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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต
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PHARMACOKINETIC/PHARMACODYNAMIC APPROACH TO EVALUATE ANTIMICROBIAL ACTIVITY OF AZITHROMYCIN

Mr. Wanchai Treyaprasert

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmaceutics
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Thesis Title	PHARMACOKINETIC/PHARMACODYNAMIC APPROACH TO EVALUATE ANTIMICROBIAL ACTIVITY OF AZITHROMYCIN
By	Mr. Wanchai Treyaprasert
Field of Study	Pharmaceutics
Thesis Advisor	Associate Professor Uthai Suvanakoot, Ph.D.
Thesis Co-Advisor	Professor Hartmut Derendorf, Ph.D.
Thesis Co-Advisor	Associate Professor Sumana Chompootaweep, M.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pornpen Pramyothin, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Porntip Nimmannitya, M.Sc. in Pharm.)

..... Thesis Advisor

(Associate Professor Uthai Suvanakoot, Ph.D.)

..... Thesis Co-Advisor

(Associate Professor Sumana Chompootaweep, M.D.)

.....Member

(Associate Professor Busba Chindavijak, Ph.D.)

..... Member

(Associate Professor Vimolmas Lipipun, Ph.D.)

..... Member

(Associate Professor Parkpoom Tengamnuay, Ph.D.)

วันชัย ตรียะประเสริฐ: วิธีทางเภสัชจลนศาสตร์/เภสัชพลศาสตร์ ที่ใช้ประเมินฤทธิ์ต้านจุลชีพของอะซิโทรมัยซิน (PHARMACOKINETIC/PHARMACODYNAMIC APPROACH TO EVALUATE ANTIMICROBIAL ACTIVITY OF AZITHROMYCIN) อ.ที่ปรึกษา: รศ.ดร. อุทัย สุวรรณกูฏ, อ.ที่ปรึกษาร่วม: Professor Hartmut Derendorf, อ.ที่ปรึกษาร่วม: รศ.พญ.สุมนา ชมพูทวีป, 161 หน้า.

ศึกษาประสิทธิผลของอะซิโทรมัยซินในการรักษาโรคติดเชื้อจากจุลชีพที่พบบ่อย โดยใช้วิธีทางเภสัชจลนศาสตร์/เภสัชพลศาสตร์ ซึ่งนำข้อมูลเภสัชจลนศาสตร์ที่ได้จากความเข้มข้นยาในรูปอิสระในร่างกายกับข้อมูลเภสัชพลศาสตร์ที่ได้จากความสัมพันธ์การฆ่าเชื้อกับเวลาในหลอดทดลองมาใช้รวมกัน แบบจำลองการติดเชื้อในหลอดทดลองถูกนำมาใช้หาความสัมพันธ์การฆ่าเชื้อกับเวลาของอะซิโทรมัยซินต่อเชื้อแบคทีเรีย 4 ชนิด (*สเตรปโตคอคคัส นิวโมนิเอ*ชนิดไวต่อเพนนิซิลิน *สเตรปโตคอคคัส นิวโมนิเอ*ชนิดไวปานกลางต่อเพนนิซิลิน *ฮีโมฟีรูส อินฟลูเอนเซ* และ*มอราเซลลา คาทาราลิส*) รูปแบบเภสัชจลนศาสตร์/เภสัชพลศาสตร์ที่แตกต่างกันจำนวน 12 แบบถูกนำมาเปรียบเทียบกับผลการฆ่าเชื้อกับเวลาโดยใช้โปรแกรมสำเร็จรูป ผลการศึกษาพบว่า รูปแบบธรรมดาของเภสัชจลนศาสตร์/เภสัชพลศาสตร์ไม่เหมาะที่จะอธิบายผลทางเภสัชพลศาสตร์ได้ รูปแบบที่เหมาะสมต้องเพิ่มตัวแปรที่เกี่ยวข้องเข้าไปด้วย ได้แก่ การอิ่มตัวของจำนวนเชื้อ ระยะเวลาเจริญเติบโตของเชื้อที่ล่าช้า และ/หรือ ระยะเวลาเริ่มฤทธิ์ต้านเชื้อ รวมทั้งตัวแปรปรับลาดความชันของเส้นกราฟ ค่าทางเภสัชพลศาสตร์ที่หาได้จากรูปแบบที่เหมาะสมบ่งชี้ว่าอะซิโทรมัยซินมีประสิทธิผลสูงต่อ *สเตรปโตคอคคัส นิวโมนิเอ* และ *มอราเซลลา คาทาราลิส* ส่วน*ฮีโมฟีรูส อินฟลูเอนเซ* มีประสิทธิผลน้อย ข้อมูลเภสัชจลนศาสตร์ได้จากอาสาสมัครสุขภาพดีจำนวน 8 คน หลังจากได้รับประทานอะซิโทรมัยซินขนาด 250 มิลลิกรัม 2 แคปซูลวันละ 1 ครั้งติดต่อกัน 3 วัน พบว่าค่าเฉลี่ยพารามิเตอร์ทางเภสัชจลนศาสตร์ ได้แก่ ค่าความเข้มข้นสูงสุดของยาในพลาสมา ค่าพื้นที่ใต้เส้นโค้งระหว่างความเข้มข้นของยาในพลาสมากับเวลาตั้งแต่เวลาศูนย์ถึงเวลาสุดท้ายของการเก็บตัวอย่างเลือด และตั้งแต่เวลาศูนย์ถึงเวลาอนันต์ ของอะซิโทรมัยซินในพลาสมากับอะซิโทรมัยซินในรูปอิสระ มีค่าลดลงประมาณร้อยละ 50 คือจาก 525.94 เป็น 292.21 นาโนกรัมต่อมิลลิลิตร 7873.84 เป็น 3755.02 ชั่วโมงคูณนาโนกรัมต่อมิลลิลิตร และ 9636.96 เป็น 4572.99 ชั่วโมงคูณนาโนกรัมต่อมิลลิลิตร ตามลำดับ ซึ่งค่าพารามิเตอร์ทางเภสัชจลนศาสตร์เหล่านี้ชี้ให้เห็นว่า ผลของการจับกับโปรตีนของยาควรต้องนำมาพิจารณาประสิทธิผลของยาด้วย ดังนั้นเภสัชจลนศาสตร์ที่ได้จากความเข้มข้นยาในรูปอิสระกับเวลาจึงเหมาะสมที่จะถูกใช้ในรูปแบบเภสัจลนศาสตร์/เภสัชพลศาสตร์ที่เหมาะสม ผลการจำลองการฆ่าเชื้อด้วยความเข้มข้นของอะซิโทรมัยซินในรูปอิสระกับเวลาในรูปแบบเภสัจลนศาสตร์/เภสัชพลศาสตร์ช่วยทำนายว่า อะซิโทรมัยซิน ขนาด 250 มิลลิกรัม 2 แคปซูลวันละ 1 ครั้ง มีประสิทธิผลฆ่า *สเตรปโตคอคคัส นิวโมนิเอ*ทั้งชนิดไวและไวปานกลางต่อเพนนิซิลินได้ดี แต่ไม่ค่อยมีผลต่อการลดจำนวนของ*ฮีโมฟีรูส อินฟลูเอนเซ* และ *มอราเซลลา คาทาราลิส* ดังนั้นวิธีทางเภสัชจลนศาสตร์/เภสัชพลศาสตร์ซึ่งนำข้อมูลทางเภสัชพลศาสตร์ที่ได้จากความสัมพันธ์การฆ่าเชื้อกับเวลามาใช้ จึงเป็นวิธีหนึ่งที่เหมาะสมสำหรับใช้ประเมินฤทธิ์ต้านจุลชีพของอะซิโทรมัยซิน

สาขาวิชา....เภสัชกรรม....
ปีการศึกษา....2549.....

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4576968733 MAJOR : PHARMACEUTICS
KEYWORD :AZITHROMYCIN/PHARMACOKINETIC/PHARMACODYNAMIC/ MODELING/EFFICACY

WANCHAI TREYAPRASERT : PHARMACOKINETIC/ PHARMACODYNAMIC APPROACH TO EVALUATE ANTIMICROBIAL ACTIVITY OF AZITHROMYCIN. THESIS ADVISOR: ASSOC.PROF. UTHAI SUVANAKOOT,Ph.D., THESIS CO-ADVISOR: PROF. HARTMUT DERENDORF,Ph.D., THESIS CO-ADVISOR: ASSOC.PROF. SUMANA CHOMPOOTAWEEP, M.D. 161 pp.

The efficacy of azithromycin in the treatment of infections caused by the common bacteria, using a PK/PD approach which combines *in vivo* PK data from the free drug concentration versus time profile and *in vitro* PD from the time-kill curve was studied. The bacterial time-kill curves of azithromycin against four bacterial strains (*Streptococcus pneumoniae*/penicillin-sensitive, *Streptococcus pneumoniae* /penicillin-intermediate, *Haemophilus influenzae* and *Moraxella catarrhalis*) were determined by *in vitro* infection models. Twelve different PK/PD models were fitted and compared to the time-kill curve data using software Scientist®. Results show that a simple PK/PD model was not sufficient to describe the pharmacodynamic effects for these four bacterial strains. Appropriate models that gave good curve fits included additional terms for saturation of the number of bacteria (N_{max}), delay in the initial bacterial growth phase and/or the onset of anti-infective activity ($1-e^{-zt}$) as well as a Hill factor (h) that captures the steepness of the concentration-response profile. The determined PD parameters from the curve fit of bacterial time-kill curves showed that azithromycin was highly effective against *S. pneumoniae* strains and *M. catarrhalis* while the efficacy of azithromycin against *H. influenzae* was poor. The pharmacokinetic of azithromycin was obtained from eight healthy volunteers after once-daily oral administration of 2x250 mg of azithromycin capsules for 3-day regimen. The mean values of C_{max}, AUC_{last}, AUC_{inf}, and AUMC were decreased approximately by 50% from 525.94 to 292.21 ng/mL, 7873.84 to 3755.02 h.ng/mL, 9636.96 to 4572.99 h.ng/mL, and 447865.88 to 194602.11 h.h.ng/mL for total plasma drug and free drug, respectively. These pharmacokinetic parameters indicated that the effect of protein binding should be taken into consideration. Therefore, PK profile from free plasma of azithromycin was properly fitted with appropriate PK/PD models. Simulated kill curves with the PK profile of free plasma concentration of azithromycin into the PK/PD models predicted that 2x250 mg of azithromycin capsules orally once-daily showed a good bactericidal effect for *S. pneumoniae* (both penicillin-sensitive and penicillin-intermediate) whereas the same dose appeared to be insufficient to decrease bacterial counts for *H. influenzae* and *M. catarrhalis*. Therefore, PK/PD approach based on time-kill curve could be a suitable method to evaluate antimicrobial activity of azithromycin.

Field of study...Pharmaceutics...

Academic year...2006...

Student's signature.....

Advisor's signature.....

Co-advisor's signature.....

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LIST OF ABBREVIATIONS

ALT	=	alanine aminotransferase
a.m.	=	before noon
AST	=	aspartate aminotransferase
ATCC	=	american type culture collection
AUC	=	area under the concentration-time curve
AUC_{24}	=	area under the concentration-time curve from zero to 24 hours
AUC_{last}	=	area under the concentration-time curve from zero to 192 hours
AUC_{inf}	=	area under the concentration-time curve from zero to infinite time
AUMC	=	area under the first moment curve
^{0}C	=	degree celcius
CFU	=	colony forming unit
CNS	=	central nervous system
Conc	=	concentration
C_{max}	=	peak plasma drug concentration
Da	=	dalton
e.g.	=	for example
g	=	gram
GI	=	gastrointestinal
h	=	hour
HPLC	=	high performance liquid chromatography
HQC	=	high quality control concentration
i.e.	=	that is
IV	=	intravenous
kDa	=	kilodalton
kg	=	kilogram
KV	=	kilovolt
L	=	liter
LC-MS	=	liquid chromatographic mass spectrometry
LQC	=	low quality control concentration

MAC	=	minimal antibiotic concentration
MIC	=	minimal inhibitory concentration
mg	=	milligram
min	=	minute
mL	=	milliliter
MQC	=	medium quality control concentration
ng	=	nanogram
Pen-I	=	penicillin-intermediate
Pen-S	=	penicillin-sensitive
PD	=	pharmacodynamic
PK	=	pharmacokinetic
PK/PD	=	pharmacokinetic/pharmacodynamic
QC	=	quality control concentration
r^2	=	coefficient of determination
rpm	=	revolution per minute
S.D.	=	standard deviation
$t_{1/2}$	=	half-life
t_{max}	=	time to peak plasma drug concentration
µg	=	microgram
µL	=	microliter

CHAPTER I

INTRODUCTION

A. Background and Rationale of the Study

Drug dosing regimens are often based on trial and error rather than rational design. Giving the right antibiotic agent at the right dosing regimen has become a very crucial issue for the treatment of infections, since improper antibiotic therapy may result not only in therapeutic failure but also in acceleration in the rate of bacterial resistance development. In order to optimize the effect and minimize the risk of resistance developing during treatment, pharmacokinetics (PK) and pharmacodynamics (PD) play an important role for drug development and drug evaluation. In general, PK/PD model is the link between PK parameters (what the body does to the drug) with the PD parameters (what the drug does to the body). PK/PD model is employed to establish correlation of the concentration time relationship (PK) with effect-concentration relationship (PD) in order to provide a better understanding of the time course of the drug and effect (PK/PD) after drug administration [1-3]. Currently, there are two main trends for antibiotic PK/PD models; PK/PD models based on the minimal inhibitory concentrations (MIC) and PK/PD models based on a time-kill curve approach [4,5].

The MIC is the most commonly used to determine the efficacy and potency of anti-infection drug. It is defined as the lowest concentration that completely inhibits visible growth of the micro-organism as detected by unaided eye after a 18-24 h incubation period with standard inoculum of approximately 10^5- 10^6 colony forming unit (CFU)/mL [6]. The MIC is a standard PD parameter routinely determined in both research and clinical microbiology laboratories and guidelines for its determination are published and updated regularly. Consequently, the MIC serves as the PD input while the serum concentration of antimicrobial agent serves as the PK input for the most widely used PK/PD approaches for antimicrobials. The PK/PD indices which have been used in the most studies are the ratio of the peak antibiotic concentration and MIC (C_{max}/MIC), the area under the concentration-time curve and MIC ratio (AUC/MIC), and the time above the MIC (t > MIC) [7-10]. For example, the antibacterial effect of aminoglycosides has been shown to be related to peak concentration, and C_{max}/MIC is the appropriate index [11]; the efficacy of

fluoroquinolones correlates best with ratio of AUC_{24}/MIC which is obtained by dividing 24 h steady-state AUC by MIC [12]. For β-lactams, the PK/PD index that best correlates with bacteriological eradication, is the time above MIC [13].

However, PK/PD model based on MIC approach has a few limitations, both from PK and PD perspectives. From the PK perspective this approach has two major limitations : protein binding and tissue distribution. First, the effect of protein binding is often taken into consideration. All drugs bind to some extent to plasma proteins and may also bind to tissue proteins or structures. Binding is important because only the free fraction of the drug is available to extent its pharmacological action. If the total plasma concentration is used instead of the free pharmacologically active fraction, the expected outcome for a specific dose may be overestimated, and the antibiotic treatment may fail or lead to the development of resistance. To determine the free drug concentration, ultrafiltration method has been commonly applied and used in clinical applications [14-16]. This method is based on the removal of proteins and other endogenous large macromolecules from biological samples without extraction or protein precipitation. Ultrafiltration uses cone-shaped membranes that fit on the top of centrifuge vials. Biological samples are placed into the cones and centrifuged. Filtrate pass through the membrane (cut-off 25,000-50,000 Da). The ultrafiltrate concentration represents the free drug concentration. Second, most infections do not take place in plasma. For the determination of PK/PD indices the outcome of the therapy may be, once again, overestimated if the drug is not able to penetrate the tissue where the infecting agent is. Tissue penetration has to be taken into account and measured directly by techniques such as microdialysis [17-23]. This technique is based on the sampling of substance from interstitial fluid at the target site by means of a semipermeable membrane, placed on the tip of a microdialysis probe. The probe is constantly perfused with a physiological solution. Once the probe is implanted the substances present in the interstitial fluid are dialyzed into the probe, where they can be collected and analyzed. Since microdialysis monitors the free tissue concentrations directly and continuously, it is a suitable method to evaluate free drug concentration in the interstitial fluid. Although many studies have shown that microdialysis is a reliable technique for investigating the kinetics of many drugs in various human organs and tissues such as brain, lung, myocardium, tumors and soft tissue [24-29], the limitation of microdialysis is associated with lipophilic drugs sticking to tubing

and probe components, thereby complicating the relation between dialysate and extracellular concentration [24]. From pilot study, azithromycin, a lipophilic drug, could not be measured by microdialysis.

From the PD perspective, the MIC is very crude indicator of antimicrobial efficacy and potency. First of all, since it is determined against static concentrations of the antibiotic it does not reflect the *in vivo* scenario where concentration fluctuate between doses. It is used as a threshold value that implies an all-or-nothing concentration-effect relationship. There is no distinction between the effects of different concentrations either above or below the MIC. The MIC also does not provide any information on the time-dependency of the antibacterial effect and bacterial growth rates. In fact, it can be shown that different combinations of bacterial growth and killing rates may result in the same MIC, and therefore the same PK/PD index, but not necessarily the same outcome [30]. Finally, the MIC is a very imprecise parameter due to the way in which it is normally determined. A two-fold variability in the results is acceptable and it is not uncommon to see the MIC reported as a range of values instead of a single number. Hence, any PK/PD approach based on MIC approach will carry with it the same amount of variability and uncertainty.

A better approach to evaluate the PD of anti-infective agents, bacterial time-kill curves, can offer detailed information about the antibacterial efficacy as a function of both time and antibiotic concentration. Time-kill curves provide a more dynamic and detailed description of the effect of antimicrobials hence allowing for more precise prediction [4,5]. Subsequently, the resulting kill curves can be analyzed with appropriate PK/PD models. Finally the PD parameters derived from time-kill experiments can be combined with *in vivo* PK data in an integrated PK/PD model that describes the activity of antibiotic as a function of time and concentration.

The concept of dynamic PK/PD approach is to use the full concentration profile versus effect rather than the static MIC approach. Therefore, PK/PD model based on the time-kill curve which combines *in vivo* PK data and the time kill curve may have better potential for the evaluation of antibiotic effect than PK/PD model based on MIC.

The American Thoracic Society and the Infections Diseases Society of America have recommended a macrolide as viable first-line option for treating community-acquired pneumonia [31-33]. Azithromycin, a 15-membered macrolide, is commonly prescribed antibacterials used to treat respiratory tract infections.

Azithromycin also displays extensive cellular uptake and prolonged retention and tissue concentrations are highly long after the last dose of the treatment regimen [34,35]. In this study, the efficacy of azithromycin in the treatment of infections caused by the common bacteria, using an integrated PK/PD approach which combines free drug concentrations and the time-kill curve will be evaluated.

B. Objectives

The overall objective of this study is to evaluate the efficacy of azithromycin in the treatment of infections caused by four common bacteria, using an integrated PK/PD approach which combines free plasma concentrations and the time-kill curve. Specific aims of this study are as follows:

Specific Aim # 1 – Pharmacodynamics

To investigate the *in vitro* antibacterial efficacy of azithromycin against four common bacterial strains by using the time-kill curve.

Specific Aim # 2 – PK/PD modeling

To establish a PK/PD model to describe the PK/PD relationship of azithromycin.

Specific Aim # 3 – Pharmacokinetics

To investigate the pharmacokinetic of azithromycin in human by analyzing plasma data, and to evaluate and compare total plasma drug and free drug concentration.

Specific Aim # 4 – Integrated PK/PD approach

To develop an integrated PK/PD model that will predict the antimicrobial effect of azithromycin by integrating the free drug concentration versus time profile into the PK/PD model.

C. Significance of the Study

1. This study will provide a PK/PD model that could predict the antimicrobial effect of azithromycin by integrating the free drug concentration versus time profile into the PK/PD model.
2. This study will provide the answer of efficacy of azithromycin in the treatment of infections caused by four common bacteria.

CHAPTER II

LITERATURES REVIEW

A. PK/PD Models for Anti-infective Agent

The selection of the correct dose and dosing regimen is a fundamental step for therapeutic success with any pharmacological agent. For antimicrobial agents the selection of the best drug and dosing scheme for a specific pathogen not only increases the chances of cure while preventing toxic side effects, but also decreases the probability of the infecting agent becoming resistant to the drug [36,37].

However, deciding on the best drug and most effective dosing scheme is not an easy task and is often done based on trial and error rather than on rational design. Today, rational drug design is strongly based on principles of pharmacokinetic/ pharmacodynamic (PK/PD) model (Figure 1). PK/PD model is employed to establish correlation of the concentration time relationship (PK) with effect-concentration relationship (PD) in order to provide a better understanding of the time course of the drug and effect (PK/PD) after drug administration [38]. PK/PD model has also become an extremely important tool in dose optimization [39]. In order to optimize the effect and minimize the risk of resistance developing during treatment, pharmacokinetics and pharmacodynamics play an important role for drug development and drug evaluation [40]. In addition, PK/PD model allows for an educated selection of the best drug and dosing regimen, good predictions of therapeutic success and safety, and minimization of costly trial and error approaches [41].

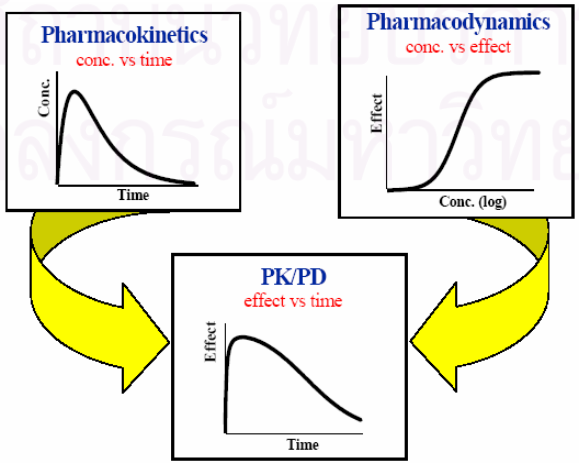


Figure 1 Integration of the concentration versus time relationship (PK) and concentration versus effect relationship (PD) into PK/PD model.

Currently there are two main trends for antibiotic PK/PD models ; PK/PD models based on the MIC and PK/PD models based on a time-kill curve approach, both of which will be described in detail as follows **:**

1. PK/PD Models Based on MIC

The MIC is the most commonly used to determine the efficacy and potency of anti-infection drug. It is defined as the lowest concentration that completely inhibits visible growth of the micro-organism as detected by unaided eye after a 18-24 h incubation period with standard inoculum of approximately 10^5- 10^6 colony forming unit (CFU)/mL [6]. The MIC is a standard PD parameter routinely determined in both research and clinical microbiology laboratories and guidelines for its determination are published and updated regularly. Consequently, the MIC serves as the PD input while the serum concentration of antimicrobial agent serves as the PK input for the most widely used PK/PD approaches for antimicrobials. The PK/PD indices which have been used in the most studies are the ratio of the peak antibiotic concentration and MIC (C_{max}/MIC), the 24 h area under the concentration-time curve and MIC ratio (AUC/MIC), and the time above the MIC (t > MIC) as shown in Figure 2 [42-44].

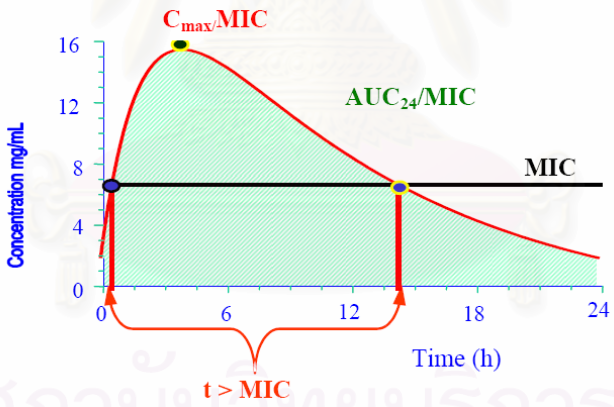


Figure 2 PK/PD indices for the evaluation of anti-infective agents

1.1 Patterns of Antimicrobial Activity

The different patterns of bacterial killing rate is illustrated in Figure 3 by showing the effect of increasing drug concentrations on the *in vitro* antimicrobial activity of tobramycin, ciprofloxacin and ticarcillin against standard strain of *Pseudomonas aeruginosa* [45-47]. Increasing concentrations of tobramycin and ciprofloxacin produced more rapid and extensive bacterial killing, as exhibited by the steeper slopes of killing curves. With ticarcillin, there was a change in slope as the concentration was increased from one to four times the MIC. However, higher

concentrations did not alter the slope. The slight reduction in bacterial numbers at the higher doses is due to an earlier onset of bacterial killing.

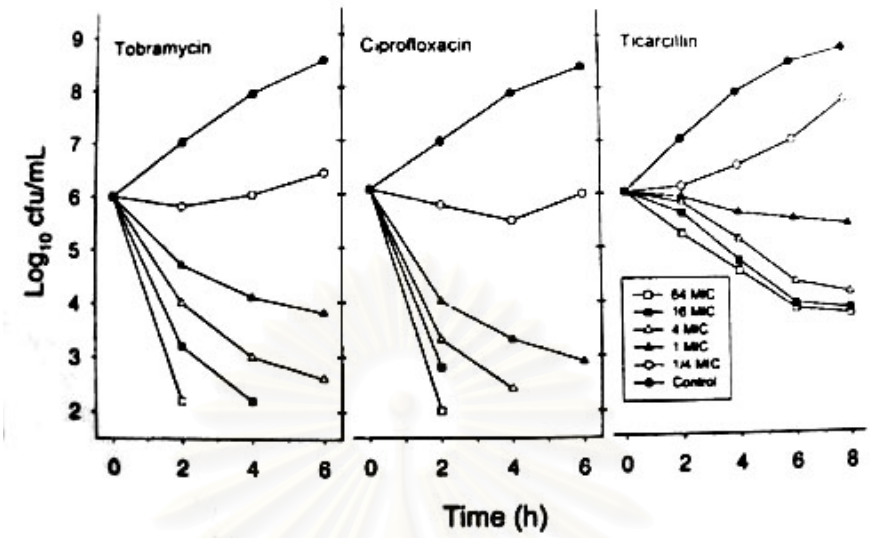


Figure 3 Time-kill curves for *P. aeruginosa* with exposure to tobramycin, ciprofloxacin and ticarcillin at concentrations from one-fourth to 64 times the MIC.

In general, antibiotics exhibit three major patterns of antimicrobial activity with their pharmacodynamic characteristics as shown in Table 1.

Table 1 Three patterns of antimicrobial activity

Category	Pattern 1	Pattern 2	Pattern 3
Pharmcodynamic characteristics	Concentration-dependent killing and moderate to prolonged persistent effects	Time-dependent killing and minimal to moderate persistent effects	Time-dependent killing and prolonged persistent effects
Antimicrobial class	Aminoglycosides, ketolides, fluoroquinolones, daptomycin, metronidazole, amphotericin B	β-lactams, macrolides, clindamycin, oxazolidinediones, flucytosine	Azithromycin, tetracyclines, glycopeptides, fluconazole
Goal of dosing regimen	Maximize concentrations	Maximize duration of exposure	Optimize amount of drug
PK parameter determining efficacy	Peak level and AUC	Time above MIC	AUC

The first pattern is characterized by concentration-dependent killing and moderate to prolonged persistent effects. Higher concentrations would kill organisms more rapidly and more extensively than lower levels. The prolonged persistent effects would allow for infrequent administration of larger doses. This pattern is observed with aminoglycosides, ketolides, fluoroquinolones, daptomycin, metronidazole, and amphotericin B [48-52]. The peak level and the AUC should be the pharmacokinetic parameter that would determine efficacy [53-55].

The second pattern is characterized by time-dependent killing and minimal to moderate persistent effects. With this pattern, saturation of the killing rate occurs at low multiples of the MIC, usually around four to five times the MIC. Drug concentrations above these values do not kill microbes faster or more extensively. In addition, organism regrowth would start very soon after serum levels fall below the MIC. This pattern is observed with β-lactams, macrolides, clindamycin, and flucytosine [53,56-61]. The duration of time that serum levels exceed some minimal value such as MIC should be the major pharmacokinetic parameter determining the efficacy of these drugs [47].

The third pattern is also characterized by time-dependent killing, but the duration of the persistent effects is much prolonged. The goal of a dosing regimen is to optimize the amount of drug administered to ensure that killing occurs for part of the time and there is no regrowth during the dosing interval. This pattern is observed with azithromycin, tetracyclines and fluconazole. The AUC should be the primary pharmacokinetic parameter that would determine efficacy [62-66].

1.2 PK/PD indices

Based on these killing patterns three different PK/PD indices have been developed to evaluate efficacy and to optimize dosing regimens of antibiotics which are the ratio of the peak antibiotic concentration and MIC (C_{max}/MIC), the 24 h area under the concentration-time curve and MIC ratio (AUC_{24}/MIC), and the time above the MIC (t > MIC). These PK/PD indices are illustrated in Figure 2. A list of different antibiotics with their respective PK/PD indices is given in Table 2.

Table 2 PK/PD indices associated with the efficacy of several antimicrobials

PK/PD index	Antimicrobials
t >MIC	Penicillins, cephalosporins, macrolides, clindamycin, penem antibiotics
C_{max}/MIC	Aminoglycosides, amphotericin B
AUC_{24}/MIC	Fluoroquinolones, aminoglycosides, daptomicin, vancomycin, ketolides, tetracyclines, azithromycin

1.2.1 Time above MIC (t>MIC)

The time above the MIC refers to the time during which the concentration of the drug in plasma remains above the MIC of the pathogen. This index is associated with the efficacy of drugs that exhibit time-dependent killing pattern, such as β-lactams and macrolides [41]. These agents achieve bacterial eradication when the serum concentrations exceed the MIC of the pathogen above 40-50% of the dosing interval for gram-negative bacteria. For gram-positive bacteria such as *Staphylococcus aureus*, maximum kill has been reported with concentrations exceeding the MIC for as short as 25% of the dosing interval.

1.2.2 C_{max}/MIC ratio

This index is obtained by dividing the peak plasma concentration in steady-state by the MIC of the pathogen. The C_{max}/MIC relates to the efficacy of drugs that exhibit a concentration-dependent killing pattern such as aminoglycosides, fluoroquinolones and daptomycin. The target is to achieve a C_{max}/MIC of at least 10-12 in order to maximize clinical response. Maximizing this index suggested to be particularly important when there is a high risk for the emergence of resistant subpopulations.

1.2.3 AUC_{24}/MIC ratio

This index is obtained by dividing the 24 h steady-state AUC by the MIC. It is similar to the area under the inhibitory curve (AUIC), which is calculated as the AUC that remains above the MIC during a 24 h interval [42]. Although some authors use both terms indiscriminately they are only the same when the concentrations are above the MIC during the entire dosing interval [67]. However, AUC_{24}/MIC is preferred over the AUIC since it is easier to calculate than the AUIC and does not depend on the concentrations being above the MIC. The AUC_{24}/MIC has

been used to evaluate the efficacy of azithromycin [8], fluoroquinolones [68], aminoglycosides [68] and daptomicin [69].

The AUC_{24}/MIC target for fluoroquinolone therapy has been subject of debate in recent years. Forrest et al, initially proposed a target of AUC_{24}/MIC > 125 for the treatment of gram-negative infections with ciprofloxacin in elderly hospitalized patients [70]. This value has become since then a standard clinical breakpoint for the treatment of gram-negative agents with fluoroquinolones although values between 100-25 are suggested to be sufficient. The target for treating infections caused by gram positive agents with fluoroquinolones is believed to be lower. For *S. pneumoniae* an AUC_{24}/MIC > 30 has been associated with high probability of success in patients with community-acquired infections. On the other hand, Schentag et al suggested that there should be no differentiation between the targets for gram-negative and gram positive bacteria and that AUIC values should be above 250 to assure rapid killing of the infecting agent [9]. However, this suggestion has been criticized by some authors who support the use of different targets for gram-negative and gram-positive bacteria [71].

For the older macrolides (such as erythromycin and clarithromycin), t >MIC is predictive of bacteriological efficacy. Although the azalide, azithromycin, does not exhibit concentration-dependent killing, the AUC_{24}/MIC correlates with bacteriological efficacy. This may be because the much longer post-antibiotic effects produced by azithromycin. For azithromycin, AUC_{24}/MIC ratio needs to exceed 25 for optimal efficacy [8].

1.3 Limitations of PK/PD Model Based on MIC Approach

PK/PD model based on MIC approach has a few limitations, both from PK and PD perspectives. From the PK perspective this approach has two major limitations : protein binding and tissue distribution . First, the effect of protein binding is often taken into consideration. All drugs bind to some extent to plasma proteins and may also bind to tissue proteins or structures. Binding is important because only the free fraction of the drug is available to extent its pharmacological action. If the total plasma concentration is used instead of the free pharmacologically active fraction, the expected outcome for a specific dose may be overestimated, and the antibiotic treatment may fail or lead to the development of resistance. Second, most infections occur not in plasma but rather in tissue sites (extracellular fluid), the ability of antibiotics to reach the target sites is a key determinant of clinical outcome. As we

know, the tissues are not homogenous compartments, and the distribution of drug molecules in plasma and tissue depends on their physical-chemical properties as shown in Figure 4 [4]. In plasma, some of the drug binds to plasma proteins or blood cells, or diffuse into the blood cells. Some drug also remains unbound in plasma and can move freely in the body. A similar scenario also occurs in the tissue cells and some stay unbound in the tissue fluid. The difference between the total plasma concentrations and the free tissue concentrations can be quite significant when the protein binding of the antibiotic is high. Therefore, the total plasma concentration is not an ideal PK parameter for rational dosing of antibiotics and the unbound drug concentration at the infection site should be preferred.

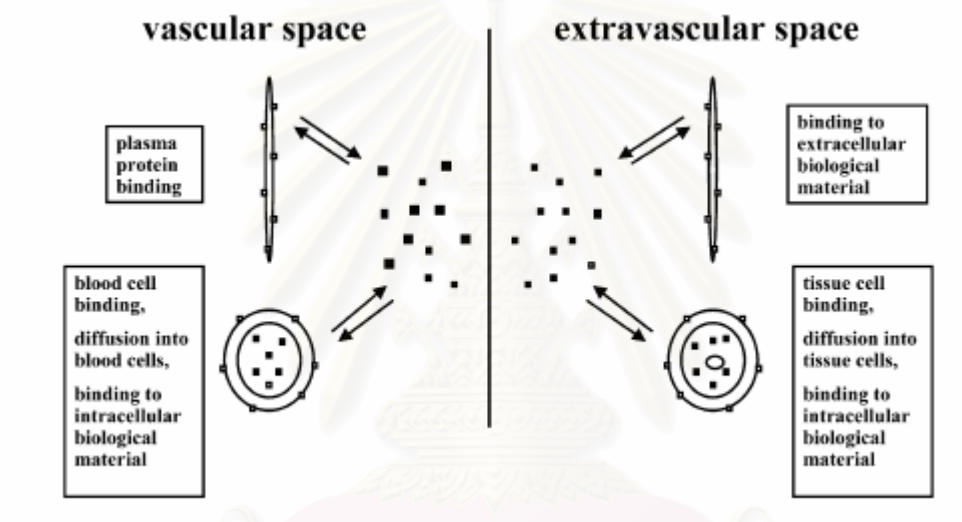


Figure 4 Schematic diagram of drug distribution in plasma and tissue

From the PD perspective, the MIC provides only limited information on the kinetics of the drug action. For instance, the MIC does not provide information on the rate of bactericidal activity and whether increasing antimicrobial concentrations can enhance this rate [72]. Since the MIC determination depends on the number of bacteria at a single time point, many different combinations growth and rates can result in the same MIC. In addition, the MIC are conventionally measured for constant antibiotic concentrations and therefore represent threshold concentrations. This implies the existence of an all-or-nothing concentration-effect relationship. All concentrations below the MIC are treated equally and similarly no quantitative distinction is made for all concentration above MIC. This is a very crude way of using antimicrobial information. Moreover, the MIC is determined against static

concentrations which by no means reflects the *in vivo* scenario, where bacteria are not being exposed to constant but constantly changing antibiotic concentrations [5].

2. PK/PD Models Based on Time-Kill Curve

A different approach to assess the antibiotic efficacy is to use PK/PD models based on time-kill curves. Time-kill curves can follow microbial killing and growth as a function of both time and antibiotic concentration. Antibiotic concentration can either be held constant or changed to mimic an *in vivo* concentration profile, be it either in plasma or at the infection site. The resulting kill curves can be subsequently analyzed with appropriate PK/PD models. Finally these PK/PD models then aid to optimize dosage regimens based on rational approach [73]. Time-kill curves can be obtained either from *in vitro* models or from animals infection models [74].

2.1 *In vitro* Models for the Development of Time-Kill Curve

Several different *in vitro* models have been developed to study kill curves of antibiotics. There are basically two different types of models : constant concentration models and dynamic concentration models.

Constant concentration models study the number of bacteria exposed to a constant antibiotic concentration. *In vitro* model normally consist of simple vented cap tissue culture flasks in which the drug and the bacteria are incubated together. Samples are taken at pre-determined time points and plated and the time-kill curves are obtained by plotting the number of viable bacteria as CFU/mL versus time. This model simulates the steady-state concentration obtained *in vivo* after constant-rate infusion.

The models described above only consider situations in which the drug concentration remains constant. However, after drug administration, the concentrations of the drug fluctuate due to drug distribution and elimination. Dynamic concentration models expose to the concentration changes, normally according to the *in vivo* half-life of the drug. These model can operate by using pumps or dialysers to dilute the antibiotic in the system [75]. Several different types of dynamic models have been developed that allow the simulation of a single and multiple compartment kinetics, and effect site PK of the drug. Models for combined therapy have also been developed. Since these models incorporate the PK of the drug they provide a better method for comparing various dosage regimens or schedules of administration and are very useful in preclinical drug development.

Single-compartment models have the simplest setups and they simulate the PK of the drug after intravenous bolus administration. They can consist of the same tissue culture flask used for the constant concentration models. To obtain the *in vivo* half-life, the drug is diluted manually by taking a fixed volume of broth from the flask with a syringe and replacing it with the same volume of fresh broth at regular times, generally every 15 min [76]. Dilution of bacteria is prevented by a 0.2 μm filter adapted to the syringe. This model has been used to evaluate the effects of several drugs, such as piperacillin, piperacillin-tazobactam combinations, cefpodoxime, and cefaclor [76-78].

However, automated models that use peristaltic pumps for the dilution of the drug are more practical. In this type of model the drug is diluted according to its *in vivo* half-life by means of a pump that continuously replaces the broth in the infection compartment with fresh broth from a reservoir flask. To prevent the dilution of the bacteria, a filter is placed at the bottom of the infection compartment flask. A magnetic stirrer is used to homogenize the medium and to prevent the filter from clogging. If the filter is not used, the concentration of bacteria in the flask can be corrected mathematically, although recent findings suggest it is not necessary. This model has been used to simulate the free cefpodoxime and cefixime interstitial concentration, measured in humans by microdialysis, against *S.pneumoniae, H. influenzae,* and *M.catarrhalis* [79].

An *in vitro* combined therapy dynamic concentration model has been developed by Liu, Rand and Derendorf to evaluate the impact of half-life of tazobactam on the outcome of piperacillin/tazobactam combination therapy [78]. This model was setup to simulate the different half-lives of piperacillin and tazobactam in the same system. With this model the authors were able to observe that despite earlier assumptions that the PK of tazobactam was not important for the overall effect of piperacillin, there is a significant difference in the bacterial kill and regrowth when the different *in vivo* half-lives of both drugs are taken into account.

Several multiple compartment models have been developed to study antimicrobial PD in systems that resemble very closely the PK *in vivo*. A more complete description of these models and their applications can be found elsewhere [5,80].

2.2 Mathematical Modeling of Time-Kill Curve

Although experimental kill curves enable a dynamic interpretation of drug-bacteria interactions, the strength of this approach is not fully exploited until the data are analyzed by mean of mathematical models. An E_{max} model has been successfully applied to evaluated antimicrobial activity by using the non-linear regression software program Scientist®. The model most commonly used to describe the change in the number of bacteria as a function of time and concentration is shown below:

$$\frac{dN}{dt} = \left[k_0 - \left(\frac{k_{max} \cdot C}{EC_{50} + C} \right) \right] \cdot N$$

where dN/dt is the change in number of bacteria as a function of time (pharmacodynamic effect). N is the number of bacteria in CFU/mL. k_{max} (h^{-1}) is the maximum killing effect. C is the concentration of antibiotic at time t. EC_{50} (μg/mL) is the concentration required for 50% of the maximum effect. k_0 (h^{-1}) is the generation rate constant in absence of any drug.

One of the greatest advantages of kill curves over MIC-based approaches is that the PD effect can be normally described by at least three parameters instead of a single threshold value: the maximum kill rate constant (k_{max}), the concentration at half-maximum effect (EC_{50}), and the growth rate constant of the bacteria (k_0) [80].

These parameters are usually obtained by modeling the kill curves with modified indirect response E_{max} models. Once these models have been established and validated, it is then possible to simulate the expected kill curves for different doses and dosing regimens of the antibiotic against the bacteria of interest by substituting the concentration term in the mathematical model by the fitted concentration-time profile of the drug.

Over the years, different mathematical models have been developed for several antibiotics as follows:

2.3 Models for β-lactam Antibiotics

The kill curves of several β-lactam antibiotics have been successfully modeled by modified indirect response E_{max} model [72,76,79,81].The model most commonly used to describe the change in the number of bacteria as a function of time and concentration is shown below:

$$\frac{dN}{dt} = \left[k_0 - \left(\frac{k_{max} \cdot C^h}{EC_{50}^h + C^h} \right) \right] \cdot N$$

Where dN/dt is the change in number of bacteria as a function of time, k_0 (h^{-1}) is the generation rate constant in absence of antibiotic, k_{max} (h^{-1}) is the maximum killing effect, C is the concentration of the drug at time t, EC_{50} (μg/mL) is the concentration required for 50% of the maximum effect, h is the Hill or shape factor and N is the number of bacteria in CFU/mL.

An additional exponential correction factor ($1-e^{-zt}$) may be included to the model sometimes to account for the fact that the bacteria are still not in logarithmic growth phase at time zero. The pharmacodynamic effect is the reduction of number of bacteria resulting in inhibition of growth or killing. In absence of any drug, bacteria grow at their normal growth rate k_0. In this case, the term $(k_{max} \times C)/(EC_{50}+C)$ equals zero. The maximum kill rate constant is $(k_0 - k_{max})$. The constant z is only relevant of the beginning of most bacteria growth. During this period of time, the growth rate is not constant but gradually increasing. Therefore, the impact of z on k_0 is greatest for the first two hours. After this time period, the expression disappears since it approaches zero.

This model has been used by Derendorf et al to describe the effects of several different dosing regimens of piperacillin in a dynamic concentration *in vitro* model of infection (Figure 5) [72]. It could also be shown that E_{max} model performed the goodness of kill curve fitting. The two concentrations shown correspond to doses of 2 and 4 g of piperacillin. The results of this study show that 2 g of piperacillin every 4 h should produce the same effect as 4 g every 4 h and is the best regimen for this strain.

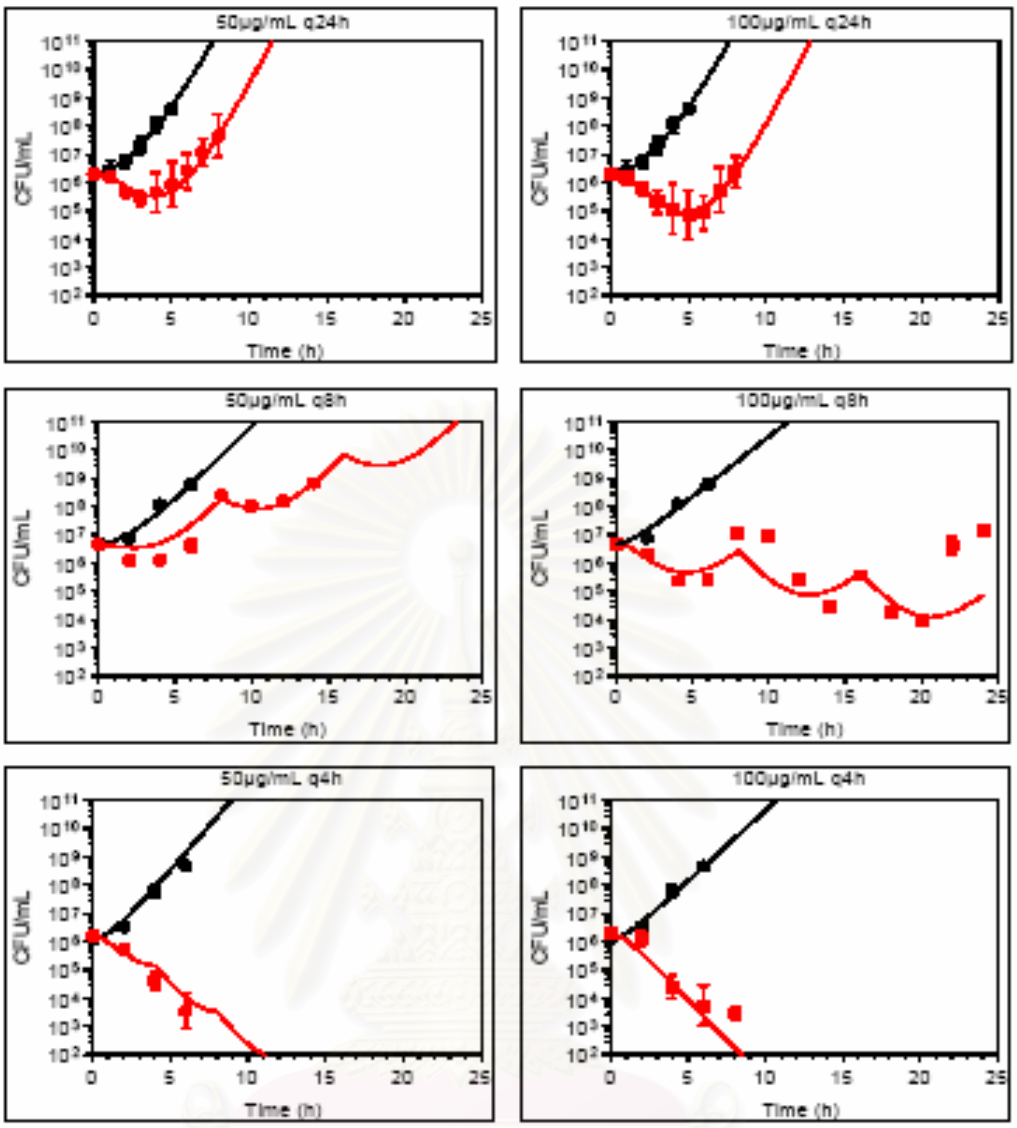


Figure 5 Kill curves of several different dosing regimens of piperacillin versus *E. coli*, obtained in a dynamic concentration *in vitro* model. The lines represent the curve fits obtained with an indirect response E_{max} model. Data presented as Means ± SD.

De la Peña et al also used this model to describe the kill curves of cefaclor against *E. coli, M. catarrhalis, H. influenzae* and *S. pneumoniae* [76]. The integration of the PK and PD properties of cefaclor in the E_{max} model shown above allowed for the selection of a twice-daily dosing regimen of a modified-release (MR) formulation of cefaclor as a safe and convenient alternative to the traditional TID treatment (Figure 6).

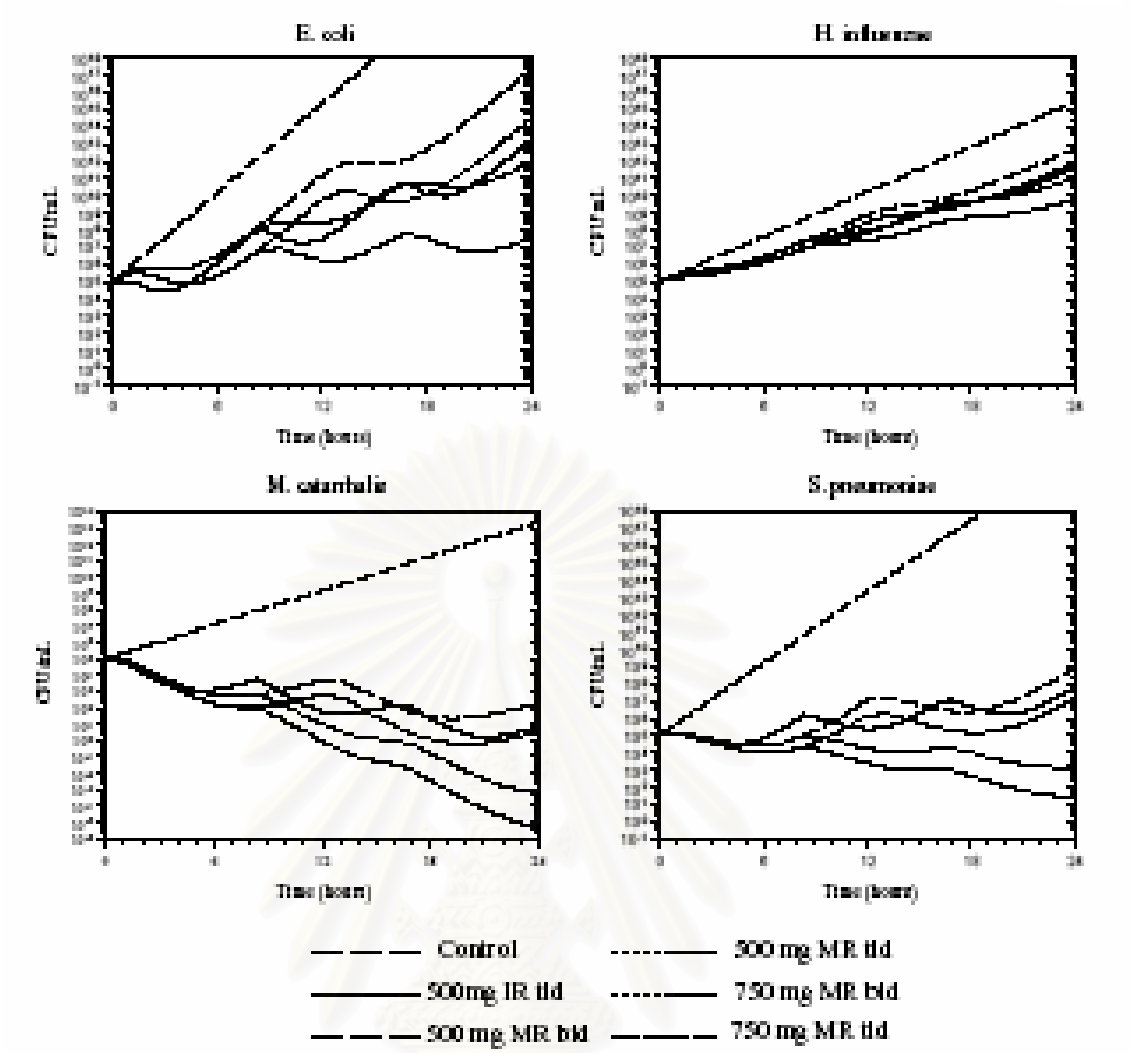


Figure 6 PK/PD simulations of the effects of the different cefaclor formulations and dosing regimens on the four bacterial strains studied, taking into account mean pharmacokinetic parameters. IR, Immediate release; MR, modified release.

This same model, with a few variations, has been used to model kill curves of other β-lactams such as cefpodoxime and cefixime [79] , faropenem [81] , and ceftriaxone [82] ,among others.

2.4 Models for Fluoroquinolones

Modeling the kill curves of fluoroquinolones presents a different challenge due to certain characteristics of their killing kinetics. This class of drugs has been reported to exhibit a biphasic killing pattern in *in vitro* systems, very fast at the beginning and then slower at later time points. The mechanism responsible for this killing pattern is not understood yet. Although the model used for β-lactam antibiotics

has been applied successfully to explain the effects of ciprofloxacin against *P. aeruginosa* in the past, it is believed that more elaborate models are needed to describe the killing kinetics of fluoroquinolones. In recent years, Schuck and Derendorf have purposed a new adaptive E_{max} model for fluoroquinolones using ciprofloxacin as the model drug [80]. This model was developed to describe the time-kill profiles of ciprofloxacin against *E. coli*. The model included a theoretical resistance compartment (C_r) to account for the change in the killing rate of ciprofloxacin after approximately 4 h. The equation describing the new adaptive E_{max} model is presented below:

$$\frac{dN}{dt} = \left[k - \frac{\left[k_1 \left[1 - \frac{C_r}{IC_{50} + C_r} \right] + k_2 \right] \cdot C}{EC_{50} + C} \right] \cdot N \cdot (1 - e^{-zt})$$

where N is the number of bacteria (CFU), k is the growth rate constant in absence of the antibiotic, k_1 is the initial fast contribution to the maximum kill rate constant, k_2 is the permanent maximum kill rate constant, C_r is the drug concentration inducing adaptive resistance, IC_{50} is the concentration at 50% of maximum resistance, EC_{50} (μg/mL) is the concentration required for 50% of the maximum effect, C is the active drug concentration, and z is an adjustment factor at the beginning of the experiment accounting for the fact that the bacteria are not instantaneously in their logarithmic growth phase.

This model was used to evaluate the expected outcome of a new ciprofloxacin dosage form (ciprofloxacin extended release tablets) against *E. coli* in comparison to that of the traditional immediate release dosage form, which is given twice a day (Figure 7) [83].

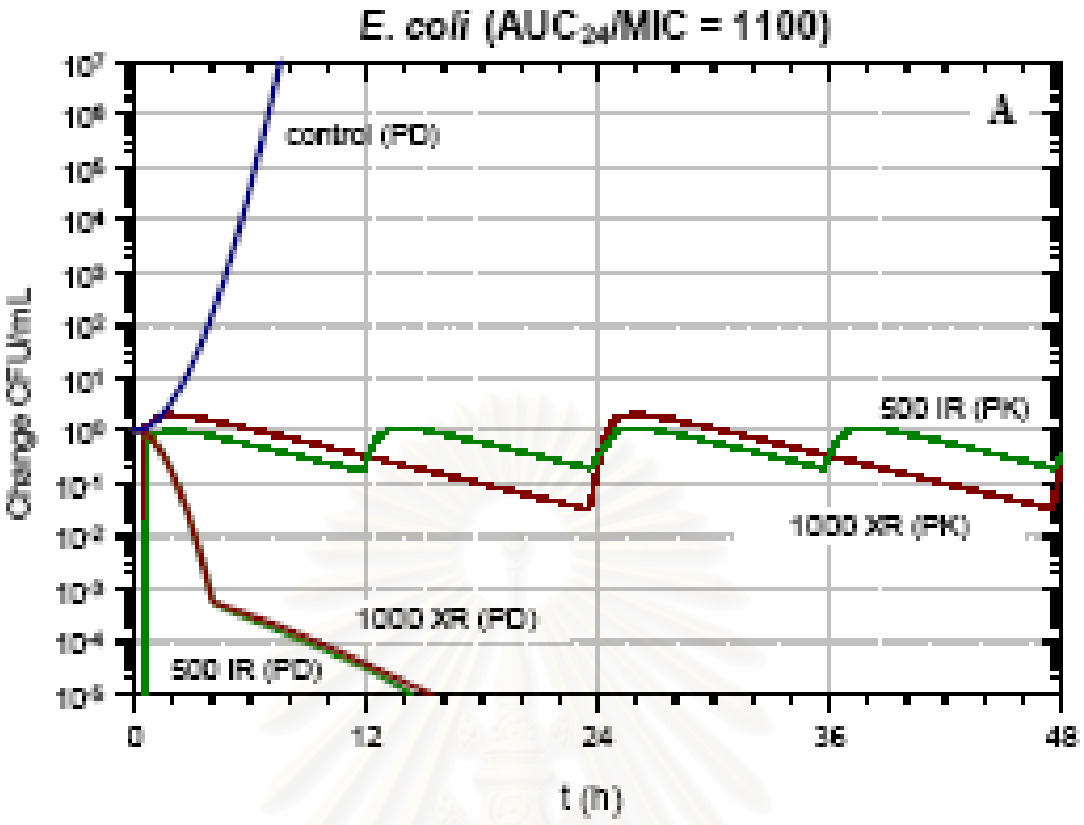


Figure 7 Simulation of the plasma concentrations (PK) and bacterial counts (PD) of ciprofloxacin after administration of 1000 mg XR once-a-day and 500 mg IR twice-a-day, respectively.

2.5 Limitations of the Time-Kill Curve Approach

The major limitation associated to the kill-curve approach is that it is not a very practical method. Obtaining a complete set of kill-curves that would allow a good evaluation of the concentration-effect and time-effect relationships between drug and bacteria is very labor-intensive and time-consuming. Moreover, the mathematical modeling of the kill-curves can be very challenging. However, once these relationships have been established and validated, much more information can be extracted from them than from simple MIC determinations.

Another limitation of this method is that the experimental conditions do not reflect the *in vivo* situation where the drug is bound to proteins and the immune system plays an important role in eradicating the infection. Therefore, the antimicrobial effect can be overestimated when extrapolating results obtained *in vitro* to *in vivo* scenarios. While this may be true in most cases, there are still situations in which the results from these *in vivo* experiments may have direct applications, such as

in infections in AIDS patients or patients undergoing cancer chemotherapy, where the function of the immune system is compromised. In addition, the impact of protein binding can be evaluated by simulating in the model unbound concentrations, measured by microdialysis at the site of infection (i.e. interstitial fluid), instead of total concentrations in plasma [29,84-86].

B. Microdialysis Method

Microdialysis is a technique used to determine the concentration of exogenous and endogenous substances in the extracellular fluid of different tissues. This technique is suitable for measuring the unbound concentration in the tissue fluid. The principle of microdialysis is to mimic the function of a capillary blood vessel by perfusing a thin dialysis tube implanted into the tissue with a physiological solution (Figure 8) [87]. A physiological buffer solution is continuously perfused through microdialysis probe at a constant flow rate by a microinfusion pump. The fluid entering the probe is called perfusate and, after getting in contact with the probe membrane and interacting with the tissue, the fluid coming out from the outlet of the probe is called dialysate. Once introduced in the tissue, the perfusate flows through the probe with no net delivery or removal of the fluid from the tissue. Unbound substances present in the tissue surrounding the probe will diffuse through the probe membrane into the perfusate and will be carried out of the probe by the constant flow. The dialysate concentration will represent the concentration attained in the tissue under investigation. Any changes in the tissue levels over time will be reflected in the dialysate concentration. Many studies have shown that microdialysis is reliable technique for investigating the kinetics of antibiotics in different tissues [21,23,26,85,88-100].

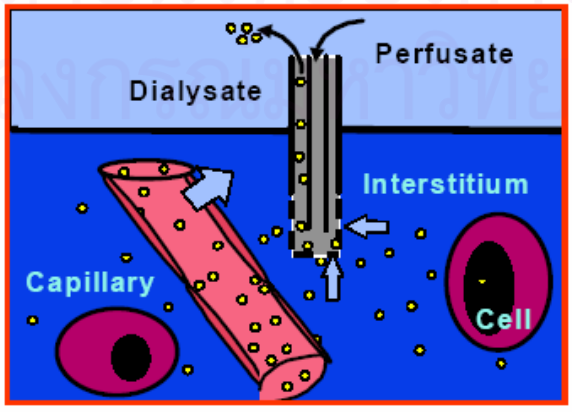


Figure 8 Scheme of a microdialysis probe implanted in a tissue.

C. Ultrafiltration Method

It is well established that plasma protein binding can have the significant impact on the pharmacokinetics and pharmacodynamics of drugs. Total plasma drug consists of unbound drugs and protein bound drugs, which are loosely bound to plasma proteins forming an equilibrium ratio between bound and unbound drugs. Determining the concentration of free, unbound drug in clinical plasma samples is important since generally this is the only pharmacologically active form of drugs. Protein-bound drugs cannot cross biological membranes and therefore cannot reach any internal target receptors. Drugs bind to a number of plasma proteins including albumin, alpha-acid glycoprotein, lipoproteins, and gamma globulins. The major drug-binding proteins are albumin and alpha-acid glycoprotein [101].

Several methods have been used to measure the analyte from biological sample by protein separation such as conventional protein precipitation, solid-phase extraction and liquid-liquid extraction [15,102-104]. However, these methods may not be able to distinguish the protein unbound fraction from the total fraction of drug. The techniques most commonly used for determination of free drug in serum are equilibrium dialysis and ultrafiltration. Ultrafiltration is a rapid and simple method to measure the unbound fraction from biological matrix, whereas equilibrium dialysis is more time-consuming, taking about 3 to 24 h depending on drugs, membrane materials, and devices before the sample is equilibrated. Due to this major disadvantage of equilibrium dialysis, the technique of ultrafiltration is commonly used in clinical applications [104,105].

Ultrafiltration is a reliable and efficient technique used for the determination of protein binding and free drug concentration in plasma. Ultrafiltration has been used for many years in clinical laboratory to monitor the free concentration of drugs such as valproic acid, carbamazepine, disopyramide, lidocaine and phenytoin[104,106]. In ultrafiltration, a pressure gradient forces the aqueous component of plasma containing the free drug through a permeability selective membrane. Ultrafiltration membrane filters at the nominal molecular weight limit (NMWL) of 10,000-30,000 Da have been used to separate free drugs from protein-bound drugs. The major drug binding proteins are albumin (MW 67,000 Da) and alpha-acid glycoprotein (MW 42,000 Da), which are captured, along with other endogenous large molecules, from plasma by the ultrafilter. Since most drugs are small molecules (<500 Da), they pass freely through the 10,000-30,000 NMWL ultrafiltration membrane; drug recovery in the ultrafiltrate

is high and non-specific binding to the membrane and device is low [101]. There are a number of ultrafiltration devices on the market that process one sample at a time. For example, a centrifugal filter device with 10,000-30,000 NMWL (Microcon®, Millipore Corporation, USA) (Figure 9) uses cone-shaped membranes that fit on the top of centrifuge vials [107]. Plasma samples are placed into the cones and centrifuged pass through the membrane. The ultrafiltrate concentration represents the free drug concentration [14].

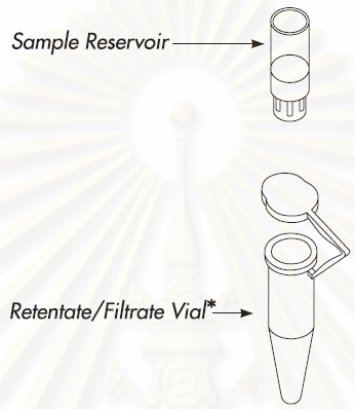


Figure 9 Diagram of centrifugal filter device

Protein Binding Studies

A study of pharmacokinetic/pharmacodynamic relationship of danofloxacin against *Mannheimia haemolytica* was carried out in a tissue-cage model. The determination of protein binding of danofloxacin in tissue-cage fluid was investigated. The pooled uninfected tissue-cage fluid was adjusted to pH 7.2 and spiked with different standard danofloxacin concentrations. Protein-bound drug was removed by ultrafiltration using centrifugal filter devices and centrifugation at 4,000 g for 25 min at room temperature. The concentration of danofloxacin before and after ultrafiltration was determined using standards in protein-free tissue-cage fluid.

Effect of protein binding on the *in vitro* activity and pharmacodynamic of faropenam was studied [108]. The protein binding of faropenem was determined in pooled inactivated human serum and in human albumin by ultrafiltration. Ultrafiltrates were assayed against faropenem phosphate buffer (pH 6) calibrators by microbiological plate assay using antibiotic medium No 1 and *S. aureus* NCTC 6571 as the indicator organism.

To determine the unbound drug concentrations of amphotericin B, pooled human EDTA plasma was spiked with amphotericin B in dimethyl sulfoxide to final concentrations of 0, 1, 5, 15, 30, and 75 μg/mL (the final dimethyl sulfoxide concentration was <0.8%) and incubated for 1 h at 37°C. The spiked plasma sample was centrifuged in a micropartition device with a 30-kDa cutoff for 30 min at 2,500 rpm. Unbound amphotericin B concentrations were determined in ultrafiltrates [109].

The protein binding of cefpodoxine in rat plasma was determined using ultrafiltration. Different concentrations (ranging from 5 to 70 mg/L) of cefpodoxime in fresh rat plasma were tested. Briefly, the plasma samples spiked with cefpodoxime were incubated at 37°C for 30 min, then an aliquot of 500 μL of plasma was added into the upper part of the centrifuged at 4,700 x g for 8 min at room temperature. Approximately 60-70 μL of ultrafiltrate sample represented the unbound concentrations of cepodoxime in plasma [110].

The protein binding of cefdinir was determined in rat plasma by the ultrafiltration method with 3-kDa cutoff. Aliquots (0.4 mL) of plasma containing the drugs were pipetted into the filter cup and were centrifuged at 4,000 x g for 3 h, thus yielding 0.2 mL of ultrafiltrate. Cefdinir concentrations in the ultrafiltrate and retentate were determined by liquid chromatography [111].

Ultrafiltration has been used in the determination of unbound ropivacaine and bupivacaine in human plasma. The plasma sample (1 mL) was transferred to an ultrafilatration device and centrifuged at 37°C and 2,000 rpm for 15 min to give 200-250 μL of filtrate. The ultrafiltrate was injected into column liquid chromatography without further clean-up pretreatment [14].

The protein binding of ciprofloxacin in human plasma was determined using ultrafiltration. Plasma ultrafiltrates were obtained from each collected plasma samples. After thawing the plasma samples at room temperature, a 300 μL aliquot of plasma samples from each time point was transferred to an ultrafiltration device and centrifuged at 10,000 rpm for 10 min. A 37 μL aliquot of ultrafiltrate was collected for analysis [30].

Serum protein binding of clindamycin in patient with AIDS was determined by ultrafiltration [112]. Serum samples were bubbled with CO_2 to a target pH of 7.3, and 1.0 mL of serum was added to the ultrafiltration device and allowed to equilibrate for approximately 30 min on a rotor prior to centrifugation. In situations in which, 1.0 mL of serum was available, the entire sample was added to the ultrafiltration device,

and then the concentration in the ultrafiltrate was adjusted to the initial volume of serum used. Samples were filtered at 2,500 rpm for 20 min at 37°C.

D. Azithromycin

Azithromycin is a semisynthetic macrolide antibiotic and is the prototype of a class of 15-membered macrolides known as the azalides. Azithromycin differs structurally from erythromycin by the addition of a methyl-substituted nitrogen atom at the 9a position of the macrolide ring (Figure 10) [32]. Azithromycin is commercially available as the dihydrate; potency is calculated on the anhydrous basis. Azithromycin anhydrous and dihydrate are a molecular weight of 749.0 and 785.0, respectively.

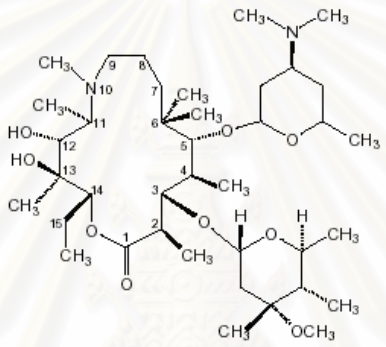


Figure 10 Structural formula of azithromycin

Azithromycin is now widely available for the treatment of a range of adult and pediatric infections, including those of upper and lower respiratory tract, skin and soft tissue, as well as sexually- transmitted diseases.

1. Mechanism of Action

Azithromycin inhibits protein synthesis in bacterial cells by binding to the 50S subunit of bacterial ribosomes [33]. It has been proposed that the high ribosome binding affinity of azithromycin may account for its enhanced activity against gram-negative organisms. The action of azithromycin is generally bacteriostatic but can be bactericidal in high concentrations against susceptible organisms. Azithromycin is more active against gram-negative organisms but has less activity against streptococci and staphylococci than does erythromycin.

2. Microbiology

Azithromycin is generally active against organisms that are usually susceptible to erythromycin. These include gram-positive organisms, such as

Staphylococcus aureus, Streptococcus agalactiae, Streptococcus pyogenes, and *Streptococcus pneumoniae*, and gram-negative *Haemophilus influenzae* and *Moraxella catarrhalis*. *Chlamydia trachomatis* is also susceptible to azithromycin. Other organisms that have shown *in vitro* susceptibility include streptococci (Groups C, F, G), *Streptococcus viridans* group, *Bordetella pertussis, Campylobacter jejuni, Haemophilus ducreyi, Legionella pneumophilia, Bacteroides bivius, Clostridium perfringens, Peptostreptococcus* species, *Mycoplasma pneumoniae, Treponema pallidum*, and *Ureaplasma urealyticum*. The excellent tissue penetration and very low MIC of azithromycin against *Borrelia burgdorferi* (the causative agent of Lyme disease) suggest it may be highly useful in treating this serious disease [113]. Clinicians are advised to consult susceptibility data at the institution in which they practice to determine azithromycin's activity.

3. Pharmacokinetics

3.1 Absorption

Azithromycin is administered orally and intravenously. Azithromycin is rapidly absorbed from the GI tract after oral administration. The absolute bioavailability of azithromycin is reported to be approximately 34-52% with single doses of 500 mg to 1.2 g administered as various oral dosage forms (such as capsules, tablets, oral suspension) [32,34,114]. Following oral administration of azithromycin 500 mg (as two 250 mg capsules or tablets) in 12 healthy volunteers, peak, trough level, and AUC_{24} were reported to be 0.5 μg/mL at about 2 h, 0.05 μg/mL, and 2.6 μg.h/mL, respectively [115]. Azithromycin plasma concentrations following IV administration of azithromycin 500 mg are substantially higher than those following oral administration of the same dose. In healthy individuals receiving 500 mg oral dose of azithromycin, peak, trough concentraion and AUC_{24} were 38, 83, and 53% of value in individuals receiving azithromycin 500 mg IV over 3 h [116]. Studies in adults, the C_{max} of azithromycin of only 0.9 mg/L was achieved after a single 500 mg oral dose. Administration of multiple doses produced only a slight increase. C_{max} values were somewhat lower in pediatric patients receiving a suspension formulation of azithromycin : 0.224 mg/L after 5 days of therapy for otitis media and 0.383 mg/L after 5 days of treatment of streptococcal pharyngitis [117]. Studies that used a research capsule formulation of azithromycin indicated decrease in both the rate and extent of absorption when the drug was taken with food. For this reason

administration of azithromycin capsules at least 1 h or 2 h after meal is recommended. Subsequent investigations of azithromycin provided no evidence of significant effect on bioavailability when the drug was taken after high fat meal. In fact the C_{max} of the tablet and the suspension was slightly increased in the fed state however the AUC remained unchanged [33].

3.2 Distribution

Azithromycin appears to be distributed into most body tissue and fluids after oral or IV administration. Azithromycin exhibits significant intracellular penetration and concentrates within fibroblasts and phagocytes. In addition, azithromycin is released slowly from phagocytic cells, substantial azithromycin concentrations are maintained for prolonged periods within these cell. Spontaneous release of azithromycin from phagocytes may be enhanced by exposure of the cell membrane to bacteria. Although release of azithromycin from fibroblasts is not enhanced by cell membrane exposure to pathogens, fibroblasts may act as drug reservoirs, releasing the drug to phagocytes for subsequent transport to the site of infection. Because of rapid distribution into tissues and high intracellular concentrations of azithromycin, tissue concentrations of the drug generally exceed plasma concentrations by 10-100 fold following single dose administration [113]. For example, peak concentrations of azithromycin exceeded 3 mg/kg in prostate, tonsil, lung , kidney, gastric and gynecologic tissue after administration of two 250 mg doses at 12 h intervals (Figure 11) [34]. High concentrations of azithromycin persisted in tissue for several days. Indeed, the reported values for volume of distribution have been large 23 to 31 L/kg. The serum protein binding of azithromycin varies with plasma concentration; 52% of the drug is bound at low concentration (0.02 μg/mL) and decreasing to 7% at higher concentrations (2 μg/mL).

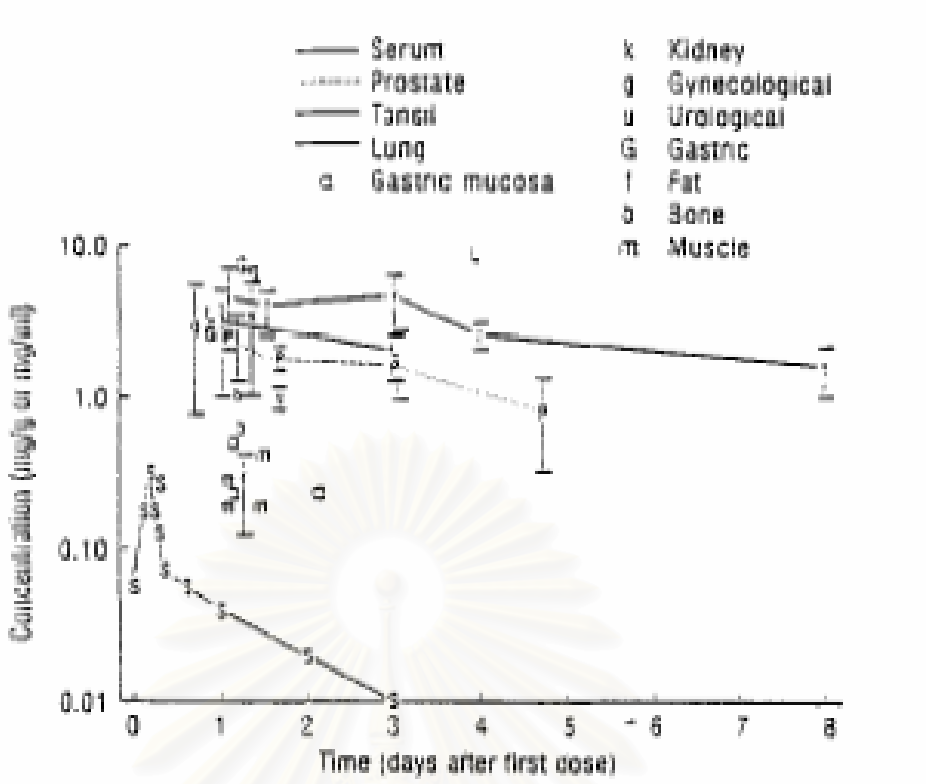


Figure 11 Mean (± SD) tissue concentrations in man after administration of azithromycin. S, serum; P, prostate; t, tonsil; k, kidney; g, gynaecol- gical; u, urological; G, gastric; m, muscle; f, fat; b, bone; *gastric mucosa.

3.3 Metabolism

The principal route of biotransformation involves *N*-demethylation of the desosamine sugar or at the 9a position on the macrolide ring. Other pathways of metabolism include *O*-demethylation and hydrolysis and/or hydroxylation of the cladinose and desosamine sugar moieties and the macrolide ring. Pharmacokinetic studies of azithromycin 500 mg administered orally in patients with mild to moderate hepatic dysfunction compared with healthy volunteers established a statistically significant ($p \leq 0.05$) increase for the patients with hepatic impairment versus controls in the terminal elimination half-life (68 vs 54 h) [116]. These results were not considered clinically significant and suggested that dosage adjustment is unnecessary in patients with mild to moderate hepatic impairment; however, it is unknown if dosage adjustment are necessary in patients with severe hepatic impairment.

3.4 Elimination

Azithromycin is mainly eliminated unchanged, principally in the feces and to lesser extent via the urinary route. In addition, it has been suggested that transintestinal excretion may be the primary route of elimination for the unchanged

compound. This transintestinal route is believed to account for elimination of 30 to 35% of the total administered dose [115]. Plasma azithromycin concentrations following a single 500-mg oral or IV dose decline in a polyphasic manner reflecting initial rapid distribution into tissues followed by slow release with a terminal elimination half-life averaging 68 h [113]. An elimination half-life of 54.5 h has been reported in children 4 months to 15 years of age receiving single or multiple oral doses of azithromycin.

4. Dosage and Administration

The recommended oral dosage regimen for azithromycin in the treatment of infections in adults, excluding those transmitted sexual, is 500 mg once daily for 3 days or a single 500 mg dose on the first day followed by 250 mg once daily for additional 4 days [118]. In pediatric patients a dosage regimen of 10 mg/kg on the first day of treatment and 5 mg/kg for a further 4 days in recommended. However, children weighing more than 45 kg should receive the adult dosage [33]. Chlamydial urethritis or cervicitis may be treated with a single oral dose of 1 g ; clinical trials have indicated that this dose is also appropriate for the treatment of chancroid and uncomplicated gonorrhoea. In the treatment of Lyme disease, a standard 5-day regimen or azithromycin 250 mg twice daily for 2 days followed by 250 mg daily for 8 days has proven effective. Preliminary studies indicate that azithromycin 500 mg dialy for 10 to 30 days is of benefit in the treatment of *M. avium* complex infection in HIV-infected patients [32].

Dosage adjustment does not appear to be necessary in elderly patients with mild renal impairment, or in patients with mild to moderate hepatic dysfunction. Although no published data are available for patients with more severe renal or hepatic impairment, it is recommended by the manufacture that azithromycin be used with caution in patients with creatinine clearance rates of <2.4 L/h, and patients with severe hepatic disease should not receive azithromycin treatment [32].

Azithromycin is contraindicated in patients with known hypersensitivity to macrolide antimicrobials and in patients receiving ergot alkaloids. Azithromycin should be administered at least 1 h before or 2 h after antacids or food [32].

5. Adverse Drug Reactions [119]

Azithromycin generally is well tolerated. In clinical studies, most adverse effects were mild to moderate in severity and were reversible upon discontinuance of the drug.

Adverse GI effects (e.g., nausea, vomiting, diarrhea, abdominal pain) and rash are the most frequent adverse effects requiring discontinuance of the drug. The manufacturer states that rate of discontinuance of azithromycin was approximately 0.7% in adults or children receiving a 5-day oral regimen; 0.4% in adults receiving a 3-day oral regimen (500 mg daily); or 1% in children receiving a single 30-mg/kg oral dose or a 3-day oral regimen (10 mg/kg daily). In addition, 1.2-2.4% of adults receiving a regimen that included both IV and oral azithromycin discontinued therapy because of adverse effects.

5.1 GI Effects

The most frequent adverse effects of azithromycin involve the GI tract (i.e., diarrhea/loose stools, nausea, abdominal pain). While these adverse effects generally are mild to moderate in severity and occur less frequently than with oral erythromycin therapy, adverse GI effects are the most frequent reason for discontinuing azithromycin therapy. Adverse GI effects have occurred more frequently in patients receiving azithromycin as a single oral dose of 1, 1.2, or 2 g than in those receiving a 5-day oral regimen.

5.2 Dermatologic and Sensitivity Reactions

Serious allergic (i.e., angioedema, anaphylaxis, bronchospasm) and dermatologic (i.e., erythema multiforme, Stevens-Johnson syndrome, toxic epidermal necrolysis) reactions, sometimes resulting in death, have been reported rarely in patients receiving azithromycin. If allergic reactions occur, azithromycin should be discontinued and appropriate therapy initiated. Rash, urticaria, pruritus, and photosensitivity have been reported in 1% or less of patients receiving a 5-day regimen of oral azithromycin. Rash or pruritus occurred in 1.9%, and urticaria in 1% or less of adults receiving a regimen that included both IV and oral azithromycin.

5.3 Hepatic Effects

Elevations in ALT (SGPT), AST (SGOT), or γ-glutamyltransferase (GGT, GGTP) have been reported in 1-2% of adults receiving oral azithromycin; elevations in serum alkaline phosphatase, lactic dehydrogenase (LDH), and/or total bilirubin concentration have been reported in less than 1% of such patients. Available follow-up data have revealed that liver function test abnormalities in patients receiving azithromycin therapy generally are reversible. However, azithromycin therapy was discontinued in clinical trials because of treatment-related liver enzyme abnormalities in at least 3 patients receiving a 5-day regimen of oral azithromycin and in less than

2% of patients receiving a regimen that included both IV and oral azithromycin. Abnormal liver function, including cholestatic jaundice and hepatitis, and pancreatitis has been reported infrequently in clinical trials or during postmarketing studies with azithromycin. Hepatic necrosis and hepatic failure, sometimes resulting in death, have occurred rarely.

5.4 Renal and Genito-urinary Effects

Elevation in serum potassium concentration has been reported in 1-2% of adults receiving azithromycin in clinical trials. Elevation in BUN, serum creatinine, or serum phosphate concentration has been reported in less than 1% of adults receiving oral azithromycin, while elevated serum creatinine concentration has been reported in 4-6% of patients receiving IV azithromycin. Available follow-up data revealed that these elevations generally were reversible. Nephritis has been reported in 1% or less of adults receiving azithromycin in clinical studies. Interstitial nephritis and acute renal failure have been reported during postmarketing studies with the drug. Azithromycin was discontinued because of an unspecified renal function abnormality in at least one patient receiving the drug in clinical trials.

5.5 Cardiovascular Effects

Palpitations, chest pain, edema, hypotension, or syncope has been reported in 1% or less of patients receiving oral azithromycin. While not directly attributed to azithromycin therapy, arrhythmia (including ventricular tachycardia) has been reported in at least one patient receiving the drug. In one patient with a history of arrhythmia, torsades de pointes with subsequent myocardial infarction occurred following completion of azithromycin therapy.

5.6 Nervous System Effects

Adverse CNS effects reported in 1% or less of adults receiving azithromycin include dizziness, headache, vertigo, or somnolence, and those reported in 1% or less of children include headache, hyperkinesia, dizziness, agitation, nervousness, fatigue, malaise, and insomnia. Fatigue or malaise has been reported and has occurred in 2-4 or about 1%, respectively, of patients receiving azithromycin 1.2 g weekly. Seizures also have been reported during azithromycin therapy. Dizziness or headache has occurred in about 1-4% of patients receiving azithromycin (1.2 g weekly) for the prevention of disseminated MAC infections. Asthenia, aggressive reaction, anxiety, or paresthesia has been reported during postmarketing studies with azithromycin.

5.7 Hematologic Effects

Leukopenia, neutropenia, neutrophilia, or thrombocytopenia, has been reported in less than 1% of adults receiving azithromycin, although a causal relationship to the drug has not been established.

5.8 Otic Effects

While audiometric testing revealed no drug-related hearing abnormalities in a limited number of individuals receiving short-term therapy with oral azithromycin (1.5 g over 5 days or 1 g as a single dose), hearing loss has been reported in some patients receiving long-term high-dose azithromycin therapy (i.e., 500-600 mg daily for up to 9 months). Hearing loss generally develops within 1.5-20 weeks and generally resolves within 5 weeks following discontinuance of azithromycin.

5.9 Other Adverse Effects

Fever or conjunctivitis has been reported in 1% or less of children receiving azithromycin. Arthralgia also has been reported during postmarketing studies with azithromycin. Hypothermia has occurred in a few patients receiving azithromycin. Increases in serum creatine kinase (CK), creatine phosphokinase (CPK) have occurred in 1-2% of patients receiving oral azithromycin, and increases in blood glucose concentration have been reported in less than 1% of patients. Hyponatremia and/or the syndrome of inappropriate antidiuretic hormone secretion, has been reported rarely with azithromycin therapy; a causal relationship to the drug has not been established.

CHAPTER III

MATERIALS AND METHODS

Materials

A. Drug

1. Azithromycin dihydrate standard powder (provided by Pfizer Inc, Groton, CT, USA)

2. Azithromycin dihydrate 250 mg capsule (Zithromax®, Pfizer Thailand Ltd.)

B. Bacteria

Streptococcus pneumoniae (Penicillin-Intermediate) ATCC® 49616, *Streptococcus pneumoniae* (Penicillin-Sensitive) ATCC® 6303, *Haemophilus influenzae* ATCC® 10211 and *Moraxella catarrhalis* ATCC® 8176 were obtained from the Microbiology laboratory, Shands Hospital at University of Florida, Gainesville, Florida, USA.

C. Broth Media

1. Mueller-Hinton Broth (Becton Dickinson, Franklin Lakes, NJ, USA)

2. Todd Hewitt Broth (Difco, Detroit, USA)

3. Haemophilus Test Media (Remel Microbiology Products, Lenexa, KS, USA)

D. Agar Plates

1. Sheep blood agar plates (Remel Microbiology Products, Lenexa, KS, USA)

2. Chocolate agar plates (Remel Microbiology Products, Lenexa, KS, USA)

E. Reagents

1. Sterile saline solution 0.9% (Shands Hospital, University of Florida, USA)

2. Turbidity standard scale solution (McFarland, Remel Microbiology Products, Lenexa, KS, USA)

3. Working standard azithromycin powder (provided by Siam Bheasach, Thailand)

4. Working standard clarithromycin powder (provided by Siam Bheasach, Thailand)

5. Acetonitrile HPLC grade (Lab Scan, Thailand)

6. Ammonium acetate AR (Merck, Germany)

7. tert-Butyl methyl ether (Merck, Germany)

8. Pooled drug free plasma (Thai Red Cross Society, Thailand)

F. Apparatus

1. Turbidimeter (A-JUST, Abbott Laboratories, North Chicago, IL, USA)
2. Tissue culture plate, 24-well (Corning Incorporated, NY, USA)
3. Tissue culture plate, 96-well (Corning Incorporated, NY, USA)
4. Tissue culture flask (Corning Incorporated, NY, USA)
5. Micropipette (Eppendorf AG, Hamburg, Germany)
6. Vortex mixer (Vortex-Genie, Scientific Industries Inc, USA)
7. Analytical balance (Mettler Model AT 201, Switzerland)
8. Sonicator (Ultrasonic bath XB6, England)
9. Glassware (Pyrex, USA)
10. Centrifuge (Mistral 3000E, UK)
11. Refrigerated centrifuge (Hettich EBA 12R, Germany)
12. Liquid chromatographic mass spectrometer (Shimadzu 2010A, Japan)
    - HPLC pump
    - Autosampler
    - Column (Inersil ODS-3, 150 x 2.1 mm, i.d., C_{18}, 5 µm)
    - Guard column (Inersil ODS-3, 50 x2.1 mm)
    - Column oven
    - UV detector
    - Masspectrometer
13. Nylon syringe filters, 13mm, 0.2µm (Whatman, England)
14. Nylon membrane filter, 47mm, 0.2µm (Whatman, England)
15. EDTA tube 9 mL (Greiner BioOne, Australia)
16. Centrifugal filter device (Microcon YM-30, USA)
17. Vacuum centrifuge (Maxi Dry Plus, Denmark)
18. Freezer (FC-27, Sharp, Japan)

Methods

A. Pharmacodynamic Studies

1. Drugs and Bacteria

Azithromycin dihydrate was provided from Pfizer Inc, Groton, CT. The compound was stored in room temperature in the original vial. Azithromycin dihydrate stock solutions were freshly prepared in distilled water prior to use. Four

different bacterial strains: *Streptococcus pneumoniae* (Penicillin-Intermediate) ATCC® 49616 , *Streptococcus pneumoniae* (Penicillin-Sensitive) ATCC® 6303, *Haemophilus infuenzae* ATCC® 10211 and *Moraxella catarrhalis* ATCC® 8176 were obtained from the microbiology laboratory, Shands Hospital at University of Florida, Gainesville, Florida.

2. Broth Preparation and Microbiological Media

Mueller-Hinton Broth (MHB) for *M. catarrhalis* and Todd Hewitt Broth (THB) for *S. pneumoniae* were both prepared according to the manufacturer's instructions and autoclaved prior to use at 121 ^{o}C (40 min per 500 mL). Haemophilus Test Media (HTM) Broth for *H. influenzae* was purchased sterile and ready to use and stored in a refrigerator at approximately 7 ^{o}C. 5% Sheep blood agar plates was used for *S. pneumoniae* and *M. catarrhalis*, and Chocolate agar plate was used for *H. influenzae*.

3. Bacterial Inoculation

The bacterial inoculum was prepared from colonies incubated overnight on appropriate agar plates (5% Sheep blood agar plate for *S. pneumoniae* and *M. catarrhalis*, Chocolate agar plate for *H. influenzae*). The microorganisms were suspended in sterile normal saline to a concentration equivalent to a 0.5 value in the McFarland scale with a turbidimeter. This value on the the McFarland scale of 0.5 is equivalent to a number of $1x10^{8}$ CFU/mL. Further dilution steps to reach a final working inoculum of approximately $5x10^{5}$ CFU/mL were done in broth.

4. Determination of MIC

The MICs for all strains were determined by serial two-fold microdilution method. Briefly, a sterile, flat bottom 24-well plate with a lid was used for the preparation of the serial two-fold dilutions. Each plate was divided in half so that two replicates were done per plate. Each one of the wells was added with 1 mL of broth, except for the first and last wells. The last well received 2 mL of broth and served as negative control. To the first well was added with 2 mL of the highest azithromycin concentration of the drug in broth. Next, with the aid of an automatic pipette, 1 mL of solution from the first well was transferred to the second well and mixed. Subsequently, 1 mL was taken from the second well into the third well and mixed. This procedure was systematically repeated up to the 10^{th} well from which 1 mL was taken and discarded. Thus, each well held a solution of azithromycin in broth that was half the concentration of the previous one. The 11^{th} well did not receive azithromycin

and served as a positive control. Finally, 1 mL of the working inoculum was added to each well and the plates were incubated at 37 ^{o}C (for *M. catarrhalis*) and 5% CO_2 (for *S. pneumoniae* and *H. influenzae*) for 18-20 h. The MIC was determined at the concentration of azithromycin that prevented visual growth of the bacteria after this incubation period.

5. Constant Concentration Time Kill Curves

An *in vitro* kinetic model was used to investigate the antibacterial efficacy of constant drug concentrations during 6 h. It consisted of a 50 mL vented cap tissue culture flask with a canted neck, containing 20 mL of the appropriate broth media, incubated at 37 ^{o}C (for *M. catarrhalis*) and 5% CO_2 (for *S. pneumoniae* and *H. influenzae*).

An aliquot of a suspension (100 mL) of the initial inoculation, ($1x10^8$ CFU/mL) was added to the *in vitro* model to produce a final inoculum of approximately $5x10^5$ CFU/mL. The model was incubated for 2 h to allow the bacteria to reach the log-growth phase before the addition of different azithromycin concentrations. Immediately before the addition of the drug, a 20 µL sample of each tissue culture flask at time-zero was taken into a sterile, flat bottom 96-well plate with lid that was used for the preparation of the serial 10-fold dilutions. Subsequently, 20 µL samples were collected at 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h.

The selection of azithromycin concentrations tested in each bacterial strain was based on their determined MIC values. At least seven different concentrations were exposed, which covered the entire range including minimum inhibition of bacterial growth (0.25xMIC, 0.5xMIC, 1xMIC), efficient bacterial killing (2xMIC, 4xMIC) and maximum bacteria killing (8xMIC, 16xMIC). A control experiment with bacteria and no drug was run simultaneously.

6. Bacterial Quantification

Bacterial counts were determined by plating 50 µL of the serial 10-fold dilutions, on an appropriate agar plate using an adapted drop-plate method. Briefly, an agar plate was divided into four quadrants. With an automatic pipette, 5 x 10 µL drops of the chosen dilution were equidistantly plated onto one of the quadrants. A duplicate was plated onto the adjacent quadrant. Then, the plates were incubated at 37 ^{o}C (for *M. catarrhalis*) and 5% CO_2 (for *S. pneumoniae* and *H. influenzae*) for 16-24 h before reading. The procedure was repeated at least 3 times per bacterial strain and each concentration. Positive controls with bacteria but no drug were run simultaneously.

Following incubation, the number of CFUs were counted in each duplicate quadrant at each time point and averaged.

Time-kill curves for each dose and bacterial strain were constructed by plotting the log CFU/mL versus time. The data from experiment were entered in the Microsoft Excel spreadsheets.

B. PK/PD Modeling

1. PK/PD Analysis

Time-kill curves analysis and mathematical modeling of the time-kill data were performed with the non-linear regression software program Scientist® (Micromath, Salt Lake City, UT, USA, used under licence of University of Florida). By using this program, the experimental time-kill data were fitted to the following 12 differential equations:

First, a simple E_{max} model was fitted to the PD data :

$$\frac{dN}{dt} = \left[k_0 - \left(\frac{k_{\max} \cdot C}{EC_{50} + C} \right) \right] \cdot N \qquad \text{(model 1)}$$

Where dN/dt is the change in number of bacteria as a function of time, k_0 (h^{-1}) is the bacterial growth rate constant in the absence of antibiotic, k_{max} (h^{-1}) the maximum killing rate constant (maximum effect), EC_{50} (μg/mL) the concentration of antibiotic necessary to produce 50% of maximum effect, C (μg/mL) the concentration of antibiotic at time t and N the number of viable bacteria expressed as CFU/mL.

Next, five different models with increasing complexity were fitted and compared.

The first parameter, adaptation rate (z) may be incorporated in the model 1. If the bacterial are not in the logarithmic growth phase at time zero, an exponential correction factor ($1-e^{-zt}$) may be necessary to compensate for this delay. This parameter was incorporated in a second model as follows:

$$\frac{dN}{dt} = \left[k_0 - \left(\frac{k_{\max} \cdot C}{EC_{50} + C} \right) \right] \cdot \left(1 - e^{-zt}\right) \cdot N \qquad \text{(model 2)}$$

Also, the onset of effect may show a delay. Therefore, the exponential correction factor can also be included at E_{max} parameter as a third model :

$$\frac{dN}{dt} = \left[k_0 - \left(\frac{k_{\max} \cdot C}{EC_{50} + C} \right) \cdot \left(1 - e^{-zt}\right) \right] \cdot N \qquad \text{(model 3)}$$

Another important parameter which may be taken into model is N_{max} because there are limitation of space and nutrients. Therefore, a fourth model can be modified :

$$\frac{dN}{dt} = \left[k_0 \left(1 - \frac{N}{N_{\max}} \right) - \left(\frac{k_{\max} \cdot C}{EC_{50} + C} \right) \right] \cdot N \qquad \text{(model 4)}$$

If the two adaptation rate terms from model 2 and 3 are included as well, two more models can be constructed as follows :

$$\frac{dN}{dt} = \left[k_0 \left(1 - \frac{N}{N_{\max}} \right) - \left(\frac{k_{\max} \cdot C}{EC_{50} + C} \right) \right] \cdot \left(1 - e^{-zt} \right) \cdot N \qquad \text{(model 5)}$$

$$\frac{dN}{dt} = \left[k_0 \left(1 - \frac{N}{N_{\max}} \right) - \left(\frac{k_{\max} \cdot C}{EC_{50} + C} \right) \cdot \left(1 - e^{-zt} \right) \right] \cdot N \qquad \text{(model 6)}$$

In addition, a Hill factor (h) may be used to modify the steepness of the concentration effect for models 1 to 6. This factor is taken into E_{max} parameter term. Another models 7-12 can be modified as follows:

$$\frac{dN}{dt} = \left[k_0 - \left(\frac{k_{\max} \cdot C^h}{EC_{50}^h + C^h} \right) \right] \cdot N \qquad \text{(model 7)}$$

$$\frac{dN}{dt} = \left[k_0 - \left(\frac{k_{\max} \cdot C^h}{EC_{50}^h + C^h} \right) \right] \cdot \left(1 - e^{-zt} \right) \cdot N \qquad \text{(model 8)}$$

$$\frac{dN}{dt} = \left[k_0 - \left(\frac{k_{\max} \cdot C^h}{EC_{50}^h + C^h} \right) \cdot \left(1 - e^{-zt} \right) \right] \cdot N \qquad \text{(model 9)}$$

$$\frac{dN}{dt} = \left[k_0 \left(1 - \frac{N}{N_{\max}} \right) - \left(\frac{k_{\max} \cdot C^h}{EC_{50}^h + C^h} \right) \right] \cdot N \qquad \text{(model 10)}$$

$$\frac{dN}{dt} = \left[k_0 \left(1 - \frac{N}{N_{\max}} \right) - \left(\frac{k_{\max} \cdot C^h}{EC_{50}^h + C^h} \right) \right] \cdot \left(1 - e^{-zt} \right) \cdot N \qquad \text{(model 11)}$$

$$\frac{dN}{dt} = \left[k_0 \left(1 - \frac{N}{N_{\max}} \right) - \left(\frac{k_{\max} \cdot C^h}{EC_{50}^h + C^h} \right) \cdot \left(1 - e^{-zt} \right) \right] \cdot N \qquad \text{(model 12)}$$

Finally, the resulting total of 12 different PK/PD models were fitted to the time-kill data and compared for best fit. For each bacterial strain, the initial estimates for k_0 and k_{max} were determined using the data for the growth rate (positive control) and the data for the maximum kill curves. The k_0 and k_{max} in each model were fixed at

the actual value whereas N_{max}, EC_{50}, z and h were fitted simultaneously to the experimental data.

2. Curve Fits of Bacterial Time-Kill Curve

2.1 Determination of k_0 from positive control data

From model 1, in the absence of drug, bacteria are grown at their normal growth rate and the E_{max} parameter, (k_{max} x C)/(EC_{50}+C) equal zero. The resultant equation for determination of k_0 is $dN/dt = k_0 \times N$. In the similar way, the equation for determination of k_0 in another models 2-12 can be modified. The estimation of k_0 for each bacterial strain was determined using the experimental data from positive control fitted to their equations.

2.2 Determination of k_{max} from maximum effect data

From model 1, at the maximum effect, C value is much higher than EC_{50}. Therefore, the term C in E_{max} parameter cancels out and the resultant kill rate is $k_0 - k_{max}$. Equation for determination of k_{max} is $dN/dt = (k_0 - k_{max}) \times N$. The equation for determination of k_{max} in another models 2-12 can be constructed in the similar way. The estimation of k_{max} for each baterial strain was determined using the experimental data from the highest azithromycin concentration whereas the determined k_0 value was fixed.

2.3 Determination of EC_{50}, N_{max}, z and h from all data of time-kill curve

The initial estimates of k_0 and k_{max} were fixed in each model at their determined values, whereas EC_{50}, N_{max}, z and h were fitted simultaneously to positive control data and data from all azithromycin concentrations in experimental time-kill curve.

3. Criteria for the Goodness of the Fit

For the mathematical evaluation, 12 different PK/PD models were determined which one fits the data best. To determine which model performed best, the Model Selection Criterion (MSC), the coefficient of determination (r^2), the correlation between observed and calculated values as well as the visual inspection were provided by Scientist® computer program and were used as criteria for the goodness of the fit. Increased MSC values indicate more appropriate fits. The resulting models were compared for best fit.

C. Pharmacokinetic Studies

1. Subjects

Eight healthy male volunteers were selected to participate in the study based on screening examination including medical history, physical examination, and clinical laboratory record such as complete blood count, blood urea nitrogen, serum creatinine, SGOT/SGPT, total bilirubin, alkaline phosphatase, hepatitis B virus antigen, and human immuno-deficiency virus antibody. All subjects were briefed on the study details and a written informed consent was obtained prior to the beginning of the study. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

2. Inclusion and Exclusion Criteria

Healthy volunteer 18-45 years of age was eligible for this study. Volunteers were included if they are male, healthy, drug free, and have a normal body mass index. Subjects were excluded if they were taking any prescription medication or OTC drugs within a period of one week prior to the study. Subjects would also be excluded if any abnormality were found as part of the pre-treatment screening or in any of the laboratory tests performed that the investigators consider as clinically significant. Furthermore pre-existent known drug allergy represented an exclusion criterion.

3. Study Protocol

Azithromycin was administered orally as two 250 mg capsules once daily for three consecutive days as treatment schedule. The initial dose was taken under direct supervision in the study center. Following the next drug administration was conducted by telephone. Subjects were instructed to take azithromycin in the morning (approximately 7 a.m.) after at least 10 h fasting period and continued fasting for 4 h after each dose. In the morning of day 3, following an overnight fast, volunteers were admitted to the clinical research ward at Department of Pharmacology, Faculty of Medicine, Chulalongkorn University. During the study-period the subjects were constantly observed and monitored by a physician. Blood samples (5 mL) were collected via an indwelling catheter placed in the antecubital vein at times 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168, and 192 h after the administration of the last dose. Plasma was immediately separated by centrifugation at 3,000 rpm for 15 min and stored at -20º C until analysis. Free plasma concentration

was determined using the ultrafiltration method. The plasma ultrafiltrate concentration represents the free plasma concentration.

4. Determination of Azithromycin in Plasma and in Ultrafiltrate

Plasma and ultrafiltrate azithromycin concentrations were determined by liquid chromatographic masspectrometer (LC-MS) using a method modified from that reported by Zhong et al [120]. The procedure was described as follows :

4.1 Sample Preparation

Preparation of plasma sample : An aliquot (1mL) of plasma sample was transferred to a glass test tube, 50 µL of internal standard (0.15 mg/mL of clarithromycin in acetonitrile) was added and mixed for 1 min in a vortex mixer. The mixture was added with 6 mL of tert-Butyl methyl ether for drug extraction and vortex mixed for 2 min. The sample was centrifuged at 14,000 rpm for 15 min. Supernatant was then separated and evaporated in a vacuum centrifuge. Residue was reconstituted with 5 mL of acetonitrile and filtered through a 0.2 µm nylon membrane. 10 µL aliquot of solution was injected into the LC-MS system.

Preparation of plasma ultrafiltrate sample : An aliquot of 500 µL of plasma was added into the upper part of the centrifugal filter device (cutoff value : 30 kDa) and centrifuged at 14,000 rpm, 25 ^{o}C for 15 min. An aliquot 70 µL of plasma ultrafiltrate was transferred to a glass test tube, 50 µL of internal standard (0.03 mg/mL of clarithromycin in acetonitrile) and mixed for 1 min in a vortex mixer. The solution was diluted with 880 µL of acetonitrile and filtered though a 0.2 µm nylon membrane. 20 µL aliquot of solution was injected into the LC-MS system.

4.2 LC-MS Systems

Instrument	: LC-MS, Shimadzu 2010A
Detector	: MS, m/z for Azithromycin = 749.45, for Clarithromycin = 748.40
Column	: Inersil ODS-3, C_{18}, 5 µm, 2.1 x 150 mm
Guard column	: Inersil ODS-3, 2.1 x 50 mm
Column oven	: 40 ^{o}C
Flow rate	: 0.2 mL/min
CDL temperature	: 250 ^{o}C
Block temperature	: 200 ^{o}C
Nebulizer gas flow	: 1.5 L/min
Detector gain	: 1.7 KV

Mobile phase : A=Acetonitrile, B=0.02 M Ammonium acetate

Gradient flow rate :

Time	Flow	A	B
0 min	1.2 mL/min	55%	45%
5 min	1.2 mL/min	80%	20%
7 min	1.2 mL/min	80%	20%
7.5 min	1.2 mL/min	55%	45%
10 min	stop		

Retention time : Azithromycin was approximately 5 min
Clarithromycin was approximately 7 min

4.3 Preparation of Standard Solution

Stock standard solutions of azithromycin were prepared. Azithromycin working standard was accurately weighted about 0.0275 g and dissolved in 25 mL of acetonitrile to give a nominal concentration of 1.0 mg/mL azithromycin. Dilutions of this solution were made with acetonitrile to give working solutions of 0.1, 1, 10 μg/mL, respectively.

Stock standard solutions of clarithromycin (internal standard) were prepared by accurately weighing 0.0300 g of clarithromycin and dissolved in 100 mL of acetonitrile to give a nominal concentration of 0.3 mg/mL of clarithromycin. Dilutions of stock solution were made with acetonitrile to give working solutions of 0.15, 0.03 mg/mL, respectively.

The stock solution and working solutions for azithromycin and claritromycin were prepared on a daily basis.

4.4 Preparation of Standard Calibration Curve

Azithromycin plasma calibration curve were prepared by spiking working solution of azithromycin in blank plasma to produce a set of standard solutions of 10, 20, 30, 50, 100, 200, 400, 600 and 800 ng/mL, respectively. All these standard solutions were analyzed following the same procedure as described earlier.

Azithromycin plasma ultrafiltrate calibration curve were prepared by spiking working solution of azithromycin in blank plasma ultrafiltrate to provide the standard solutions of 5, 10, 30, 50, 70, 100, 200, 400 and 600 ng/mL, respectively. An aliquot of 70 μL of plasma ultrafiltrate was analyzed following the same procedure as described earlier. The blank plasma ultrafiltrates were obtained as follows. Blank plasma was transferred to the upper part of centrifugal filter device on an eppendorf

tube and centrifuged at 14,000 rpm, 25 ^{o}C for 15 min. The solution passed through this device is the blank plasma ultrafiltrate.

The ratio of peak area of azithromycin to that of clarithromycin versus known azithromycin concentrations were fitted to a straight line using linear regression analysis.

4.5 Bioanalytical Method Validation

The methods were validated in accordance with the Guidance for Industry : Bioanlytical Method Validation of Center for Drug Evaluation and Research (CDER) and Center of Veterinary Medicine (CVM), U.S. Department of Health and Human Services, Food and Drug Administration, 2001. The details of validation were described as follows:

4.5.1 Selectivity/Specificity

It was determined by analyzing samples prepared from blank plasma and blank plasma ultrafiltrate of six sources with and without azithromycin and clarithromycin (internal standard). Any peaks due to the presence of plasma protein and/or endogenous substances should not interfere with the drug and the internal standard.

4.5.2 Lower Limit of Quantification (LLOQ)

Five determination of lowest concentration of standard azithromycin in plasma and in ultrafiltrate were analyzed. The LLOQ were established by examination of the accuracy and precision data. Analyte peak of these concentrations should be identifiable, discrete, and reproducible with accuracy of 80-120%, a precision not exceed 20%.

4.5.3 Linearity and Standard Calibration Curve

Nine azithromycin concentrations of standard solution of 10, 20, 30, 50, 100, 200, 400, 600 and 800 ng/mL in plasma as well as nine azithromycin concentrations of standard solution of 5, 10, 30, 50, 70, 100, 200, 400 and 600 ng/mL in plasma ultrafiltrate were analyzed. The peak area ratio of azithromycin to that of internal standard were fitted to straight line using linear regression analysis.The coefficient of determination should be more than 0.99. The percent recovery for accuracy and percent coefficient of variation for precision of the LLOQ, and standards other than LLOQ should not more than 20% and 15% respectively.

4.5.4 Accuracy

Five determinations of quality control samples (QC samples) which include three standard concentrations (low, medium, high) of azithromycin in plasma (30,300,700 ng/mL) and in ultrafiltrate (15, 300, 500 ng/mL) were analyzed. Accuracy of analytical method was estimated by the percent recovery of each concentration level using the following equation.

$$\% \text{ Recovery} = \frac{\text{Estimated concentration}}{\text{Known concentration}} \times 100$$

The mean value of the percent recovery of each concentration level should be within 15% of nominal concentration.

4.5.5 Precision

4.5.5.1 Within-run precision

Five determinations of three QC samples of azithromycin in plasma and in ultrafiltrate were prepared and analyzed in the same day. Precision of the determination is estimated by calculating percent coefficient of variation (%CV) of each concentration level using the following equation.

$$\% \text{ CV} = \frac{\text{SD}}{\bar{X}} \times 100$$

The percent coefficient of variation of each concentration level should not exceed 15%.

4.5.5.2 Between-run precision

Five determinations of three QC samples of azithromycin in plasma and in ultrafiltrate were analyzed on five different days. The percent coefficient of variation of each concentration level should not exceed 15%.

4.5.6 Stability

4.5.6.1 Freeze-thaw Stability

Three aliquots of two concentrations of QC samples in plasma and in ultrafiltrate (low and high) were analyzed and stored at -20 ^{o}C for 24 h and thawed unassisted at room temperature. This was one freeze-thaw cycle. After complete thaw, the freeze-thaw cycles were repeated two more times under the same conditions, samples were then prepared and analyzed on the third cycle. The percent deviation of the mean estimated concentration from the time zero should be within $\pm$ 15%.

4.5.6.2 Short-term Room Temperature Stability

Three aliquots of two concentrations of QC samples in plasma and in ultrafiltrate (low and high) were analyzed and stored at -20 ^{o}C for 24 h. Afterward, they were thawed at room temperature and analyzed after being kept at this temperature for 6 and 12 h. The percent deviation of the mean estimated concentration from the time zero should be within $\pm$ 15%.

4.5.6.3 Long-term Stability

Three aliquots of two concentrations of QC samples in plasma and in ultrafiltrate (low and high) were analyzed and stored at -20 ^{o}C for 6 weeks. Each QC sample was prepared and analyzed every 2 weeks. The percent deviation of the mean estimated concentration from the time zero should be within $\pm$ 15%.

4.5.6.4 Post-preparative Stability

Three aliquots of two concentrations of QC samples in plasma and in ultrafiltrate (low and high) were prepared and analyzed. The prepared samples were analyzed after being kept in autosampler for 6 and 12 hr. The percent deviation of the mean estimated concentration from the time zero should be within $\pm$ 15%.

4.5.7 Recovery of Extraction

Five determinations of three concentration of QC samples (low, medium and high) and one concentration of clarithromycin (internal standard) in plasma and in water were analyzed. The percent recovery was calculated by comparing the peak area of extracted plasma samples at each concentration with unextracted standards that represent 100% recovery. The percent recovery was determined as follows :

$$\%\text{Recovery} = \frac{\text{Peak area of analyte extracted from plasma}}{\text{Peak area of analyte unextracted in water}} \times 100$$

The percent recovery of each concentration level need not be 100%, but the extent of recovery of an analyte and of internal standard should be consistent, precise, and reproducible. The determination of extraction recovery of azithromycin in ultrafiltrate was not conducted because the extraction was not required for analysis of the ultrafiltrate.

In Process Validation of Analytical Method for Determination of Azithromycin in Plasma and in Ultrafiltrate

A standard/calibration curve was generated for each analyte to assay samples in each analytical run and used to calculate the concentration of the analyte in unknown samples in the run. An analytical run consisted of calibration standard, QC samples and all processed samples to be analyzed as one batch. Accuracy and precision was monitored by analyzing QC samples with processed test samples at intervals based on the total number of samples. The QC samples in duplicate at three concentrations (low, medium, high) were incorporated in each assay run. The results of the QC samples provided the basis of accepting or rejecting the run.

Acceptance Criteria for the Run

The acceptance criteria were followed with the Guidance for Industry : Bioanlytical Method Validation of Center for Drug Evaluation and Research (CDER) and Center of Veterinary Medicine (CVM), U.S. Department of Health and Human Services, Food and Drug Administration, 2001. The details of acceptance criteria were described as follows:

1. Standard calibration samples : 75% or a minimum of six from eight standards when back-calculated should fall within $\pm$15%, except for LLOQ, when it should be $\pm$20% of the nominal value.

2. Quality control samples : At least 67% (four out of six) of the QC samples should be within 15% of their respective nominal values ; 33% of the QC samples (not all replicates at the same concentration) can be outside $\pm$15% of the nominal value.

5. Pharmacokinetic Analysis

5.1 Non-compartmental Pharmacokinetic Analysis

Non-compartmental PK analysis in plasma (total and free) was performed by using WinNonlin® (Pharsight Corporation, Mountain View, CA, USA, under licence of University of Florida). The pharmacokinetic parameters were calculated for each subject. The primary non-compartmental pharmacokinetic parameters determined were area under the curve (AUC), maximum concentration of drug (C_{max}), time of maximum concentration (t_{max}), and the elimination rate constant (k_e). AUC from zero to the last measured time plot (AUC_{last}) was calculated by linear trapezoidal rule. AUC from zero to infinite time (AUC_{inf}) was calculated as $AUC_{last} + AUC_{extra}$

where AUC_{extra} is calculated as the last concentration (C_{last})/k_e. The k_e was obtained by linear regression of the terminal log linear phase of the concentration-time curve. Both C_{max} and t_{max} were obtained from the plots of concentration-time curve. The area under the first moment curve (AUMC) was calculated from a plot of concentration x time (C·t) versus time using the trapezoidal rule up to the last data point at time t_{last} and adding the extrapolated terminal area, calculated as $C_{last} \cdot t_{last} + C_{last} / k_e^2$. The mean residence time was calculated as AUMC/AUC.

5.2 Compartmental Pharmacokinetic Analysis

Compartmental pharmacokinetic analysis was performed with non-linear regression software program Scientist®. The mean total plasma drug and free drug concentrations were fitted to a one-compartment body model and two-conpartment body model according to the following equations :

$$C_t = A \cdot e^{-k_e t} + B \cdot e^{-k_a t}$$

Where C_t is the concentration of drug at time point t, A and B the back-extrapolated intercepts, k_e the elimination rate constant, k_a the absorption rate constant.

$$C_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} - (A + B) \cdot e^{-k_a t}$$

Where C_t is the concentration of drug at time point t, A and B the back-extrapolated intercepts, Alpha (α) and Beta (β) the hybrid rate constants, k_a the absorption rate constant.

To determine which model fits the data best. Goodness of fit was determined by the coefficient of determination and the Model Selection Criteria (MSC). The closer the coefficient of determination is to 1, the better the correlation between observed and predicted values. The higher the MSC, the more appropriate the selected model.

D. Integrated PK/PD Approach

1. PK/PD simulations

From PK/PD analysis in the experimental time-kill curve, the appropriate PK/PD models show a result in the best fit were used to simulate and predict changes in the number of bacteria for common therapeutic dosing regimens of azithromycin in human. Simulated time-kill profiles of dosing regimens of azithromycin against four

bacterial strains were generated by integrating the fitted free concentration versus time profiles into these PK/PD models with the software program Scientist®.

2. Data analysis

The simulated time-kill curves were shown to exhibit the efficacy of azithromycin against four bacterial strains after once-daily oral administration of 2x250 mg azithromycin capsules.

CHAPTER IV

RESULTS AND DISCUSSION

A. Pharmacodynamic Studies

1. MIC Values and Azithromycin Time-Kill Curve Concentrations

The determined MIC values and azithromycin concentrations used in the time-kill curve experiments against these four bacterial strains are summarized in Table 3. Overall, the results of MIC values obtained in this study were consistent with the reported MIC values [121-123]. The MIC of azithromycin was 0.06, 0.008 and 1 μg/mL for *S. pneumoniae* (both penicillin-sensitive and penicillin intermediate), *M. catarrhalis* and *H. influenzae*, respectively.

The results of the selected azithromycin concentrations tested in each bacterial strain based on their determined MIC in the time-kill curve experiments indicated that seven concentrations were enough to cover the entire range including minimum inhibition of bacterial growth (0.25xMIC, 0.5xMIC, 1xMIC), efficient bacterial killing (2xMIC, 4xMIC) and maximum bacterial killing (8xMIC, 16xMIC) for *S. pneumoniae* both penicillin-sensitive and penicillin-intermediate whereas *M. catarrhalis* and *H. influenzae* needed concentrations more than 16xMIC to reach at the maximum bacterial killing effect for determination of maximum killing rate constant (k_{max}).

Table 3 Azithromycin MIC values and concentrations in time-kill curve.

Strain	MIC(μg/mL)	Tested concentrations (μg/mL)
S. pneumoniae(Pen-S)	0.06	0.015, 0.03, 0.06, 0.12, 0.24, 0.48, 0.96
S. pneumoniae (Pen-I)	0.06	0.015, 0.03, 0.06, 0.12, 0.24, 0.48, 0.96
M. catarrhalis	0.008	0.002, 0.004, 0.008, 0.016, 0.032, 0.064, 0.128, 0.256, 0.512, 1.024
H. influenzae	1	0.25, 0.5, 1, 2, 4, 8, 16, 32, 64

2. Time-Kill Curves

The antimicrobial effect was evaluated by time-kill curves which determine the number of bacteria over time (during 6 h) when exposed to different azithromycin

concentrations covered the entire range. The results of the selected azithromycin concentrations tested in time-kill curve are shown in Table 3. The following Tables 4-7 showed the results of bacterial viable counts in the 6-h time-kill curves which compose of bacterial growth without drug (positive control) and bacterial kill data in the bacteria density (CFU/mL) versus time for the azithromycin concentrations against each bacteria strain, expressed as Mean±S.D. Data from Tables 4-7 were used to construct time-kill curves (Figures 12-15) by plotting the log CFU/mL (Mean±S.D) at 0, 0.5, 1, 1.5, 2, 3, 4, 5 and 6 h to their time during expose to tested azithromycin concentrations (times the MIC) for *S. pneumoniae* both penicillin-sensitive and penicillin-intermediate , *M. catarrhalis*, and *H. influenzae* , respectively.

Results from Tables 4-7 and Figures 12-15 showed that after *in vitro* model was incubated with the initial inoculum of approximately $5x10^5$ CFU/mL for 2 h to allow the bacteria to reach the log-growth phase before the addition of different azithromycin concentrations, the bacteria density (CFU/mL) at time zero in each model had approximately 10^6 CFU/mL. Therefore, number of bacteria density at time zero in model of bacterial growth without drug (positive control) and model of tested azithromycin concentrations for *S. pneumoniae* both penicillin-sensitive and penicillin-intermediate, *M. catarrhalis*, and *H. influenzae* are nearly started at the same point.

Results from Table 4 and Figure 12 for *S. pneumoniae* ATCC 6303 (penicillin-sensitive) showed that number of bacteria density and curve for positive control rising up from time zero according to rate of bacterial growth. Number of bacteria density and curve for tested azithromycin concentrations at 0.25xMIC, 0.5xMIC and 1xMIC also rised up because these concentrations just showed effect of bacterial growth inhibition and not enough to show effect of bacterial killing. Number of bacteria density but curve for tested azithromycin concentrations at 2xMIC, 4xMIC, 8xMIC and 16xMIC declined because these concentrations show effect of bacterial killing.

Results from Table 5 and Figure 13 for *S. pneumoniae* ATCC 49619 (penicillin-intermediate) showed that number of bacteria density and curve for positive control rising up from time zero according to rate of bacterial growth. Number of bacteria density and curve for tested azithromycin concentrations at 0.25xMIC, 0.5xMIC and 1xMIC also rised up because these concentrations just showed effect of bacterial growth inhibition but not enough to show effect of bacterial

killing. Number of bacteria density and curve for tested azithromycin concentrations at 2xMIC, 4xMIC, 8xMIC and 16xMIC declined because these concentrations show efficient bacterial killing.

Results from Table 6 and Figure 14 for *M. catarrhalis* ATCC 8176 showed that number of bacteria density and curve for positive control rising up from time zero according to rate of bacterial growth. After seven azithromycin concentrations at 0.25-16 times the MIC were tested for two times (n=2), it is found that number of bacteria density and curve for tested azithromycin concentrations at 0.25xMIC, 0.5xMIC, 1xMIC 2xMIC and 4xMIC still rised up because these concentrations just showed effect of bacterial growth inhibition but not enough to show effect of bacterial killing. Number of bacteria density and curve for tested azithromycin concentrations at 8xMIC and 16xMIC declined because these concentrations show effect of bacterial killing. Therefore, to reach the maximum bacterial effect, new seven azithromycin concentrations at 2-128 times the MIC were later tested to cover the entire range : minimum inhibition of bacterial growth (2xMIC, 4xMIC, 8xMIC), efficient bacterial killing (16xMIC, 32xMIC) and maximum bacterial killing (64xMIC, 128xMIC). Results showed that number of bacteria density and curve for tested azithromycin concentrations at 64xMIC and 128xMIC could provide the effect of maximum bacterial killing.

Results from Table 7 and Figure 15 for *H. influenzae* ATCC 10211 showed that number of bacteria density and curve for positive control rising up from time zero according to rate of bacterial growth. After seven azithromycin concentrations at 0.25-16 times the MIC were tested for three times (n=3), it is found that number of bacteria density and curve for tested azithromycin concentrations at 0.25xMIC, 0.5xMIC and 1xMIC still rised up because these concentrations just showed effect of bacterial growth inhibition and not enough to show effect of bacterial killing whereas number of bacteria density and curve for tested azithromycin concentrations at 2xMIC, 4xMIC, 8xMIC and 16xMIC declined because these concentrations show effect of bacterial killing. However, the tested azithromycin concentrations at 16xMIC may not reach the maximum bacterial killing effect because slope of killing curves still decreased. Therefore, azithromycin concentrations at 32xMIC and 64xMIC were later tested to prove that these concentrations reached the maximum bacterial killing effect. Results showed that azithromycin concentrations at 32xMIC

and 64xMIC were the maximum bacterial killing effect because slope of killing curves for tested azithromycin concentrations at 32xMIC and 64xMIC did not alter.

Concerning pattern of bacterial killing activity for *S. pneumoniae* (both penicillin-sensitive and penicillin-intermediate) and *M. catarrhalis* seems that bacterial killing activity characteristics is time-dependent killing effect. Because the extent of bacterial killing is primarily dependent on the duration of exposure (no increase in the rate of bacterial killing with increasing concentration). Hence, saturation of killing rate occurred at low concentration (4xMIC) as observed by an excessive changing in slope of killing curves at concentration ranged from 0.25xMIC-4xMIC whereas at higher concentrations (8xMIC-16xMIC), the changing in slope of killing curves was very slight, indicating time-dependent killing effect for *S. pneumoniae* (both penicillin-sensitive and penicillin-intermediate). For *M. catarrhalis*, saturation of the killing rate occurred at low concentration (8xMIC) as observed by an excessive changing in slope of killing curves at concentration ranged from 0.25xMIC-8xMIC whereas higher concentrations (16xMIC-128xMIC), the changing in slope of killing curves was very slight, indicating time-dependent killing effect. In case *H. influenzae*, it is characterized by concentration-dependent killing effect. Because increasing concentrations of azithromycin produced more rapid and extensive bacterial killing as exhibited by the steeper slopes of killing curves. There was more rapid change in slope as the concentration increased from 1xMIC-32xMIC.

Since determined MIC of azithromycin could provide only a single static value (Table 3) whereas the time-kill curve could provide antibacterial efficacy (number of bacteria density) with more dynamic as function of both time and azithromycin concentrations (Tables 4-7 and Figures 12-15). Therefore, time-kill curve could better provide the patterns of bacterial killing activity of azithromycin and more information on rate of bacterial activity than a single static MIC parameter.

Table 4 Mean ± SD of bacteria density (10^6 CFU /mL) versus time for azithromycin concentrations against *S. pneumoniae* ATCC 6303

Time (h)	Control n=3[a]	0.015 µg/mL (0.25MIC) n=3	0.03 µg/mL (0.5MIC) n=3	0.06 µg/mL (MIC) n=3	0.12 µg/mL (2MIC) n=3	0.24 µg/mL (4MIC) n=3	0.48 µg/mL (8MIC) n=3	0.96 µg/mL (16MIC) n=3
0	0.05±0.14	0.53±0.06	0.57±0.11	0.68±0.24	0.70±0.32	0.53±0.13	0.75±0.25	0.58±0.10
0.5	0.75±0.06	0.91±0.60	1.04±0.42	1.36±1.11	0.81±0.10	0.70±0.23	0.62±0.09	0.65±0.19
1	1.08±0.27	1.16±0.12	1.51±0.73	1.13±0.19	1.00±0.31	0.79±0.01	0.90±0.56	0.70±0.16
1.5	1.93±0.33	2.04±0.14	4.41±1.30	1.54±0.43	1.34±0.79	0.80±0.16	0.57±0.10	0.61±0.19
2	5.22±3.19	4.61±1.01	7.17±3.96	2.19±0.81	1.45±0.98	0.74±0.14	0.70±0.24	0.55±0.09
3	15.73±3.77	15.57±3.58	12.07±5.01	3.25±1.80	1.49±1.03	0.64±0.33	0.43±0.13	0.36±0.19
4	67.33±30.02	47.80±20.30	27.07±5.01	6.81±5.26	1.08±0.70	0.63±0.31	0.50±0.10	0.24±0.05
5	111.00±67.09	81.67±52.54	130.73±161.71	11.60±9.08	1.40±1.59	0.54±0.33	0.22±0.09	0.20±0.04
6	125.00±83.52	88.33±51.48	99.67±69.90	26.93±27.90	1.48±1.13	0.32±0.28	0.16±0.11	0.07±0.06

[a] n = Number of time-kill curve experiment

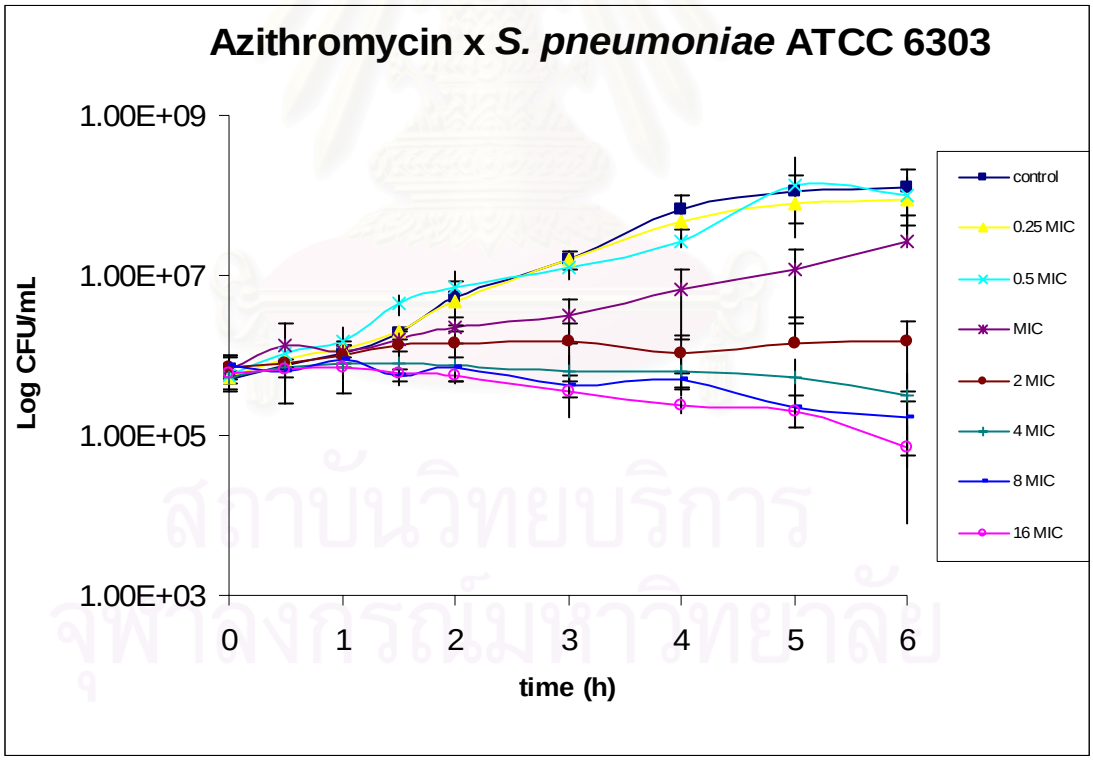


Figure 12 Time-kill curve of azithromycin against *S. pneumoniae* ATCC 6303. Data presented as Mean±SD.

Table 5 Mean ± SD of bacteria density (10^6 CFU /mL) versus time for azithromycin concentrations against *S. pneumoniae* ATCC 49619

Time (h)	Control n=3[a]	0.015 µg/mL (0.25MIC) n=3	0.03 µg/mL (0.5MIC) n=3	0.06 µg/mL (MIC) n=3	0.12 µg/mL (2MIC) n=3	0.24 µg/mL (4MIC) n=3	0.48 µg/mL (8MIC) n=3	0.96 µg/mL (16MIC) n=3
0	0.62±0.16	1.05±0.70	0.63±0.26	0.96±0.58	0.56±0.07	0.60±0.03	0.58±0.17	0.72±0.02
0.5	0.73±0.21	0.95±0.47	0.79±0.04	1.11±0.41	0.65±0.17	0.62±0.32	0.67±0.13	0.69±0.25
1	1.15±0.23	1.16±0.31	0.93±0.12	1.63±0.96	0.84±0.61	0.69±0.18	0.60±0.13	0.84±0.66
1.5	3.47±2.31	1.71±0.01	1.84±0.78	2.03±0.02	0.52±0.11	1.28±0.38	0.56±0.04	0.65±0.11
2	5.28±0.60	3.37±2.16	2.92±0.97	2.44±1.74	0.82±0.26	0.86±0.30	0.50±0.05	0.45±0.04
3	31.00±7.07	20.95±22.56	7.15±5.73	4.49±5.11	0.58±0.07	0.64±0.16	0.34±0.17	0.34±0.06
4	106.50±14.85	14.80±10.32	11.55±11.10	4.47±5.51	0.65±0.14	0.38±0.00	0.35±0.16	0.19±0.03
5	106.00±67.18	30.75±30.05	12.05±9.55	10.65±12.80	0.45±0.24	0.44±0.36	0.15±0.04	0.11±0.03
6	276.00±231.93	45.20±49.21	24.25±22.27	13.60±17.68	0.34±0.29	0.16±0.06	0.08±0.01	0.08±0.03

[a] n = Number of time-kill curve experiment

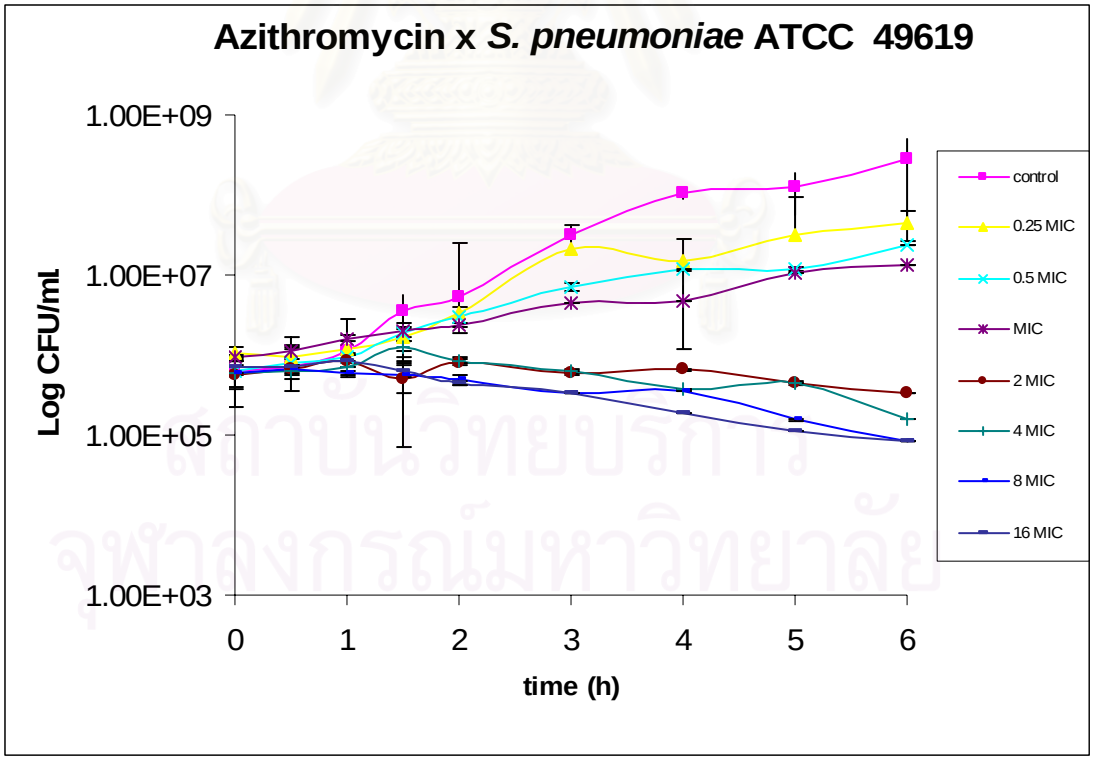


Figure 13 Time-kill curve of azithromycin against *S. pneumoniae* ATCC 49619. Data presented as Mean±SD.

Table 6 Mean ± SD of bacteria density (10^6 CFU /mL) versus time for azithromycin concentrations against *M. catarrhalis* ATCC 8176

Time (h)	Control n=4[a]	0.002 µg/mL (0.25MIC) n=2	0.004 µg/mL (0.5MIC) n=2	0.008 µg/mL (MIC) n=2	0.016 µg/mL (2MIC) n=4	0.032 µg/mL (4MIC) n=4	0.064 µg/mL (8MIC) n=4	0.128 µg/mL (16MIC) n=4	0.256 µg/mL (32MIC) n=2	0.512 µg/mL (64MIC) n=2	1.024 µg/mL (128MIC) n=2
0	0.42±0.03	0.44±0.02	0.33±0.03	0.37±0.03	0.59±0.28	0.42±0.09	0.04±0.10	0.42±0.11	0.50±0.07	0.35±0.01	0.49±0.11
0.5	0.56±0.09	0.49±0.00	0.52±0.33	0.31±0.10	0.48±0.10	0.46±0.18	0.37±0.20	0.50±0.16	0.44±0.16	0.41±0.00	0.32±0.13
1	0.85±0.23	0.44±0.06	0.42±0.06	0.60±0.12	0.44±0.16	0.56±0.24	0.41±0.09	0.43±0.08	0.38±0.03	0.35±0.08	0.33±0.07
1.5	1.12±0.27	0.45±0.06	0.55±0.01	0.39±0.05	0.56±0.26	0.41±0.06	0.45±0.08	0.36±0.20	0.44±0.02	0.29±0.11	0.27±0.07
2	1.56±0.30	0.55±0.14	0.72±0.69	0.52±0.16	0.58±0.20	0.53±0.16	0.38±0.09	0.35±0.12	0.33±0.11	0.25±0.08	0.27±0.07
3	4.32±1.49	1.16±0.06	1.20±0.59	1.05±0.31	1.90±1.58	0.97±0.32	0.44±0.17	0.36±0.12	0.33±0.05	0.22±0.06	0.18±0.03
4	20.88±12.42	6.30±1.13	11.50±6.65	4.59±3.27	5.32±3.04	1.65±0.81	0.62±0.21	0.33±0.15	0.32±0.06	0.20±0.06	0.13±0.03
5	60.70±29.52	20.20±7.35	17.25±2.05	14.80±12.45	24.50±20.92	8.28±6.26	0.62±0.14	0.29±0.18	0.19±0.03	0.15±0.03	0.12±0.01
6	201.00±179.88	85.00±26.87	94.50±84.15	60.00±0.00	124.50±149.91	15.83±7.26	0.78±0.32	0.19±0.18	0.18±0.02	0.17±0.04	0.10±0.02

[a] n = Number of time-kill curve experiment

Table 7 Mean ± SD of bacteria density (10^6 CFU /mL) versus time for azithromycin concentrations against *H. influenzae* ATCC 10211

Time (h)	Control n=4[a]	0.25 µg/mL (0.25MIC) n=3	0.5 µg/mL (0.5MIC) n=3	1 µg/mL (MIC) n=4	2 µg/mL (2MIC) n=4	4 µg/mL (4MIC) n=4	8 µg/mL (8MIC) n=4	16 µg/mL (16MIC) n=4	32 µg/mL (32MIC) n=1	64 µg/mL (64MIC) n=1
0	0.94±0.27	0.94±0.51	1.15±0.43	0.86±0.36	1±0.31	0.93±0.23	1.03±0.32	0.95±0.11	0.74	0.53
0.5	1.55±0.66	1.71±0.77	1.56±0.78	1.04±0.86	1.49±0.54	1.40±0.63	1.29±0.29	1.10±0.28	1.14	1.14
1	4.46±4.37	2.29±0.79	2.93±1.35	2.44±1.09	2.09±0.38	1.97±0.84	1.73±0.48	1.05±0.34	0.65	0.65
1.5	4.32±2.89	5.00±2.32	3.97±1.27	4.09±4.13	3.52±1.83	2.72±1.90	1.83±1.17	1.09±0.51	0.45	0.09
2	10.73±5.19	8.60±3.32	10.17±1.52	8.11±3.90	3.35±0.82	1.59±0.39	1.52±0.59	0.64±0.24	0.14	0.06
3	29.78±18.01	46.20±38.29	53.00±42.11	19.65±15.16	3.96±1.04	1.65±0.56	0.64±0.31	0.12±0.03	0.01	0.04
4	135.50±61.95	124.67±52.55	61.50±42.11	30.86±16.82	7.07±5.03	0.71±0.34	0.21±0.09	0.01±0.01	0	0
5	559.50±493.67	435.00±243.77	243.67±32.44	55±36.97	2.85±0.96	0.40±0.32	0.07±0.02	0	0	0
6	1062.50±599.08	1400.00±597.33	1200±626.98	177.25±122.38	1.90±0.82	0.14±0.04	0	0	0	0

[a] n = Number of time-kill curve experiment

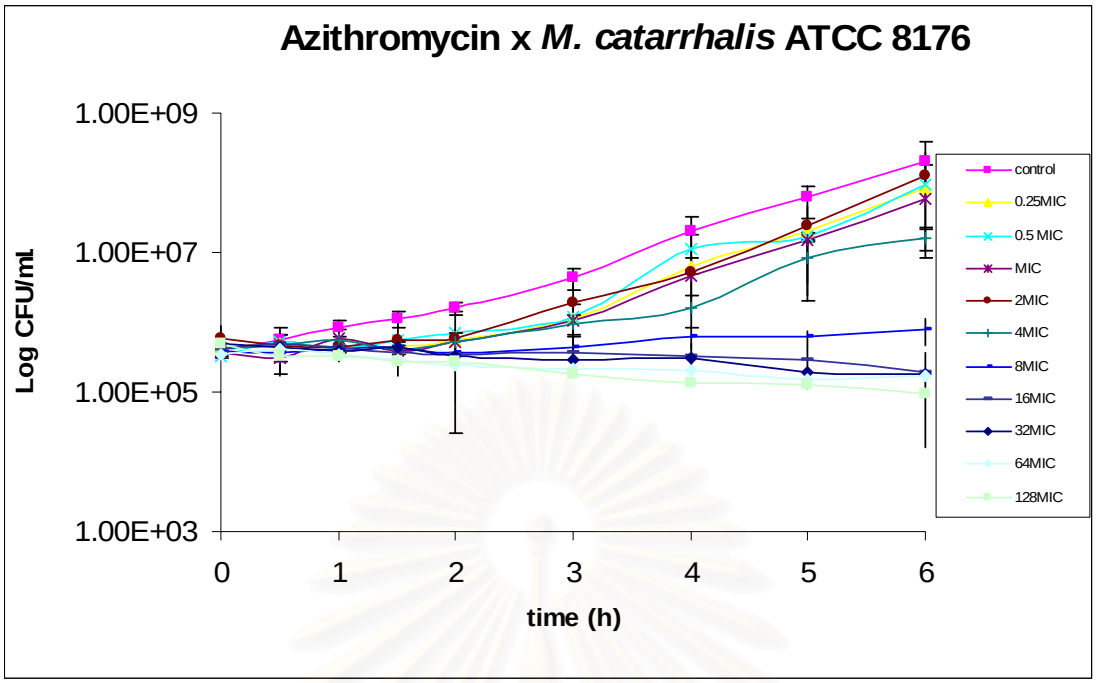


Figure 14 Time-kill curve of azithromycin against *M. catarrhalis* ATCC 8176

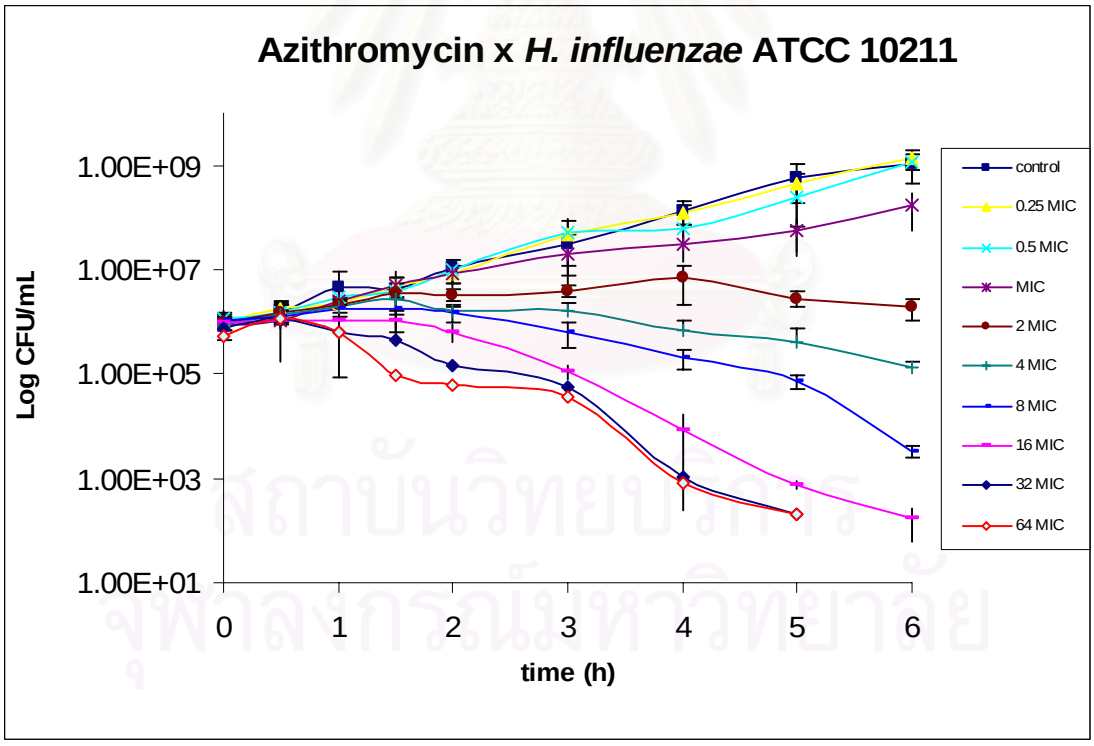


Figure 15 Time-kill curve of azithromycin against *H. influenzae* ATCC 10211. Data presented as Mean±SD.

B. PK/PD Modeling

1. Curve Fits of Bacterial Time-Kill Curves

Time-kill curves can follow microbial killing and growth as a function of both time and azithromycin concentration. The time-kill curve data in the mean bacteria density (log CFU/mL) versus time provide the change of bacteria concentration according to the time of expose to azithromycin. PK/PD models are used to describe the change in the number of bacteria as a function of time and concentration. The resulting time-kill curves can be analyzed by mean of PK/PD models. To determine a suitable PK/PD model for fitting all the time-kill data including bacterial growth and kill curves, the twelve PK/PD models were compared and fitted to the experimental time-kill curve data by using Scientist® computer program. The pharmacodynamic parameters for each PK/PD models, at least three pharmacodynamic parameters : the maximum kill rate constant (k_{max}), the concentration at half-maximum effect (EC_{50}), and the growth rate constant of the bacteria (k_0), were obtained subsequently after curve fitting. Examples of determination of pharmacodynamic parameters and curve fits are displayed in Appendix A. Results of the determined pharmacodynamic and goodness of fit criteria for twelve PK/PD models in four bacterial strains are shown in Tables 8-11. Results of curve fits using twelve PK/PD models in four bacterial strains are shown in Figures 16-23.

To determine the best PK/PD model, the following criteria were taken into consideration: goodness of fit statistics (MSC and r^2) as well as visual inspection for curve fitting. The most suitable model being the best model selected should have both criteria.

For *S. pneumoniae* ATCC 6303, summary of the determined pharmacodynamic parameters and goodness of fit criteria for each PK/PD model are shown in Table 8 and the fitted curves of each PK/PD model are shown in Figures 16 and 17. Comparison of twelve models with goodness of fit statistics (MSC and r^2 in Table 8) as well as visual inspection (curve fitting in Figures 16 and 17) indicate that model 4 was the best model with both higher values of MSC (1.98), r^2 (0.87) and the best fitted curve (model 4 in Figure 16) as observed by its line passed almost all the time-kill data (both bacterial growth and kill curves). Although models 5, 10-12 had values of MSC and r^2 equal to and/or higher than those of model 2, but their curves fitting appear to be inferior.

For *S. pneumoniae* ATCC 49619, summary of the determined pharmacodynamic azithromycin parameters and goodness of fit criteria for each PK/PD model are shown in Table 9 and the fitted curve of each PK/PD model are shown in Figures 18 and 19. Comparison of twelve models with goodness of fit statistics (MSC and r^2 in Table 9) as well as visual inspection (curve fitting in Figures 18 and 19) indicate that model 4 was the best model with both higher values of MSC (3.24), r^2 (0.96) and the best fitted curve (model 4 in Figure 18) as observed by its line passed almost all the time-kill data (both bacterial growth and kill curves). Although model 10 had values of MSC and r^2 higher than those of model 4, but their curves fitting appear to be inferior.

For *M. catarrhalis* ATCC 8176, summary of the determined pharmacodynamic azithromycin parameters and goodness of fit criteria for each PK/PD model are shown in Table 10 and the fitted curve of each PK/PD model are shown in Figures 20 and 21. Comparison of twelve models with goodness of fit statistics (MSC and r^2 in Table 9) as well as visual inspection (curve fitting in Figures 20 and 21) indicate that model 2 was the best model with both higher values of MSC (2.08), r^2 (0.88) and the best fitted curve (model 2 in Figure 20) as observed by its line passed almost all the time-kill data (both bacterial growth and kill curves). Although models 7-12 had values of MSC and r^2 higher than those of model 4 but their curves fitting appear to be inferior.

For *H. influenzae* ATCC 10211, summary of the determined pharmacodynamic azithromycin parameters and goodness of fit criteria for each PK/PD model are shown in Table 11 and the fitted curve of each PK/PD model are shown in Figures 22 and 23. Comparison of twelve models with goodness of fit statistics (MSC and r^2 in Table 11) as well as visual inspection (curve fitting in Figures 22 and 23) indicate that model 9 was the best model with both higher values of MSC (2.55), r^2 (0.92) and the best fitted curve (model 9 in Figure 23) as observed by its passing almost all the time-kill data (both bacterial growth and kill curves). Although model 12 had values of MSC and r^2 higher than those of model 9, but their curves fitting appear to be inferior.

In summary, models 4, 2 and 9 were the best models for the best fitting the time-kill curve data for *S. pneumoniae* (both penicillin-sensitive and penicillin intermediate), *M. catarrhalis* and *H. influenzae*, respectively. The mathematic equations and parameters from these models can then be integrated with the time-

concentration profiles of azithromycin in pharmacokinetic studies, to describe and predict the antimicrobial effect.

Table 8 Pharmacodynamic parameters of activity of azithromycin against *S. pneumoniae* ATCC 6303 and goodness of fit criteria.

Model	k_0 (h^{-1})	k_{max} (h^{-1})	EC_{50} (μg/mL)	z (h^{-1})	N_{max} (10^8CFU/mL)	h	MSC	r^2
1	0.95	1.34	0.30	-	-	-	1.21	0.71
2	1.03	1.50	0.28	2.63	-	-	1.12	0.69
3	0.95	2.09	0.43	1.54	-	-	1.15	0.71
4	1.36	1.80	0.34	-	1.52	-	1.98	0.87
5	3.31	3.73	0.35	0.27	1.41	-	1.98	0.87
6	1.36	2.52	0.45	1.99	1.51	-	1.96	0.87
7	0.95	1.34	0.11	-	-	2.18	1.28	0.74
8	1.03	1.84	0.23	2.74	-	1.27	1.12	0.70
9	0.95	1.85	0.16	1.33	-	1.70	1.25	0.74
10	1.36	1.49	0.08	-	1.13	4.52	2.41	0.92
11	3.30	3.65	0.22	0.27	1.27	1.37	2.16	0.89
12	1.36	4.31	0.09	0.98	1.13	4.67	2.36	0.92

Pharmacodynamic parameter

k_0 = growth rate constant of the bacteria

k_{max} = maximum kill rate constant

EC_{50} = concentration at half-maximum effect

z = adaptation rate

h = Hill factor

N_{max} = maximum number of bacteria at the end of the growth phase

Goodness of fit criteria

MSC = Model Selection Criterion

r^2 = coefficient of determination

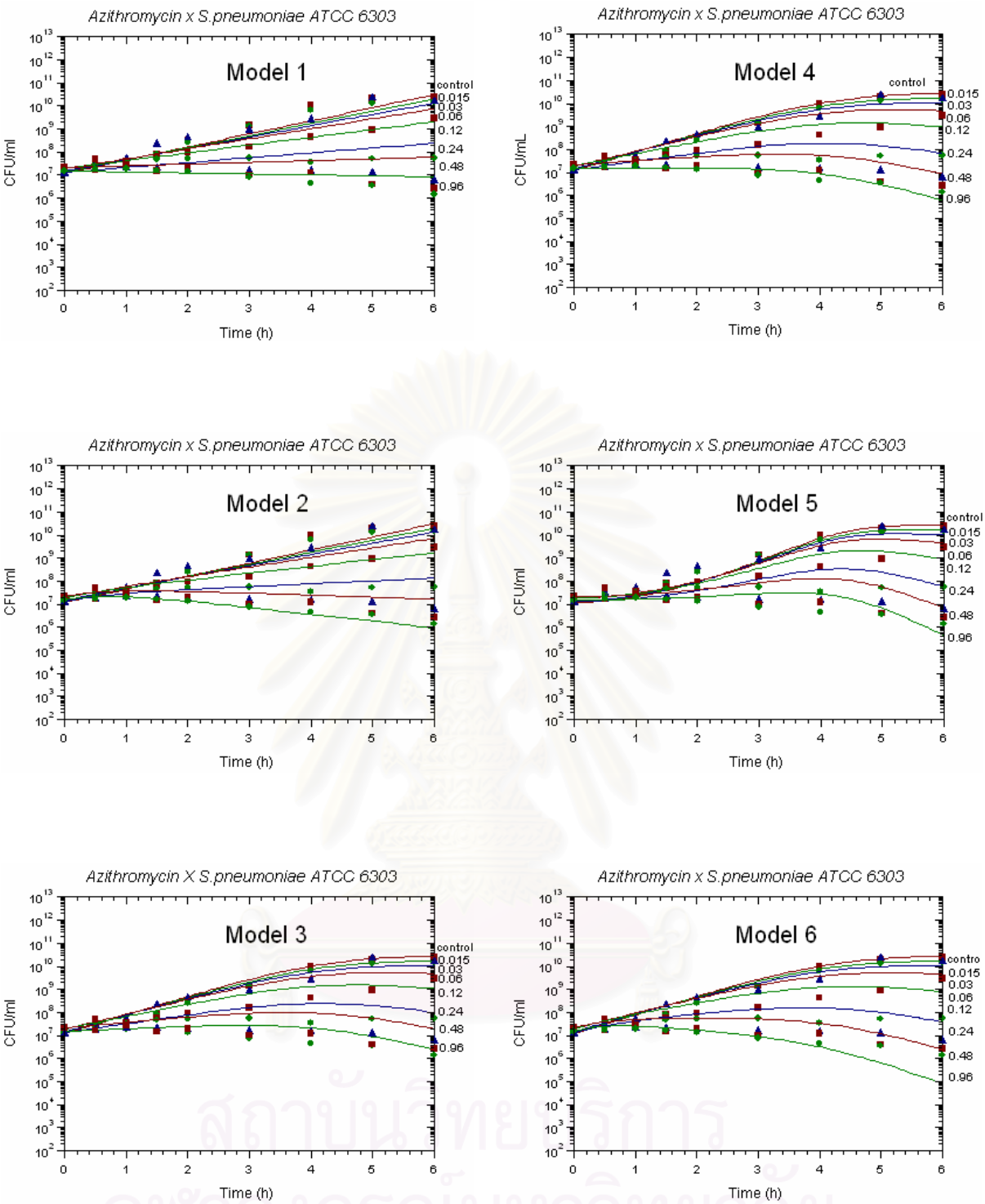


Figure 16 The curve fits using models 1-6 for various constant azithromycin concentrations (µg/mL) against *S. pneumoniae* ATCC 6303. The lines represent the fitted curve to bacterial viable counts in the 6 h time-kill curves.

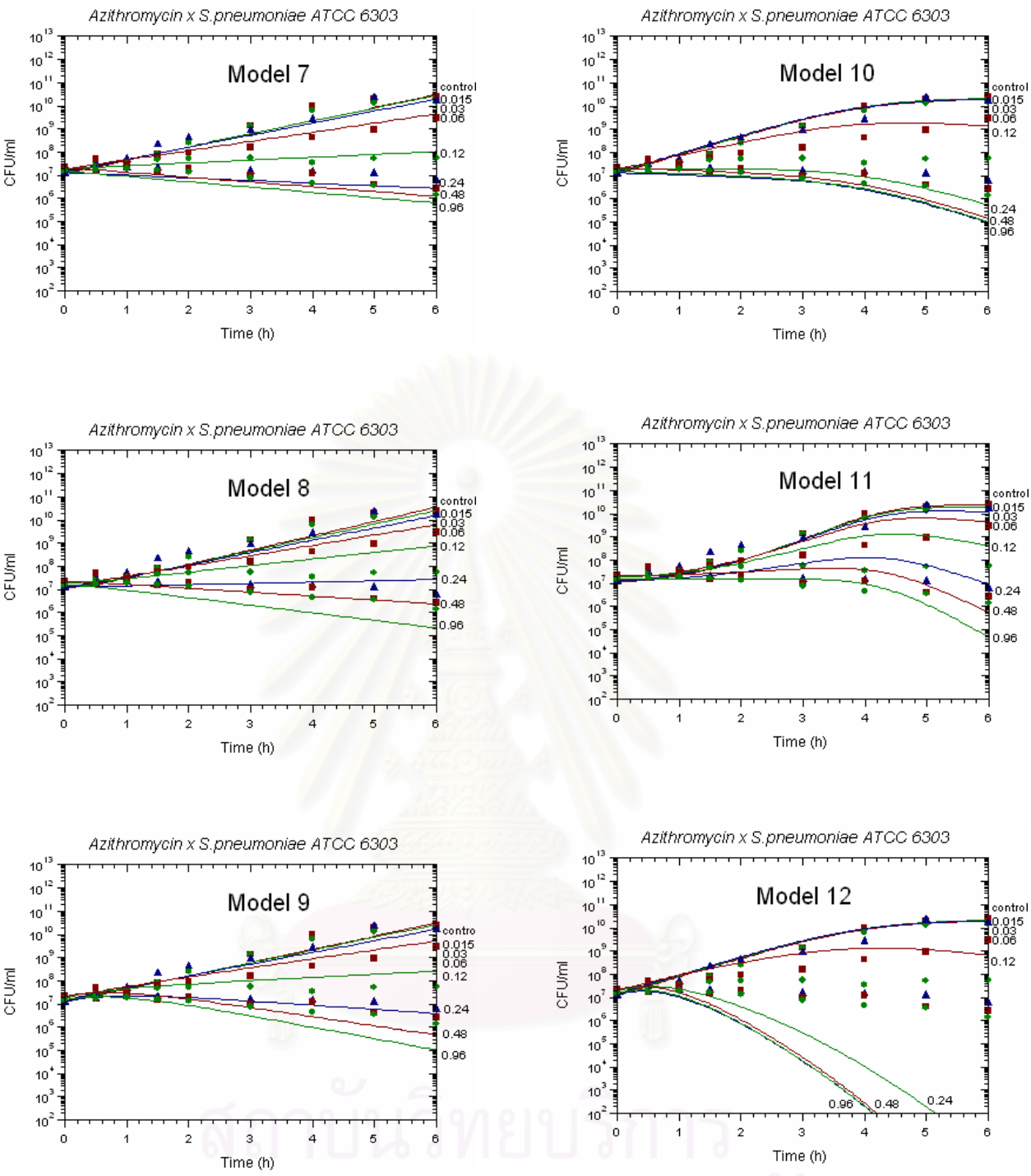


Figure 17 The curve fits using models 7-12 for various constant azithromycin concentrations (μg/mL) against *S. pneumoniae* ATCC 6303. The lines represent the fitted curve to bacterial viable counts in the 6 h time-kill curves.

Table 9 Pharmacodynamic parameters of activity of azithromycin against *S. pneumoniae* ATCC 49619 and goodness of fit criteria.

Model	k_0 (h^{-1})	k_{max} (h^{-1})	EC_{50} (μg/mL)	z (h^{-1})	N_{max} (10^8CFU/mL)	h	MSC	r^2
1	1.02	1.27	0.04	-	-	-	2.71	0.93
2	1.12	2.20	0.07	2.01	-	-	2.44	0.92
3	1.02	2.32	0.04	0.35	-	-	2.70	0.94
4	1.21	1.61	0.05	-	3.86	-	3.24	0.96
5	2.21	3.22	0.06	0.39	3.09	-	2.64	0.93
6	1.21	2.56	0.06	0.90	3.83	-	3.17	0.96
7	1.02	1.57	0.20	-	-	0.44	2.74	0.94
8	1.12	1.80	0.15	2.03	-	0.53	2.48	0.92
9	1.02	2.57	0.31	1.50	-	0.54	2.71	0.94
10	1.21	1.54	0.08	-	3.84	0.64	3.31	0.97
11	2.21	3.35	0.10	0.39	3.01	0.75	2.16	0.89
12	1.21	2.79	0.10	0.61	3.86	0.74	3.19	0.96

Pharmacodynamic parameter

k_0 = growth rate constant of the bacteria

k_{max} = maximum kill rate constant

EC_{50} = concentration at half-maximum effect

z = adaptation rate

h = Hill factor

N_{max} = maximum number of bacteria at the end of the growth phase

Goodness of fit criteria

MSC = Model Selection Criterion

r^2 = coefficient of determination

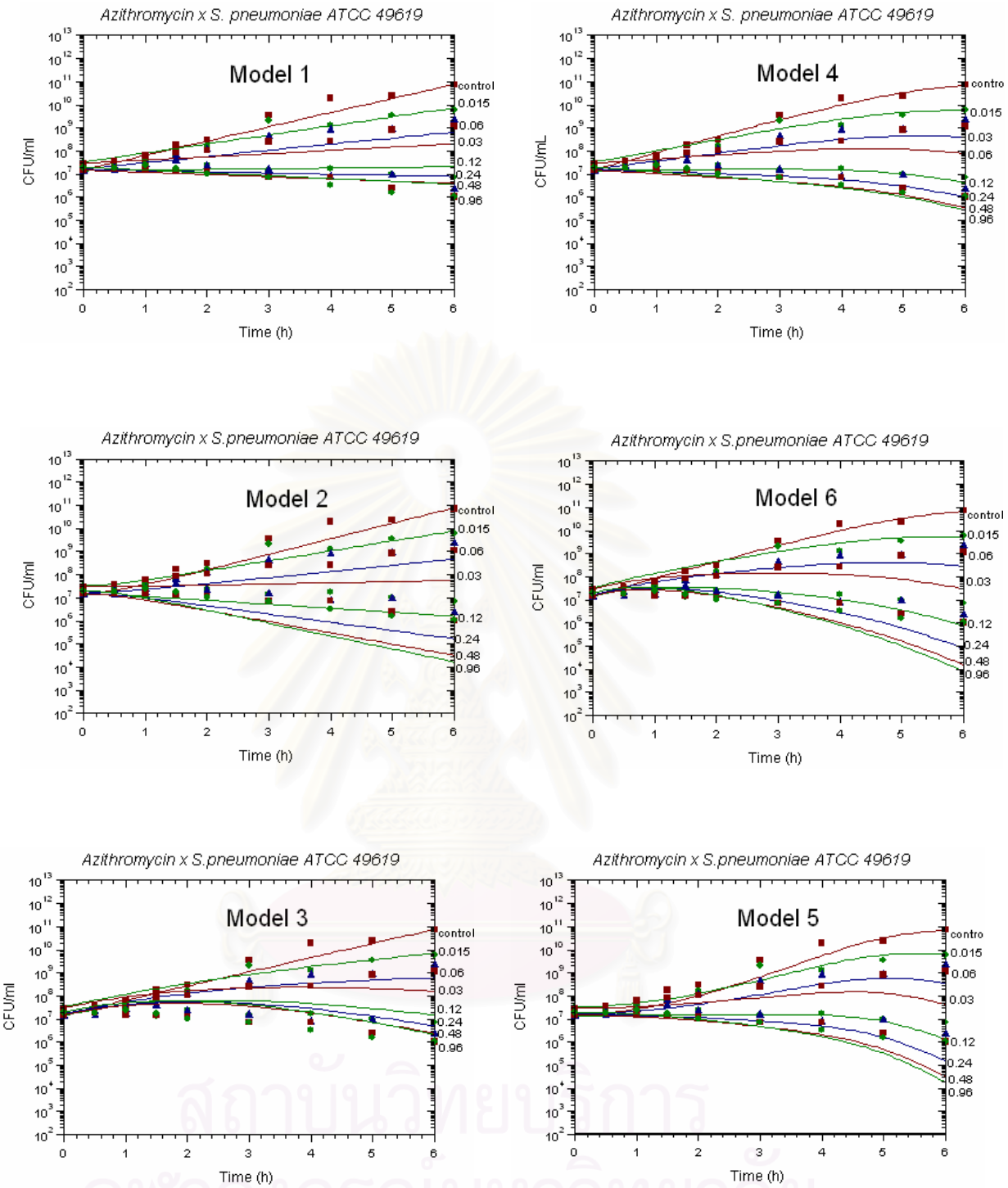


Figure 18 The curve fits using models 1-6 for various constant azithromycin concentrations (µg/mL) against *S. pneumoniae* ATCC 49619. The lines represent the fitted curve to bacterial viable counts in the 6 h time-kill curves.

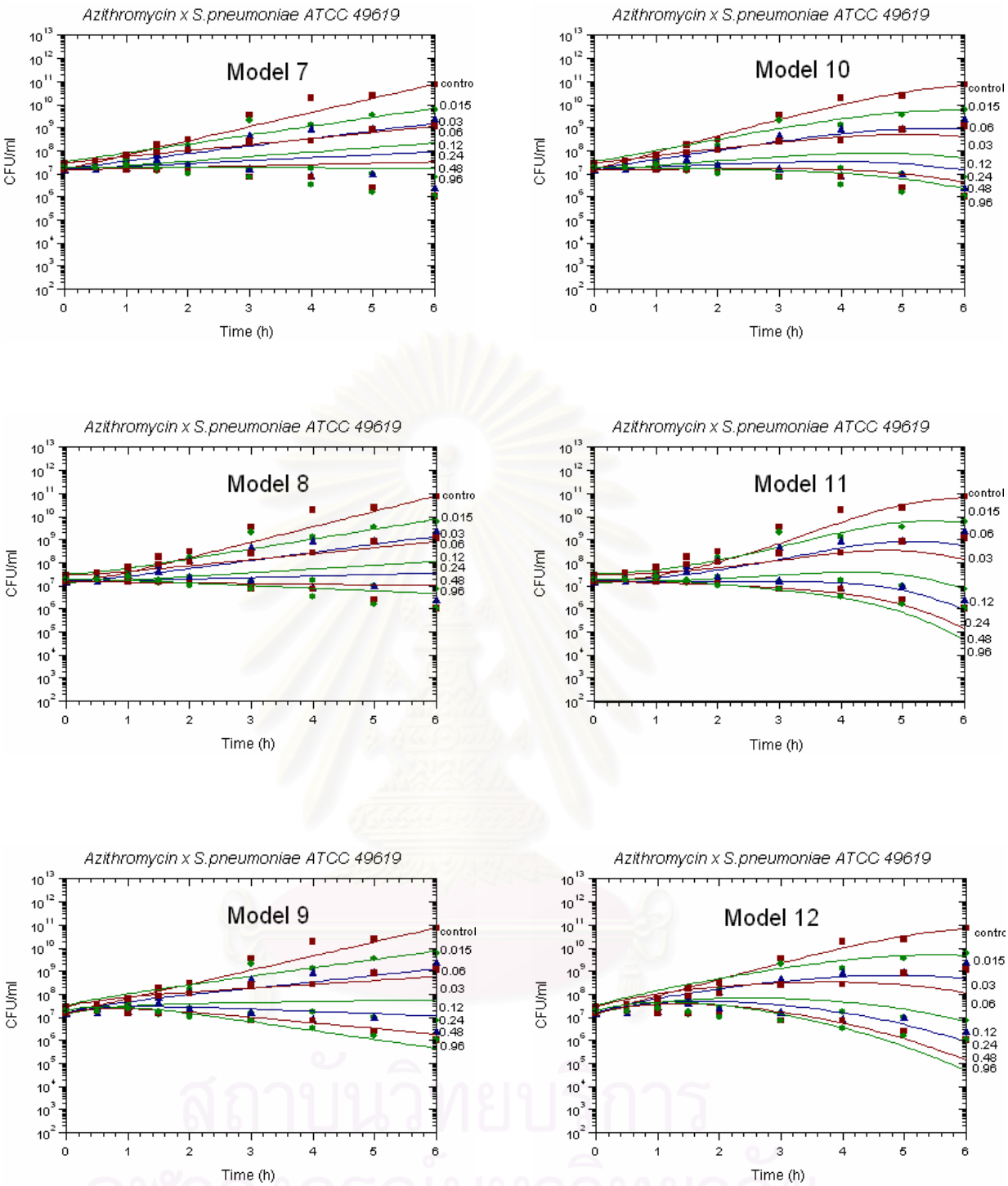


Figure 19 The curve fits using models 7-12 for various constant azithromycin concentrations (μg/mL) against *S. pneumoniae* ATCC 49619. The lines represent the fitted curve to bacterial viable counts in the 6 h time-kill curves.

Table 10 Pharmacodynamic parameters of activity of azithromycin against *M. catarrhalis* ATCC 8176 and goodness of fit criteria.

Model	k_0 (h^{-1})	k_{max} (h^{-1})	EC_{50} (μg/mL)	z (h^{-1})	N_{max} (10^8CFU/mL)	h	MSC	r^2
1	1.03	1.45	0.07	-	-	-	1.66	0.81
2	1.62	2.50	0.37	2.01	-	-	2.08	0.88
3	1.03	2.27	0.11	2.48	-	-	1.60	0.81
4	1.08	1.50	0.11	-	3.64	-	1.69	0.82
5	2.44	3.20	0.13	0.22	3.30	-	2.05	0.88
6	1.08	2.41	0.17	2.28	3.54	-	1.65	0.82
7	1.03	1.66	0.92	-	-	0.45	2.35	0.91
8	1.62	2.50	0.16	0.39	-	0.83	2.18	0.89
9	1.03	2.60	5.18	58.97	-	0.40	2.35	0.91
10	1.08	1.55	0.59	-	8.10	0.46	2.13	0.89
11	2.44	4.33	0.69	0.21	1.06	0.54	2.43	0.92
12	1.08	2.50	1.50	1.44	8.17	0.46	2.09	0.87

Pharmacodynamic parameter

k_0 = growth rate constant of the bacteria

k_{max} = maximum kill rate constant

EC_{50} = concentration at half-maximum effect

z = adaptation rate

h = Hill factor

N_{max} = maximum number of bacteria at the end of the growth phase

Goodness of fit criteria

MSC = Model Selection Criterion

r^2 = coefficient of determination

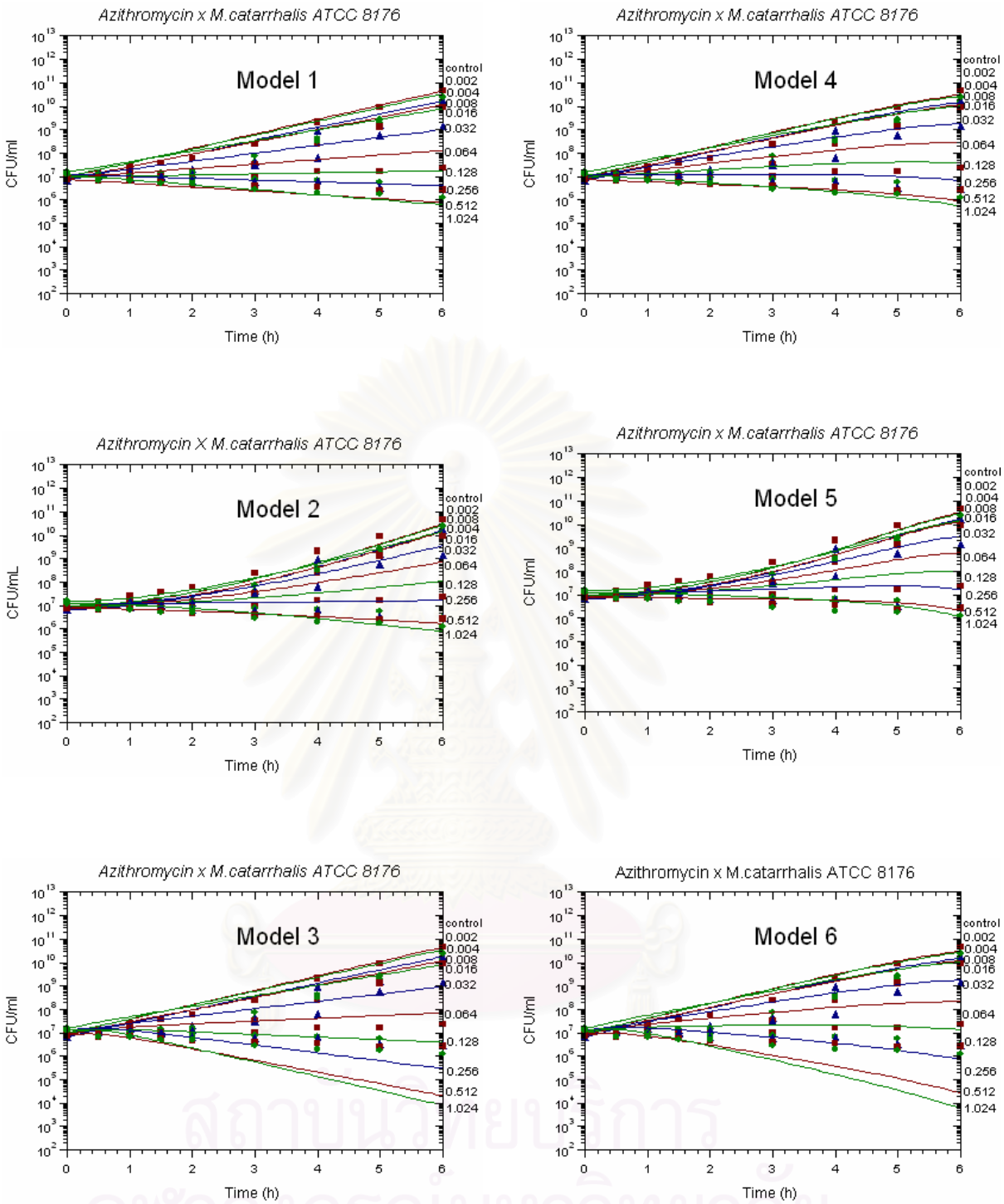


Figure 20 The curve fits using models 1-6 for various constant azithromycin concentrations (µg/mL) against *M. catarrhalis* ATCC 8176. The lines represent the fitted curve to bacterial viable counts in the 6 h time-kill curves.

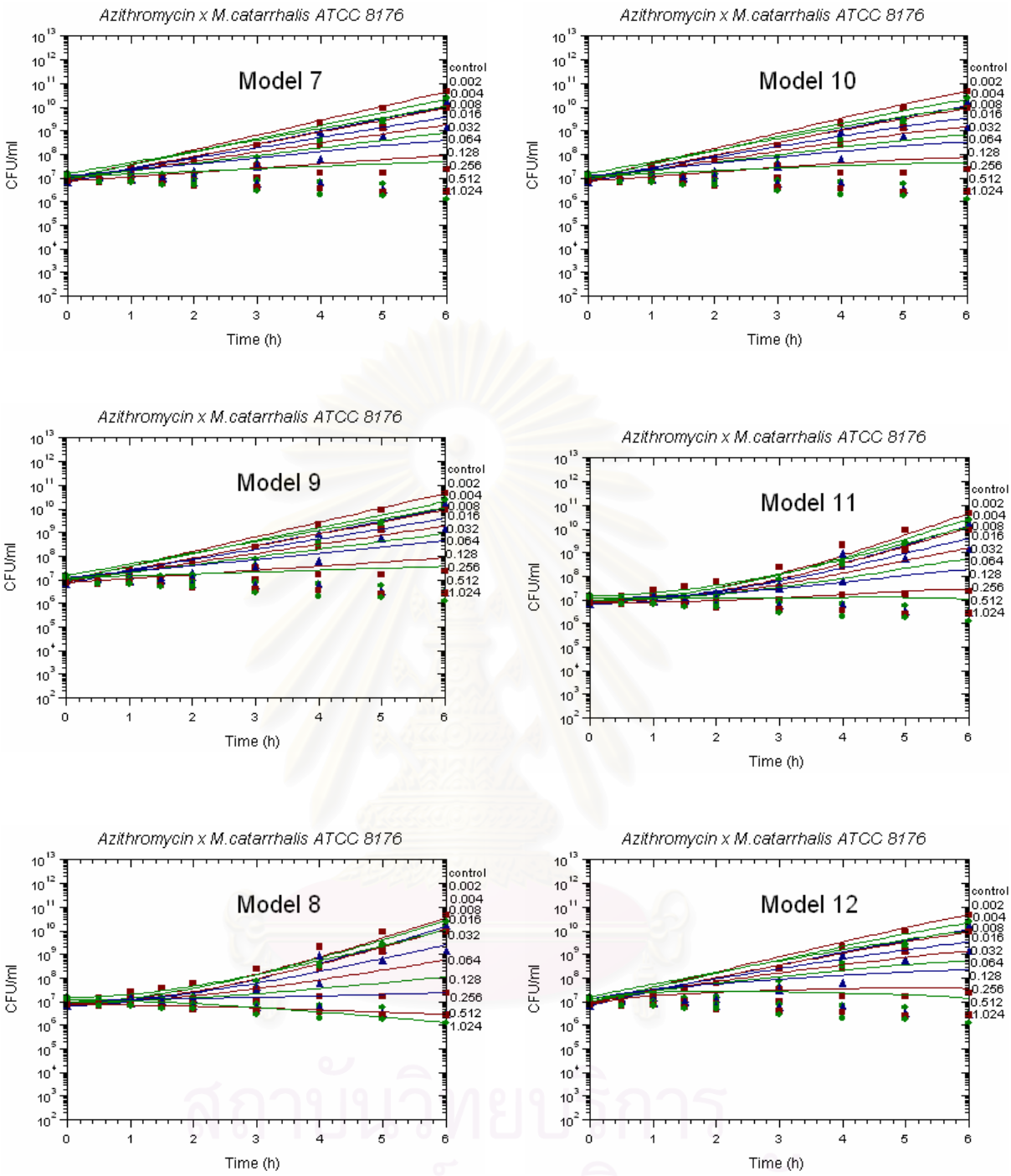


Figure 21 The curve fits using models 7-12 for various constant azithromycin concentrations (μg/mL) against *M. catarrhalis* ATCC 8176. The lines represent the fitted curve to bacterial viable counts in the 6 h time-kill curves.

Table 11 Pharmacodynamic parameters of activity of azithromycin against *H. influenzae* ATCC 10211 and goodness of fit criteria.

Model	k_0 (h^{-1})	k_{max} (h^{-1})	EC_{50} (μg/mL)	z (h^{-1})	N_{max} (10^8CFU/mL)	h	MSC	r^2
1	1.18	2.55	17.01	-	-	-	1.90	0.85
2	2.08	5.50	17.85	0.36	-	-	1.91	0.86
3	1.18	6.23	42.17	4.03	-	-	1.90	0.86
4	1.33	2.50	12.18	-	28.69	-	2.06	0.88
5	3.07	7.00	17.29	0.25	18.75	-	2.08	0.88
6	1.33	11.95	57.08	2.21	28.35	-	2.08	0.88
7	1.18	2.54	10.41	-	-	1.27	2.07	0.88
8	2.08	5.50	21.72	0.35	-	0.96	1.85	0.85
9	1.18	3.71	3.68	0.79	-	2.08	2.55	0.92
10	1.33	2.44	10.01	-	27.88	1.13	2.13	0.89
11	3.07	8.03	22.54	0.22	27.61	0.96	1.93	0.87
12	1.33	14.44	4.67	0.32	26.58	2.15	2.70	0.94

Pharmacodynamic parameter

k_0 = growth rate constant of the bacteria

k_{max} = maximum kill rate constant

EC_{50} = concentration at half-maximum effect

z = adaptation rate

h = Hill factor

N_{max} = maximum number of bacteria at the end of the growth phase

Goodness of fit criteria

MSC = Model Selection Criterion

r^2 = coefficient of determination

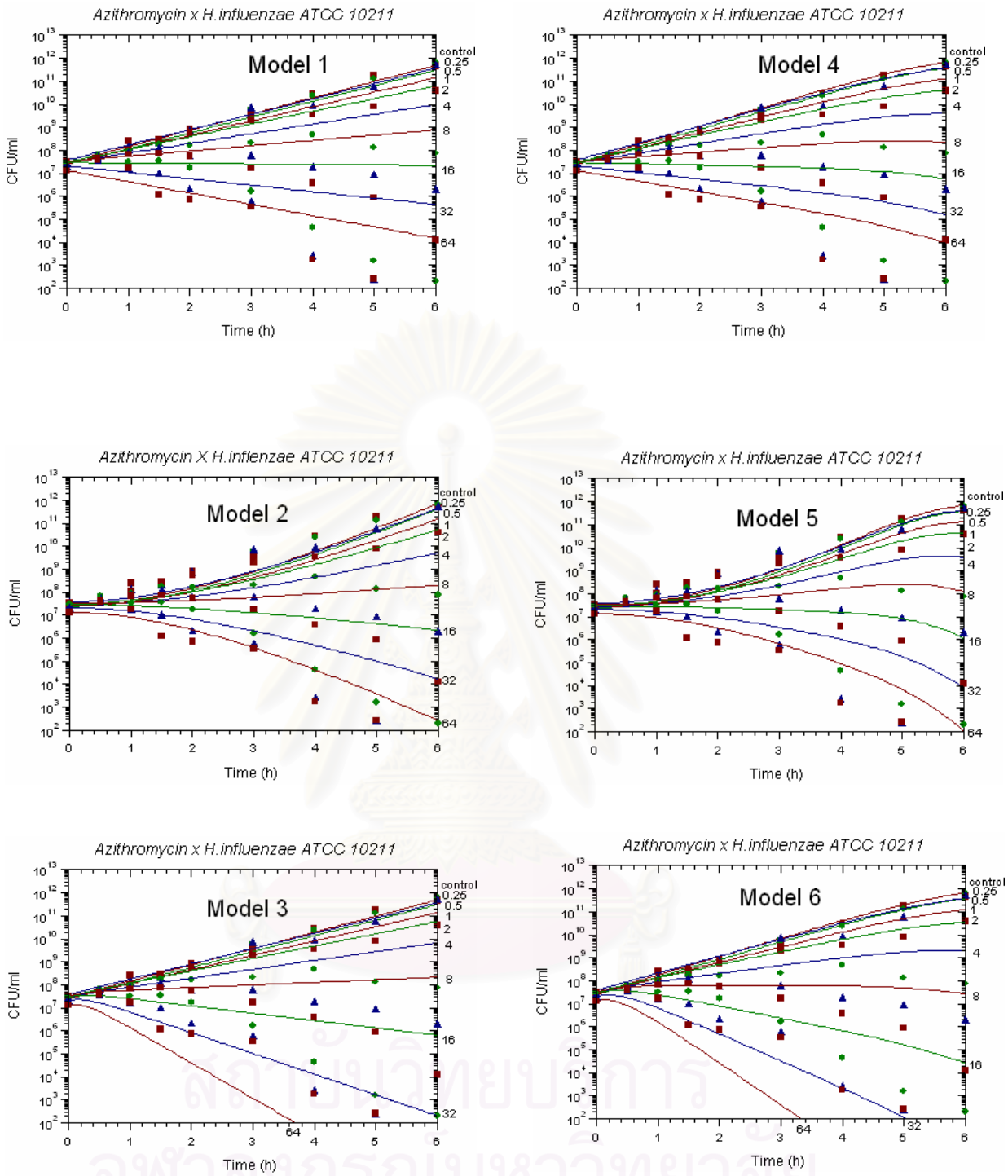


Figure 22 The curve fits using models 1-6 for various constant azithromycin concentrations (μg/mL) against *H. influenzae* ATCC 10211. The lines represent the fitted curve to bacterial viable counts in the 6 h time-kill curves.

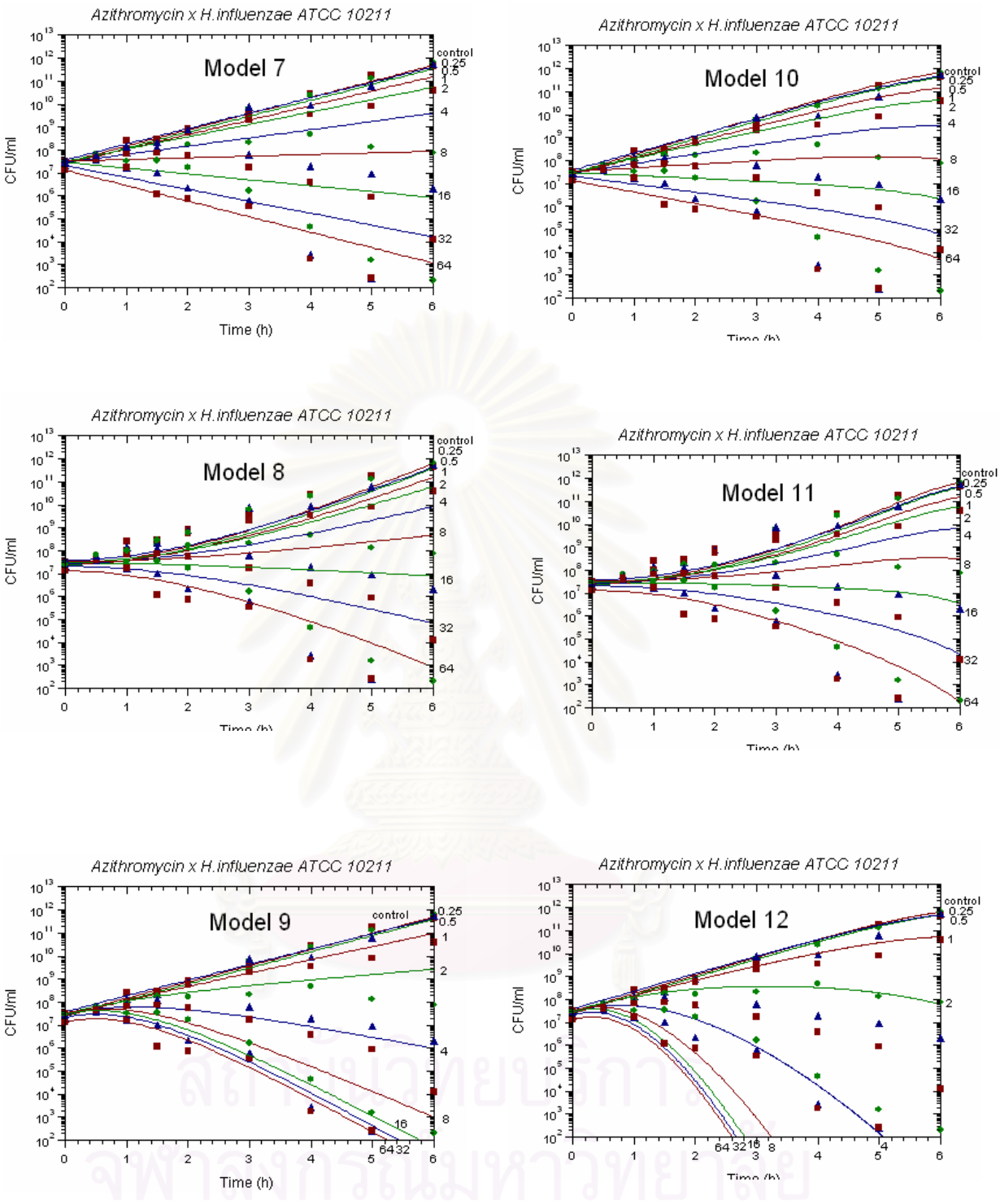


Figure 23 The curve fits using models 7-12 for various constant azithromycin concentrations (µg/mL) against *H. influenzae* ATCC 10211. The lines represent the fitted curve to bacterial viable counts in the 6 h time-kill curves.

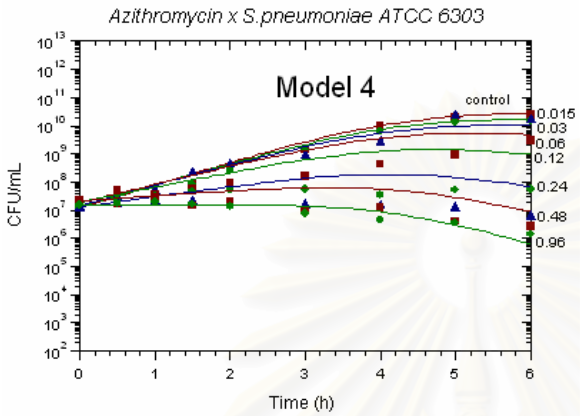
2. PK/PD Analysis

From summarizing curve fits of bacterial time-kill curves, the models 4, 2 and 9 were the best models for best fitting the time-kill curve data for *S. pneumoniae* (both penicillin-sensitive and penicillin-intermediate), *M. catarrhalis* and *H. influenzae*, respectively. For both *S. pneumoniae* penicillin-sensitive (ATCC 6303) and penicillin-intermediate (ATCC 49619), the data were best explained by PK/PD model 4 that incorporates an N_{max} term. Model 2 that incorporates an adaptation rate constant was found appropriate to describe the data for *M. catarrhalis*. For *H. influenzae*, model 3 could not explain the data well. Model 9 that incorporates additional Hill (h) factor displayed the better fit. The results indicated that a simple PK/PD model (model 1) was not sufficient to describe the observed pharmadynamic effects for four bacterial strains. It is necessary to add some additional terms into the model. For both penicillin-sensitive and penicillin-intermediate *S. pneumoniae*, the limitation of space and nutrients (N_{max}) had an effect on the growth rate. Therefore, the addition of a saturation term into the simple model appeared to be necessary. The growth of *M. catarrhalis* was delayed and not in the logarithmic growth phase at time zero. Therefore, an adaptation rate constant ($1-e^{-zt}$) was necessary to account for these effects. For *H. influenzae*, the maximum kill rate showed delay while the bacterial growth did not. Therefore, an adaptation term was used only for the bacterial killing effect. In constrast to other antibiotic classes like β-lactams, no correlation was found between the delay in the onset of growth and kill due to the mechanism of action.

Results of the best fitted curves of azithromycin against four bacterial strains are summarized in Figure 24. The determined pharmacodynamic parameters of azithromycin and goodness of fit from using the best PK/PD model as mentioned above for each individual bacterial strain are summarized in Table 12. Results from the concentration at half-maximum effect (EC_{50}) indicate that azithromycin showed high efficacy against *S. pneumoniae* strains (EC_{50} /ATCC 6303: 0.34 μg/mL; EC_{50} /ATCC 49619: 0.05 μg/mL) and *M. catarrhalis* (EC_{50}:0.15 μg/mL) but low efficacy against *H. influenzae* (EC_{50}:3.68 μg/mL).

Figure 24 Summary of curve fits for four bacterial strains with various constant concentrations (µg/mL) (A) azithromycin against *S. pneumoniae* ATCC 6303 (B) azithromycin against *S. pneumoniae* ATCC 49619 (C) azithromycin against *M. catarrhalis* ATCC 8176 (D) azithromycin against *H. influenzae* ATCC 10211

A



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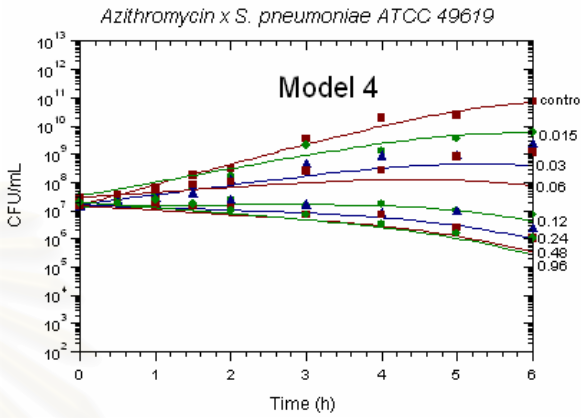

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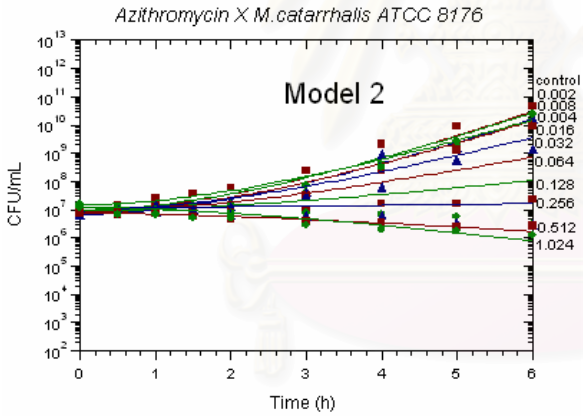

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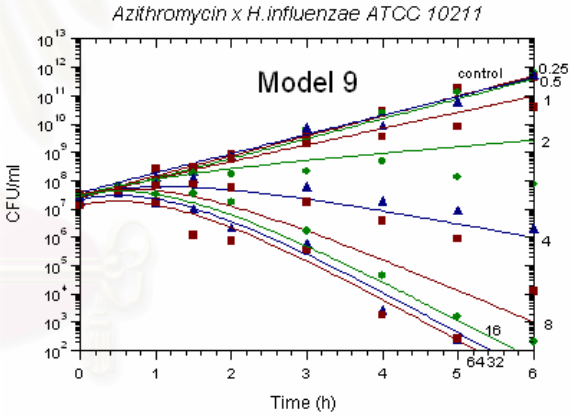


Table 12 Summary of pharmacodynamic azithromycin parameters and goodness of fit criteria

Parameters	*S. pneumoniae* ATCC 6303	*S. pneumoniae* ATCC 49619	*M. catarrhalis* ATCC 8176	*H. influenzae* ATCC 10211
k_0 (h^{-1})	1.36	1.21	1.63	1.18
k_{max} (h^{-1})	1.80	1.61	2.50	3.71
EC_{50} (µg/mL)	0.34	0.05	0.15	3.68
N_{max} (10^8CFU/mL)	1.52	3.86	-	-
z(h^{-1})	-	-	0.37	0.79
h	-	-	-	2.08
MSC / r^2	1.98 / 0.8	3.24 / 0.96	2.08 / 0.88	2.55 / 0.92

C. Pharmacokinetic Studies

1. Bioanalytical Method Validation of Determining Azithromycin in Plasma and in Ultrafiltrate

1.1 Selectivity/Specificity

Chromatograms of analytical method for determination of azithromycin and clarithromycin in plasma and in ultrafiltrate are shown in Figures 25 and 26, respectively. The retention times of azithromycin and clarithromycin were approximately 5 and 7 min, respectively. No any interfering peaks due to the presence of plasma protein and/or endogenous substances affecting the peaks of azithromycin and internal standard are observed. This indicates the selectivity of the analytical method.

1.2 Lower Limit of Quantification (LLOQ)

The lower limit of quantification of the analysis method of azithromycin in plasma and in ultrafiltrate were found to be 10 and 5 ng/mL, respectively. The accuracy of azithromycin at 10 and 5 ng/mL in plasma and in ultrafiltrate were 105.56% with a precision of 8.63% and 99.68% with a precision of 6.47%, respectively. These findings were acceptable for accuracy ($\pm$20%) and for precision (<20%) as shown in Tables 13-14.

1.3 Linearity and Standard Calibration Curve

Typical linearity data and plots utilizing linear regression analysis of azithromycin in plasma and in ultrafiltrate are presented in Tables 15-16 and Figures 27-28. These demonstrate that a nine points calibration curve of peak area ratios of azithromycin to clarithromycin were linear covering the range of concentrations employed (10-800 ng/mL in plasma and 5-600 ng/mL in ultrafiltrate) with the coefficient of determination better than 0.999.

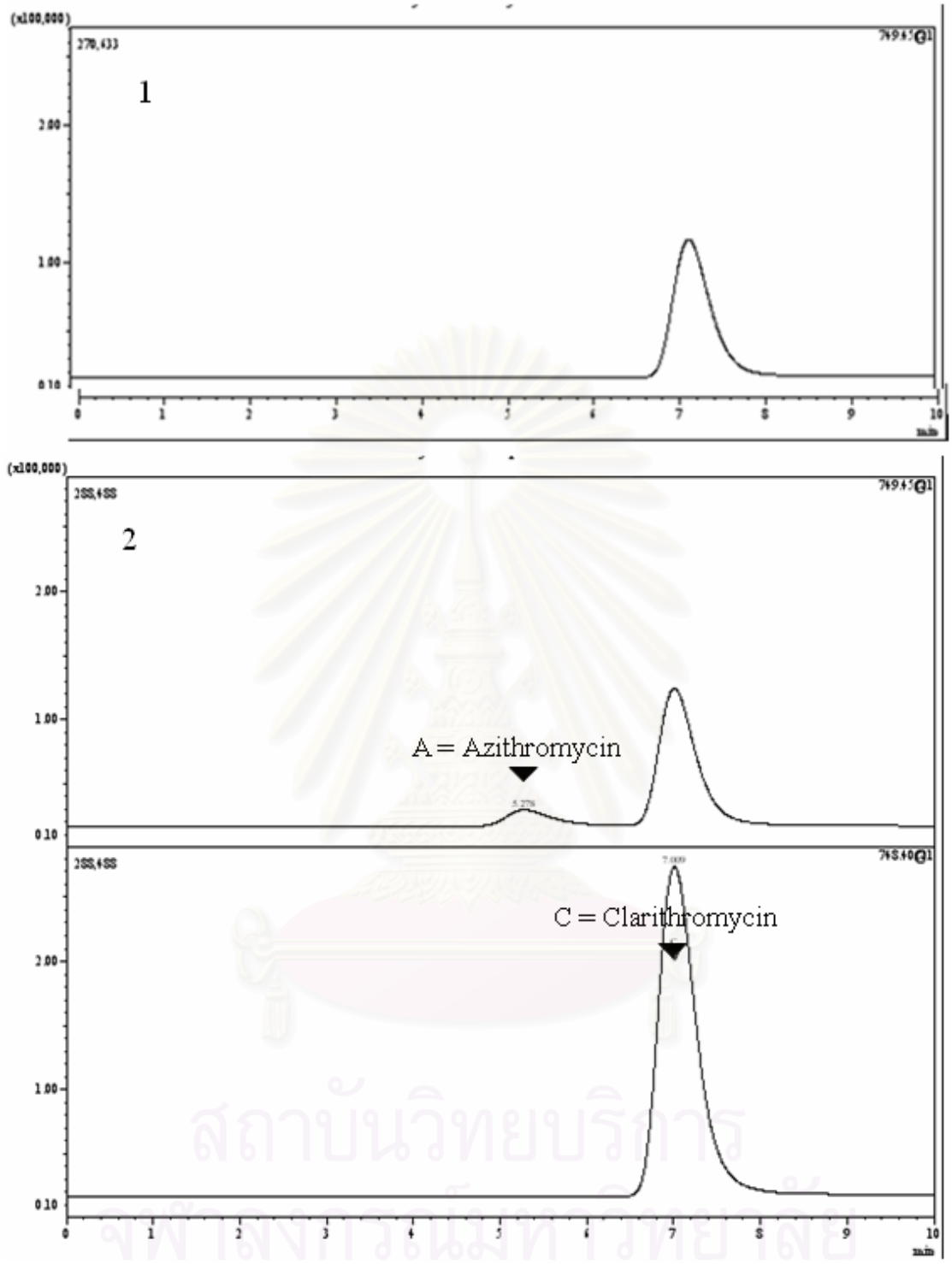


Figure 25 Chromatograms of analytical method for determination of azithromycin in plasma. 1 = Blank plasma, 2 = Plasma spiked with azithromycin (A) 200 ng/mL and clarithromycin (C) 50 µL of 0.15 mg/mL.

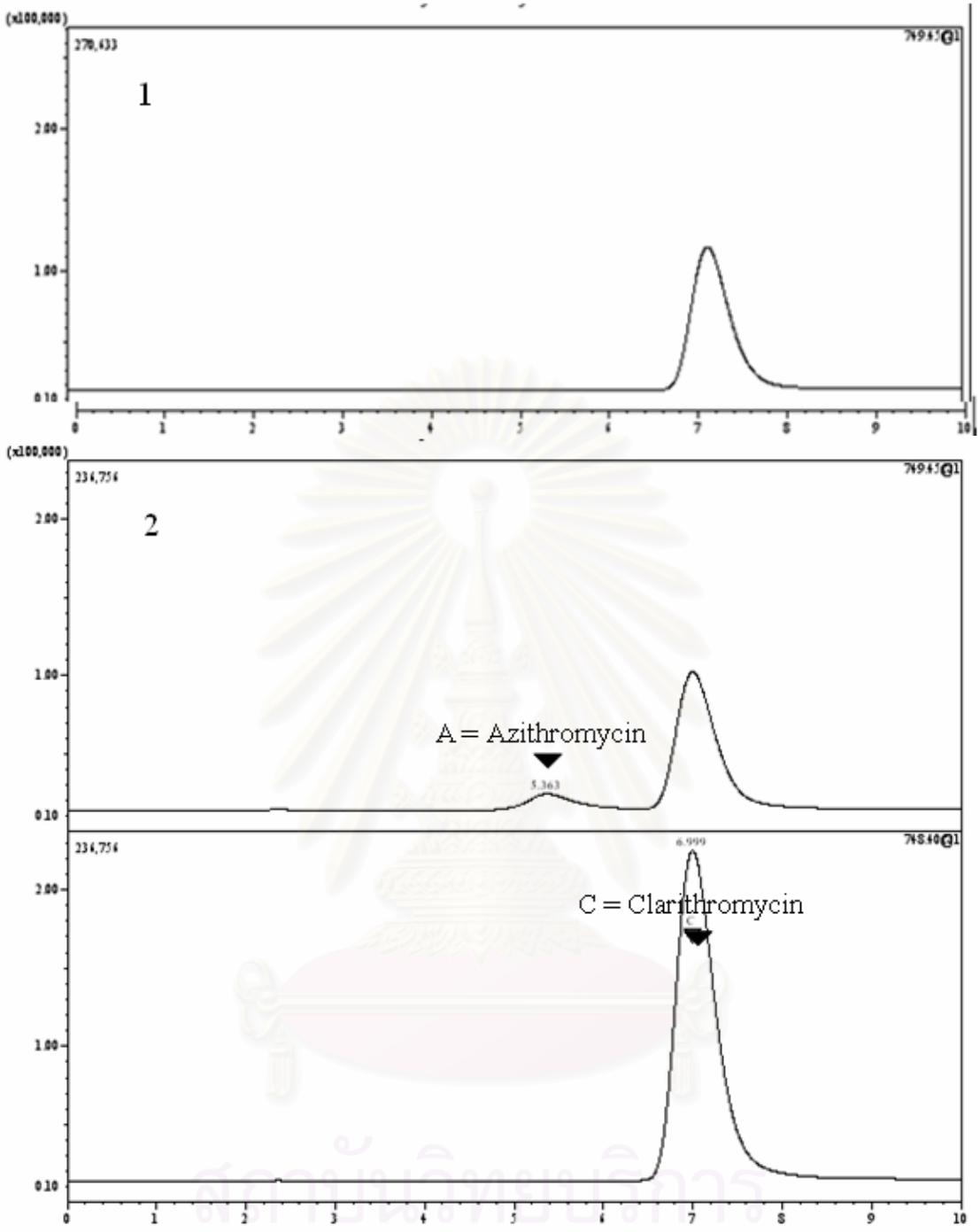


Figure 26 Chromatograms of analytical method for determination of azithromycin in ultrafiltrate. 1 = Blank ultrafiltrate, 2 = Ultrafiltrate spiked with azithromycin (A) 200 ng/mL and clarithromycin (C) 50 µL of 0.03 mg/mL.

Table 13 Lower limit of quantification of analytical method for determination of azithromycin in plasma.

Replication no	Known conc (ng/mL)	Estimated conc (ng/mL)	% Recovery
1	10	11.08	110.80
2	10	10.08	100.80
3	10	9.60	96.00
4	10	10.15	101.50
5	10	11.87	118.70
Mean	-	10.55	105.52
S.D.	-	0.91	9.11
% C.V.	-	8.64	8.64

Table 14 Lower limit of quantification of analytical method for determination of azithromycin in ultrafiltrate

Replication no	Known conc (ng/mL)	Estimated conc (ng/mL)	% Recovery
1	5	4.78	96.60
2	5	4.97	99.40
3	5	5.20	104.00
4	5	4.89	97.90
5	5	4.52	90.40
Mean	-	4.87	97.66
S.D.	-	0.25	4.93
% C.V.	-	5.13	5.04

Table 15 Linearity and standard curve of analytical method for determination of azithromycin in plasma.

Line 1

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	10	0.001268	11.08	110.80
2	20	0.003109	17.16	85.80
3	30	0.007566	31.87	106.23
4	50	0.013882	52.72	105.44
5	100	0.029305	103.64	103.64
6	200	0.056292	192.74	96.37
7	400	0.121124	406.78	101.69
8	600	0.175025	584.73	97.45
9	800	0.242988	809.10	101.14

Peak area ratio = 0.0003029 Conc - 0.0020885
r^2 = 0.9993

Line 2

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	10	0.001461	10.08	100.80
2	20	0.004110	19.10	95.50
3	30	0.008064	32.58	108.60
4	50	0.013519	51.17	102.34
5	100	0.028069	100.76	100.76
6	200	0.057254	200.24	100.12
7	400	0.114663	395.90	98.98
8	600	0.172656	593.56	98.93
9	800	0.235137	806.52	100.82

Peak area ratio = 0.0002934 Conc - 0.0014953
r^2 = 0.9998

Line 3

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	10	0.002082	9.60	96.00
2	20	0.004910	19.88	99.40
3	30	0.007579	29.59	98.63
4	50	0.013023	49.38	98.76
5	100	0.027847	103.29	103.29
6	200	0.057373	210.66	105.33
7	400	0.106111	387.89	96.97
8	600	0.160730	586.50	97.75
9	800	0.223040	813.08	101.64

Peak area ratio = 0.000275 Conc - 0.000558
r^2 = 0.9991

Line 4

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	10	0.002097	10.13	101.30
2	20	0.004694	19.11	95.55
3	30	0.007904	30.21	100.70
4	50	0.013198	48.52	97.14
5	100	0.029795	105.91	105.91
6	200	0.059151	207.41	103.71
7	400	0.110120	383.66	95.92
8	600	0.171857	597.13	99.52
9	800	0.232739	807.65	101.96

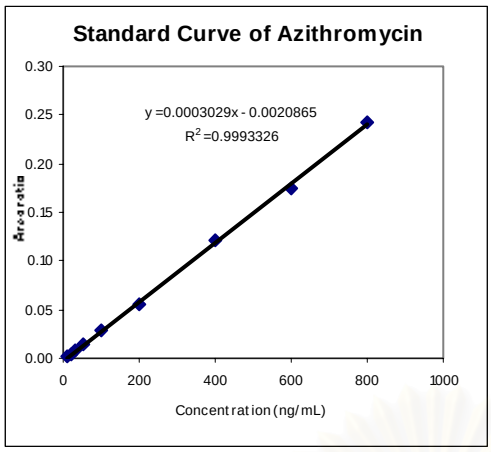
Peak area ratio = 0.0002892 Conc - 0.000833
r^2 = 0.9994

Line 5

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	10	0.001602	11.87	118.70
2	20	0.004650	22.76	113.80
3	30	0.006681	30.01	100.03
4	50	0.013883	55.72	111.44
5	100	0.025839	98.40	98.40
6	200	0.052558	193.80	96.90
7	400	0.108240	392.59	98.15
8	600	0.166080	599.09	99.85
9	800	0.223906	805.53	100.69

Peak area ratio = 0.0002801 Conc - 0.0017243
r^2 = 0.9997

Line 1



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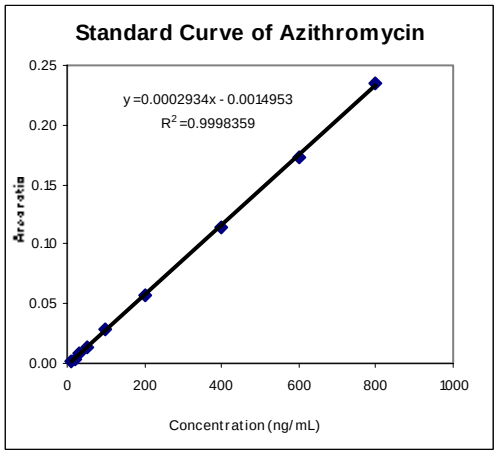

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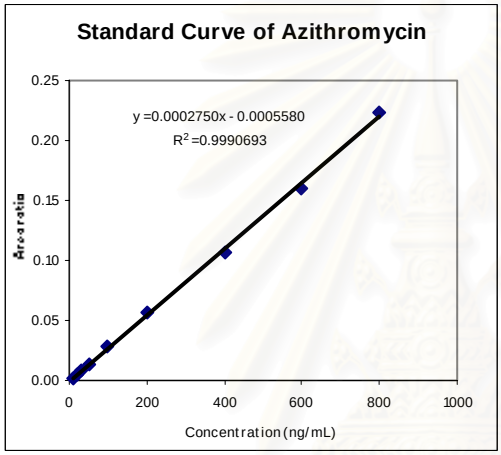

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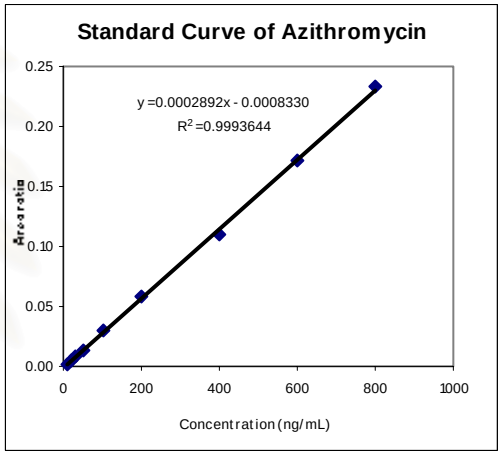

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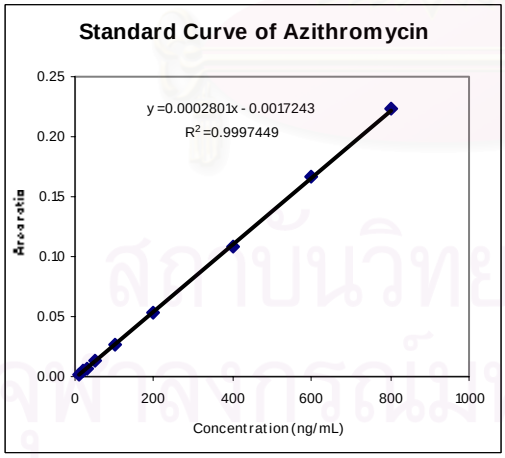


Figure 27 Standard curve for determination of azithromycin in plasma

Table 16 Linearity and standard curve of analytical method for determination of azithromycin in ultrafiltrate.

Line 1

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	5	0.00124	4.78	96.60
2	10	0.00254	10.47	104.70
3	30	0.00657	28.11	93.70
4	50	0.01172	50.66	101.32
5	70	0.01579	68.48	97.83
6	100	0.02162	94.01	94.01
7	200	0.04802	209.59	104.80
8	400	0.09217	402.90	100.73
9	600	0.13633	596.24	99.37

Peak area ratio = 0.0002284 Conc + 0.0001488
r^2 = 0.9995

Line 2

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	5	0.00145	4.97	99.40
2	10	0.00266	10.06	100.60
3	30	0.00686	27.72	92.40
4	50	0.01284	52.87	105.74
5	70	0.01598	66.08	94.40
6	100	0.02343	97.40	97.40
7	200	0.04890	204.51	102.26
8	400	0.09696	406.61	101.65
9	600	0.14176	595.01	99.17

Peak area ratio = 0.0002378 Conc + 0.0002672
r^2 = 0.9996

Line 3

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	5	0.00178	5.20	104.00
2	10	0.00295	10.12	101.20
3	30	0.00742	28.88	96.27
4	50	0.01324	53.35	106.70
5	70	0.01649	67.01	95.73
6	100	0.02343	96.17	96.17
7	200	0.04878	202.68	101.34
8	400	0.09696	405.12	101.28
9	600	0.14248	596.38	99.40

Peak area ratio = 0.0002380 Conc + 0.0005426
r^2 = 0.9997

Line 4

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	5	0.00229	4.89	97.80
2	10	0.00348	9.85	98.50
3	30	0.00827	29.85	99.50
4	50	0.01416	54.43	108.86
5	70	0.01689	65.82	94.03
6	100	0.02514	100.26	100.26
7	200	0.04878	198.92	99.46
8	400	0.09728	401.34	100.33
9	600	0.14473	599.38	99.90

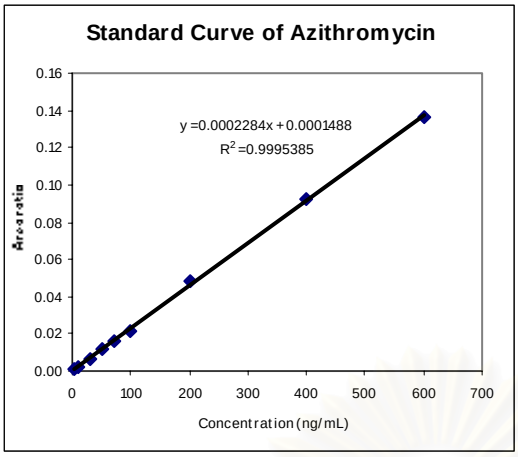
Peak area ratio = 0.0002396 Conc + 0.001119
r^2 = 0.9998

Line 5

Standard no	Known conc (ng/mL)	Peak area ratio	Estimated conc (ng/mL)	% Recovery
1	5	0.00164	4.52	90.40
2	10	0.00269	9.06	90.60
3	30	0.00827	33.12	110.40
4	50	0.01247	51.33	102.66
5	70	0.01519	63.08	90.11
6	100	0.02438	102.80	102.80
7	200	0.04764	203.32	101.66
8	400	0.09217	395.75	98.94
9	600	0.13983	601.72	100.29

Peak area ratio = 0.0002314 Conc + 0.000593
r^2 = 0.9997

Line 1



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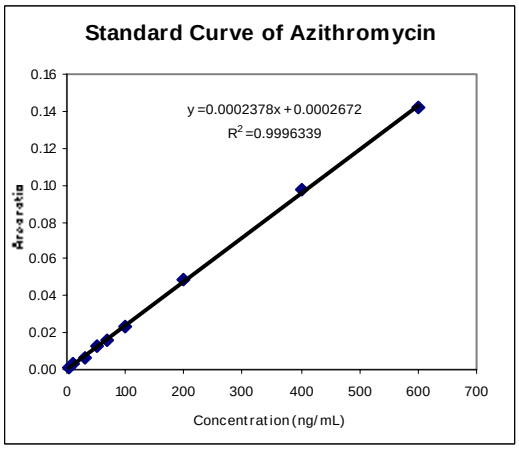

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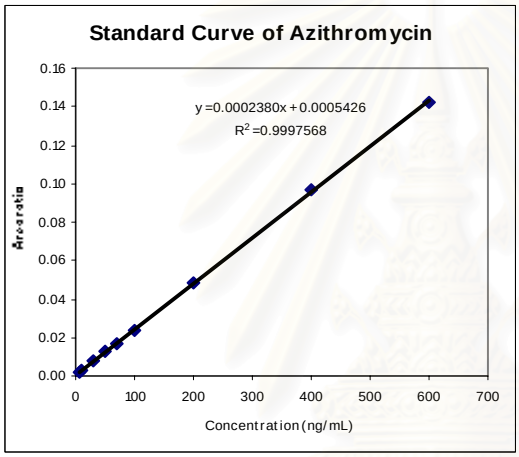

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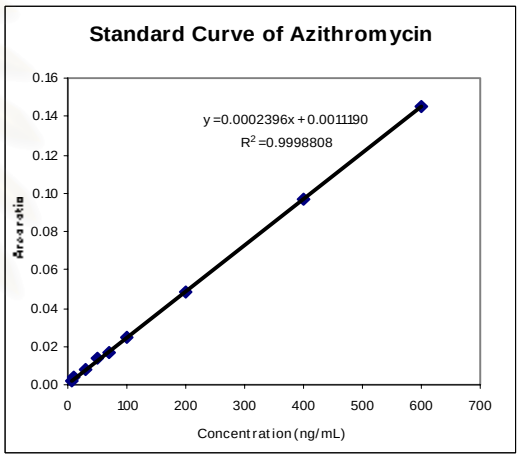

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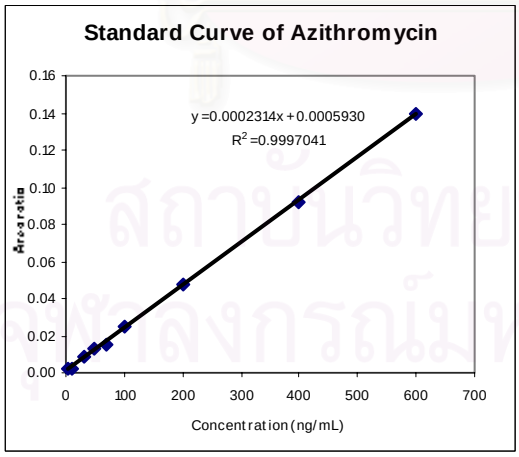


Figure 28 Standard curve for determination of azithromycin in ultrafiltrate

1.4 Accuracy and Precision

The accuracy, within-run and between-run precision were assessed using 3 quality control concentrations. Results are shown in Tables 17-22. The percent recovery of the method in plasma was between 91.05-94.86 % , and that of method in ultrafiltrate was 97.86-107.78%. The % C.V. for within- and between-run precision of azithromycin in plasma and in ultrafiltrate were less than 15%. These results were within acceptance criteria for accuracy (recovery $\pm$15%) and precision (%C.V.<15%).

1.5 Stability

1.5.1 Freeze-thaw Stability

The two QC samples in plasma and in ultrafiltrate were stored at –20 ^{o}C for 24 h and thawed at room temperature unassisted. After completion of three freeze-thaw cycles, the two QC samples were still stable in plasma and ultrafiltrate under this stressed condition. All data are shown in Tables 23-24. The percent deviation of azithromycin concentrations compared to those before processing were within the acceptable ranges ($\pm$ 15%).

1.5.2 Short-term Room Temperature Stability

Results demonstrates that the two QC samples were found to be well stable after they were kept at room temperature for 12 h as shown in Tables 25-26. No tendency of degradation of azithromycin could be observed. The percent decrease of the azithromycin concentrations from the time zero were within $\pm$ 15%.

1.5.3 Long-term Stability

The long-term stability of azithromycin in plasma and in ultrafiltrate data are presented in Tables 27-28. The results revealed that azithromycin samples were stable for 6 weeks at 20 ^{o}C. These results were within the acceptable criteria ($\pm$ 15%). Hence, these storage times were sufficient for completion of drug analysis.

1.5.4 Post-preparative Stability

As presented in Tables 29-30, the processed samples could be stored in autosampler upto 12 h without any problems. All percent decreases of azithromycin were within the acceptance range (<15%).

1.6 Recovery of Extraction

Results are presented in Table 31. High recoveries were obtained for azithromycin ranging from 100.09-108.31%, indicating specificity of the extraction procedure. Thus, the extraction method used is appropriate for this study.

Table 17 Accuracy of analytical method for determination of azithromycin in plasma

Replication no	Estimated concentration (ng/mL)					
	LQC (30 ng/mL)		MQC (300 ng/mL)		HQC (700 ng/mL)	
	Est.conc[a]	% Recovery	Est.conc	% Recovery	Est.conc	% Recovery
1	27.30	91.00	271.01	90.34	651.19	93.03
2	27.82	92.73	281.36	93.79	669.03	95.58
3	27.09	90.30	271.89	90.63	651.46	93.07
4	27.42	91.40	277.15	92.38	679.16	97.02
5	26.96	89.83	273.13	91.04	656.29	93.76
Mean	27.32	91.05	273.13	91.64	663.99	94.86
S.D.	0.34	1.12	4.30	1.44	12.54	1.79
% C.V.	1.23	1.23	1.58	1.57	1.89	1.89

[a] Est.conc = Estimated concentration

Table 18 Accuracy of analytical method for determination of azithromycin in ultrafiltrate

Replication no	Estimated concentration (ng/mL)					
	LQC (15 ng/mL)		MQC (300 ng/mL)		HQC (500 ng/mL)	
	Est.conc	% Recovery	Est.conc	% Recovery	Est.conc	% Recovery
1	14.65	97.63	309.55	103.18	518.79	103.76
2	14.20	94.66	306.79	102.26	545.33	109.07
3	14.48	96.55	313.63	104.54	539.36	107.87
4	14.58	97.19	304.96	101.65	547.37	109.47
5	14.98	99.86	305.23	101.74	523.63	104.73
Mean	14.58	97.86	308.03	102.68	538.92	107.78
S.D.	0.28	1.86	3.62	1.21	10.75	2.15
% C.V.	1.94	1.94	1.18	1.18	1.99	1.99

[a] Est.conc = Estimated concentration

Table 19 Within-run precision of analytical method for determination of azithromycin in plasma

Replication no	Estimated concentration (ng/mL)		
	LQC (30 ng/mL)	MQC (300 ng/mL)	HQC (700 ng/mL)
1	27.30	271.01	651.19
2	27.82	281.36	669.03
3	27.09	271.89	651.46
4	27.42	277.15	679.16
5	26.95	273.13	656.39
Mean	27.32	273.13	663.99
S.D.	0.34	4.30	12.54
% C.V.	1.23	1.58	1.89

Table 20 Within-run precision of analytical method for determination of azithromycin in ultrafiltrate

Replication no	Estimated concentration (ng/mL)		
	LQC (15 ng/mL)	MQC (300 ng/mL)	HQC (500 ng/mL)
1	14.65	309.55	518.79
2	14.20	306.79	545.33
3	14.48	313.63	539.36
4	14.58	304.96	547.37
5	14.98	305.23	523.63
Mean	14.58	308.03	538.92
S.D.	0.28	3.62	10.75
% C.V.	1.94	1.18	1.99

Table 21 Between-run precision of analytical method for determination of azithromycin in plasma

Replication no	Estimated concentration (ng/mL)		
	LQC (30 ng/mL)	MQC (300 ng/mL)	HQC (700 ng/mL)
1	28.20	284.63	662.53
2	28.17	293.06	670.28
3	29.07	277.68	671.07
4	28.98	280.90	655.28
5	28.82	277.65	691.77
Mean	28.65	277.78	670.19
S.D.	0.43	6.42	13.67
% C.V.	1.51	2.27	2.04

Table 22 Between-run precision of analytical method for determination of azithromycin in ultrafiltrate

Replication no	Estimated concentration (ng/mL)		
	LQC (15 ng/mL)	MQC (300 ng/mL)	HQC (500 ng/mL)
1	13.88	284.39	506.76
2	14.32	288.83	527.72
3	14.22	296.48	496.71
4	14.44	306.60	502.01
5	14.63	295.06	504.27
Mean	14.30	294.67	507.49
S.D.	0.28	8.61	11.90
% C.V.	1.93	2.92	2.35

Table 23 Freeze-thaw stability of analytical method for determination of azithromycin in plasma

Replication no	LQC (30 ng/mL)			HQC (700 ng/mL)		
	0 Cycle	3 Cycle		0 Cycle	3 Cycle	
	Init.conc	Est.conc	%Dev	Init.conc	Est.conc	%Dev
1	31.39	27.67	-11.85	748.51	674.11	-9.94
2	32.99	28.52	-13.55	712.08	629.52	-11.59
3	32.16	30.07	-6.50	711.08	642.64	-9.62
Mean	32.18	28.75	-10.63	723.89	648.76	-10.39
S.D.	0.80	1.22	3.68	21.33	22.92	1.06

Table 24 Freeze-thaw stability of analytical method for determination of azithromycin in ultrafiltrate

Replication no	LQC (15 ng/mL)			HQC (500 ng/mL)		
	0 Cycle	3 Cycle		0 Cycle	3 Cycle	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Init.conc	Est.conc	%Dev
1	15.28	14.18	-7.20	529.48	497.24	-6.09
2	15.34	14.15	-7.76	518.97	482.16	-7.09
3	15.17	14.07	-7.25	521.35	479.34	-8.06
Mean	15.26	14.13	-7.40	523.27	486.25	-7.08
S.D.	0.09	0.06	0.31	5.51	9.62	0.98

[a] Init.conc = Initial concentration
[b] Est.conc = Estimated concentration
[c] % Dev = % Deviation = (Est.conc – Init.conc) x100 / Init.conc

Table 25 Short-term room temperature stability of analytical method for determination of azithromycin in plasma

Replication no	LQC (30 ng/mL)				
	0 h	6 h		12 h	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev
1	34.70	32.75	-5.62	29.53	-14.90
2	34.01	32.62	-4.09	30.03	-11.70
3	34.31	32.43	-5.48	29.87	-12.94
Mean	34.34	32.60	-5.06	29.81	-13.18
S.D.	0.35	0.16	0.85	0.26	1.61

Replication no	HQC (700 ng/mL)				
	0 h	6 h		12 h	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev
1	797.10	779.92	-2.16	698.82	-12.33
2	798.52	772.89	-3.21	695.77	-12.87
3	798.48	776.42	-2.76	698.82	-12.48
Mean	798.03	776.41	-2.71	697.80	-12.56
S.D.	0.81	3.52	0.53	1.76	0.28

[a] Init.conc = Initial concentration
[b] Est.conc = Estimated concentration
[c] % Dev = % Deviation = (Est.conc – Init.conc) x100 / Init.conc

Table 26 Short-term room temperature stability of analytical method for determination of azithromycin in ultrafiltrate

Replication no	LQC (15 ng/mL)				
	0 h	6 h		12 h	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev
1	14.78	14.64	-0.95	14.26	-3.52
2	15.76	15.24	-3.30	14.86	-5.71
3	15.42	15.14	-1.82	14.72	-4.54
Mean	15.32	15.01	-2.02	14.61	-4.59
S.D.	0.50	0.32	1.19	0.31	1.10

Replication no	HQC (500 ng/mL)				
	0 h	6 h		12 h	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev
1	535.24	502.29	-6.16	471.67	-11.88
2	529.42	486.28	-8.15	443.22	-16.28
3	543.16	512.47	-5.65	479.32	-11.75
Mean	535.94	500.35	-6.65	464.74	-13.30
S.D.	6.90	13.20	1.32	19.02	2.58

[a] Init.conc = Initial concentration
[b] Est.conc = Estimated concentration
[c] % Dev = % Deviation = (Est.conc – Init.conc) x100 / Init.conc

Table 27 Long-term stability of analytical method for determination of azithromycin in plasma

Replication no	LQC (30 ng/mL)						
	0 Week	2 Week		4 Week		6 Week	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev	Est.conc	%Dev
1	29.98	29.14	-2.80	28.91	-3.57	28.35	-5.44
2	29.30	29.15	-0.57	28.88	-1.43	28.57	-2.49
3	29.77	29.28	-1.65	28.87	-3.02	28.54	-4.13
Mean	29.68	29.19	-1.65	28.89	-2.68	28.49	-4.02
S.D.	0.35	0.08	1.14	0.02	1.11	0.12	1.48

Replication no	HQC (700 ng/mL)						
	0 Week	2 Week		4 Week		6 Week	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev	Est.conc	%Dev
1	699.69	682.89	-2.40	671.67	-4.00	642.95	-8.10
2	703.29	677.09	-3.73	650.17	-7.55	647.74	-7.89
3	700.69	642.62	-8.29	654.35	-6.61	641.85	-8.39
Mean	701.22	667.53	-4.80	658.73	-6.06	644.18	-8.13
S.D.	1.86	21.77	3.09	11.40	1.84	3.13	0.25

[a] Init.conc = Initial concentration
[b] Est.conc = Estimated concentration
[c] % Dev = % Deviation = (Est.conc – Init.conc) x100 / Init.conc

Table 28 Long-term stability of analytical method for determination of azithromycin in ultrafiltrate

Replication no	LQC (15 ng/mL)						
	0 Week	2 Week		4 Week		6 Week	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev	Est.conc	%Dev
1	14.91	14.64	-1.81	14.29	-4.16	13.86	-7.04
2	14.25	14.07	-1.26	13.87	-2.67	13.46	-5.54
3	14.38	13.97	-2.85	13.62	-5.29	13.29	-7.58
Mean	14.51	14.23	-1.98	13.93	-4.04	13.54	-6.72
S.D.	0.35	0.36	0.81	0.34	1.31	0.29	1.06

Replication no	HQC (500 ng/mL)						
	0 Week	2 Week		4 Week		6 Week	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev	Est.conc	%Dev
1	518.34	504.59	-2.65	495.61	-4.39	475.64	-8.24
2	521.49	506.28	-2.92	491.27	-5.79	468.47	-10.17
3	533.18	514.85	-3.44	502.37	-5.78	482.49	-9.51
Mean	524.34	508.57	-3.00	496.42	-5.32	475.53	-9.30
S.D.	7.82	5.50	0.40	5.59	0.81	7.01	0.98

[a] Init.conc = Initial concentration
[b] Est.conc = Estimated concentration
[c] % Dev = % Deviation = (Est.conc – Init.conc) x100 / Init.conc

Table 29 Post-preparative (autosampler) stability of analytical method for determination of azithromycin in plasma

Replication no	LQC (30 ng/mL)				
	0 h	6 h		12 h	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev
1	28.18	27.42	-2.70	27.45	-2.59
2	28.18	27.19	-3.51	27.02	-4.12
3	28.21	27.89	-1.13	27.66	-1.95
Mean	28.19	27.50	-2.45	27.38	-2.89
S.D.	0.02	0.36	1.21	0.33	1.11

Replication no	HQC (700 ng/mL)				
	0 h	6 h		12 h	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev
1	769.21	799.72	3.97	725.53	-5.68
2	793.47	795.06	0.20	720.75	-9.16
3	793.40	800.15	0.85	724.12	-8.73
Mean	785.36	798.31	1.67	723.47	-7.86
S.D.	13.99	2.82	2.01	2.46	1.90

[a] Init.conc = Initial concentration
[b] Est.conc = Estimated concentration
[c] % Dev = % Deviation = (Est.conc – Init.conc) x100 / Init.conc

Table 30 Post-preparative (autosampler) stability of analytical method for determination of azithromycin in ultrafiltrate

Replication no	LQC (15 ng/mL)				
	0 h	6 h		12 h	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev
1	14.98	14.64	-2.27	14.42	-3.74
2	15.16	15.08	-0.53	14.82	-2.24
3	15.12	14.94	-1.19	14.74	-2.51
Mean	15.09	14.89	-1.33	14.66	-2.83
S.D.	0.09	0.22	0.88	0.21	0.80

Replication no	HQC (500 ng/mL)				
	0 h	6 h		12 h	
	Init.conc[a]	Est.conc[b]	%Dev[c]	Est.conc	%Dev
1	525.36	503.27	-4.20	482.74	-8.11
2	539.72	498.54	-7.63	463.22	-14.17
3	543.12	518.37	-4.56	479.32	-11.75
Mean	535.94	506.73	-5.46	475.09	-11.34
S.D.	6.90	10.36	1.88	10.42	3.05

[a] Init.conc = Initial concentration
[b] Est.conc = Estimated concentration
[c] % Dev = % Deviation = (Est.conc – Init.conc) x100 / Init.conc

Table 31 Recovery of extraction of analytical method for determination of azithromycin and clarithromycin(IS) in plasma

Replication no	LQC (30 ng/mL)			MQC (300 ng/mL)		
	Peak area		% Recovery of extraction	Peak area		% Recovery of extraction
	Plasma	Aqueous		Plasma	Aqueous	
1	24533	21289	115.24	245710	225802	108.82
2	22502	21991	102.32	252478	238359	105.92
3	26845	26254	102.25	269417	270992	99.42
4	25514	24425	104.46	279473	298734	93.55
5	21352	18206	117.28	277615	296203	93.72
Mean	24149.20	22433.00	108.31	264948.60	266018.00	100.29
S.D.	2225.70	3079.76	7.35	15141.02	33123.10	6.96
% C.V.	9.22	13.73	6.78	5.71	12.45	6.94

Replication no	HQC (700 ng/mL)			IS (0.15 µg/mL)		
	Peak area		% Recovery of extraction	Peak area		% Recovery of extraction
	Plasma	Aqueous		Plasma	Aqueous	
1	580555	542049	107.11	4112969	4134754	99.47
2	583291	544471	107.13	4402119	4360230	100.96
3	662770	682651	97.09	4222813	4216521	100.15
4	626413	597882	104.77	4235814	4214264	100.51
5	672750	707717	95.06	4150775	4177311	99.36
Mean	625155.80	614952.20	102.23	4224898.00	4220616.00	100.09
S.D.	43079.89	77077.26	5.57	111302.64	84848.09	0.68
% C.V.	6.89	12.53	5.62	2.63	2.01	0.68

2. Total Plasma and Free Drug Concentrations

Eight male subjects participated in the study. They were healthy based on passing physical examination as well as clinical blood/urine biochemistry laboratory tests. Their demographic data and laboratory test are presented in Tables 41 and 42 (Appendix B). None withdrew from the study or exhibited signs of allergy and adverse drug reactions to azithromycin (Table 43 in Appendix B).

Total plasma and free drug concentration-time profiles on day 3 of each individual subject who received once-daily 2x250 mg of azithromycin capsules for 3-day regimen are reported in Tables 32 and 33, respectively. It is clearly observed from data presented in both Tables that there are wide intersubject variations. These variations are possible differences in the extent of drug absorption due to its low oral bioavailability (37%) [32], differences in the rate of distribution of drug into tissues and subsequent release from tissues (good to excellent) because azithromycin is essentially nonpolar, lipophilic and able to penetrate cell membranes [32], or differences in drug elimination because the liver is a major route of elimination [35]. In addition, intersubjects variability may be taken into account for this variation. Within 2 h of oral administration, mean of total plasma and free drug concentration reached the peak concentrations of 461.20 and 236.19 ng/mL in Tables 32 and 33, respectively. Peak drug concentration (461.20 ng/mL) observed in this study is consistent with the value reported in the literature [34]. The plots of azithromycin concentration-time profile as pairwise comparison of total plasma and free drug concentrations of each individual subject are shown in Figures 29-36. The mean of total plasma and free drug concentration-time profiles of all subjects are summarized in Table 34 and displayed in Figure 37. Results showed that total plasma and free drug concentration-time profiles were similar. The pharmacokinetic profiles appeared to reflect a rapid and extensive uptake from the circulation into intracellular compartments followed by slow release from tissues to plasma (Figure 37). After drug concentrations reached to peak concentration, concentrations declined in a polyphasic manner with a prolonged elimination half-life reflecting initial rapid distribution into tissues and then slowed return to plasma from tissues [113]. Concerning protein binding, percent protein binding of each individual subject was determined and presented in Tables 44-52 (Appendix C). Results show that the average protein binding of azithromycin was 47.08%. This value is consistent with the value reported in the literature [124].

Table 32 Total plasma azithromycin concentration (ng/mL) on day 3 of 8 subjects after once-daily oral administration of 2x250 mg of azithromycin capsules for 3-day regimen.

Time	Subject no.										
(h)	1	2	3	4	5	6	7	8	Mean	S.D.	%CV
0	57.49	46.74	43.81	73.79	57.44	72.84	51.55	70.56	59.28	11.86	20.01
0.5	94.35	215.40	108.58	107.64	67.92	115.07	92.77	102.84	113.07	43.80	38.74
1	146.77	398.92	153.55	101.83	130.81	317.61	623.44	124.42	249.67	184.43	73.87
1.5	165.43	354.84	214.92	140.72	274.09	409.14	710.93	530.26	350.04	195.73	55.92
2	452.72	314.46	375.86	407.00	321.76	574.96	510.84	731.97	461.20	141.41	30.66
2.5	511.48	296.62	372.77	581.64	243.74	398.53	410.80	667.90	424.19	158.20	37.29
3	254.69	240.14	295.72	262.60	174.52	283.79	251.01	531.94	275.55	109.12	39.60
3.5	195.97	181.30	217.45	258.88	130.33	210.94	133.78	416.53	218.15	90.83	41.64
4	117.03	129.60	143.54	206.48	115.17	159.37	204.57	300.58	172.04	63.02	36.63
6	88.76	116.79	71.38	177.36	117.89	121.32	152.07	211.74	132.16	46.15	34.92
9	79.27	120.54	82.73	142.64	103.76	72.16	99.04	153.22	106.67	29.84	27.98
12	45.64	69.42	61.94	126.45	72.97	106.13	88.49	129.37	87.55	30.62	34.97
24	33.66	62.10	44.14	69.12	79.82	60.43	69.97	102.75	65.25	21.15	32.42
48	32.48	41.38	33.17	57.16	40.05	43.65	67.66	58.69	46.78	12.87	27.51
72	21.27	30.11	31.24	39.83	28.11	35.84	39.36	45.86	33.95	7.80	22.98
96	24.54	25.42	19.46	19.26	24.39	26.37	31.12	33.94	25.57	5.09	19.89
120	15.46	15.24	19.35	28.24	18.75	19.36	26.02	29.82	21.53	5.70	26.47
144	14.86	15.84	14.32	21.60	15.75	20.12	19.42	22.52	18.05	3.23	17.88
168	13.73	14.54	15.53	23.89	15.27	15.39	16.70	25.71	17.60	4.55	25.87
192	13.86	11.60	15.72	18.16	12.22	16.21	17.13	23.80	16.09	3.88	24.09

Table 33 Free drug concentration (ng/mL) on day 3 of 8 subjects after once-daily oral administration of 2x250 mg of azithromycin capsules for 3-day regimen.

Time	Subject no.										
(h)	1	2	3	4	5	6	7	8	Mean	S.D.	%CV
0	11.27	25.07	30.41	30.89	25.88	22.32	15.50	21.88	22.90	6.82	29.76
0.5	41.64	183.41	100.48	48.72	27.99	69.75	30.96	82.13	73.13	51.28	70.11
1	69.26	350.50	133.04	55.79	84.77	149.74	270.46	78.59	149.02	106.73	71.62
1.5	100.53	262.39	145.32	72.98	156.02	123.60	339.14	409.38	201.17	121.82	60.55
2	160.51	279.49	375.24	236.06	132.08	129.08	238.30	338.78	236.19	92.41	39.12
2.5	211.50	184.79	338.13	284.10	172.40	148.68	242.26	286.92	233.60	65.71	28.13
3	138.86	231.02	165.61	189.45	146.26	195.45	191.34	276.40	191.80	45.16	23.55
3.5	95.18	136.25	170.86	162.98	126.26	154.21	111.97	119.19	134.61	26.40	19.62
4	65.28	100.72	111.64	119.42	97.06	84.37	98.30	174.66	106.43	32.11	30.17
6	45.89	79.95	51.93	89.11	63.55	69.75	69.75	69.35	67.41	13.94	20.68
9	28.78	32.80	43.64	80.56	59.87	56.08	86.08	49.11	54.61	20.66	37.82
12	24.30	36.36	56.21	74.41	45.27	45.64	45.64	46.93	46.84	14.50	30.94
24	15.69	28.47	33.43	39.42	47.45	37.39	37.96	32.79	34.15	9.33	27.32
48	12.31	14.58	17.94	32.13	11.40	20.28	20.28	15.82	18.09	6.57	36.32
72	9.91	12.74	13.23	27.30	9.38	9.23	15.23	13.17	13.76	5.84	42.44
96	8.81	10.13	11.77	12.30	6.34	7.13	14.13	12.47	10.38	2.76	26.61
120	6.80	9.02	11.31	11.69	6.22	6.66	10.66	12.49	9.36	2.52	26.96
144	6.01	9.89	10.62	10.10	6.34	6.10	8.10	12.23	8.67	2.38	27.38
168	5.24	7.95	7.60	12.89	6.22	5.23	6.23	9.76	7.64	2.61	34.18
192	5.86	5.37	6.33	8.35	6.04	5.95	5.95	8.40	6.53	1.17	17.86

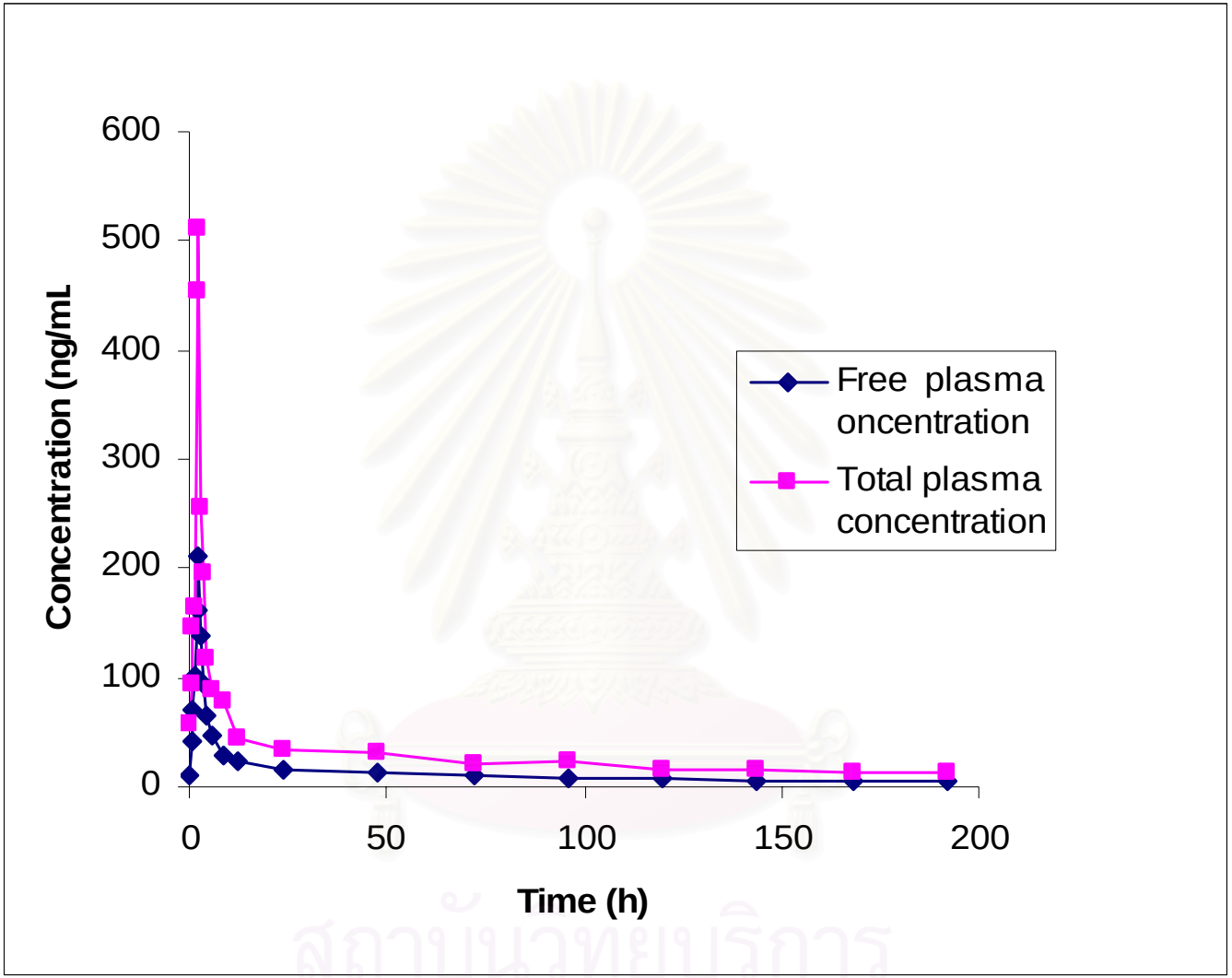


Figure 29 Azithromycin concentration-time profile of subject no.1

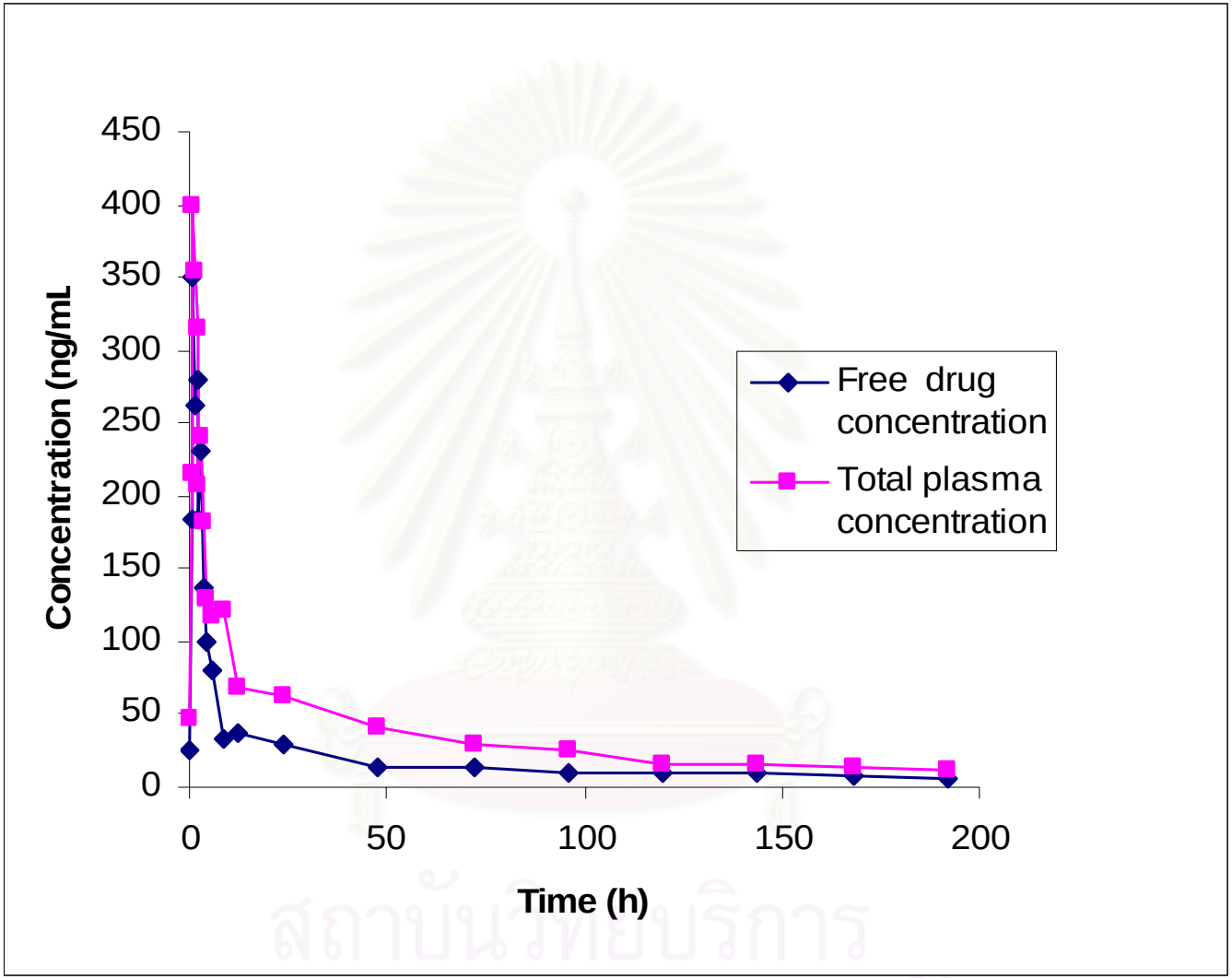


Figure 30 Azithromycin concentration-time profile of subject no.2

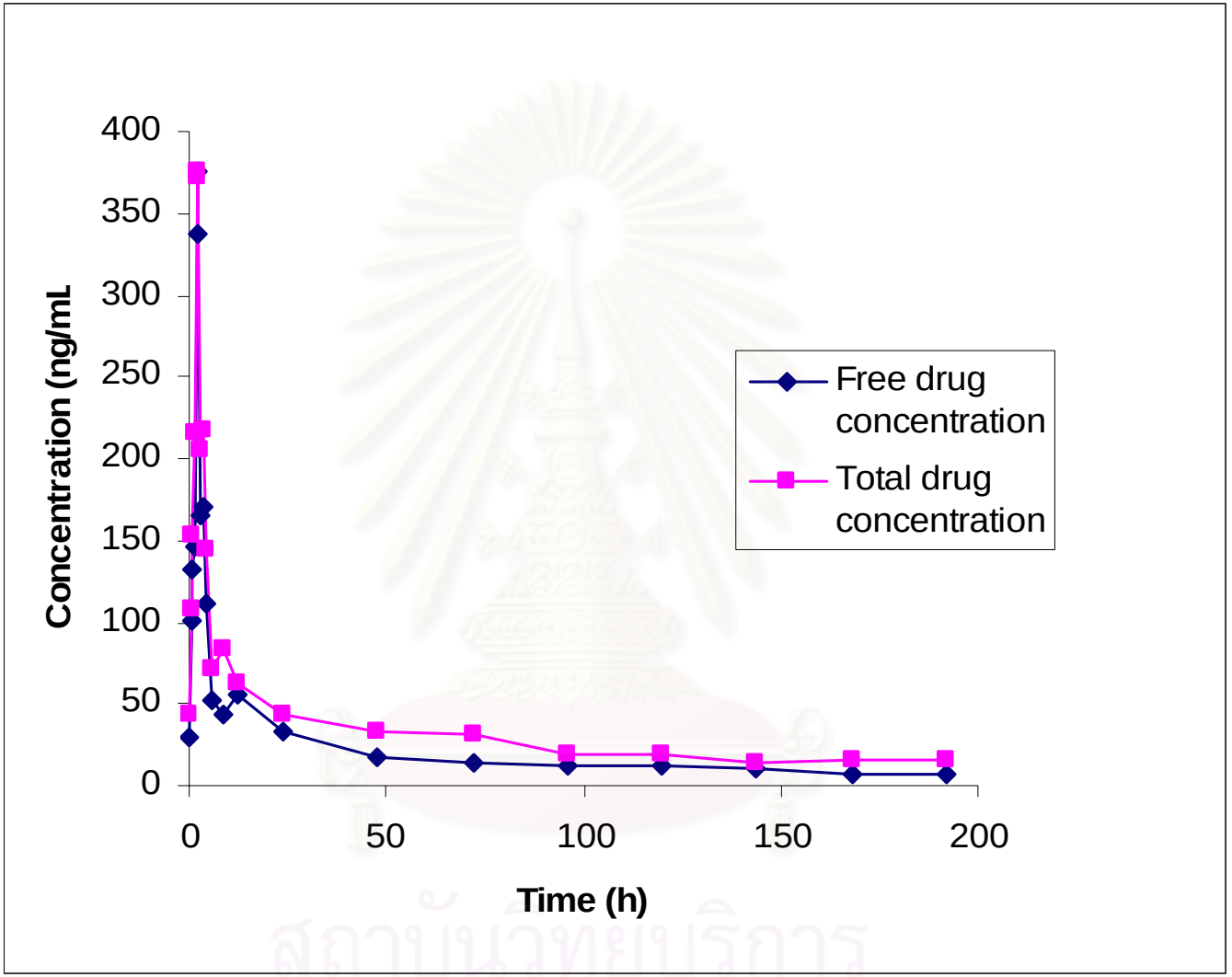


Figure 31 Azithromycin concentration-time profile of subject no. 3

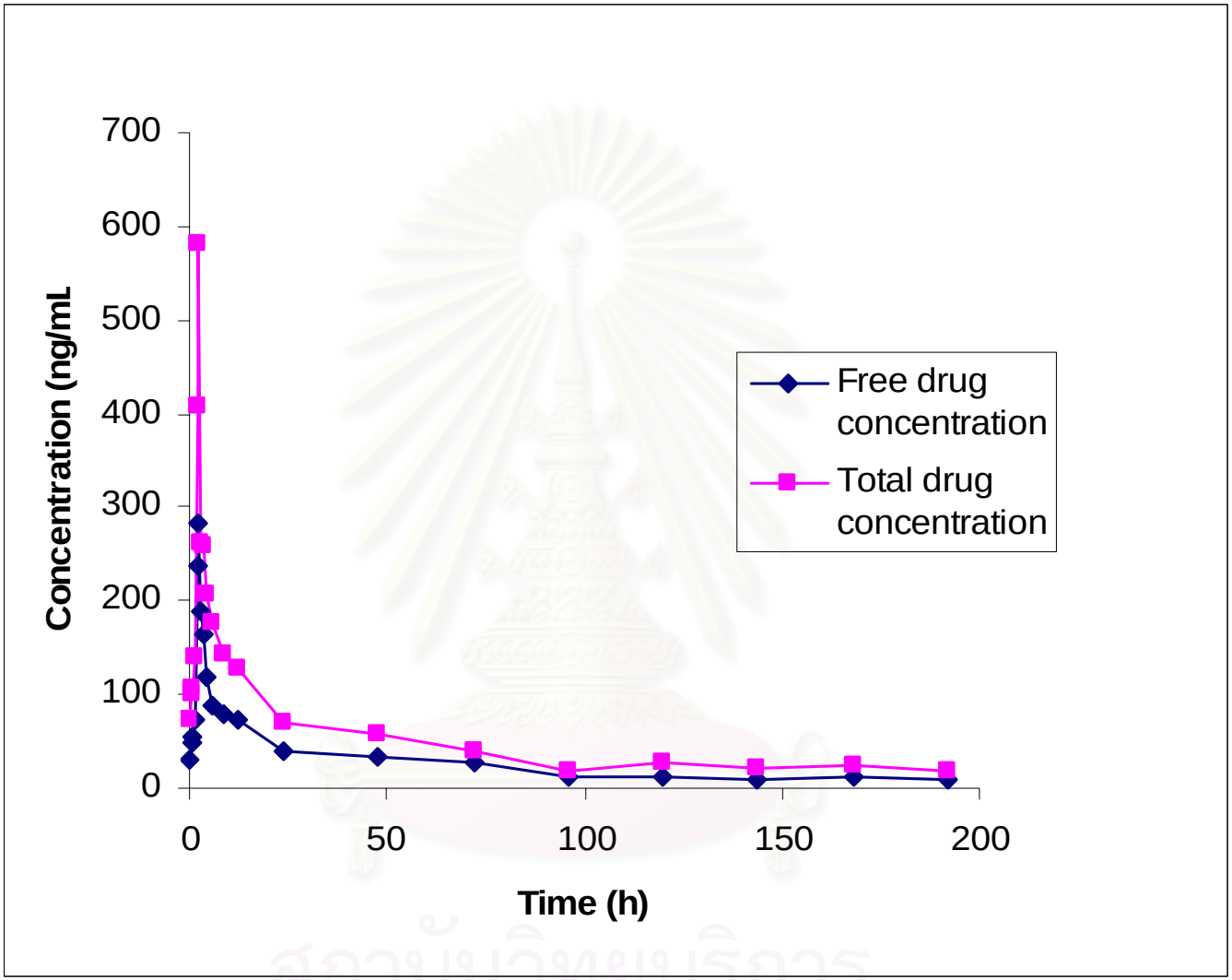


Figure 32 Azithromycin concentration-time profile of subject no. 4

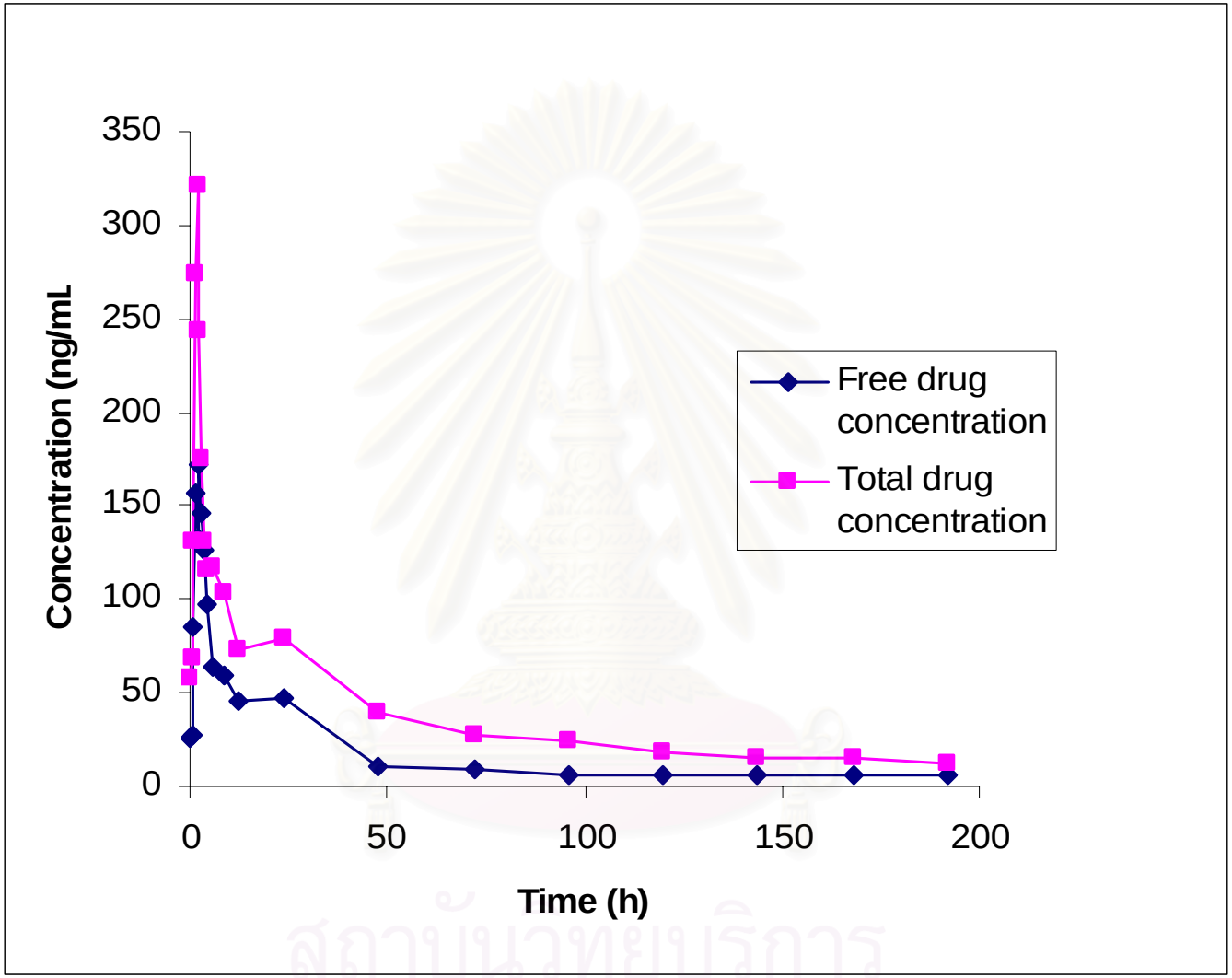


Figure 33 Azithromycin concentration-time profile of subject no.5

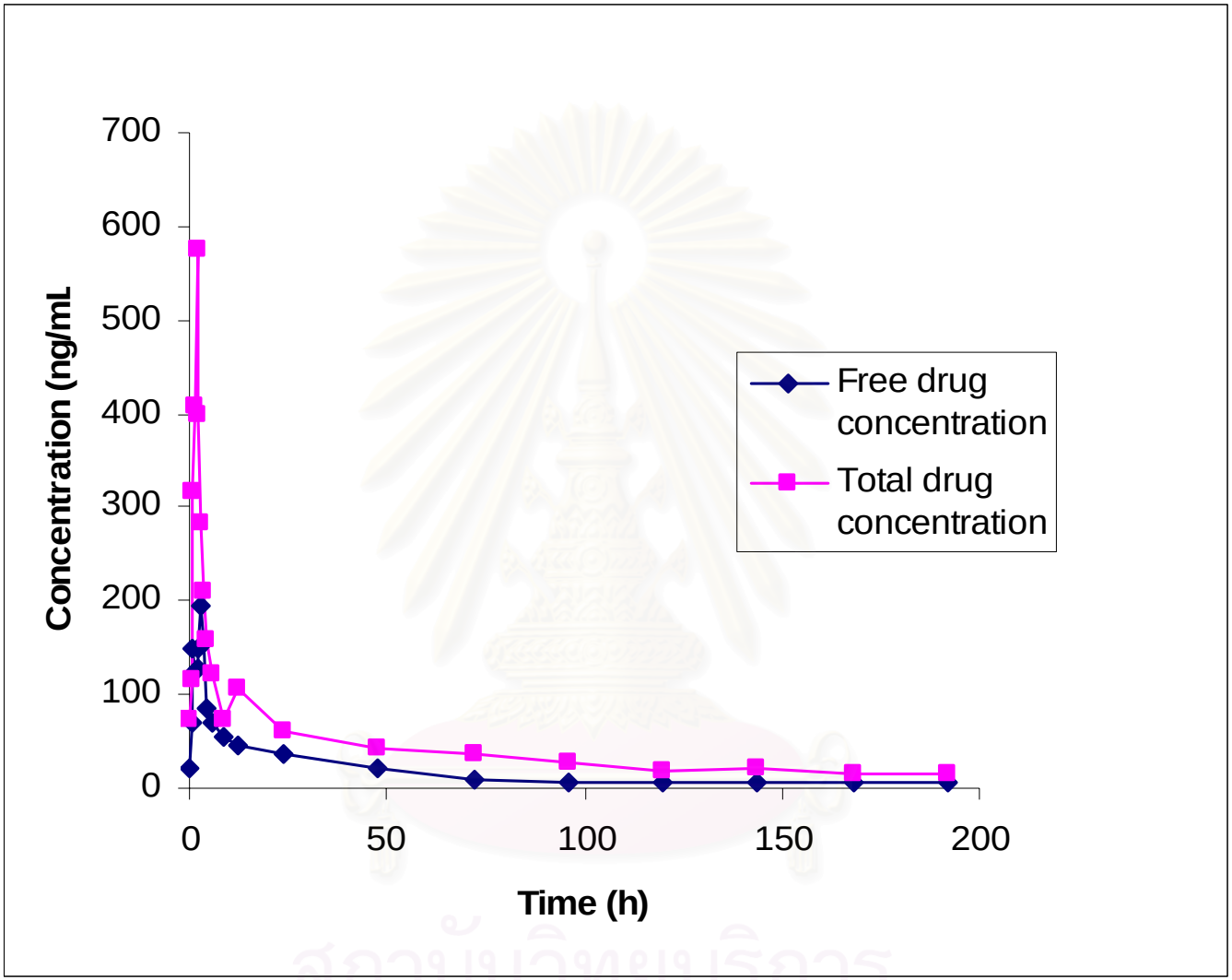


Figure 34 Azithromycin concentration-time profile of subject no. 6

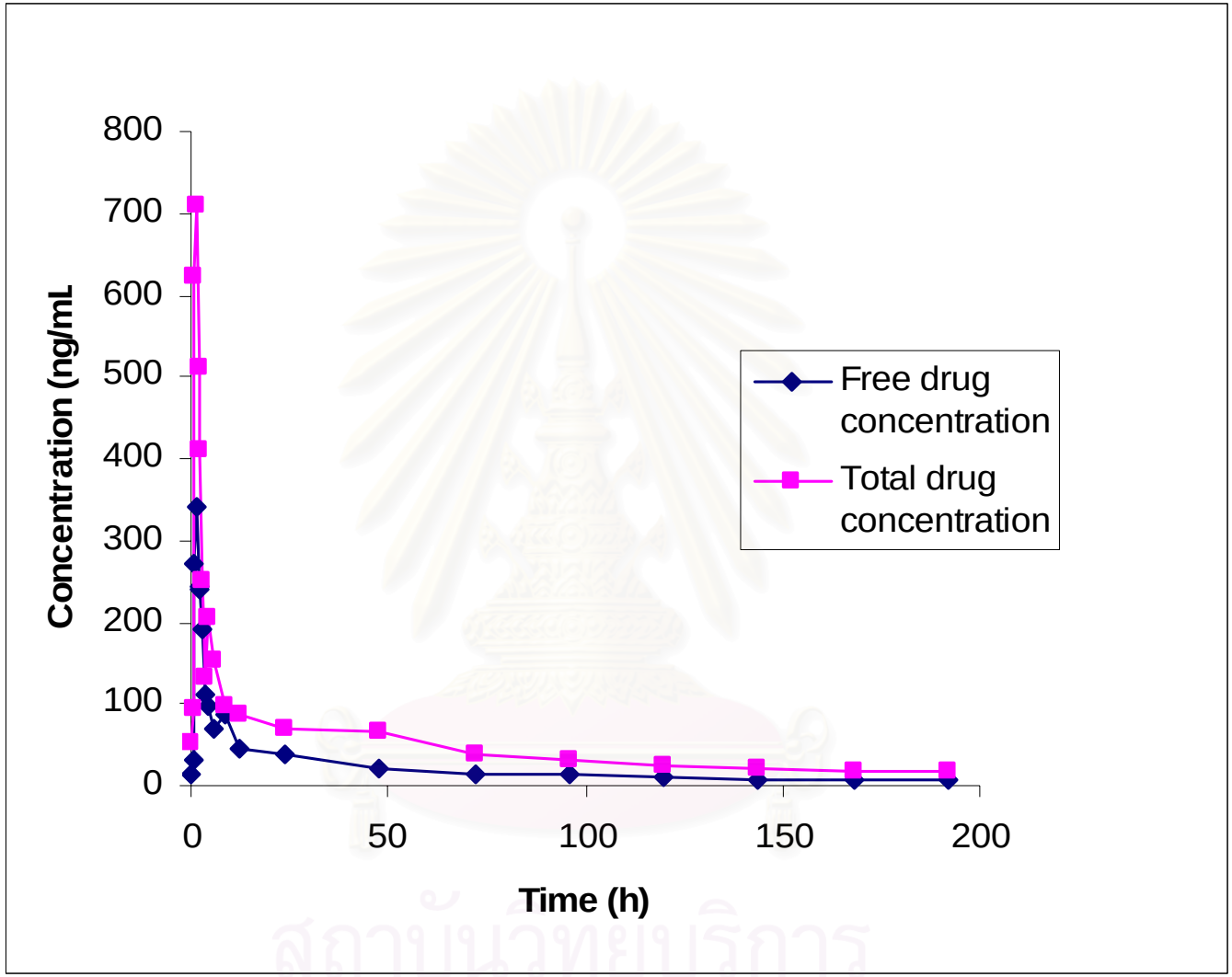


Figure 35 Azithromycin concentration-time profile of subject no.7

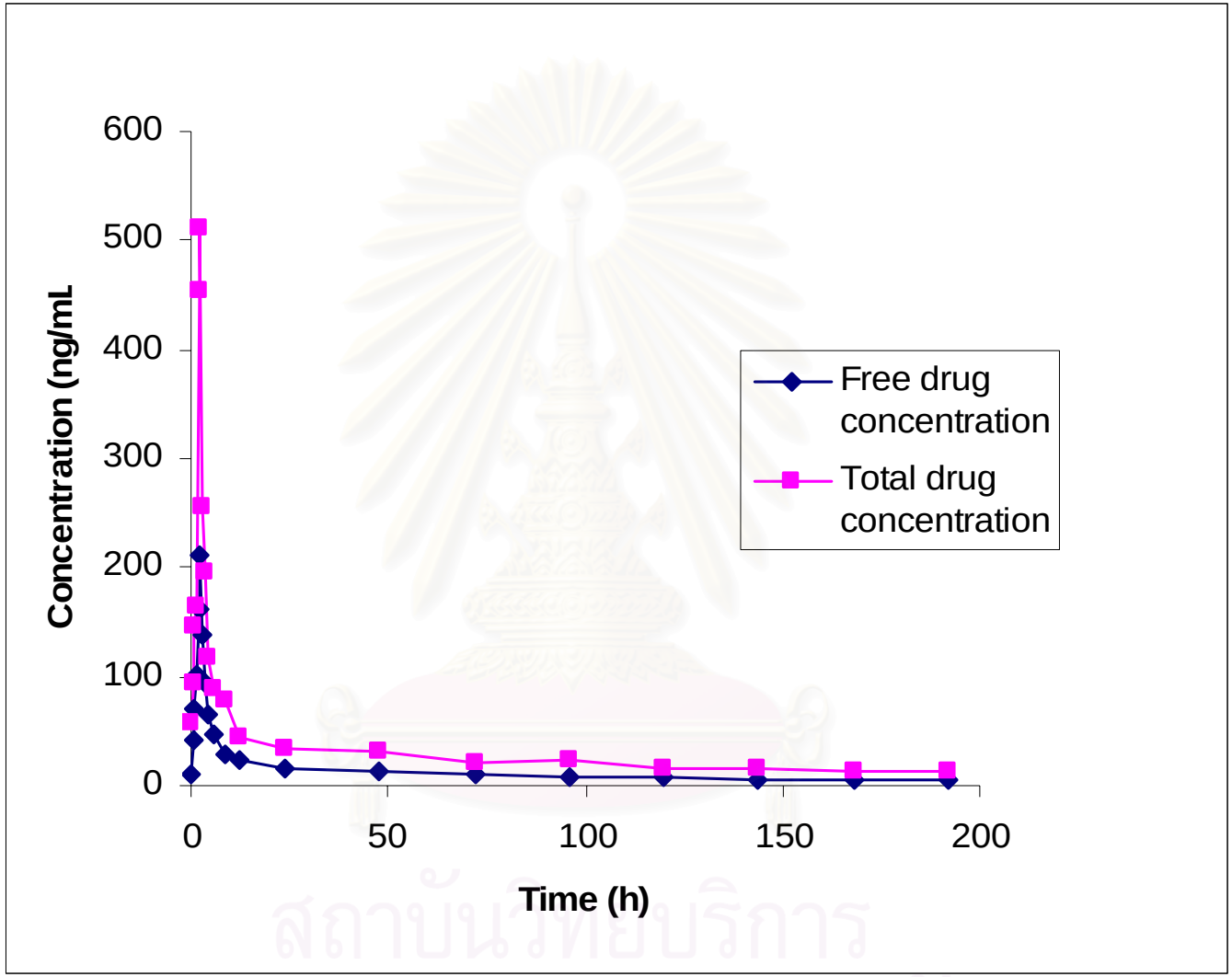


Figure 36 Azithromycin concentration-time profile of subject no.8

Table 34 Mean total plasma and free concentration of azithromycin. Results expressed as Mean±SD (n=8).

Time (h)	Plasma concentration (ng/mL)	
	Total	Free
0	59.28 ± 11.86	22.90 ± 6.82
0.5	113.07 ± 43.80	73.13 ± 51.26
1	249.67 ± 184.43	149.02 ± 106.73
1.5	350.04 + 195.73	201.17 ± 121.82
2	461.20 ± 141.41	236.19 + 92.41
2.5	424.19 ± 158.20	233.60 ± 65.71
3	275.55 ± 109.12	191.80 ± 45.18
3.5	218.15 ± 90.83	134.61 ± 26.40
4	172.04 ± 63.02	106.43 ± 32.11
6	132.16 ± 46.15	67.41 ± 13.94
9	106.67 ± 29.84	54.61 ± 20.66
12	87.55 ± 30.62	46.84 ± 14.50
24	65.25 ± 21.15	34.15 ± 9.33
48	46.78 ± 12.87	18.09 ± 6.57
72	33.95 ± 7.80	13.76 ± 5.84
96	25.57 ± 5.09	10.38 ± 2.76
120	21.53 ± 5.70	9.36 ± 2.52
144	18.05 ± 3.23	8.67 ± 2.38
168	17.60 ± 4.55	7.64 ± 2.61
192	16.09 ± 3.88	6.53 ± 1.17

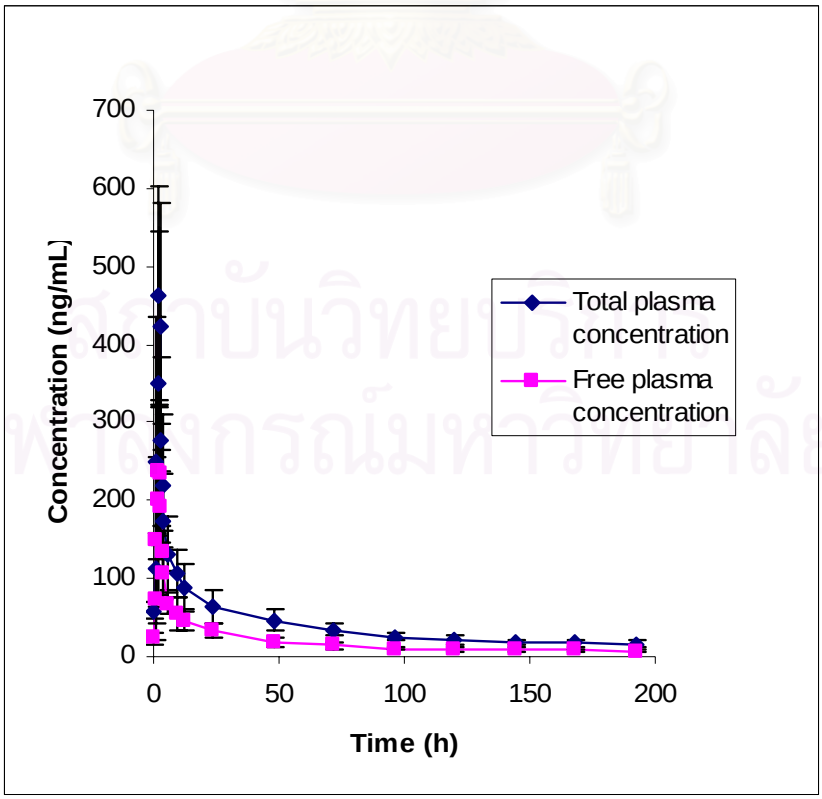


Figure 37 Mean total plasma and free drug concentration-time of 8 subjects.

3. Pharmacokinetic Analysis

3.1 Non-compartmental pharmacokinetic analysis

The results of non-compartmental PK analysis performed with the software WinNonlin® are shown in Tables 35 and 36 for total plasma drug and free drug, respectively. Summary of pharmacokinetic parameter of azithromycin in plasma are presented in Table 37. As mentioned earlier, there are wide variations of total plasma and free drug concentrations among subjects. The pharmacokinetic data obtained for total plasma drug and free drug also gave wide variations. Results in Table 37 indicate that the PK parameters obtained for both total plasma and free drug concentrations were similar. The mean t_{max} of total plasma drug and free drug was 1.94 and 2.06 h, respectively. The mean MRT of total plasma drug and free drug was 57.16 and 51.73 h, respectively. The mean half-life of total plasma drug and free drug was 77.38 and 85.83 h, respectively. For the mean values of C_{max}, AUC_{last}, AUC_{inf}, and AUMC were decreased approximately by 50% from 525.94 to 292.21 ng/mL, 7873.84 to 3755.02 h.ng/mL, 9636.96 to 4572.99 h.ng/mL, and 447865.88 to 194602.11 h.h.ng/mL for total plasma drug and free drug, respectively. This results are consistent with average protein binding of azithromycin (47.08%) which showed effect of protein binding on these parameters. Because if the protein binding is 50%, the free drug AUC would be 50% of total plasma AUC. In addition, this result offers support that the expected outcome of therapy from determination of PK/PD indices (C_{max}/MIC or AUC/MIC) may be overestimated if the total plasma drug is used instead of the free drug. This confirms that protein binding is important and should be taken into consideration because only the free plasma drug is available to exert its pharmacological action. Therefore, the free drug concentration should be used instead of the total plasma concentration to evaluate antimicrobial activity.

Table 35 Non-compartmental pharmacokinetic analysis of total azithromycin in plasma.

Subject no	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h.ng/mL)	AUC_{inf} (h.ng/mL)	AUMC (h.h.ng/mL)	MRT (h)	k_e (h^{-1})	$t_{1/2}$ (h)
1	2.5	511.48	5582.45	7435.56	338698.22	60.67	0.0075	92.67
2	1	398.92	6981.49	8186.17	382752.40	54.82	0.0096	71.98
3	2	375.86	6082.83	7793.58	371545.40	61.08	0.0092	75.43
4	2.5	581.64	9047.99	11461.13	524144.29	57.38	0.0075	92.11
5	2	321.76	6982.27	8555.73	396661.26	56.81	0.0078	89.25
6	2	574.96	7827.98	9565.83	440249.45	56.24	0.0093	74.31
7	1.5	710.93	9248.09	10845.07	513186.12	55.49	0.0107	64.62
8	2	731.97	11237.64	13252.61	615689.90	54.79	0.0118	58.68
Mean	1.94	525.94	7873.84	9636.96	447865.88	57.16	0.0092	77.38
S.D.	0.50	152.47	1875.93	2048.80	94802.13	2.47	0.0016	12.80
% C.V.	25.57	28.99	23.82	21.26	21.17	4.32	4.32	16.54

Table 36 Non-compartmental pharmacokinetic analysis of free azithromycin in plasma.

Subject no	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h.ng/mL)	AUC_{inf} (h.ng/mL)	AUMC (h.h.ng/mL)	MRT (h)	k_e (h^{-1})	$t_{1/2}$ (h)
1	2.5	211.50	2407.13	3179.17	138264.59	57.44	0.0076	91.32
2	1	350.50	3637.41	4732.08	186407.95	51.25	0.0077	89.66
3	2	375.24	3960.79	4941.67	208711.48	52.69	0.0065	107.41
4	2.5	284.10	5065.53	5977.79	281211.56	55.52	0.0092	75.73
5	2.5	172.40	3256.87	3909.70	151350.70	46.47	0.0093	74.92
6	3	195.45	3347.01	3916.62	155349.64	46.41	0.0104	66.36
7	1.5	339.14	4159.68	4729.54	205547.72	49.41	0.0104	66.39
8	1.5	409.38	4205.75	5597.39	229973.26	54.68	0.0060	114.83
Mean	2.06	292.21	3755.02	4572.99	194602.11	51.73	0.0084	85.83
S.D.	0.68	89.86	790.29	930.04	47344.54	4.11	0.0017	18.24
% C.V.	32.88	30.75	21.50	20.34	24.50	7.94	20.21	21.26

Table 37 Summary of pharmacokinetic parameter of azithromycin in plasma. Results expressed as Mean±SD (n=8).

PK parameter	Unit	Total plasma drug	Free drug
t_{max}	h	1.94 ± 0.50	2.06 ± 0.68
C_{max}	ng/mL	525.94 ± 152.47	292.21 ± 89.86
AUC_{last}	h.ng/mL	7873.84 ± 1875.93	3755.02 ± 790.29
AUC_{inf}	h.ng/mL	9636.96 ± 2048.80	4572.99 ± 930.04
AUMC	h.h.ng/mL	447865.88 ± 94802.13	194602.11 ± 47344.54
MRT	h	57.16 ± 2.47	51.73 ± 4.11
k_e	h^{-1}	0.0092 ± 0.0016	0.0084 ± 0.0017
$t_{1/2}$	h	77.38 ± 12.80	85.83 ± 18.24

3.2 Compartmental pharmacokinetic analysis

The compartmental pharmacokinetic analysis was also performed using the curve-fitting program Scientist®. In order to select the most suitable compartment pharmacokinetic model to describe comcentration-time profile for azithromycin concentrations following once-daily oral administration of 2x250 mg of azithromycin capsules for 3-day regimen, the mean data of total plasma drug and free drug concentrations on day 3 which concentration levels did not reach steady state were fitted to one-compartment and two-compartment models with single dose administration. Figure 38 shows curve fits with one-compartment model to concentration-time profiles for total plasma and free drug concentration after once-daily oral administration of 2x250 mg of azithromycin capsules. After curve fitting, Scientist® provided the estimated pharmacokinetic parameters, graphic output and the goodness of fit statistic output. Example of curve fit using Scientist® is shown in Appendix A. Results of the estimated pharmacokinetic parameters and goodness of fit criteria with one-compartment model are presented in Table 38. Figure 39 showed curve fits with two-compartment model to concentration-time profile for total plasma and free drug concentration. Results of the estimated pharmacokinetic parameters and goodness of fit criteria with two-compartment model are presented in Table 39. Comparison between one-compartment and two-compartment model with goodness of fit statistic, MSC and r^2 (Tables 38-39) as well as visual inspection, fitted curve (Figures 38-39) indicate that two-compartmet model had higher values of MSC (1.60 vs

0.35), r^2 (0.88 vs 0.53) for total plasma concentration and had higher values of MSC (2.18 vs 0.42), r^2 (0.93 vs 0.56) for free drug concentration as well as the fitted curve were superior (Figure 39). Therefore, the two-compartment model was the most suitable pharmacokinetic model to describe the PK profile of both total plasma and free drug concentrations. The mathematic equation of two-compartment model and its estimated pharmacokinetic parameters (A = 377.01, B = 0.04, Alpha = 0.5762, Beta = 0.0106 and k_a = 0.5770) derived from fitting comcentration-time profile of azithromycin were used in integrated PK/PD model study.

Table 38 Pharmacokinetic parameters and goodness of fit criteria with one-compartment model.

Plasma	A (µg/mL)	B (µg/mL)	k_e (h^{-1})	k_a (h^{-1})	MSC	r^2
Total	0.02	0.25	0.0011	0.0611	0.35	0.53
Free	0.005	0.15	0.0034	0.0611	0.42	0.56

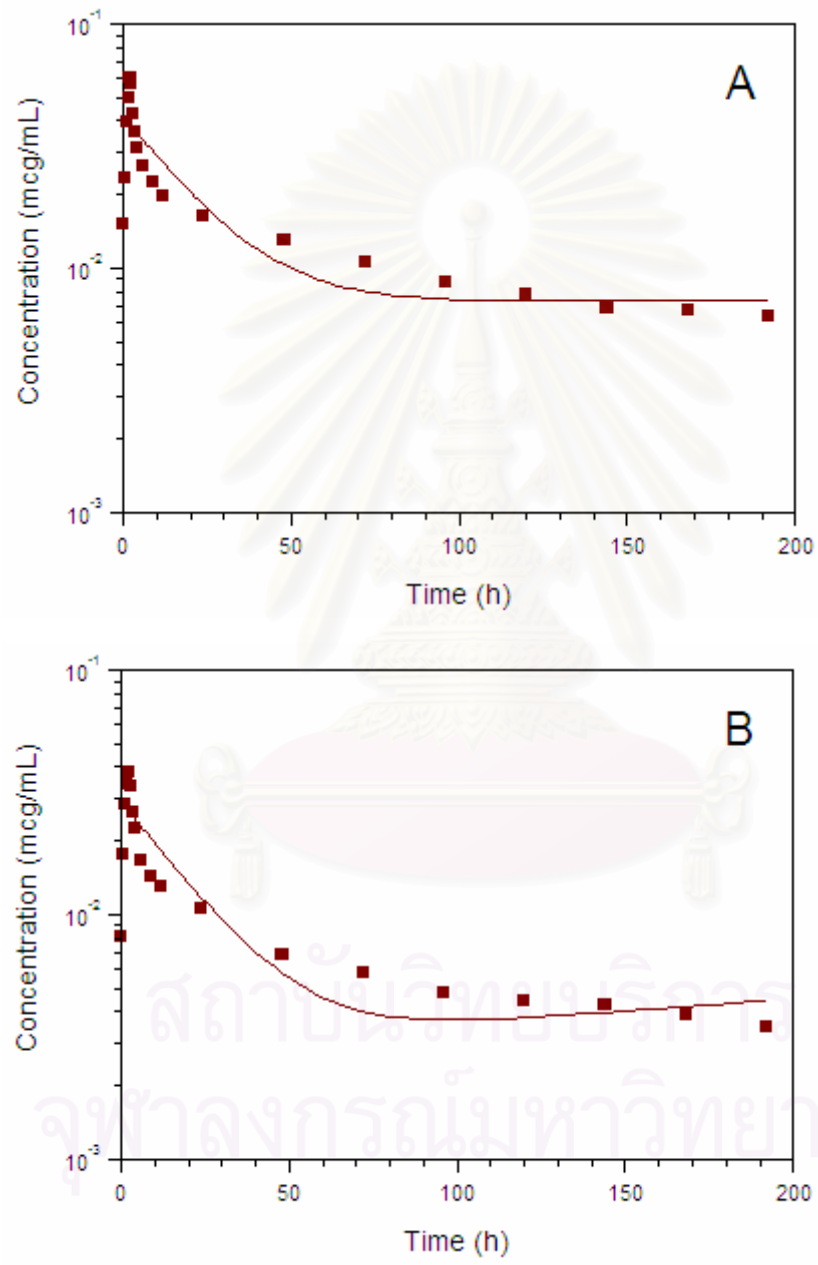


Figure 38 Curve fits with one-compartment model to plasma concentration-time profile (semi-logarithmic plots). A) Total plasma drug. B) Free drug. Symbols represent measured data and the fitted lines present predicted data.

Table 39 Pharmacokinetic parameters and goodness of fit criteria with two-compartment model.

Plasma	A (µg/mL)	B (µg/mL)	Alpha (h^{-1})	Beta (h^{-1})	k_a (h^{-1})	MSC	r^2
Total	0.07	1597.70	0.0092	0.6003	0.6005	1.60	0.88
Free	377.01	0.04	0.5762	0.0106	0.5770	2.18	0.93

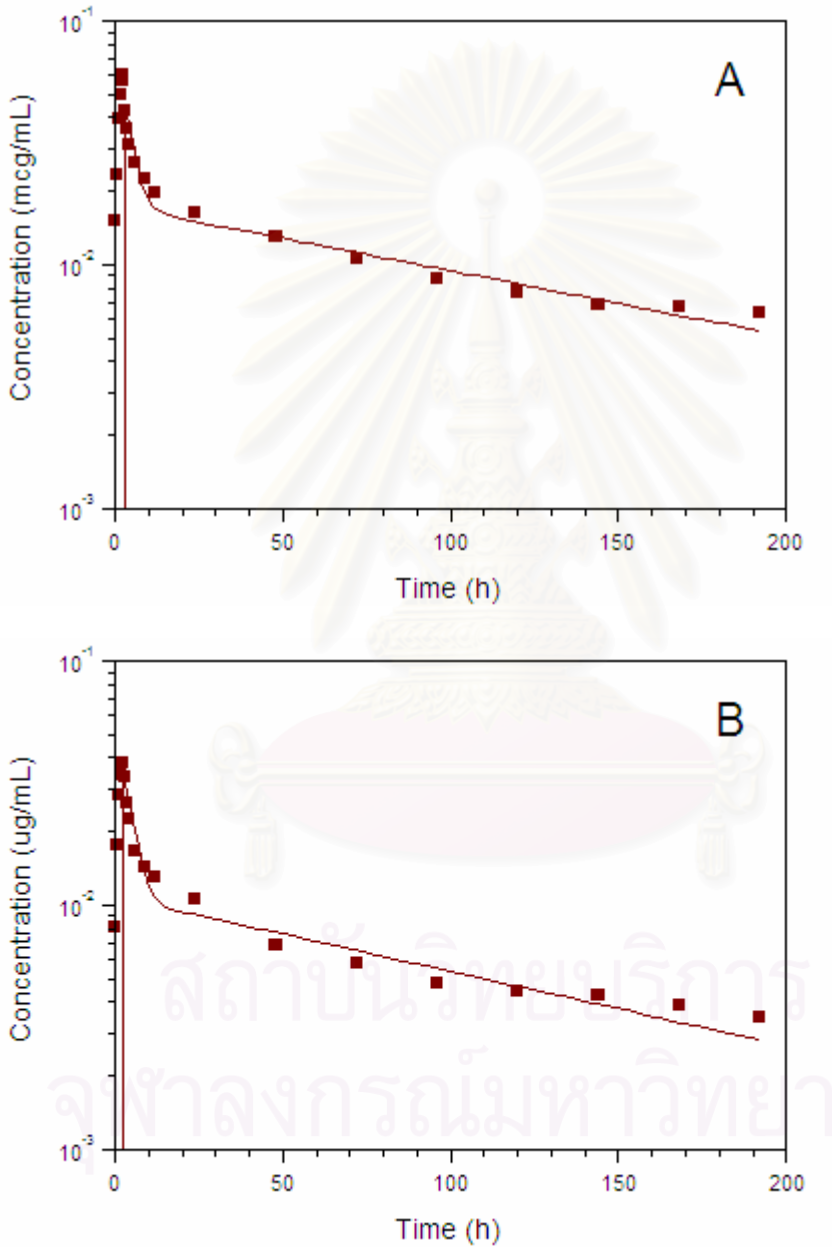


Figure 39 Curve fits with two-compartment model to plasma concentration-time profile (semi-logarithmic plots). A) Total plasma drug. B) Free drug. Symbols represent measured data and the fitted lines present predicted data.

D. Integrated PK/PD Approach

PK/PD Simulations and Evaluation of Antimicrobial Activity

The mathematic equations and parameters obtained from best fitting the time-kill curves in pharmacodynamic studies and from best fitting the concentration-time profile in pharmacokinetic studies are used in PK/PD simulation to describe and predict the antimicrobial effect. As results, the best PK/PD models of each bacterial strain were models 2, 4 and 9 for *S. pneumoniae* (both penicillin-sensitive and penicillin-intermediate), *M. catarrhalis*, *H. influenzae*, respectively. The PK parameters from Table 39 and PD parameters from Table 12 were used in the PK/PD simulations and summarized in Table 40. PK/PD simulations of azithromycin which combine PK profile from the free drug concentration and PD data from the time-kill curve are performed by using the program Scientist®. Examples of PK/PD simulation of azithromycin on day 3 for 3-day regimen are shown in Appendix A. PK profile simulation of free drug concentration on day 3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen is shown in Figure 40. PK/PD simulation of azithromycin against four bacterial strains on day 3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen are shown in Figure 41. For *S. pneumoniae* ATCC 6303, bacterial growth curve (solid line) showed line up and then constant due to the growth saturation from maximum number of bacteria with limitation of space and nutrients while kill curve (dash line) showed line down because killing effect with decrease number of bacteria occurred within 96 h. For *S. pneumoniae* ATCC 49619, bacterial growth curve (solid line) also showed line up and then constant due to the growth saturation from maximum number of bacteria with limitation of space and nutrients while kill curve (dash line) showed line down because more killing effect with decrease number of bacteria occurred within 24 h. For *M. catarrhalis* ATCC 8176, bacterial growth curve (solid line) showed line up due to without limitation of space and nutrients while kill curve (dash line) showed also line up due to effect of bacterial growth exhibition and without killing effect. For *H. influenzae* ATCC 10211, bacterial growth curve (solid line) and kill curve (dash line) showed line up and overlap due to no effect of bacterial growth exhibition and without killing effect.

In addition, PK/PD simulation of azithromycin against four bacterial strains on days 1-3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen could be performed by using Scientist®. Examples of PK/PD simulation of

azithromycin on days 1-3 for 3-day regimen are shown in Appendix A. PK profile simulation of free drug concentration on days 1-3 after once-daily after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen is shown in Figure 42. PK/PD simulation of azithromycin against four bacterial strains on days 1-3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen are shown in Figure 43. For *S. pneumoniae* ATCC 6303, bacterial growth curve (solid line) showed line up and then constant due to the growth saturation from maximum number of bacteria with limitation of space and nutrients while kill curve (dash line) showed line down with more killing effect as concentration fluctuate between dose. Therefore, decrease number of bacteria occurred within 36 h. For *S. pneumoniae* ATCC 49619, bacterial growth curve (solid line) showed also line up and then constant due to the growth saturation from maximum number of bacteria with limitation of space and nutrients while kill curve (dash line) showed line down with more killing effect as concentration fluctuate between dose. Therefore, decrease number of bacteria occurred rapidly within 24 h. For *M. catarrhalis* ATCC 8176, bacterial growth curve (solid line) showed line up with increase number of bacteria without limitation of space and nutrients while kill curve (dash line) show line up with effect of bacterial growth inhibition as concentration fluctuate between dose without killing effect. For *H. influenzae* ATCC 10211, although it was exposed to azithromycin concentrations on days 1-3, bacterial growth curve (solid line) and kill curve (dash line) showed still line up and overlap due to no effect of bacterial growth exhibition and without killing effect.

Table 40 Summary of mean of determined pharmacokinetic and pharmacodynamic parameters were used in the PK/PD simulations.

Parameters	*S. pneumoniae* ATCC 6303	*S. pneumoniae* ATCC 49619	*M. catarrhalis* ATCC 8176	*H. influenzae* ATCC 10211
Pharmacokinetic				
A(µg/mL)	377.01	377.01	377.01	377.01
B(µg/mL)	0.04	0.04	0.04	0.04
Alpha(h^{-1})	0.5762	0.5762	0.5762	0.5762
Beta(h^{-1})	0.0106	0.0106	0.0106	0.0106
k_a(h^{-1})	0.5770	0.5770	0.5770	0.5770
Pharmacodynamic	Model 4	Model 4	Model 2	Model 9
k_0(h^{-1})	1.36	1.21	1.63	1.18
k_{max}(h^{-1})	1.80	1.61	2.50	3.71
EC_{50} (µg/mL)	0.34	0.05	0.15	3.68
N_{max}(10^8CFU/mL)	1.52	3.86	-	-
z(h^{-1})	-	-	0.37	0.79
h	-	-	-	2.08

Figure 40 PK profile simulation of free plasma concentration of azithromycin on day 3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen, using mean PK parameters

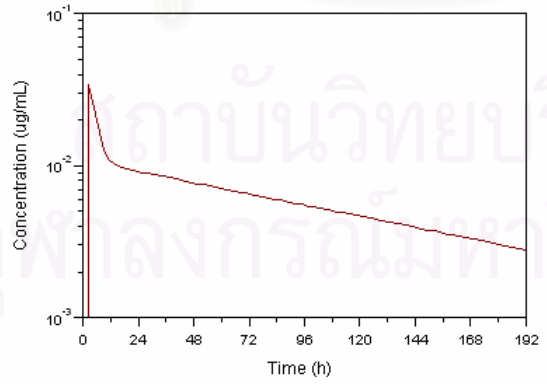


Figure 41 PK/PD simulations of azithromycin against four bacterial strains on day 3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen, using mean PK/PD parameters. Bacterial growth(——) and kill (– –)

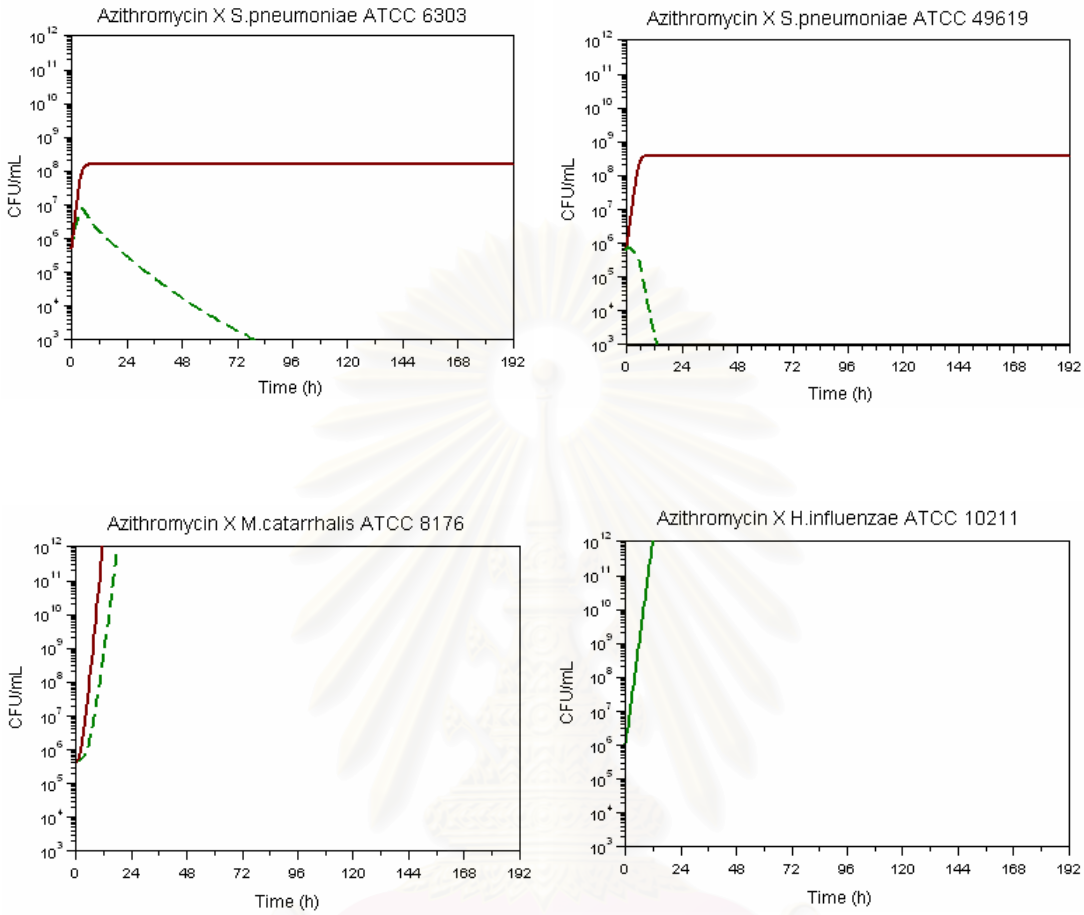


Figure 42 PK profile simulation of free plasma concentration of azithromycin on days 1-3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen, using mean PK parameters

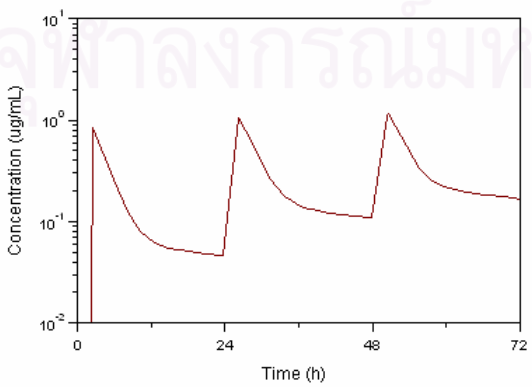
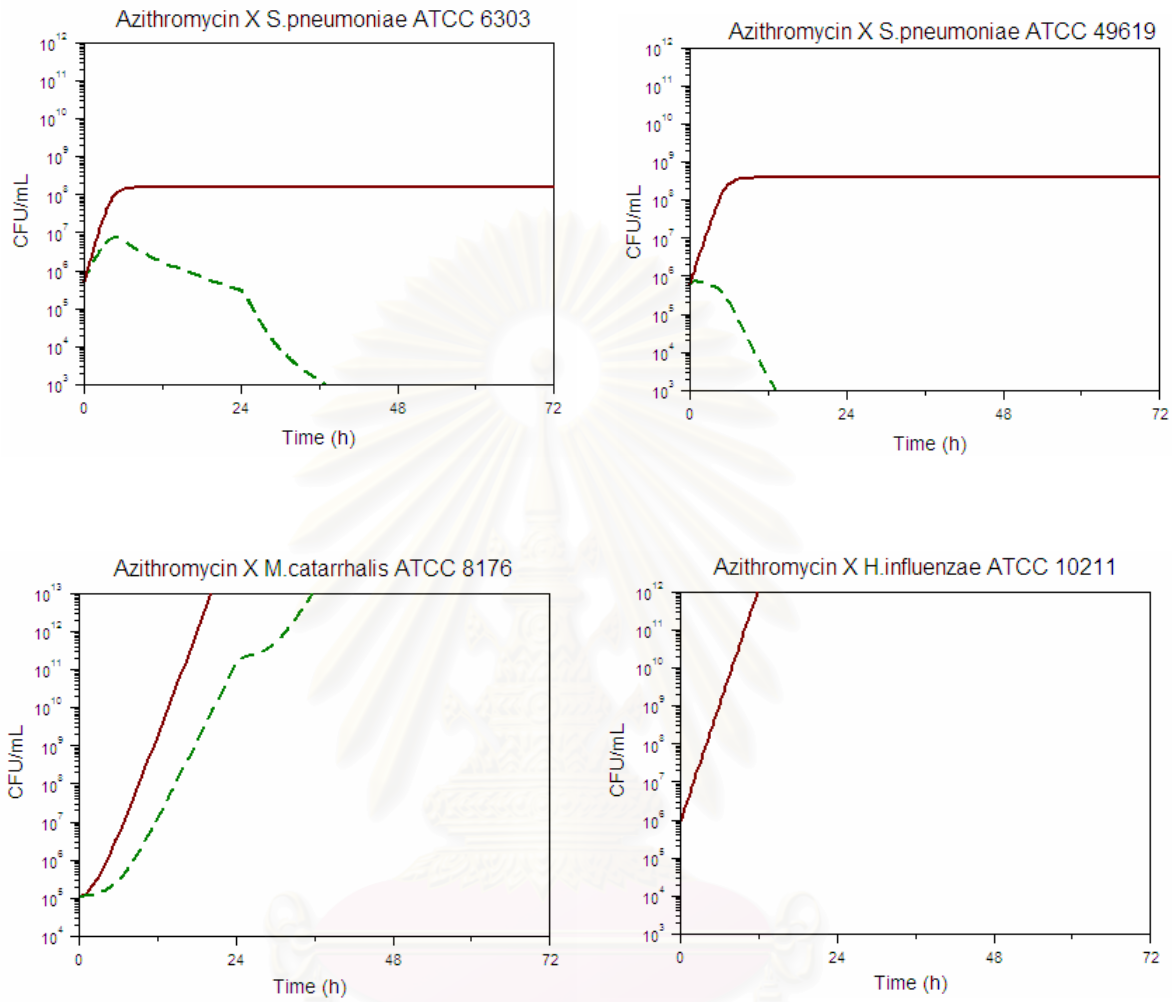


Figure 43 PK/PD simulations of azithromycin against four bacterial strains on days 1-3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen, using mean PK/PD parameters. Bacterial growth(——) and kill (– –)



Evaluation of azithromycin antimicrobial activity on day 3 and days 1-3 for 3-day regimen, for *S. pneumoniae* (both penicillin-sensitivity and penicillin-intermediate), a dose of 2x250 mg azithromycin showed a good bactericidal effect. For *H. influenzae* and *M. catarrhalis*, the same dose did not seem to be sufficient enough to decrease bacterial counts. For *H. influenzae*, the free azithromycin peak concentration was determined to be approximately 0.23 µg/mL, which is less than 1 x MIC (1 µg/mL). Therefore, free azithromycin plasma concentration do not show a bactericidal effect on *H. influenzae*. Although free azithromycin plasma concentrations do not reach MIC levels of *H. influenzae*, efficacy is shown in clinical data [125-128]. It may seem paradox that although serum concentrations are much lower than reported tissue concentrations [113,129,130], azithromycin is highly

effective against extracellular *H. influenzae*. The cellular location of *H. influenzae* in relation to tissue pharmacokinetic of azithromycin may explain this apparent paradox [7]. Nightingle [7] explained that *H. influenzae* is not an intracellular organism and therefore will not be exposed to high drug levels achieved in cellular tissue. *H. influenzae*, may reside adjacent to or attached to the exterior cell surface. As azithromycin is slowly released from within cell, it crosses the cell membrane, reaching a high concentration on the outside of membrane. Therefore, *H. influenzae* will be exposed to high azithromycin concentration. In addition, the slow release of azithromycin from macrophages, fibroblasts to sites of infection and inflammation also may explain its efficacy in infections caused by *H. influenzae*. It was a subject of controversy whether serum or tissue concentrations should be considered for antimicrobial efficacy [124,131]. Originally, it was suggested to use free serum concentrations as a predictor of extracellular fluid concentrations, as the main determinant of efficacy against extracellular pathogens [124]. However, host-defense mechanisms themselves might have been underestimated so far. Advanced-generation macrolides, and in particular azithromycin, are highly concentrated in polymorphonuclear leucocytes, which gravitate by chemotactic mechanisms to sites of infection. Following phagocytosis at the infection site, the bacteria are exposed to very high, and sometimes cidal, intracellular concentrations of the antibacterial agent [126,131]. Therefore, it seems to be more appropriate to account tissue concentrations for efficacy. Measurement of the free azithromycin concentration in the infected tissue fluid with microdialysis might help to clarify this question. Unfortunately, due to its high lipophilicity, azithromycin could not be measured by microdialysis (resulted from pilot study). Therefore, free azithromycin concentrations in plasma were used instead in this study.

For *M. catarrhalis*, saturation of the killing rate occurred at high concentrations ranged from 16xMIC to 128xMIC because the changing in slope of killing curves was very slight, indicating time-dependent killing effect (see Figure 14). This may explain that antimicrobial effect of azithromycin relates more to the exposure time of bacteria to azithromycin than to extent of drug concentration.

Results from this study showed that the developed PK/PD models which combine *in vivo* PK data from the free drug concentration versus time profile and *in vitro* PD from the time-kill curve could be used to predict the antimicrobial effect of azithromycin in the treatment of infections caused by four common bacterial strains.

The same PK/PD models could be used to azithromycin pharmacokinetic data from other clinical pharmacokinetic study. Simulation based on clinical pharmacokinetic study can predict clinical outcome and help come up with dose recommendations.

In addition, results from this study may be applied in clinical practice. When the dose of azithromycin does not enough to show killing effect because the extent of azithromycin concentration is low, the dose which produces an appropriate concentration can be estimated to pharmacokinetic model which used in PK/PD simulations.

Therefore, pharmacokinetic/pharmacodynamic approach based on time-kill curve could be a suitable method to evaluate the antimicrobial activity of azithromycin.

CHAPTER V

CONCLUSIONS

The efficacy of azithromycin in the treatment of infections caused by the common bacteria, using a PK/PD approach which combines *in vivo* PK data from the free drug concentration versus time profile and *in vitro* PD from the time-kill curve was established. The results were concluded as follows :

1. The MIC of azithromycin was 0.06, 0.008 and 1 μg/mL for *S. pneumoniae* (both penicillin-sensitive and penicillin intermediate), *M. catarrhalis* and *H. influenzae*, respectively. The results of the selected azithromycin concentrations tested in each bacterial strain based on their determined MIC in the time-kill curve experiments indicated that seven concentrations were enough to cover the entire range including minimum inhibition of bacterial growth (0.25xMIC, 0.5xMIC, 1xMIC), efficient bacterial killing (2xMIC, 4xMIC) and maximum bacterial killing (8xMIC, 16xMIC) for *S. pneumoniae* both penicillin-sensitive and penicillin-intermediate whereas *M. catarrhalis* and *H. influenzae* needed concentrations more than 16xMIC to reach the maximum bacterial killing effect.

2. The bacterial time-kill curves of azithromycin against four bacterial strains were determined in *in vitro* infection model. The results could provide the patterns of bacterial killing rate of azithromycin that it is characterized by the time-dependency and concentration-dependency of in vitro antimicrobial activity. This confirms that the time-kill curve experiment could provide more detailed information about the pharmacodynamic potency than traditional MIC values.

3. Results from curve fits of bacterial time-kill curves with twelve PK/PD models, the models 4, 2 and 9 were the most suitable PK/PD models for the best fitting the time-kill curve data for *S. pneumoniae* (both penicillin-sensitive and penicillin intermediate), *M. catarrhalis* and *H. influenzae*, respectively. Results of PK/PD model analysis, for both *S. pneumoniae* penicillin-sensitive (ATCC 6303) and penicillin-intermediate (ATCC 49619), the data were best explained by PK/PD model 4 that incorporates an N_{max} term. Model 2 that incorporates an adaptation rate constant was found appropriate to describe the data for *M. catarrhalis*. For *H. influenzae*, model 3 could not explain the data well. Model 9 that incorporates additional Hill (h) factor displayed the better fit. The results indicated that a simple PK/PD model

(model 1) was not sufficient to describe the observed pharmacokinetic effects for four bacterial strains. It is necessary to add some additional terms into the model. For both penicillin-sensitive and penicillin-intermediate *S. pneumoniae*, the limitation of space and nutrients (N_{max}) had an effect on the growth rate. Therefore, the addition of a saturation term into the simple model appeared to be necessary. The growth of *M. catarrhalis* was delayed and not in the logarithmic growth phase at time zero. Therefore, an adaptation rate constant ($1-e^{-zt}$) was necessary to account for these effects. For *H. influenzae*, the maximum kill rate showed delay while the bacterial growth did not. Therefore, an adaptation term was used only for the bacterial killing effect.

4. Results from the curve fit of bacterial time-kill curves could provide at least three PD parameters of azithromycin : the maximum kill rate constant (k_{max}), the concentration at half-maximum effect (EC_{50}), and the growth rate constant of the bacteria (k_0). These parameters were obtained by modeling the kill curves with the most suitable PK/PD models. Results show that azithromycin showed high efficacy against *S. pneumoniae* strains (EC_{50} /ATCC 6303: 0.34 µg/mL; EC_{50} /ATCC 49619: 0.05 µg/mL) and *M. catarrhalis* (EC_{50}:0.15 µg/mL) but low efficacy against *H. influenzae* (EC_{50}:3.68 µg/mL).

5. The analytical method for determination of azithromycin in plasma and in ultrafiltrate samples by liquid chromatographic masspectrometer (LC-MS) has been developed and validated. Azithromycin and clarithromycin (internal standard) were extracted with tert-Butyl methyl ether followed by centrifugation. Supernatant was then separated and evaporated in a vacuum centrifuge. Residue was reconstituted with acetonitrile. Aliquot of solution was analyzed by the LC-MS. The preparation of plasma ultrafiltrate samples which represents free drug concentration for analysis by ultrafiltration method, human plasma was added into the upper part of the centrifugal filter device that fit on the top of centrifuge vials and centrifuged to distinguish the protein unbound fraction from the total fraction of drug. The filtered ultrafiltrate was mixed with clarithromycin and diluted with acetonitrile. Aliquot of solution was injected into the LC-MS. The analysis was performed on Inersil ODS-3, C_{18} column, using acetonitrile and 0.02 M ammonium acetate as mobile phase with flow rate 0.2 mL/min based as the gradient system, column oven was set at 40 ^{o}C, CDL temperature was 250 ^{o}C, block temperature was 200 ^{o}C, nebulizer gas flow with the rate of 1.5 L/min, detector gain at 1.7 KV and m/z for azithromycin = 749.45, for

Clarithromycin = 748.40. Azithromycin and clarithromycin were clearly separated with retention times approximately 5 and 7 min, respectively. The validation of these analytical methods conformed the acceptable criteria.

6. Eight male subjects could participate throughout this study. No subjects withdrew from the experiment. No adverse effects occurred due to azithromycin. Results of determination of protein binding were found that percent protein binding of azithromycin was averaged to be 47.08% which was consistent with the reported value.

7. Results from non-compartmental PK analysis revealed that the mean values of C_{max}, AUC_{last}, AUC_{inf}, and AUMC were decreased approximately by 50% from 525.94 to 292.21 ng/mL, 7873.84 to 3755.02 h.ng/mL, 9636.96 to 4572.99 h.ng/mL, and 447865.88 to 194602.11 h.h.ng/mL for total plasma drug and free drug, respectively. These results are consistent with average protein binding of azithromycin (47.08%) showed effect of protein binding on these parameters. Therefore, the free drug concentration should be used instead of the total plasma concentration to evaluate antimicrobial activity. For compartmental PK analysis, the two-compartment model was the most suitable pharmacokinetic model to describe the PK profile of both total plasma and free drug concentrations very well.

8. PK/PD simulations of azithromycin which combines PK profile from the free drug concentration and PD data from the time-kill curve could provide the expected kill curve for 2x250 mg azithromycin casules orally once-daily as treatment schedule (3-day regimen). The results from evaluation of antimicrobial activity, for *S. pneumoniae* (both penicillin-sensitivity and penicillin-intermediate), a dose of 2x250 mg azithromycin showed a good bactericidal effect. For *H. influenzae* and *M. catarrhalis*, the same dose did not seem to be sufficient enough to decrease bacterial counts.

9. In this study, the developed PK/PD model which combines *in vivo* PK data from the free drug concentration-time profile and *in vitro* PD from the time-kill curve could be used to predict the antimicrobial effect of azithromycin in the treatment of infections caused by four common bacterial strains. Simulation based on clinical pharmacokinetic study can predict clinical outcome and help come up with dose recommendations. Therefore, pharmacokinetic/pharmacodynamic approach based on time-kill curve could be a suitable method to evaluate the antimicrobial activity of azithromycin.

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APPENDICES

APPENDIX A

Examples of Determination of Pharmadynamic Parameters and Curve Fit

Scientist is designed to provide a comprehensive solution to the problem of fitting experimental data under Microsoft Windows. It includes the capability of solving systems of model equations that can include nonlinear equations, ordinary differential equations and Laplace transforms. Scientist is commonly used in PK/PD modeling analysis. The following examples were the determination of pharmacodynamic parameters and curve fit with model 1 for *S. pneumoniae* ATCC 6303 by using Scientist.

Example of determination of k_0

The process performed through these steps.

Step 1. The equation to be fit was constructed in a model file. The model file is shown in a model window (Figure 44).

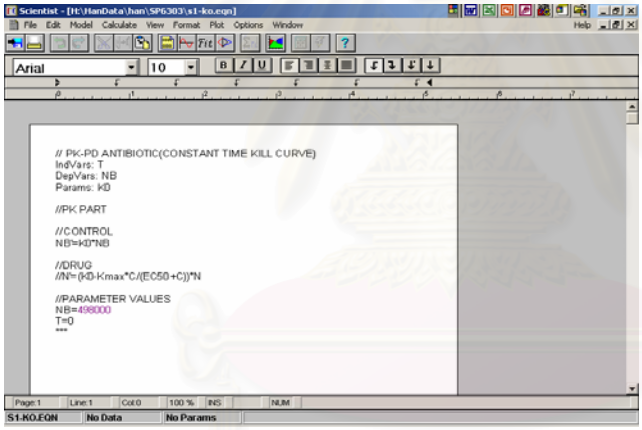


Figure 44 The model file for determination of k_0

Step 2. Data input was positive control data entered into a spreadsheet window (Figure 45).

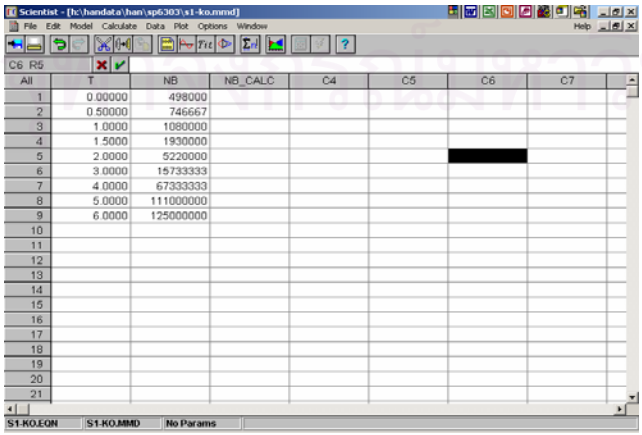


Figure 45 The spreadsheet window for determination of k_0

Step 3. To perform fitting, it is necessary to enter initial estimate of k_0 (default estimate of 0.0) into a parameter window (Figure 46).

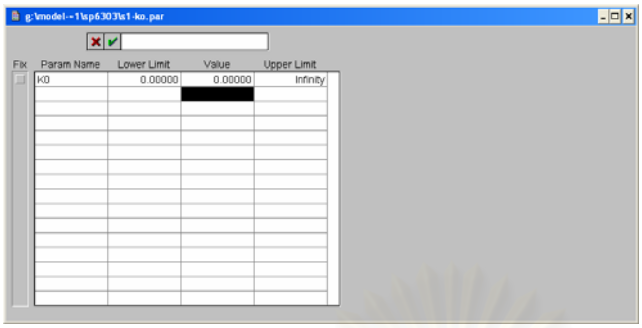


Figure 46 The parameter window for initial estimate of k_0

Step 4. After fit command was done, the final parameter value (k_0=0.94809) from the fit is displayed in the parameter window (Figure 47).

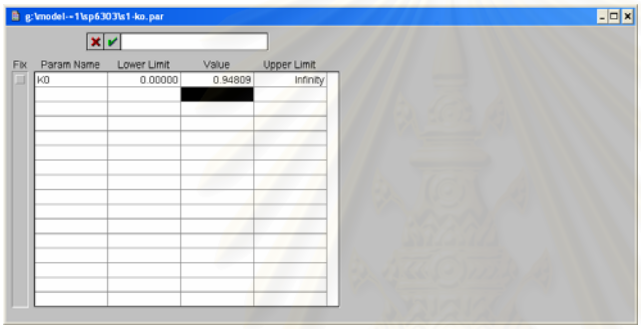


Figure 47 The parameter window for final parameter of k_0

Example of determination of k_{max}

The process performed through these steps.

Step 1. The equation to be fit is constructed in model file. The model file is displayed in a model window (Figure 48).

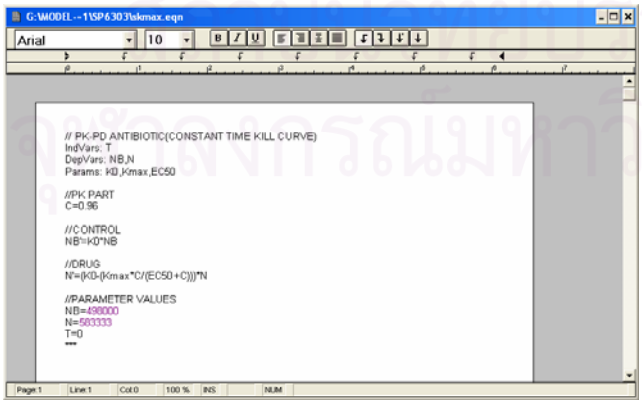
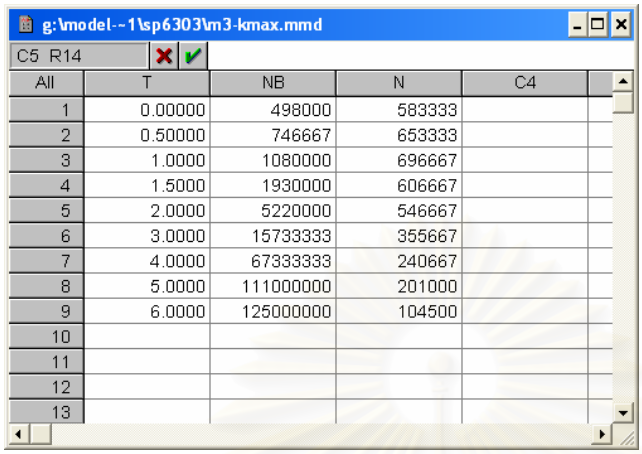


Figure 48 The model file for determination of k_{max}

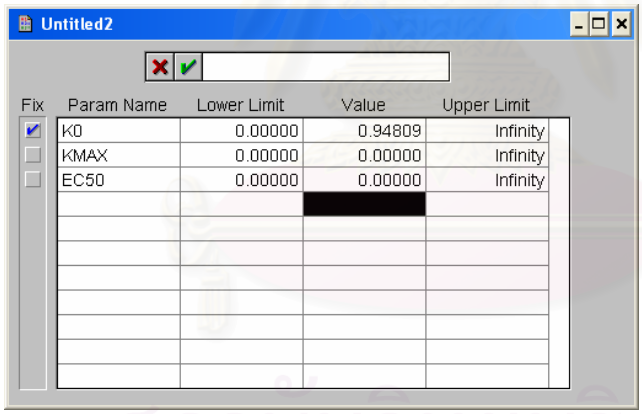
Step 2. Data input was positive control data and killing data from the highest azithromycin concentration (0.96 µg/mL) entered into a spreadsheet window (Figure 49).



All	T	NB	N	C4
1	0.00000	498000	583333	
2	0.50000	746667	653333	
3	1.0000	1080000	696667	
4	1.5000	1930000	606667	
5	2.0000	5220000	546667	
6	3.0000	15733333	355667	
7	4.0000	67333333	240667	
8	5.0000	111000000	201000	
9	6.0000	125000000	104500	
10				
11				
12				
13				

Figure 49 The spreadsheet window for determination of k_{max}

Step 3. To perform fitting, it is necessary to enter initial estimate of k_{max} (default estimate of 0.0) and initial estimate of k_0 (determined value of 0.94809) into parameter window (Figure 50).



Fix	Param Name	Lower Limit	Value	Upper Limit
☑	K0	0.00000	0.94809	Infinity
☐	KMAX	0.00000	0.00000	Infinity
☐	EC50	0.00000	0.00000	Infinity

Figure 50 The parameter window for initial estimate parameter

Step 4. After fit command was done, the final parameter values from the fit were k_0=0.94809, k_{max}=1.4794 and EC_{50}=0.31972 as shown in a parameter window (Figure 51).

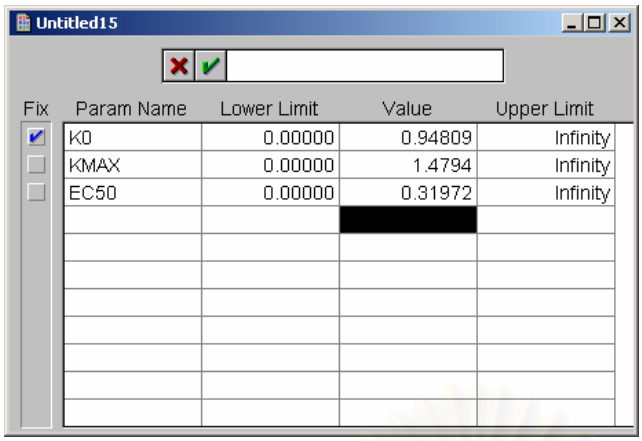


Figure 51 The parameter window for final parameter of k_{max}

Example of determination of EC_{50}

The process performed through these steps.

Step 1. The equation to be fit was constructed in model file. The model file is shown in a model window (Figure 52).

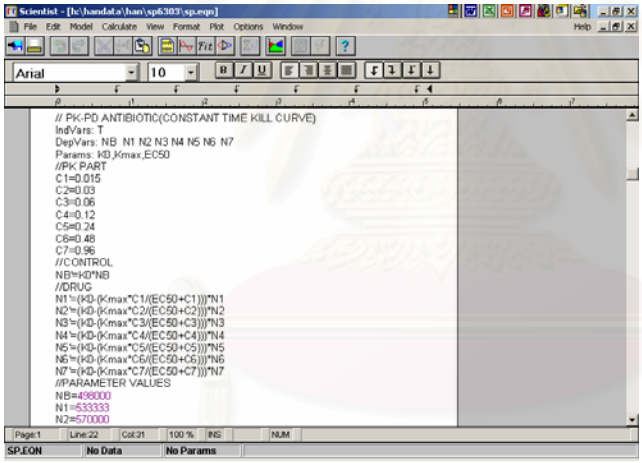


Figure 52 The model file for determination of ED_{50}

Step 2. Data input was positive control data and data from all azithromycin concentrations (0.015, 0.03, 0.06, 0.12, 0.48, and 0.96 μg/mL) entered into spreadsheet window (Figure 53).

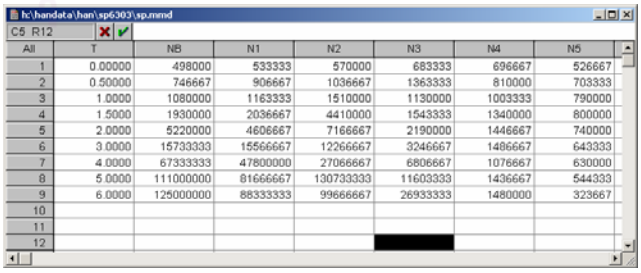


Figure 53 The spreadsheet window for determination of ED_{50}

Step 3. To perform fitting, it is necessary to enter initial estimate k_0 (determined value of 0.94809), initial estimate of k_{max} (determined value of 1.4794) and initial estimate of EC_{50} (determined value of 0.31972) into a parameter window (Figure 54).

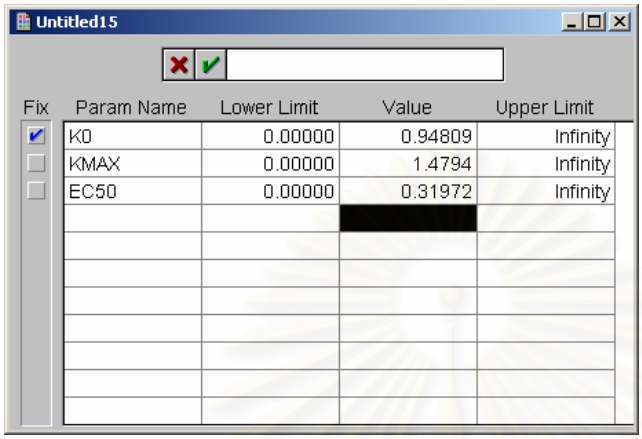


Figure 54 The parameter window for initial estimate of k_0, k_{max} , EC_{50}

Step 4. After fit command was done, the final parameter values from the fit were k_0=0.94800, k_{max}=1.3365 and EC_{50}=0.19254 as shown in the parameter window (Figure 55).

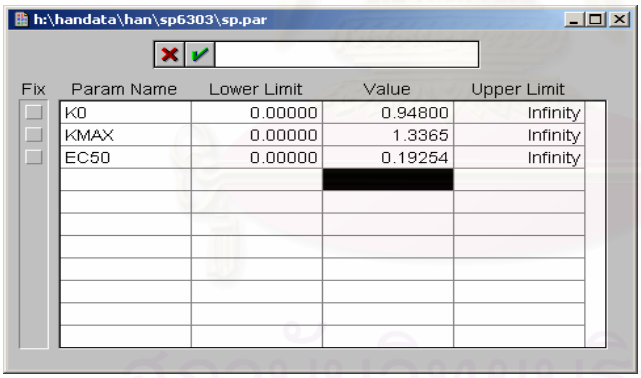


Figure 55 The parameter window for final parameter of k_0, k_{max} , EC_{50}

Example of graphic and statistic output

Scientist provides a graphic output and a broad range of statistics output, including parameter estimates, confidence limits, various measures of goodness of fit, variance-covariance and correlation information, and analysis of residuals. The goodness of fit statistic as well as visual inspection are criteria to determine which model performed best fit.

After graph and statistic command were done, graphic output and the goodness of fit statistic output obtained from curve fit with model 1 are shown in Figures 56 and 57, respectively.

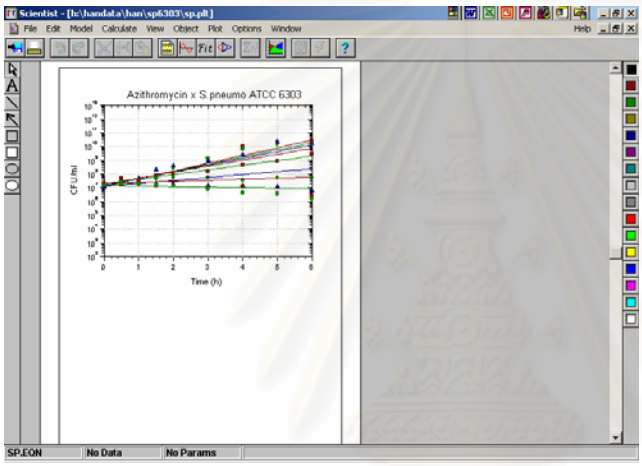


Figure 56 Graphic output of curve fit with model 1

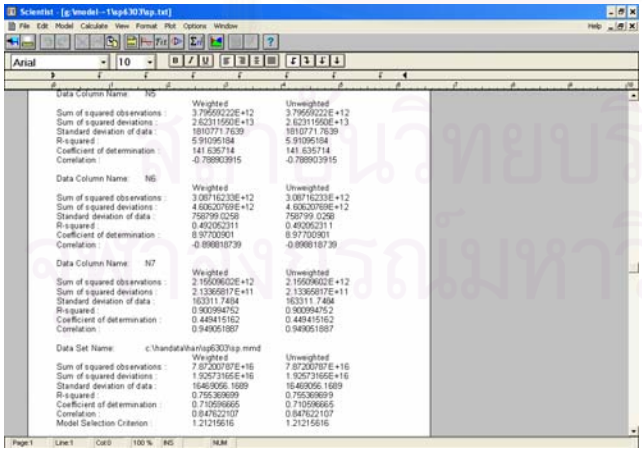


Figure 57 Goodness of fit statistic output with model 1

Examples of Curve Fit with Two Compartment Model

The following examples were curve fit to concentration-time profile of free drug concentration with two compartment model by using Scientist. The process of curve fit performed through these steps.

Step 1. The equation to be fit was constructed in a model file. The model file is shown in a model window (Figure 58).

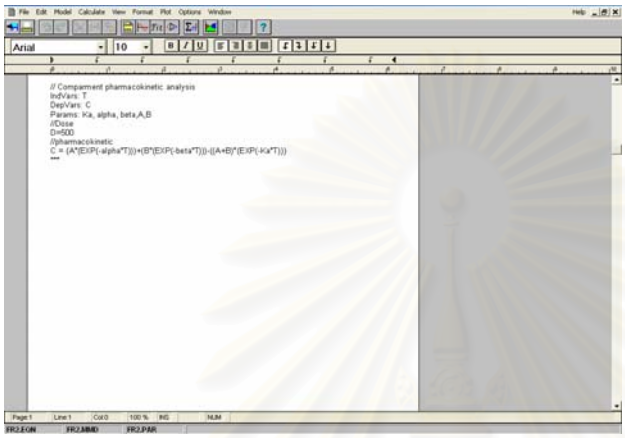


Figure 58 The model file for curve fit

Step 2. Data input was concentration-time profile of free drug concentration entered into a spreadsheet window (Figure 59).

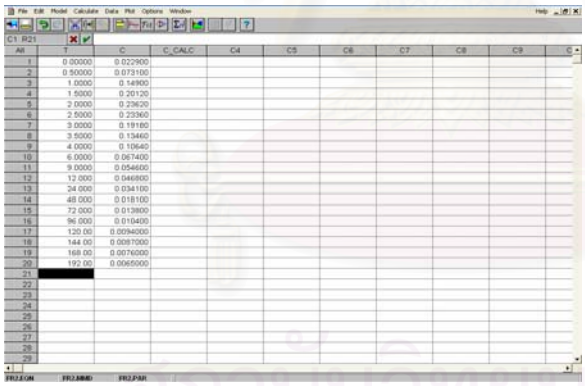


Figure 59 The spreadsheet window for curve fit

Step 3. To perform fitting, it is necessary to enter initial estimate of k_a, Alpha, Beta, A and B (default estimate of 0.0) into a parameter window (Figure 60).

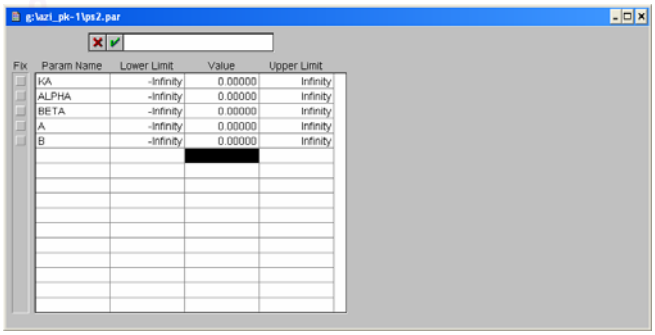


Figure 60 The parameter window for curve fit

Step 4. After fit command was done, the final parameter values (k_a= 0.5770, Alpha=0.5762, Beta=0.0106, A=377.01 and B=0.04) from the fit is displayed in the parameter window (Figure 61).

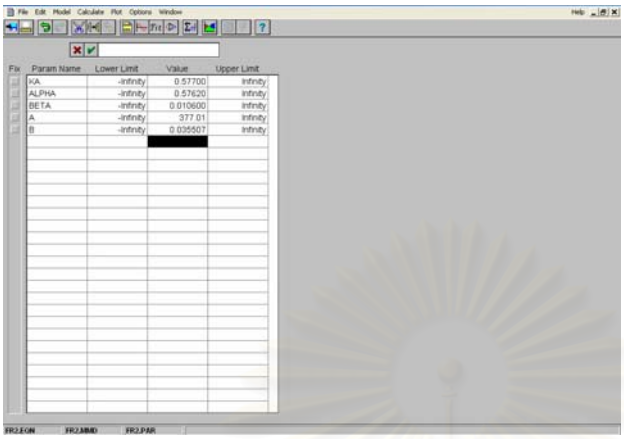


Figure 61 The parameter window for final parameter values

Result of graphic and statistic output

After fitting curve, the graphic output and the goodness of fit statistic output from curve fit are shown in Figures 62 and 63.

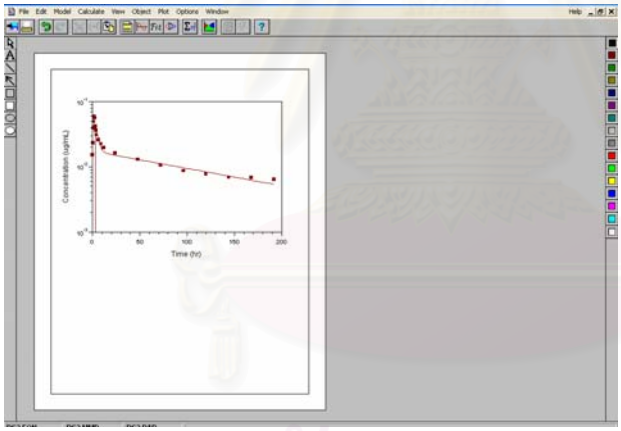


Figure 62 Graphic output of curve fit with two-compartment model

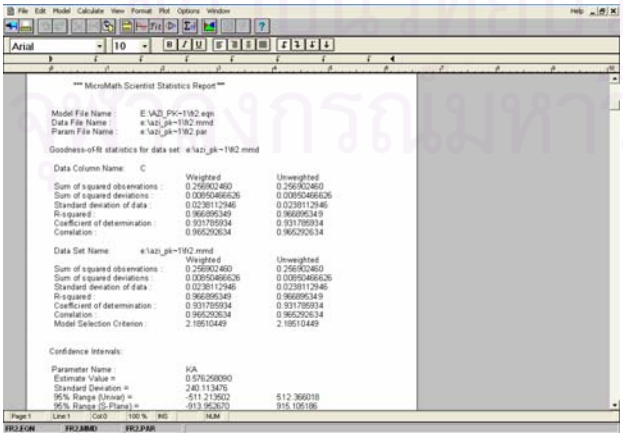


Figure 63 Goodness of fit statistic output with two-compartment model

Examples of PK/PD Simulations

Example of PK/PD simulation of azithromycin on day 3

PK/PD simulation of azithromycin against *S. pneumoniae* ATCC 6303 on day 3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen, using mean PK/PD parameters was performed by using Scientist. The process of simulation performed through these steps.

Step 1. The equations from fitting the time-kill curves (model 4) and from fitting the concentration-time profile (two-compartment model) to be simulated were constructed in a model file. The model file is shown in a model window (Figure 64).

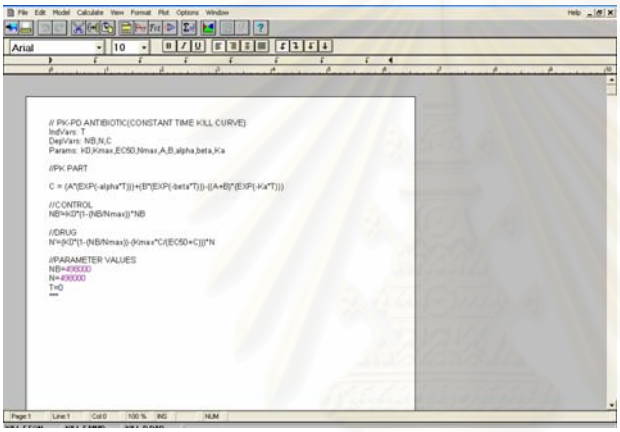
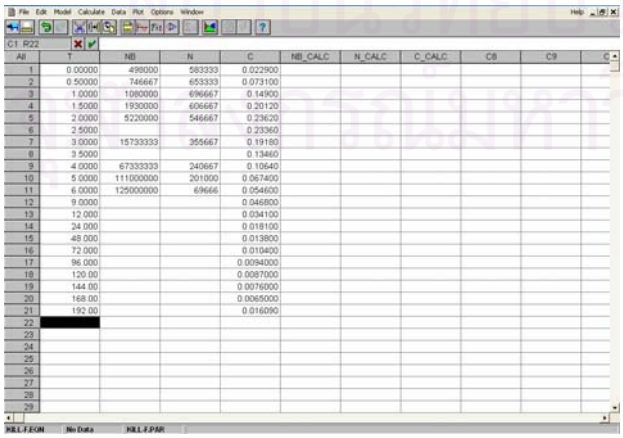


Figure 64 The model file for PK/PD simulation on day 3

Step 2. Data input were combined with concentration-time profile of mean free drug concentration from pharmacokinetic study and mean of time-kill curve data (positive control and kill curve data at azithromycin concentration 0.96 μg/mL) from pharmacodynamic study entered into an initial spreadsheet window (Figure 65).



All	T	NB	N	C	NB_CALC	N_CALC	C_CALC	C8	C9	C
1	0.00000	498000	583333	0.022900						
2	0.50000	746667	653333	0.073100						
3	1.0000	1080000	696667	0.14900						
4	1.5000	1930000	606667	0.20120						
5	2.0000	5220000	546667	0.23620						
6	2.5000			0.23360						
7	3.0000	15733333	355667	0.19180						
8	3.5000			0.13460						
9	4.0000	67333333	240667	0.10640						
10	5.0000	111000000	201000	0.067400						
11	6.0000	125000000	69666	0.054600						
12	9.0000			0.046800						
13	12.000			0.034100						
14	24.000			0.018100						
15	48.000			0.013800						
16	72.000			0.010400						
17	96.000			0.0094000						
18	120.00			0.0087000						
19	144.00			0.0076000						
20	168.00			0.0065000						
21	192.00			0.016090						
22										
23										
24										
25										
26										
27										
28										
29										

Figure 65 The initial spreadsheet window for PK/PD simulation on day 3

Step 3. To perform PK/PD simulation, the parameter values from the resulting fits were k_0=1.36, k_{max}=1.80, EC_{50}=0.34, N_{max}=1.52, k_a=0.5770, Alpha=0.5762, Beta=0.0106, A=377.01 and B=0.04 are entered into a parameter window (Figure 66).

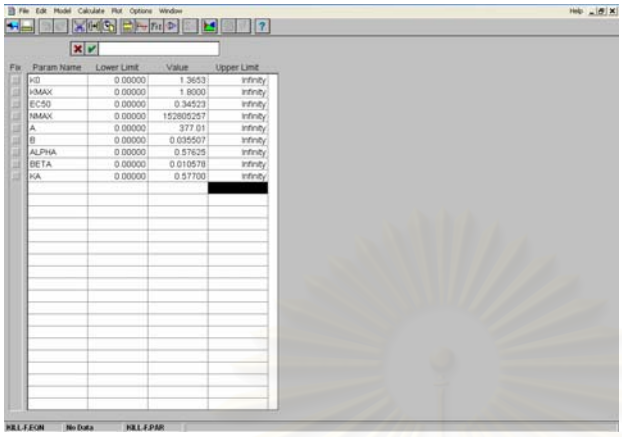


Figure 66 The parameter window for PK/PD simulation on day 3

Step 4. After simulation command was done, the calculated values obtained from simulation is displayed in a final spreadsheet window (Figure 67).

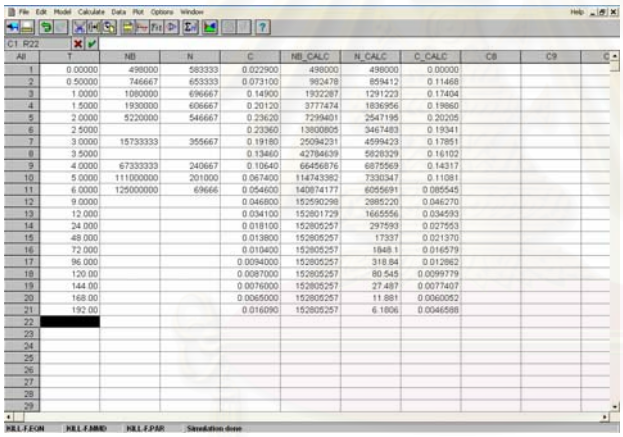


Figure 67 The final parameter window for PK/PD simulation on day 3

Step 5. After PK/PD simulation, the graphic output showed pharmacokinetic profile simulation (Figure 68) and PK/PD simulation (Figure 69).

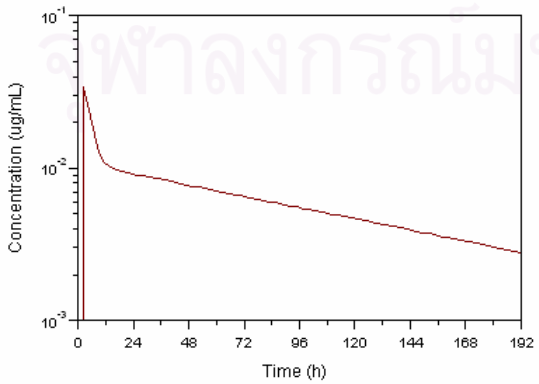


Figure 68 Graphic output of pharmacokinetic simulation on day 3

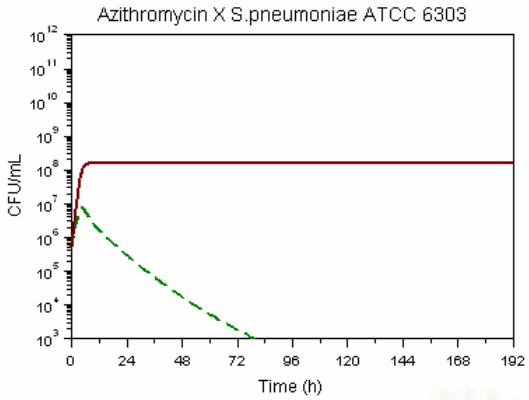


Figure 69 Graphic output of PK/PD simulation on day 3

Example of PK/PD simulation of azithromycin on days 1-3

PK/PD simulation of azithromycin against *S. pneumoniae* ATCC 6303 on days 1-3 after once-daily oral administration of 2x250 mg azithromycin for 3-day regimen, using mean PK/PD parameters was performed by using Scientist. The process of simulation performed through these steps.

Step 1. The equations from fitting the time-kill curves (model 4) and the modified equations for the concentration-time profile on days 1-3 to be simulated were constructed in a model file. The model file is shown in a model window (Figure 70).

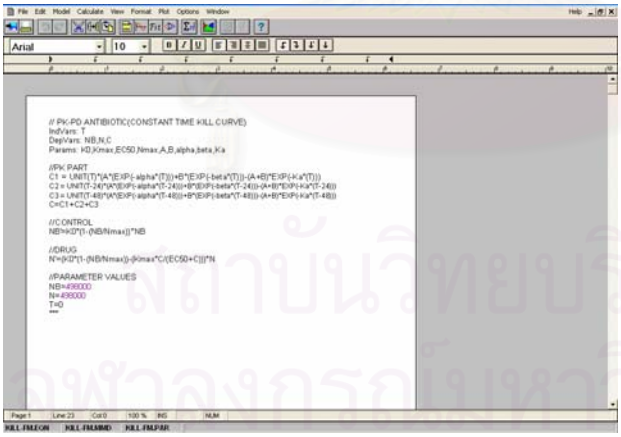


Figure 70 The model file for PK/PD simulation on days 1-3

Step 2. Data input was combined with concentration-time profile of mean free drug concentration from pharmacokinetic study and mean of time-kill curve data (positive control and kill curve data at azithromycin concentration 0.96 μg/mL) from pharmacodynamic study entered into an initial spreadsheet window (Figure 71).

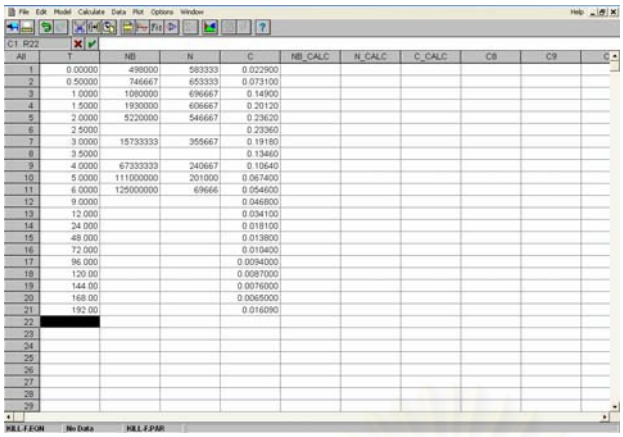


Figure 71 The initial spreadsheet window for PK/PD simulation on days 1-3

Step 3. To perform PK/PD simulation, the parameter values from the resulting fits were k_0=1.36, k_{max}=1.80, EC_{50}=0.34, N_{max}=1.52, k_a=0.5770, Alpha=0.5762, Beta=0.0106, A=377.01 and B=0.04 were entered into a parameter window (Figure 72).

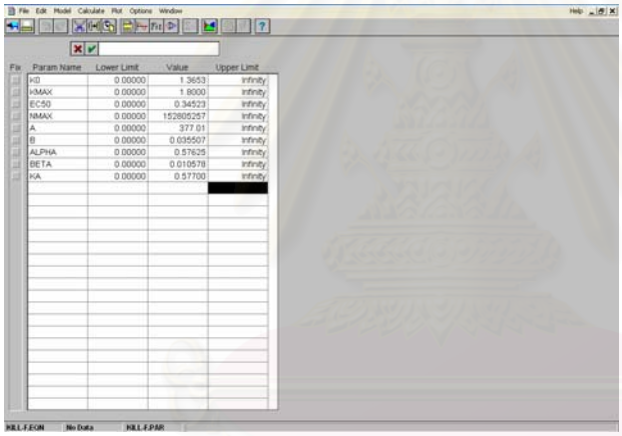


Figure 72 The parameter window for PK/PD simulation on days 1-3

Step 4. After simulation command was done, the calculated values obtained from simulation is displayed in a final spreadsheet window (Figure 73).

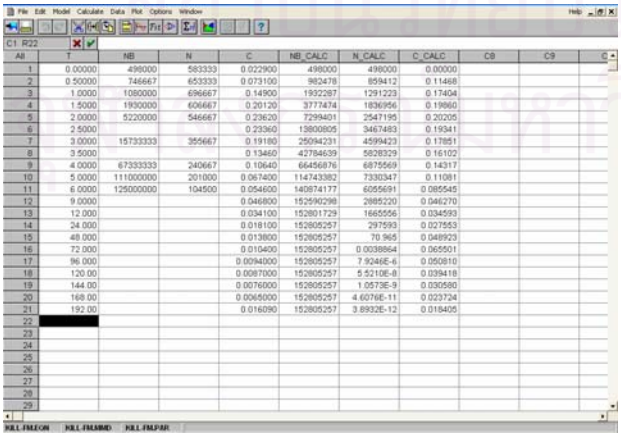


Figure 73 The final parameter window for PK/PD simulation on days 1-3

Step 5. After PK/PD simulation, the graphic output showed pharmacokinetic profile simulation (Figure 74) and PK/PD simulation (Figure 75).

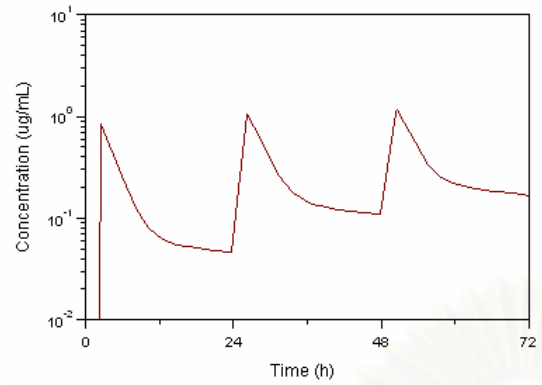


Figure 74 Graphic output of pharmacokinetic simulation on days 1-3

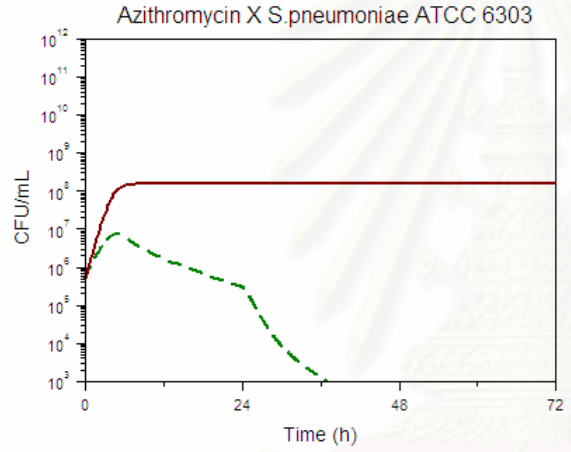


Figure 75 Graphic output of PK/PD simulation on days 1-3

APPENDIX B

Table 41 Demographic data of subjects participated in this study

Subject no.	Age(year)	Weight(kg)	Height(m)	BMI[a](kg/m^2)
1	37	80	1.75	26.12
2	29	60	1.65	22.04
3	36	63	1.67	22.59
4	41	68	1.77	21.70
5	21	76	1.74	25.10
6	23	71	1.84	20.97
7	25	63	1.72	21.29
8	29	50	1.61	19.29
Mean	29.80	65.60	1.74	21.39
S.D.	9.44	9.91	0.08	2.23
% CV	31.69	15.11	4.83	9.96

[a] BMI (Body Mass Index) = Weight(kg) / Height^2(m^2)

Table 42 Haematologic and blood/urine biochemical test of subjects participated in this study

Laboratory test	Normal range	Subject no.							
		1	2	3	4	5	6	7	8
Glucose	70-110 mg/dL	100	90	91	89	75	73	86	84
BUN	10-20 mg/dL	13	12	16	11	15	15	10	10
Creatinine	0.5-2 mg/dL	1.2	0.9	1.0	0.9	1.2	0.9	0.8	0.8
SGOT	0-38 U/L	23	14	24	19	13	17	11	17
SGPT	0-38 U/L	24	7	29	18	12	20	11	14
AP[a]	39-117 U/L	77	34	49	49	52	53	58	60
Hb	13-18 g/dL	15.1	14.3	16.7	14.3	13.7	13.7	12.1	15.8
Hct	40-54 %	43.1	41.8	46.8	41.2	44.3	40.8	39.5	47.4
HIV antigen	Neg[b]	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
HbsAg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Urine analysis									
- pH	5-8.5	6.0	6.0	6.0	6.0	6.0	6.0	6.0	5.0
- Alb	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
- Sugar	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
- RBC	0-2 Cells/HPF	-	-	-	0-1	-	0-1	-	0-1
- WBC	0-2 Cells/HPF	-	-	1-2	2-3	0-1	1-2	-	1-2

[a] AP = Alkaline phosphatase

[b] Neg = Negative

Table 43 History and monitoring of subjects participated in this study

Item	Subject no.							
	1	2	3	4	5	6	7	8
History								
Allergy macrolide	NH[a]	NH	NH	NH	NH	NH	NH	NH
Blood pressure	N[b]	N	N	N	N	N	N	N
Pulse rate	N	N	N	N	N	N	N	N
Adverse drug reaction								
Nausea	NS[c]	NS	NS	NS	NS	NS	NS	NS
Anorexia	NS	NS	NS	NS	NS	NS	NS	NS
Vomitting	NS	NS	NS	NS	NS	NS	NS	NS
Diarrhea	NS	NS	NS	NS	NS	NS	NS	NS
Constipation	NS	NS	NS	NS	NS	NS	NS	NS
Rush	NS	NS	NS	NS	NS	NS	NS	NS
Prutitus	NS	NS	NS	NS	NS	NS	NS	NS
Jaundice	NS	NS	NS	NS	NS	NS	NS	NS
Headache	NS	NS	NS	NS	NS	NS	NS	NS
Others	NS	NS	NS	NS	NS	NS	NS	NS

[a] NH = No history

[b] N = Normal

[c] NS = No symptom

APPENDIX C

Table 44 Percent protein binding of subject no.1

Time (h)	Free Conc. (ng/mL)	Total Conc. (ng/mL)	% Protein Binding[a]
0	11.27	57.49	80.40
0.5	41.64	94.35	55.87
1	69.26	146.77	52.81
1.5	100.53	165.43	39.23
2	160.51	452.72	64.55
2.5	211.50	511.48	58.65
3	138.86	254.69	45.48
3.5	95.18	195.97	51.43
4	65.28	117.03	44.22
6	45.89	88.76	48.30
9	28.78	79.27	63.69
12	24.30	45.64	46.76
24	15.69	33.66	53.39
48	12.31	32.48	62.10
72	9.91	21.27	53.42
96	8.81	24.57	64.14
120	6.80	15.46	56.03
144	6.01	14.86	59.57
168	5.24	13.73	61.84
192	5.86	13.86	57.73

[a] % Protein binding = (Total Conc.-Free Conc.) / Total Conc. X 100

Table 45 Percent protein binding of subject no.2

Time (h)	Free Conc. (ng/mL)	Total Conc. (ng/mL)	% Protein Binding[a]
0	25.07	46.74	46.36
0.5	183.41	215.40	14.85
1	350.50	398.92	12.14
1.5	262.39	354.84	26.05
2	279.49	314.46	11.12
2.5	184.79	206.62	10.57
3	231.02	240.14	3.80
3.5	136.25	181.30	24.85
4	100.72	129.60	22.28
6	79.95	116.79	31.54
9	32.80	120.54	72.79
12	36.36	69.42	47.63
24	28.47	62.10	54.15
48	14.58	41.38	64.78
72	12.74	30.11	57.67
96	10.13	25.42	60.17
120	9.02	15.24	40.85
144	9.89	15.84	37.53
168	7.95	14.54	45.33
192	5.37	11.60	53.66

[a] % Protein binding = (Total Conc.-Free Conc.) / Total Conc. X 100

Table 46 Percent protein binding of subject no.3

Time (h)	Free Conc. (ng/mL)	Total Conc. (ng/mL)	% Protein Binding[a]
0	30.41	43.81	30.57
0.5	100.48	108.58	7.46
1	133.04	153.55	13.36
1.5	145.32	214.92	32.38
2	375.24	375.86	0.17
2.5	338.13	372.77	9.29
3	165.61	205.72	19.50
3.5	170.86	217.45	21.43
4	111.64	143.54	22.22
6	51.93	71.38	27.25
9	43.64	82.73	47.26
12	56.21	61.94	9.26
24	33.43	44.14	24.27
48	17.94	33.17	45.91
72	13.23	31.24	57.67
96	11.77	19.46	39.54
120	11.31	19.35	41.57
144	10.62	14.32	25.86
168	7.60	15.53	51.04
192	6.33	15.72	59.72

[a] % Protein binding = (Total Conc.-Free Conc.) / Total Conc. X 100

Table 47 Percent protein binding of subject no.4

Time (h)	Free Conc. (ng/mL)	Total Conc. (ng/mL)	% Protein Binding[a]
0	30.89	73.79	58.14
0.5	48.72	107.64	54.74
1	55.79	101.83	45.21
1.5	72.98	140.72	48.14
2	236.06	407.00	42.00
2.5	284.10	581.64	51.16
3	189.45	262.60	27.86
3.5	162.98	258.88	37.05
4	119.42	206.48	42.16
6	89.11	177.36	49.76
9	80.56	142.64	43.52
12	74.41	126.45	41.16
24	39.42	69.12	42.97
48	32.13	57.16	43.80
72	27.19	39.83	31.74
96	12.30	19.26	36.15
120	11.69	28.24	58.59
144	10.10	21.60	53.25
168	12.89	23.89	46.04
192	8.35	18.16	54.04

[a] % Protein binding = (Total Conc.-Free Conc.) / Total Conc. X 100

Table 48 Percent protein binding of subject no.5

Time (h)	Free Conc. (ng/mL)	Total Conc. (ng/mL)	% Protein Binding[a]
0	25.88	57.44	54.94
0.5	27.99	67.92	58.78
1	84.77	130.81	35.19
1.5	156.02	274.09	43.08
2	132.08	321.76	58.95
2.5	172.40	243.74	29.27
3	146.26	174.52	16.19
3.5	126.26	130.33	3.13
4	97.06	115.17	15.73
6	63.55	117.89	46.10
9	59.87	103.76	42.30
12	45.27	72.97	37.96
24	47.45	79.82	40.55
48	11.40	40.05	71.53
72	9.38	28.11	66.61
96	6.34	24.39	74.00
120	6.22	18.75	66.85
144	6.34	15.75	59.75
168	6.22	15.27	59.30
192	6.04	12.22	50.57

[a] % Protein binding = (Total Conc.-Free Conc.) / Total Conc. X 100

Table 49 Percent protein binding of subject no.6

Time (h)	Free Conc. (ng/mL)	Total Conc. (ng/mL)	% Protein Binding[a]
0	22.32	72.84	69.35
0.5	69.75	115.07	39.39
1	149.74	317.61	52.85
1.5	123.60	409.14	69.79
2	129.08	574.96	77.55
2.5	148.68	398.53	62.69
3	195.45	283.79	31.13
3.5	154.21	210.94	26.89
4	84.37	159.37	47.06
6	69.75	121.32	42.51
9	56.08	72.16	22.28
12	45.64	106.13	56.99
24	37.96	60.43	37.18
48	20.28	43.65	53.54
72	9.23	35.84	74.25
96	7.13	26.37	72.96
120	6.66	19.36	65.58
144	6.10	20.12	69.69
168	5.23	15.39	66.01
192	5.95	16.21	63.28

[a] % Protein binding = (Total Conc.-Free Conc.) / Total Conc. X 100

Table 50 Percent protein binding of subject no.7

Time (h)	Free Conc. (ng/mL)	Total Conc. (ng/mL)	% Protein Binding[a]
0	15.50	51.55	69.93
0.5	30.96	92.77	66.63
1	270.46	623.44	56.62
1.5	339.14	710.93	52.30
2	238.30	510.84	53.35
2.5	242.26	410.80	41.03
3	191.34	251.01	23.77
3.5	111.97	133.78	16.30
4	98.30	204.57	51.95
6	69.75	152.07	54.14
9	86.08	99.04	13.09
12	45.64	88.49	48.42
24	37.96	69.97	45.75
48	20.28	67.66	70.03
72	15.23	39.36	61.31
96	14.13	31.12	54.60
120	10.66	26.02	59.02
144	8.10	19.42	58.31
168	6.23	16.70	62.68
192	5.95	17.13	65.24

[a] % Protein binding = (Total Conc.-Free Conc.) / Total Conc. X 100

Table 51 Percent protein binding of subject no.8

Time (h)	Free Conc. (ng/mL)	Total Conc. (ng/mL)	% Protein Binding[a]
0	21.88	70.56	69.00
0.5	82.13	102.84	20.14
1	78.59	124.42	36.84
1.5	409.38	530.26	22.80
2	338.78	731.97	53.72
2.5	286.92	667.90	57.04
3	276.40	531.94	48.04
3.5	119.19	416.53	71.38
4	174.66	300.58	41.89
6	69.35	211.74	67.25
9	49.11	153.22	67.95
12	46.93	129.37	63.72
24	32.79	102.75	68.09
48	15.82	58.69	73.04
72	13.17	45.86	71.29
96	12.47	33.94	63.26
120	12.49	29.82	58.12
144	12.23	22.52	45.66
168	9.76	25.71	62.03
192	8.40	23.80	64.72

[a] % Protein binding = (Total Conc.-Free Conc.) / Total Conc. X 100

Table 52 Summary of percent protein binding of 8 subjects.

Time	Subject no.									
(h)	1	2	3	4	5	6	7	8	Mean	S.D.
0	80.40	46.36	30.57	58.14	54.94	69.35	69.93	69.00	59.84	15.87
0.5	55.87	14.85	7.46	54.74	58.78	39.39	66.63	20.14	39.73	22.73
1	52.81	12.14	13.36	45.21	35.19	52.85	56.62	36.84	38.13	17.42
1.5	39.23	26.05	32.38	48.14	43.08	69.79	52.30	22.80	41.72	15.31
2	64.55	11.12	0.17	42.00	58.95	77.55	53.35	53.72	45.18	26.59
2.5	58.65	10.57	9.29	51.16	29.27	62.69	41.03	57.04	39.96	21.39
3	45.48	3.80	19.50	27.86	16.19	31.13	23.77	48.04	26.97	14.75
3.5	51.43	24.85	21.43	37.05	3.13	26.89	16.30	71.38	31.56	21.45
4	44.22	22.28	22.22	42.16	15.73	47.06	51.95	41.89	35.94	13.66
6	48.30	31.54	27.25	49.76	46.10	42.51	54.14	67.25	45.86	12.59
9	63.69	72.79	47.26	43.52	42.30	22.28	13.09	67.95	46.61	21.30
12	46.76	47.63	9.26	41.16	37.96	56.99	48.42	63.72	43.99	16.26
24	53.39	54.15	24.27	42.97	40.55	37.18	45.75	68.09	45.79	13.08
48	62.10	64.78	45.91	43.80	71.53	53.54	70.03	73.04	60.59	11.53
72	53.42	57.67	57.67	31.74	66.61	74.25	61.31	71.29	59.25	13.23
96	64.14	60.17	39.54	36.15	74.00	72.96	54.60	63.26	58.10	14.04
120	56.03	40.85	41.57	58.59	66.85	65.58	59.02	58.12	55.83	9.76
144	59.57	37.53	25.86	53.25	59.75	69.69	58.31	45.66	51.20	14.14
168	61.84	45.33	51.04	46.04	59.30	66.01	62.68	62.03	56.78	8.10
192	57.73	53.66	59.72	54.04	50.57	63.28	65.24	64.72	58.62	5.54
Grand mean									47.08	
S.D. of grand mean									3.63	

VITAE

Mr. Wanchai Treyaprasert was born on June 13, 1962 in Chachengsao, Thailand. He received his bachelor degree in Pharmacy from Faculty of Pharmacy, Prince of Songkhla University in 1985 and his master degree of science in Pharmacy from Faculty of Pharmaceutical Sciences, Chulalongkorn University in 1991. He has worked as a lecturer at Faculty of Pharmaceutical Sciences, Chulalongkorn University since 1993. Presently, he is an assistant professor at Faculty of Pharmaceutical Sciences, Chulalongkorn University.