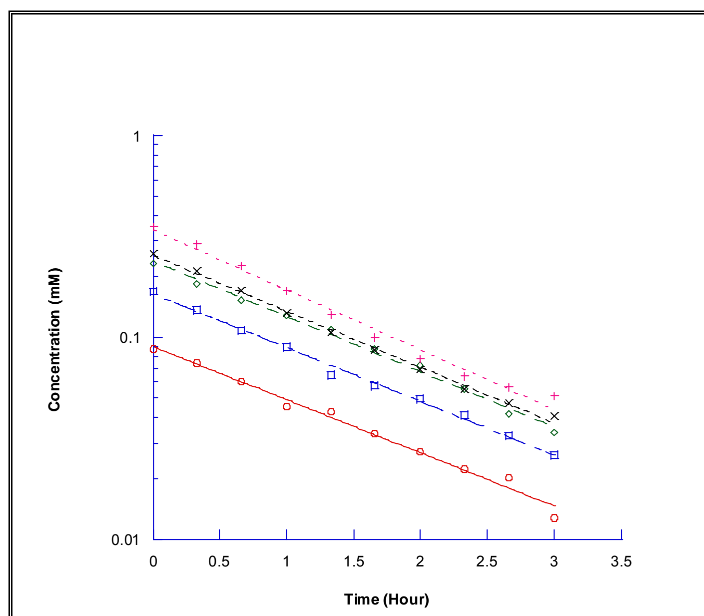


Figure 39 First order plots of oxytetracycline- Cu^{2+} complex under UV light 840 Lux.hours at different initial oxytetracycline concentrations; 0.101(∘), 0.201(□), 0.262(◊), 0.302(×), 0.402(+) mM

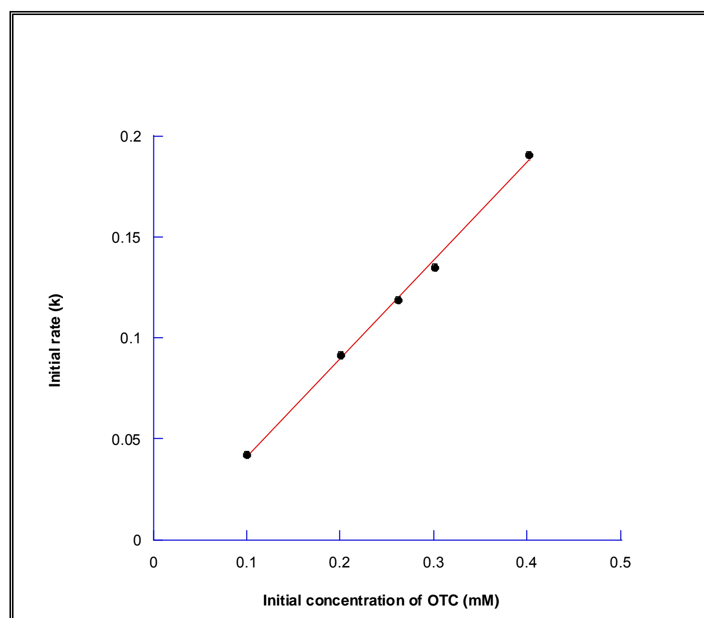


Figure 40 Correlation between initial rate of photolysis with initial concentration of oxytetracycline in oxytetracycline-Cu^{2+} complex solution

Fractional time technique was also employed in determination of reaction order. In this study, $t_{0.75}$ or time required for 25% loss was directly estimated from corresponding concentration time profiles (Conners, 1990). Then, $t_{0.75}$ value were plotted against initial concentration of oxytetracycline with a slope around zero indicated that photolysis of oxytetracycline-Cu^{2+} complex followed the first order reaction (Figure 41). In conclusion, photolysis reaction order of oxytetracycline-Cu^{2+} complex was determination using 3 techniques and was concluded to follow the first order reaction.

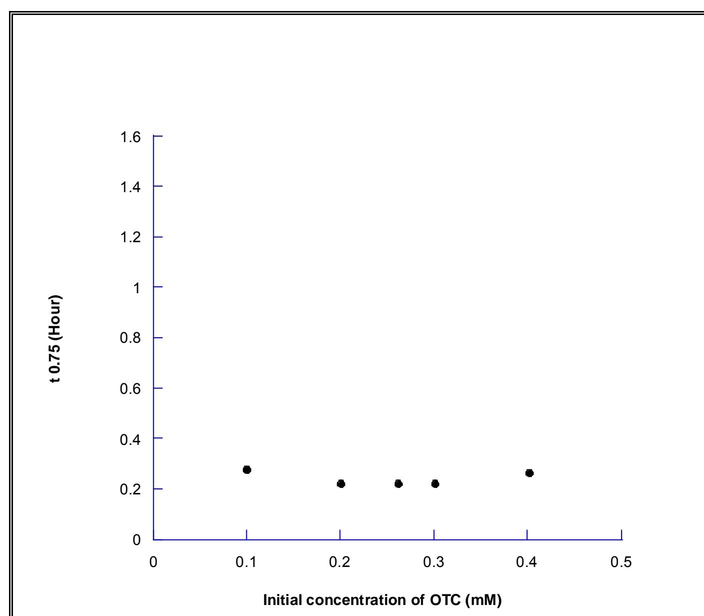


Figure 41 Correlation between initial oxytetracycline concentration (mM) with time of 75% oxytetracycline remaining ($t_{0.75}$) of oxytetracycline-Cu^{2+} complex photolysis in Tris-hydrochloride buffer pH 6.50 under UV light.

Overall, photolysis of oxytetracycline-Mg^{2+} complex was a zero order reaction while photolysis of oxytetracycline-Ca^{2+} or oxytetracycline-Cu^{2+} complex was a first order reaction. Explaination of these surprising results is unclear. Oxytetracycline-Mg^{2+} and oxytetracycline-Ca^{2+} complexes were shown to undergo the same photolysis pathway due to the fact that these cations were believed to bind and form complex with oxytetracycline at the same position giving rise to the 4-epi-N-desmethyl-oxytetracycline (E-N-DM-OTC), a common photodegradation product. However, the photolysis of oxytetracycline-Mg^{2+} complex was a zero order reaction but the photolysis of oxytetracycline-Ca^{2+} complex was a first order reaction. Oxytetracycline-Cu^{2+} complex was believed to undergo a different photolysis pathway due to the fact that Cu^{2+} ion was believed to bind to oxytetracycline at

different position resulting in difference in fluorescent characteristic of the complex and fast photolysis degradation rate.

However, photolysis of oxytetracycline-Ca^{2+} and oxytetracycline Cu^{2+} complexes accidentally was first order reactions. In addition, photolysis degraded products of oxytetracycline-Cu^{2+} complex were not detectable using a 254 nm analytical wavelength. Other analytical techniques should be developed in order to detect the degradation products. Since chemical structures of the degradation products were keys to elucidate reaction pathway.

3.3 Ionic strength effects on photolysis of oxytetracycline-divalent cation complex

Ionic strength effects on photolysis of oxytetracycline-Mg^{2+} complex

Ionic strength effect on photolysis of oxytetracycline-Mg^{2+}complex was studied by starting the reaction with 0.201 mM oxytetracycline and 2.475 mM $MgCl_2$ in 0.1 M Tris-hydrochloride buffer pH 7.80 and varying the ionic strength of the system in the range of 0.04 to 0.6 M. This study aimed to investigate effects of ionic strength concentration on photolysis rate constant of oxytetracycline-divalent cation complex. Concentration time profiles of oxytetracycline-Mg^{2+}complex under 5,040 Lux.hours of UV light was shown in Figure 42. Each profiles illustrated that oxytetracycline concentration was linearly decreased overtime consistent with the previous conclusion that photolysis of oxytetracycline-Mg^{2+} complex followed zero-order reaction. Degradation rate constants were obtained from slopes of the concentration time profiles. As ionic strength was varied from 0.04, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.4, 0.6 M, rate constant of photolysis of oxytetracycline-Mg^{2+} were

slowly increased from 0.009035, 0.009673, 0.009761, 0.009833, 0.010211, 0.010427, 0.010584, 0.012004, 0.011027 mM/hour, respectively. In this study, pH value of the sample solution was constant at 7.80±0.02 throughout the experiment course.

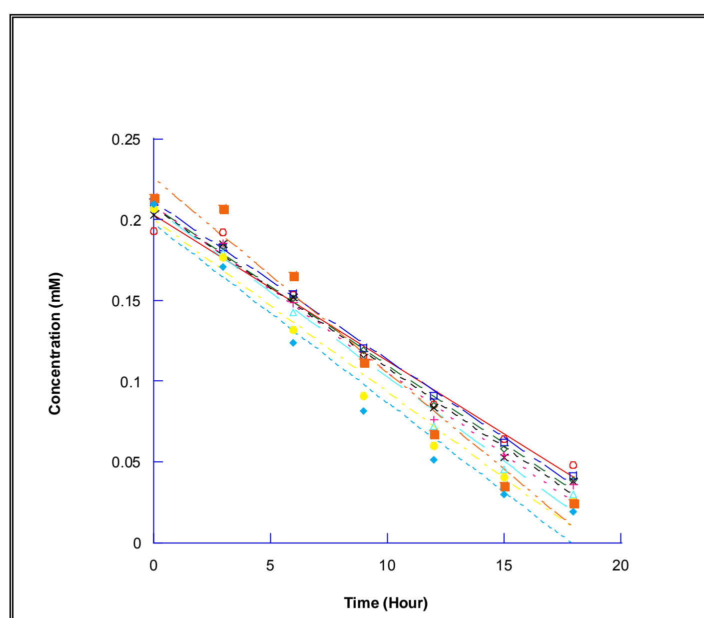


Figure 42 Concentration time profiles of oxytetracycline-Mg^{2+}complex under 5,040 Lux.hours of UV light at different ionic strength; 0.04 (ₒ), 0.06 (□), 0.07 (◊), 0.08 (×), 0.09 (+), 0.1 (Δ), 0.2 (●), 0.4 (■), 0.6 (♦) M

In general, when ionic strength is in a range of 0-0.1 M, ionic strength effects on degradation rate constant is derived from Guntelburg's equation (Carstensen, 2000).

Guntelburg equation : $\log \gamma = -AZ^2 \frac{\sqrt{u}}{1+\sqrt{u}}$ Equation (4)

Where γ is activity coefficient

A is a constant of 0.51

Z is charge of a specific ion

u is ionic strength

Degradation rate constant affected by ionic strength is

$$\log k = \log k^{o} + 2Z_A Z_B A \frac{\sqrt{u}}{1+\sqrt{u}} \qquad \text{Equation (5)}$$

Where k is first-order rate constant

k^{o} is first-order rate constant when ionic strength approached zero

Z_A is charge of substrate

Z_B is charge of reactant

Guntelburg's equation developed based on an assumption that ion size is very small so that there is no steric hindrance effect or fiction from ion movement. Guntelburg's equation can accurately calculate activity coefficient up to 0.1 M ionic strength, equation (5) is expected to describe data up to the limitation of Guntelburg's equation.

In case of oxytetracycline-Mg^{2+}complex, the reaction was a zero-order reaction. Although the reaction appeared to show ionic strength effect, equation (5) could not be used to explain the ionic strength effect.

Ionic strength effects on photolysis of oxytetracycline-Ca^{2+} complex

Ionic strength effect on photolysis of oxytetracycline-Ca^{2+}complex was investigated by mixing 0.201 mM oxytetracycline with 2.233 mM $CaCl_2$ in 0.1 M Tris-hydrochloride buffer pH 7.80 and varying ionic strength of the samples in a range of 0.04 to 0.6 M. Concentration time profiles of oxytetracycline-Ca^{2+}complex under 3,920 Lux.hours of UV light were shown in Figure 43. In this study, pH value

of the sample solution was constant at 7.80±0.01 throughout the experiment course. Exponential loss of oxytetracycline was consistent with previous results that photolysis of oxytetracycline-Ca^{2+} complex was the first order reaction. First order rate constants were estimated by fitting data with a first order integrated rate equation.

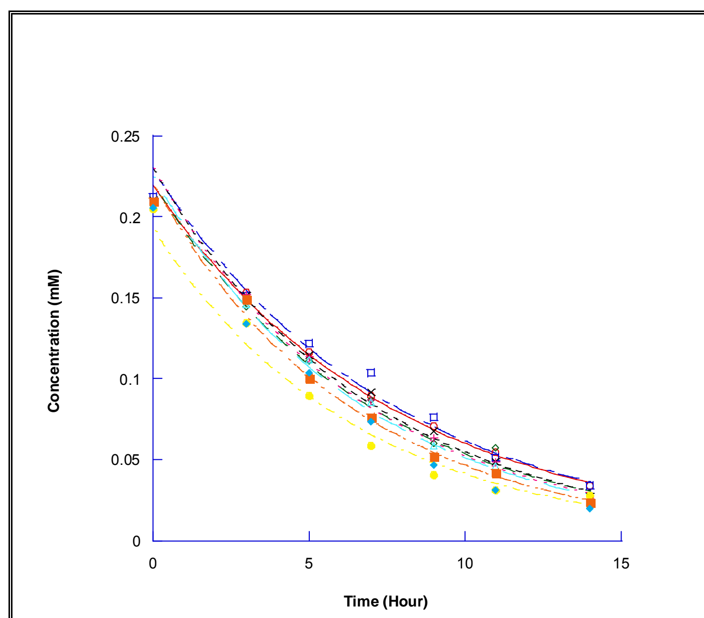


Figure 43 Concentration time profiles of oxytetracycline-Ca^{2+}complex under UV light 3,920 Lux.hours at different ionic strength; 0.04 (₀), 0.06 (□), 0.07 (◊), 0.08 (×), 0.09 (+), 0.1 (Δ), 0.2 (●), 0.4 (■), 0.6 (♦) M

According to equation (5), a plot of log k against $\frac{\sqrt{u}}{1+\sqrt{u}}$ should give a linear relationship with a slope which is a product of $2Z_AZ_BA$. In this study, photolysis of oxytetracycline-Ca^{2+} complex at various ionic strengths showed a linear increase of log k versus $\frac{\sqrt{u}}{1+\sqrt{u}}$ up to ionic strength of 0.1 M with a slope of around 1.043 (Figure 44). The plot showed a negative deviation when ionic strength was higher than 0.1 M.

The observed negative deviation was due to limitation of Guntelburg's equation that could accurately estimate activity coefficient of ions up to 0.1 M. The deviation of a slope value from its theoretical value was expected to be due to violation of Guntelburg assumption. Size of oxytetracycline-Ca^{2+} complex is not a point charge since oxytetracycline itself has more than one ionizable group. Therefore, other ionized group and chemical structure of oxytetracycline had direct influence on photolysis of oxytetracycline-Ca^{2+} complex.

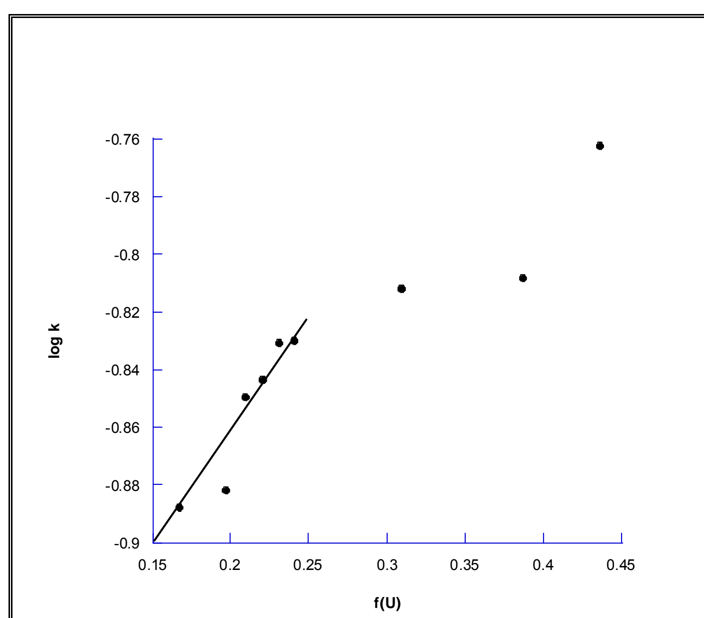


Figure 44 Ionic strength effect of oxytetracycline-Ca^{2+}complex under 3,920 Lux.hours of UV light.

Ionic strength effects on photolysis of oxytetracycline-Cu^{2+} complex

Ionic strength effect on photolysis of oxytetracycline-Cu^{2+}complex was investigated by mixing 0.201 mM oxytetracycline with 8.006 mM $CuSO_4$ in 0.1 M Tris-hydrochloride buffer pH 6.50 and varying ionic strength of the samples in a

range of 0.07 to 0.6 M. Concentration time profiles of oxytetracycline-Cu^{2+}complex after stress under 840 Lux.hours of UV light were shown in Figure 45. pH values of each samples were monitored to be constant at 6.50±0.02 throughout the experiment course. Exponential loss of oxytetracycline was consistent with the previous results that photolysis of oxytetracycline-Cu^{2+} complex was the first order reaction. First order rate constants were estimated as previously described and plotted against ionic strength of the systems (Figure 46).

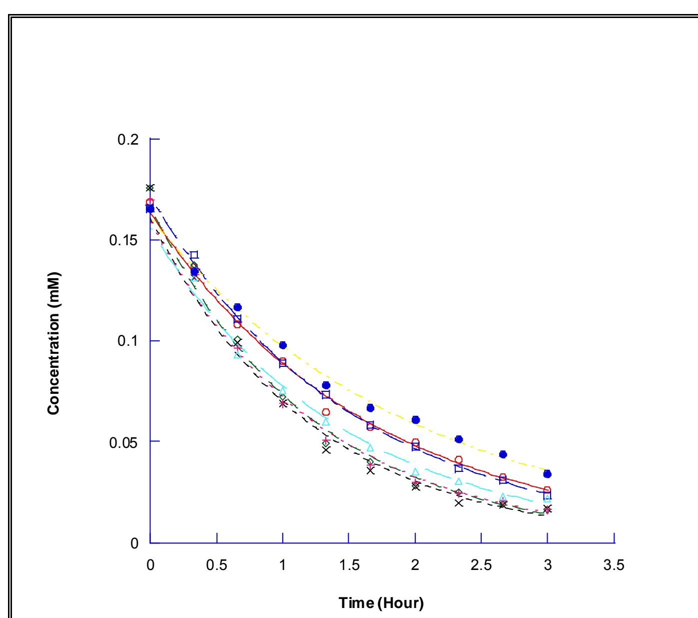


Figure 45 Concentration time profiles of oxytetracycline-Cu^{2+}complex under UV light 840 Lux.hours at different ionic strength; 0.07 (ₒ), 0.08 (□), 0.09 (◊), 0.1 (×), 0.2 (+), 0.4 (Δ), 0.6 (●) M.

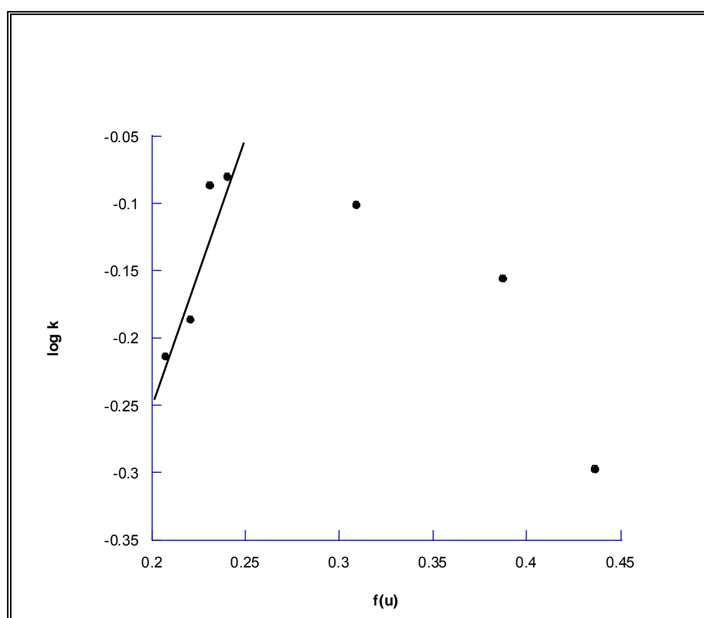


Figure 46 Ionic strength effect of oxytetracycline-Cu^{2+}complex under 840 Lux.hours of UV light.

Photolysis of oxytetracycline-Cu^{2+}complex showed a linear increase of log k with respect to $\frac{\sqrt{u}}{1+\sqrt{u}}$ up to ionic strength of 0.1 M with a slope around 1.234. The negative deviation of the profile at higher ionic strength and observed lower extent of ionic strength effects were expected to be due to limitation and violation of Guntelburg equation.

Overall, photolysis of oxytetracycline-divalent cation complex was expected to have ionic strength effect since the complex possessed charges. At low concentration of salt, photolysis rate constants were linearly increased as ionic strength increased. However, a negative deviation of photolysis rate constants at high ionic strength illustrated difficulty of oxytetracycline-divalent cation complexes to undergo photodegradation in nature such as seawater (μ=0.6 M).

Finally, photolysis of oxytetracycline free molecule and oxytetracycline-divalent cation complex in 0.1 M Tris-hydrochloride buffer pH 7.80 with an ionic strength of 0.07 M was showed in Figure 47. Photolysis rate constant of oxytetracycline free molecule, oxytetracycline-Mg^{2+} complex, oxytetracycline-Ca^{2+} complex, and oxytetracycline-Cu^{2+} complex to be 0.123 $hour^{-1}$, $8.93x10^{-3}$ mM/hour, 0.14 $hour^{-1}$, and 0.48 $hour^{-1}$, respectively. Concentration time profile and rate constant indicated that photolysis of oxytetracycline-Ca^{2+} and oxytetracycline-Cu^{2+} complex followed first order reaction with degradation faster rate than oxytetracycline free molecule. While, photolysis of oxytetracycline-Mg^{2+} complex followed zero order reaction with degradation less than oxytetracycline free molecule. Thus, the oxytetracycline-Ca^{2+} and oxytetracycline-Cu^{2+} complex can be used as a new mean of accelerating oxytetracycline degradation in the environment, with a goal to reduce oxytetracycline contamination in surface water. While, photolysis of oxytetracycline-Mg^{2+} complex occurred at a slower rate. Therefore, drainage waste water contaminated with oxytetracycline to a sea resulted in contamination of oxytetracycline in the environment for a longer time because high Mg^{2+} content in seawater can form complex with the oxytetracycline and retard photolysis of oxytetracycline.

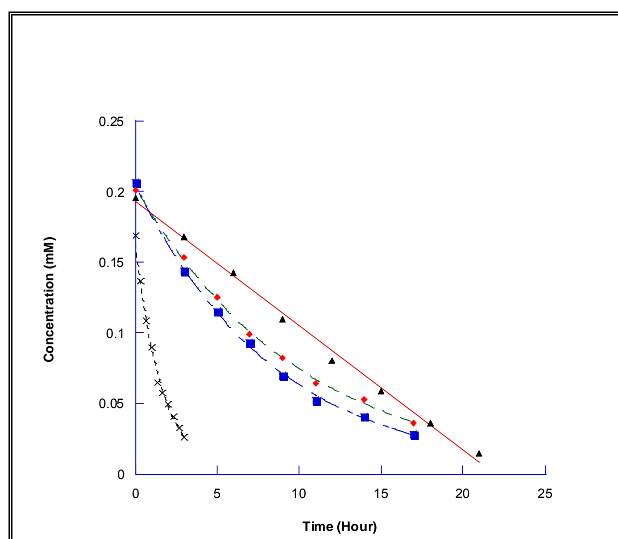


Figure 47 Concentration time profiles of photolysis of oxytetracycline free molecule (♦), oxyteratacycline-Mg^{2+} complex (▲), oxytetracycline-Ca^{2+} complex (■), and oxytetracycline-Cu^{2+} complex (×).

Future studies and suggestions

Further information are needed such as photolysis degradation pathway of oxytetracycline-Cu^{2+} complex, structure elucidation and toxicity of its degradation products. Binding site of Cu^{2+} to oxytetracycline and chemical structure of its degraded products should be identified using techniques such as NMR, MS. These information can be used to clarify photolysis pathway of oxytetracycline-Cu^{2+} complex. A preliminary study of oxytetracycline-Cu^{2+} complex photodegradation in seawater should be conducted in order to evaluate possibility of employing oxytetracycline-Cu^{2+} complex as a new technique to increase of oxytetracycline degradation. Furthermore, toxicity of its degradation products to environment is needed to evaluate before employing Cu^{2+} ion as a complexing agent in accelelation of oxytetracycline degradation.

CHAPTER V

CONCLUSION

In this study, the complex formation of oxytetracycline with three different types of divalent cations, i.e. Mg^{2+},Ca^{2+}, and Cu^{2+}, in either Tris-hydrochloride buffer pH 7.80 or phosphate buffer pH 7.80 was studied using a spectrofluorometer. Tris-hydrochloride buffer was chosen to use as a solvent for further studies because of its higher buffer capacity so that constant pH value of the systems could be maintained. The binding constants (K) of oxytetracycline with Mg^{2+},Ca^{2+}, and Cu^{2+} ions in Tris-hydrochloride buffer pH 7.80 were calculated to be 3,921.8 , 4,386.8 , and 1,149.9 M^{-1} respectively.

Photolysis of oxytetracycline-Ca^{2+} and oxytetracycline-Cu^{2+} complex was first order reaction. The complexes underwent photolysis at a faster rate than that of free molecules. While, photolysis of oxytetracycline-Mg^{2+} complex was a zero order reaction with a lower degradation rate than that of oxytetracycline free molecule. Thus, photolysis of oxytetracycline-Ca^{2+} and oxytetracycline-Cu^{2+} complex can be used as new means of accelerating oxytetracycline degradation in the environment, with a goal to reduce oxytetracycline contamination in surface water.

Ionic strength has a direct impact on photolysis of oxytetracycline-Ca^{2+} and oxytetracycline-Cu^{2+} complexes. This finding was consistent with the theory. However, negative deviation of ionic strength effects were observed due to limitation and violation of Gultelberg equation. While, rate constants of photolysis of oxytetracycline-Mg^{2+} complex were slowly increased with ionic strength. The

reaction was a zero order reaction. Gultelberg equation could not be used to explain the ionic strength effect.

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APPENDIX

APPENDIX

Table 2 Fluorescence intensities of oxytetracycline at various concentration in phosphate buffer pH 7.80. Excitation and emission wavelength of 390 and 510 nm, respectively

Concentration of oxytetracycline (M)	Fluorescence intensity
$1.00x10^{-6}$	160.8
$5.00x10^{-6}$	209.9
$1.00x10^{-5}$	283.4
$5.00x10^{-5}$	692.6
$8.00x10^{-5}$	813.2
$1.00x10^{-4}$	888.9
$3.00x10^{-4}$	785.7
$5.00x10^{-4}$	598.6
$8.00x10^{-4}$	449.5
$1.00x10^{-3}$	388.9

Table 3 Fluorescence intensities of oxytetracycline in the presence of Mg^{2+} in Tris-hydrochloride buffer pH 7.80. Excitation and emission wavelength of 390 and 510 nm, respectively

Concentration of Mg^{2+} (M)	Fluorescence intensity	
	Run1	Run2
0.00	519.9	566.4
$3.00x10^{-5}$	1078.0	1131.0
$6.00x10^{-5}$	1520.0	1590.0
$3.00x10^{-4}$	2899.0	2891.0
$6.00x10^{-4}$	3396.0	3324.0
$1.20x10^{-3}$	3798.0	3743.0
$1.80x10^{-3}$	4058.0	3968.0
$2.40x10^{-3}$	4163.0	4122.0
$3.00x10^{-3}$	4324.0	4176.0
$3.60x10^{-3}$	4415.0	4316.0
$4.20x10^{-3}$	4427.0	4376.0
$4.80x10^{-3}$	4462.0	4392.0
$6.00x10^{-3}$	4553.0	4451.0
0.012	4676.0	4588.0
0.018	4650.0	4614.0

Table 4 Fluorescence intensities of oxytetracycline in the presence of Ca^{2+} in Tris-hydrochloride buffer pH 7.80. Excitation and emission wavelength of 390 and 510 nm, respectively

Concentration of Ca^{2+} (M)	Fluorescence intensity	
	Run1	Run2
0.00	528.0	621.1
$3.00x10^{-5}$	651.9	691.6
$6.00x10^{-5}$	762.8	807.6
$3.00x10^{-4}$	1590.0	1631.0
$6.00x10^{-4}$	1995.0	2076.0
$1.20x10^{-3}$	2255.0	N/A
$1.80x10^{-3}$	2305.0	2405.0
$2.40x10^{-3}$	2318.0	N/A
$3.00x10^{-3}$	2329.0	2443.0
$3.60x10^{-3}$	2368.0	N/A
$4.20x10^{-3}$	2364.0	N/A
$4.80x10^{-3}$	2339.0	2437.0
$5.40x10^{-3}$	2316.0	N/A
$6.00x10^{-3}$	2314.0	2394.0

Table 5 Fluorescence intensities of oxytetracycline in the presence of Cu^{2+} in Tris-hydrochloride buffer pH 7.80. Excitation and emission wavelength of 390 and 510 nm, respectively

Concentration of Cu^{2+} (M)	Fluorescence intensity	
	Run1	Run2
0.00	504.8	514.2
3.00×10^{-5}	455.3	453.0
6.00×10^{-5}	430.7	425.1
3.00×10^{-4}	278.6	275.4
6.00×10^{-4}	216.5	221.1
1.20×10^{-3}	163.9	N/A
1.50×10^{-3}	167.4	N/A
1.80×10^{-3}	144.3	127.6

Table 6 Peak area of oxytetracycline in samples containing oxytetracycline-Cu^{2+}complex in 0.1 M Tris-hydrochloride buffer after exposure to UV light for 10 min and storage in the dark at 4°C for 6 hours

Time (Min)	Area	Time (Min)	Area
0	1217155	180	866331
32	1058320	240	851199
65	978824	300	825265
120	934230	360	770754

Table 7 Peak area of oxytetracycline in samples containing oxytetracycline-Cu^{2+} complex in 0.01 N HCl pH 2 after exposure to UV light for 10 min and storage in the dark at 4°C for 6 hours

Time (Min)	Area	Time (Min)	Area
0	1712030	180	1674730
34	1698301	240	1668683
68	1695461	300	1652930
120	1694408	360	1646623

Table 8 Remaining concentration of oxytetracycline after exposure of oxytetracycline-Mg^{2+}complex to UV light up to 5,880 Lux.hours when initial concentration was varied from 0.101 to 0.402 mM

Time (hr)	Lux.hr	Concentration of oxytetracycline (mM)				
0.0	0.0	0.092	0.196	0.261	0.307	0.409
3.0	840	0.065	0.168	0.221	0.282	0.391
6.0	1680	0.041	0.129	0.191	0.245	0.350
9.0	2520	0.020	0.093	0.159	0.206	0.294
12.0	3360	0.012	0.063	0.112	0.179	0.241
15.0	4200	N/A	0.044	0.083	0.134	0.195
18.0	5040	N/A	0.026	0.059	0.102	0.163
21.0	5880	N/A	0.017	0.044	0.078	0.132

Table 9 Remaining concentration of oxytetracycline after exposure of oxytetracycline-Ca^{2+}complex to UV light up to 4,760 Lux.hours when initial concentration was varied from 0.101 to 0.402 mM

Time (hr)	Lux.hr	Concentration of oxytetracycline (mM)				
0.0	0.0	0.098	0.206	0.266	0.317	0.430
2.0	560	0.067	N/A	N/A	N/A	N/A
3.0	840	N/A	0.154	0.210	0.245	0.347
4.0	1120	0.047	N/A	N/A	N/A	N/A
5.0	1400	N/A	0.115	0.161	0.202	0.302
6.0	1680	0.028	N/A	N/A	N/A	N/A
7.0	1960	N/A	0.093	0.127	0.164	0.239
8.0	2240	0.022	N/A	N/A	N/A	N/A
9.0	2520	N/A	0.069	0.100	0.129	0.183
10.0	2800	0.013	N/A	N/A	N/A	N/A
11.0	3080	N/A	0.052	0.079	0.095	0.149
14.0	3920	N/A	0.031	0.056	0.065	0.114
17.0	4760	N/A	0.018	0.028	0.035	0.060

Table 10 Remaining concentration of oxytetracycline after exposure of oxytetracycline-Cu^{2+}complex to UV light up to 840 Lux.hours when initial concentration was varied from 0.101 to 0.402 mM

Time (hr)	Lux.hr	Concentration of oxytetracycline (mM)				
0.00	0.00	0.088	0.169	0.231	0.259	0.351
0.33	92.40	0.075	0.137	0.184	0.214	0.291
0.66	184.80	0.060	0.108	0.153	0.169	0.225
1.00	280	0.045	0.089	0.129	0.132	0.169
1.33	372.40	0.043	0.065	0.109	0.105	0.129
1.66	464.80	0.033	0.057	0.088	0.086	0.100
2.00	560	0.027	0.049	0.073	0.069	0.078
2.33	652.40	0.022	0.041	0.056	0.055	0.065
2.66	744.80	0.020	0.032	0.042	0.047	0.057
3.00	840	0.013	0.026	0.034	0.041	0.052

Table 11 Remaining concentration of oxytetracycline after exposure of oxytetracycline free molecule to UV light up to 4,760 Lux.hours when initial concentration was 0.201 mM

Time (hr)	Lux.hr	Concentration of oxytetracycline (mM)
0.0	0	0.205
3.0	840	0.174
5.0	1400	0.125
7.0	1960	0.099
9.0	2520	0.071
11.0	3080	0.064
14.0	3920	0.043
17.0	4760	0.026

Table 12 Remaining concentration of oxytetracycline after exposure of 0.201 mM oxytetracycline-Mg^{2+}complex to UV light up to 5,040 Lux.hours at ionic strength of 0.04, 0.06, 0.07, 0.08, and 0.09 M

Time (hr)	Lux.hr	Ionic strength (M)				
		0.04	**0.06**	**0.07**	**0.08**	**0.09**
0.0	0.0	0.193	0.211	0.207	0.203	0.209
3.0	840	0.192	0.183	0.184	0.184	0.186
6.0	1680	0.153	0.154	0.151	0.152	0.148
9.0	2520	0.116	0.120	0.119	0.114	0.112
12.0	3360	0.086	0.091	0.085	0.083	0.076
15.0	4200	0.064	0.062	0.058	0.053	0.054
18.0	5040	0.048	0.041	0.039	0.038	0.036

Table 13 Remaining concentration of oxytetracycline after exposure of 0.201 mM oxytetracycline-Mg^{2+}complex to UV light up to 5,040 Lux.hours at ionic strength of 0.1, 0.2, 0.4, and 0.6 M

Time (hr)	Lux.hr	Ionic strength (M)			
		0.1	0.2	0.4	0.6
0.0	0.0	0.209	0.207	0.213	0.209
3.0	840	0.179	0.177	0.207	0.171
6.0	1680	0.142	0.132	0.165	0.123
9.0	2520	0.112	0.091	0.112	0.082
12.0	3360	0.072	0.060	0.068	0.052
15.0	4200	0.045	0.041	0.035	0.029
18.0	5040	0.030	0.025	0.024	0.019

Table 14 Remaining concentration of oxytetracycline after exposure of 0.201 mM oxytetracycline-Ca^{2+}complex to UV light up to 3,920 Lux.hours at ionic strength of 0.04, 0.06, 0.07, 0.08, and 0.09 M

Time (hr)	Lux.hr	Ionic strength (M)				
		0.04	**0.06**	**0.07**	**0.08**	**0.09**
0.0	0.0	0.208	0.212	0.205	0.210	0.208
3.0	840	0.153	0.152	0.145	0.151	0.152
5.0	1400	0.117	0.122	0.111	0.114	0.114
7.0	1960	0.089	0.104	0.087	0.091	0.087
9.0	2520	0.071	0.076	0.060	0.068	0.064
11.0	3080	0.055	0.052	0.058	0.049	0.049
14.0	3920	0.034	0.034	0.025	0.028	0.026

Table 15 Remaining concentration of oxytetracycline after exposure of 0.201 mM oxytetracycline-Ca^{2+}complex to UV light up to 3,920 Lux.hours at ionic strength of 0.1, 0.2, 0.4, and 0.6 M

Time (hr)	Lux.hr	Ionic strength (M)			
		0.1	**0.2**	**0.4**	**0.6**
0.0	0.0	0.209	0.205	0.209	0.206
3.0	840	0.146	0.134	0.149	0.134
5.0	1400	0.112	0.089	0.100	0.103
7.0	1960	0.085	0.059	0.076	0.073
9.0	2520	0.059	0.041	0.052	0.046
11.0	3080	0.046	0.031	0.042	0.031
14.0	3920	0.026	0.028	0.024	0.019

Table 16 Remaining concentration of oxytetracycline after exposure of 0.201 mM oxytetracycline-Cu^{2+}complex to UV light up to 840 Lux.hours at ionic strength of 0.07, 0.08, 0.09, and 0.1 M

Time (hr)	Lux.hr	Ionic strength (M)			
		0.07	**0.08**	**0.09**	**0.1**
0.00	0.00	0.169	0.166	0.176	0.176
0.33	92.40	0.137	0.142	0.138	0.131
0.66	184.80	0.108	0.111	0.100	0.099
1.00	280	0.089	0.089	0.072	0.069
1.33	372.40	0.065	0.073	0.049	0.046
1.66	464.80	0.057	0.058	0.040	0.036
2.00	560	0.049	0.048	0.028	0.027
2.33	652.40	0.041	0.037	0.025	0.019
2.66	744.80	0.032	0.031	0.019	0.019
3.00	840	0.026	0.024	0.017	0.017

Table 17 Remaining concentration of oxytetracycline after exposure of 0.201 mM oxytetracycline-Cu^{2+}complex to UV light up to 840 Lux.hr at ionic strength of 0.07, 0.08, 0.09, and 0.1 M

Time (Hr)	Lux.hr	Ionic strength (M)		
		0.2	**0.4**	**0.6**
0.00	0.00	0.169	0.166	0.165
0.33	92.40	0.133	0.131	0.134
0.66	184.80	0.096	0.093	0.117
1.00	280	0.069	0.076	0.098
1.33	372.40	0.051	0.060	0.078
1.66	464.80	0.038	0.047	0.067
2.00	560	0.029	0.035	0.061
2.33	652.40	0.025	0.031	0.051
2.66	744.80	0.021	0.023	0.044
3.00	840	0.017	0.022	0.034

VITA

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