DETERMINATION OF ATORVASTATIN IN HUMAN PLASMA WITH SOLID PHASE MICRO-EXTRACTION FOLLOWED BY LC MS/MS METHOD AND ITS APPLICATION TO PHARMACOKINETICS STUDY

PATTARAWIT RUKTHONG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE IN PHARMACY
(PHARMACEUTICS)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2007

COPYRIGHT OF MAHIDOL UNIVERSITY

Thesis
Entitled

DETERMINATION OF ATORVASTATIN IN HUMAN PLASMA WITH SOLID PHASE MICRO-EXTRACTION FOLLOWED BY LC MS/MS METHOD AND ITS APPLICATION TO PHARMACOKINETICS STUDY

……………………………………………

Mr. Pattarawit Rukthong
Candidate

……………………………………………

Assoc. Prof. Korbtham Sathirakul,
Ph.D.
Major-Advisor

……………………………………………

Assoc. Prof. Polkit Sangvanich,
Ph.D.
Co-Advisor

……………………………………………

Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies

……………………………………………

Prof. Ampol Mitrevej,
Ph.D. (Pharmaceutics)
Chair
Master of Science in Pharmacy
Programme in Clinical Pharmacy
Faculty of Pharmacy

Thesis
Entitled

DETERMINATION OF ATORVASTATIN IN HUMAN PLASMA WITH SOLID PHASE MICRO-EXTRACTION FOLLOWED BY LC MS/MS METHOD AND ITS APPLICATION TO PHARMACOKINETICS STUDY

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science in Pharmacy (Pharmaceutics)
on
May 14, 2007

……………………………………………

Mr. Pattarawit Rukthong
Candidate

……………………………………………

Assist. Prof. Gaysorn Chansiri,
Ph.D.
Chair

……………………………………………

Assoc. Prof. Polkit Sangvanich,
Ph.D.
Member

……………………………………………

Assoc. Prof. Korbtham Sathirakul,
Ph.D.
Member

……………………………………………

Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

……………………………………………

Prof. Ampol Mitrevej,
Ph.D. (Pharmaceutics)
Dean
Faculty of Pharmacy
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deepest appreciation to my major advisor, Associated Professor Dr. Korbtham Sathirakul for his valuable guidance, suggestions, supervisor, kindness and encouragement contributed to this research.

My greatest esteem goes to professor Associated Professor Dr. Polkit Sangvanich, my co-advisor, for him helpful criticism, consultation, and comment on this thesis.

I am great gratitude and thankfulness are extended to Assistant Professor Dr. Gaysorn Chansiri for her constructive comment, suggestion, and also being member for my examining committee.

I am very deep gratitude to P'Sarunyaporn for her suggestion and guidance, especially the starting of the mass spectrometry experiment.

The Thailand research fund through Cooperative Research Network (CRN) in the field of Genetic/Bio-informatics/Bioactive Compounds and Dr. Korbtham's research assistance scholarship are gratefully acknowledged for providing scholarship and research grant during my study.

My honest thanks were also extend to all volunteers participated in my study for their willingness and generous cooperation.

Specially thanks Tor, for his technical advice.

To all of my friends : P'Ju, P'Kruay, P'Noam, P'Tick, P'Took, Kae, Ni, Noon, Nook and other persons who had been mentioned here, I would like to thank all of them and would record my special appreciation for their helping and friendship all time of study.

Finally, I am pound of my mom "Jamnean Rukthong" for her being the strongest person, and most inspiration for all of her children. I also deeply thank my sisters who cheered me up from the starting until the end for this study.

Pattarawit Rukthong

DETERMINATION OF ATORVASTATIN IN HUMAN PLASMA WITH SOLID PHASE MICRO-EXTRACTION FOLLOWED BY LC MS/MS METHOD AND ITS APPLICATION TO PHARMACOKINETICS STUDY.

PATTARAWIT RUKTHONG 4637606 PYPT/M

M.Sc. in Pharm. (PHARMACEUTICS)

THESIS ADVISORS: KORBTHAM SATHIRAKUL, Ph.D., POLKIT SANGVANICH, Ph.D.

ABSTRACT

Determination of drugs in human plasma using the HPLC technique has been widely accepted as an effective method owing to its practical applicability in routine drug analysis in clinical trials. Nevertheless, the method sensitivity can be compromised once limited sample volume is available. This problem is typically encountered in pharmacokinetics studies from which small volume of plasma samples either form humans or animals can be obtained. Atorvastatin, an HMG-CoA reductase inhibitor widely prescribed for the treatment of hypercholesterolemia and the prevention of cardiovascular diseases was selected as a model drug in this study. Drug extraction from human plasma was performed with the aid of reversed-phase C18 solid phase micro-extraction. The detection was accomplished by triple quandruple mass spectrometer interfaced operated in electrospray positive ion mode. Quantitation was achieved by using multiple reaction monitoring (MRM) precursor-production transitions at m/z 559.2 and 440.1 for atorvastatin and m/z 412.2 and 224.2 for fluvastatin (internal standard). Linearity was found within the atorvastatin concentration range of 0.2-80 ng/ml. The limit of detection was found to be 0.05 ng/ml. The lower limit of quantification was 0.1 ng/ml with a relative standard deviation (%RSD) of less than 12%. Acceptable precision and accuracy were obtained for the concentrations within the calibration curve range. The average recovery at low, medium and high of atorvastatin concentrations from spiked plasma: quality control samples, were 84.16 ± 7.99%, 96.82 ± 6.14%, and 102.84 ± 6.73%, respectively. The need for less than 250 ml of plasma volume each sample made it possible to decrease sample preparation time. The method was successfully validated and proved appropriate for the analysis of atorvastatin in human plasma and can be applied for pharmacokinetics, bioavailability and bioequivalence studies.

KEY WORDS: ATORVASTATIN / LC MS MS / PLASMA / SOLID PHASE MICRO-EXTRACTION / PHARMACOKINETIC STUDY

136 P..

การวัดยาอาทอร์วาสตาตินในเลือดมนุษย์ด้วยวิธีการ SOLID PHASE MICRO-EXTRACTION ตามด้วย LC MS/MS และการประยุกต์ใช้ในการศึกษาเภสัชจลนศาสตร์ (DETERMINATION OF ATORVASTATIN IN HUMAN PLASMA WITH SOLID PHASE MICRO-EXTRACTION FOLLOWED BY LC MS/MS METHOD AND ITS APPLICATION TO PHARMACOKINETICS STUDY)

ภัทรวิทย์ รักษ์ทอง 4637606 PYPT/M

ภ.ม. (เภสัชการ)

คณะกรรมการควบคุมวิทยานิพนธ์: กอบธัม สถิรกุล, Ph.D., พลกฤษณ์ แสงวาณิช, Ph.D

บทคัดย่อ

การหาปริมาณยาในเลือดด้วยวิธี HPLC นั้นเป็นที่ยอมรับอย่างกว้างขวางในการวิเคราะห์ยาในทางคลินิก อย่างไรก็ตามเมื่อสารตัวอย่างมีปริมาณน้อยและต้องการความไว (sensitivity) ในการตรวจพบยาซึ่งเป็นปัญหาทางเภสัชจลนศาสตร์เมื่อทำการวัดระดับยาในเลือดมนุษย์และสัตว์ โดยยาอาทอร์วาสตาตินเป็นยากลุ่มยับยั้งเอนไซม์ HMG Co-A reductase ซึ่งมีการใช้อย่างแพร่หลายในการลดไขมันในเลือดและป้องกันการเกิดภาวะโรคหัวใจโดยวิธีการสกัดยาออกจากพลาสมานั้นใช้วิธีการ solid phase micro-extraction และตามด้วย LC MS/MS ในการหาปริมาณยานั้นและใช้ electrospray ด้านบวกและ multiple reaction monitoring (MRM) โดยมีมวลต่อประจุของยาอาทอร์วาสตาตินที่ 559.2 และ 440.1 สำหรับ Internal standard ใช้ยาฟลูวาสตาตินซึ่งมีประจุต่อมวลเท่ากับ 412.2 และ 224.2 ในการทดลองครั้งนี้พบว่าความสัมพันธ์เชิงเส้นอยู่ในช่วงความเข้มข้นที่ 0.2-80 นาโนกรัม/มิลลิลิตร ความเข้มข้นที่สามารถตรวจพบอยู่ที่ 0.05 นาโนกรัม/มิลลิลิตร และมีความเข้มข้นของยาต่ำสุดที่สามารถหาปริมาณได้อยู่ที่ 0.1 นาโนกรัม/มิลลิลิตรโดยมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ไม่เกิน 12 % ค่าการค้นพบยาหลังจากการสกัดที่ความเข้มข้น ต่ำ กลาง สูง พบ 84.16±7.99%, 96.8±6.14และ102.84±6.73 ตามลำดับ จากการวิเคราะห์พบว่าจะใช้ปริมาณพลาสมาไม่เกิน 250 ไมโครลิตรซึ่งสามารถลดปริมาณตัวอย่างและเวลาในการสกัดโดยสามารถนำมาประยุกต์ใช้ในการศึกษาเภสัชจลนศาสตร์, ชีวประสิทธิผลและชีวสมมูลของยาอาทอร์วาสตาติน

136 หน้า

CONTENTS

	Page
ACKNOWLEDGEMENT	iii
ABSTRACT (ENGLISH)	iv
ABSTRACT (THAI)	v
LIST OF TABLES	vii
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS	xvi
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	
Method development	3
Background information on atorvastatin	4
Bioanalytical assay methods overview of atorvastatin	9
Bioavailability and bioequivalence	11
III MATERIALS AND METHODS	
Materials	21
Equipments	21
Methods	22
IV RESULTS AND DISCUSSION	
Mass spectrometry	33
Performance of LC	33
Bioanalytical method validation	38
Pharmacokinetic of atorvastatin in healthy volunteers	55
V CONCLUSION	118
REFERENCES	119
APPENDIX	123
BIOGRAPHY	136

LIST OF TABLES

Table		Page
1.	Chromatography-mass spectrometry methods for the quantitation of atorvastatin and their metabolite in biological matrix.	12
2.	Tandem mass spectrometer main working parameter.	24
3.	Randomization table of atorvastatin products of each subject.	30
4.	The recoveries of atorvastatin.	49
5.	Precision and accuracy data of back-calculation concentrations of calibration samples for atorvastatin in human plasma.	49
6.	Between-run precision and accuracy of the method for determining atorvastatin concentrations in plasma samples.	51
7.	Within run precision and accuracy of the method for determining atorvastatin concentrations in plasma samples.	51
8.	Stability of atorvastatin in human plasma.	53
9.	Stock solution stability of atorvastatin and fluvastatin (internal standard).	54
10.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.1	58
11.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.2	59
12.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.3	60
13.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.4	61
14.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.5	62
15.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.6	63

LIST OF TABLES (continued)

Table		Page
16.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.7	64
17.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.8	65
18.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.9	66
19.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.10.	67
20.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.11.	68
21.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.12.	69
22.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.13.	70
23.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.14.	71
24.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.15.	72
25.	Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.16.	73

LIST OF TABLES (continued)

Table **Page**

26. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.17. 74
27. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.18. 75
28. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.19. 76
29. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.20. 77
30. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.21. 78
31. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.22. 79
32. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.23. 80
33. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject no.24. 81
34. Maximum concentration (C_{max}) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24). 109

LIST OF TABLES (continued)

Table		Page
35.	Area under the plasma-concentration curve ($AUC_{0\text{-}last}$) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24).	110
36.	Area under the plasma-concentration curve ($AUC_{0\text{-}inf(observe)}$) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24).	111
37.	Time to maximum concentration (T_{max}) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24).	112
38.	$AUC_{0\text{-}last}/AUC_{0\text{-}inf(observe)}$) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24).	113
39.	Descriptive statistical of pharmacokinetics parameter.	114
40.	Results from pharmacokinetic study of atorvastatin acid.	115
41.	BE statistical result (ANOVA) type III for all subjects (n=24) (balance data set).	116
42.	BE statistical result 90% Westlake confidence interval of parameters for all subject (n=24).	116
43.	BE statistical result of T max using nonparametric friedman's test for all subjects.	117
44.	Report of sequence data after LC MS/MS.	135

LIST OF FIGURES

Figure		Page
1.	Structure of HMG Co-A reductase inhibitor.	5
2.	Full scan positive ion turboIonspray Q1 mass spectra of atorvastatin.	34
3.	Full scan positive ion turboIonspray product ion mass spectra of atorvastatin.	35
4.	Full scan positive ion turboIonspray Q1 mass spectra of internal standard (fluvastatin).	36
5.	Full scan positive ion turboIonspray product ion mass spectra of internal standard (fluvastatin).	37
6.	MRM ion-chromatograms resulting from the analysis of blank no.1 (drug and internal standard free) human plasma for atorvastatin and internal standard.	39
7.	MRM ion-chromatograms resulting from the analysis of blank no.2 (drug and internal standard free) human plasma for atorvastatin and internal standard.	40
8.	MRM ion-chromatograms resulting from the analysis of blank no.3 (drug and internal standard free) human plasma for atorvastatin and internal standard.	41
9.	MRM ion-chromatograms resulting from the analysis of blank no.4 (drug and internal standard free) human plasma for atorvastatin and internal standard.	42
10.	MRM ion-chromatograms resulting from the analysis of blank no.5 (drug and internal standard free) human plasma for atorvastatin and internal standard.	43
11.	MRM ion-chromatograms resulting from the analysis of blank no.6 (drug and internal standard free) human plasma for atorvastatin and internal standard.	44

LIST OF FIGURES (continued)

Figure		Page
12.	MRM ion chromatograms resulting from the analysis of blank plasma spiked with drug only.	45
13.	MRM ion chromatogram resulting from the analysis of blank plasma spiked with standard only (Zero sample).	46
14.	Representative MRM ion chromatograms resulting from the analysis 0.1 ng/ml (LLOQ) of atorvastatin.	47
15.	Plasma Concentration Time Profile of Atorvastatin in Subject 1 Normal Plot (Above) and Semilog Plot (Below).	82
16.	Plasma Concentration Time Profile of Atorvastatin in Subject 2 Normal Plot (Above) and Semilog Plot (Below).	83
17.	Plasma Concentration Time Profile of Atorvastatin in Subject 3 Normal Plot (Above) and Semilog Plot (Below).	84
18.	Plasma Concentration Time Profile of Atorvastatin in Subject 4 Normal Plot (Above) and Semilog Plot (Below).	85
19.	Plasma Concentration Time Profile of Atorvastatin in Subject 5 Normal Plot (Above) and Semilog Plot (Below).	86
20.	Plasma Concentration Time Profile of Atorvastatin in Subject 6 Normal Plot (Above) and Semilog Plot (Below).	87
21.	Plasma Concentration Time Profile of Atorvastatin in Subject 7 Normal Plot (Above) and Semilog Plot (Below).	88
22.	Plasma Concentration Time Profile of Atorvastatin in Subject 8 Normal Plot (Above) and Semilog Plot (Below).	89
23.	Plasma Concentration Time Profile of Atorvastatin in Subject 9 Normal Plot (Above) and Semilog Plot (Below).	90
24.	Plasma Concentration Time Profile of Atorvastatin in Subject 10 Normal Plot (Above) and Semilog Plot (Below).	91
25.	Plasma Concentration Time Profile of Atorvastatin in Subject 11 Normal Plot (Above) and Semilog Plot (Below).	92

LIST OF FIGURES (continued)

Figure		Page
26.	Plasma Concentration Time Profile of Atorvastatin in Subject 12 Normal Plot (Above) and Semilog Plot (Below).	93
27.	Plasma Concentration Time Profile of Atorvastatin in Subject 13 Normal Plot (Above) and Semilog Plot (Below).	94
28.	Plasma Concentration Time Profile of Atorvastatin in Subject 14 Normal Plot (Above) and Semilog Plot (Below).	95
29.	Plasma Concentration Time Profile of Atorvastatin in Subject 15 Normal Plot (Above) and Semilog Plot (Below).	96
30.	Plasma Concentration Time Profile of Atorvastatin in Subject 16 Normal Plot (Above) and Semilog Plot (Below).	97
31.	Plasma Concentration Time Profile of Atorvastatin in Subject 17 Normal Plot (Above) and Semilog Plot (Below).	98
32.	Plasma Concentration Time Profile of Atorvastatin in Subject 18 Normal Plot (Above) and Semilog Plot (Below).	99
33.	Plasma Concentration Time Profile of Atorvastatin in Subject 19 Normal Plot (Above) and Semilog Plot (Below).	100
34.	Plasma Concentration Time Profile of Atorvastatin in Subject 20 Normal Plot (Above) and Semilog Plot (Below).	101
35.	Plasma Concentration Time Profile of Atorvastatin in Subject 21 Normal Plot (Above) and Semilog Plot (Below).	102
36.	Plasma Concentration Time Profile of Atorvastatin in Subject 22 Normal Plot (Above) and Semilog Plot (Below).	103
37.	Plasma Concentration Time Profile of Atorvastatin in Subject 23 Normal Plot (Above) and Semilog Plot (Below).	104
38.	Plasma Concentration Time Profile of Atorvastatin in Subject 24 Normal Plot (Above) and Semilog Plot (Below).	105

LIST OF FIGURES (continued)

Figure		Page
39.	Average ± S.D. of Plasma Concentration Time Profile of Atorvastatin in the Study Normal Plot (Above) and Semilog Plot (Below) for all subjects (n=24).	106
40.	Spaghetti Plot for Plasma Concentration Time Profile of Reference Treatment Atorvastatin for All Subjects Normal Plot (Above) and Semilog Plot (Below) (n=24).	107
41.	Spaghetti Plot for Plasma Concentration Time Profile of Test Treatment Atorvastatin for All Subjects Normal Plot (Above) and Semilog Plot (Below) (n=24).	108
42.	MRM ion-chromatograms resulting from the analysis of subject No.5 at 60 minutes for test product in human plasma for atorvastatin and internal standard.	124
43.	MRM ion-chromatograms resulting from the analysis of subject No.5 at 60 minutes for reference product in human plasma for atorvastatin and internal standard.	125
44.	MRM ion-chromatograms resulting from the analysis of subject No.14 at 240 minutes for test product in human plasma for atorvastatin and internal standard.	126
45.	MRM ion-chromatograms resulting from the analysis of subject No.14 at 240 minutes for reference product in human plasma for atorvastatin and internal standard.	127
46.	MRM ion-chromatograms resulting from the analysis of subject No.18 at 60 minutes for test product in human plasma for atorvastatin and internal standard.	128

LIST OF FIGURES (continued)

Figure **Page**

47. MRM ion-chromatograms resulting from the analysis of subject No.18 at 60 minutes for reference product in human plasma for atorvastatin and internal standard. 129
48. MRM ion-chromatograms resulting from the analysis of subject No.22 at 90 minutes for test product in human plasma for atorvastatin and internal standard. 130
49. MRM ion-chromatograms resulting from the analysis of subject No.22 at 90 minutes for reference product in human plasma for atorvastatin and internal standard. 131
50. MRM ion-chromatograms resulting from the analysis of subject No.23 at 120 minutes for test product in human plasma for atorvastatin and internal standard. 132
51. MRM ion-chromatograms resulting from the analysis of subject No.23 at 120 minutes for reference product in human plasma for atorvastatin and internal standard. 133
52. MRM ion-chromatograms resulting from the analysis of lower limit of detection (LOD) in human plasma for atorvastatin and internal standard. 134

LIST OF ABBREVIATIONS

°C	Celsius degree
ng	nanogram
μl	microliter
ANOVA	analysis of variance
AUC	area under the plasma concentration time curve
$AUC_{0\text{-}last}$	area under the plasma concentration time curve from time zero to the last time
$AUC_{0\text{-}inf(observe)}$	area under the plasma concentration time curve from time zero to the infinity time from observed
C_{max}	maximum plasma concentration
CV	coefficient of variation
g	gram
HPLC	High performance liquid chromatography
IS	internal standard
l	liter
LOD	lower limit of detection
LLOQ	lower limit of quantitation
M	molar
mg	milligram
min	minute
ml	milliliter
MS	mass spectrometry
no.	number
pH	the negative logarithm of the hydrogen ion concentration
pKa	the negative logarithm of the dissociation constant
rpm	revolution per minute
RSD	relative standard deviation

LIST OF ABBREVIATIONS (continued)

r^2	square of correlation coefficient
SD	standard deviation
SEM	standard error of mean
SPME	solid phase micro-extraction
T_{max}	time to maximum plasma concentration
$t_{½}$	half-life
λz	the termination elimination rate constant

CHAPTER I
INTRODUCTION

Atorvastatin is a HMG-coA reductase inhibitor, which has a widespread use in the prevention of cardiovascular event. Atorvastatin is rapidly absorbed after oral administration however, due to pre-systematic clearance in gastro-intestinal mucosa and metabolism in the liver, it absolute bioavailability is approximately 12% and low plasma concentration is achieved following administration of the drug (1). Thus, quantification methods of the drug in pharmacokinetic studies need to be sensitivity and specific. For atorvastatin, six HPLC methods for the determination of drug in human plasma have previously published. The most of extraction method use liquid-liquid extraction (LLE) for sample preparation and this method have been applied in clinical studies, with atorvastatin dose regimen 40 and 80 mg (2). Another one was used solid phase extraction (SPE) sample in low dose (10 mg) of atorvastatin.

However, sensitivity can be compromised when limited sample volumes are available, which is common in pharmacokinetics studies involving small volume of plasma from human or animal. A reverse phase C18 SPE sorbent material was pack in a 100 μl pipette tip to create a micro volume SPE device (OMIX 100 μl C18). The monolithic nature of the SPE sorbent allows less restricted bi-directional flow of liquid though the bed, unlike traditional silica bed-based materials. Thus, the aim of this study is to develop a chromatographic method for determining the atorvastatin in human plasma, using solid phase micro-extraction and to compare the bioequivalence of locally made oral tablet.

Twenty-four Thai healthy volunteers participate in the study which is a crossover design, with a one week wash-out period. After an overnight fast, a single 40 mg atorvastatin tablet of either the reference product or the test product is orally administered to each subject. A venous blood sample of five milliliters is drawn prio to dosing and at 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 48 hours after dosing. The plasma sample is analyzed for total atorvastatin concentration by LC-MS/MS

method. Pharmacokinetic parameters of atorvastatin from 24 healthy Thai subjects are calculated using the WinNonlin professional edition version 3.1. The plasma concentration time profile in each subject is fitted using noncompartmental model of extravascular input.

The specific aims of this study were:

1. To develop and validate LC-MS/MS assay for the quantitation of atorvastatin in human plasma by solid phase micro-extraction (Omix ® pipette tip).
2. To demonstrate the bioequivalence of generic oral tablets with innovator's oral tablets.

CHAPTER II
LITERATURE REVIEW

1. Method development

A bioanalytical method is a set of all of the procedures involved in the collection, processing, storing, and analysis of a biological matrix for an analyze (3). Analytical methods employed for quantitative determination of drugs and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data that in turn are used in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetics. Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification (4, 5).

Choice of extraction procedure, which is time economical, gives the highest possible recovery without interference at the elution time of the analyte of interest and has acceptable accuracy and precision. Method performance is determined primarily by the quality of the procedure itself. The two factors that are most important in determining the quality of the method are selective recovery and standardization.

1.1 The limits of detection and quantitation (LLOD and LLOQ)

The US pharmacopoeia (USP) defines the limit of detection (LLOD) as the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated.

They also define the lowest limit of quantification (LLOQ), as the lowest amount of a sample that can be determined (quantitated) with acceptable precision and accuracy under the stated operational condition of the method. The limits are commonly associated with the signal to noise ratio (S/N). In the case of LLOD, analysts often use S/N (signal to noise ratio) of 2:1 or 3:1, while a S/N of 10:1 or 5:1 is often considered to be necessary for the LLOQ. Typically the signal is measured from the base line to

peak apex and divided by the peak-to-peak noise, which is determined from the blank plasma injection.

2 Calibration line

A calibration line is a curve showing the relation between the concentration of the analyte in the sample and the detected response. It is necessary to use a sufficient number of standards to define adequately the relationship between response and concentration. The relationship between response and concentration must be demonstrated to be continuous and reproducible. The number of standards to be used will be a function of the dynamic range and nature of the concentration-response relationship. In many cases, five to eight concentrations (excluding blank values) may define the standard curve. More standard concentrations may be necessary for non-linear relationships than would be for a linear relationship (3).

2. Background information on atorvastatin

2.1 Structure and chemical properties

Atorvastatin calcium is a synthetic lipid-lowering agent. Atorvastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis.

The empirical formula of atorvastatin is $C_{33}H_{35}FN_2O_5$ and its molecular weight is 558.64 g/mol. The calcium salt of atorvastatin is a white to off-white crystalline powder that is insoluble in aqueous solutions of pH 4 and below. Atorvastatin calcium is very slightly soluble in distilled water, phosphate buffer pH 7.4, and acetonitrile, slightly soluble in ethanol, and freely soluble in methanol

2.2 Pharmacological

Atorvastatin is a selective, competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, a precursor of sterols, including cholesterol (6). Cholesterol and triglycerides circulate in the bloodstream as part of lipoprotein complexes. With ultracentrifugation, these complexes separate into HDL (high-density lipoprotein),

IDL (intermediate-density lipoprotein), LDL (low-density lipoprotein), and VLDL (very-low-density lipoprotein) fractions.

Figure 1. Structure of HMG-CoA reductase inhibitors.

Triglycerides (TG) and cholesterol in the liver are incorporated into VLDL and released into the plasma for delivery to peripheral tissues.LDL is formed from VLDL and is catabolized primarily through the high-affinity LDL receptor(6). Clinical and pathologic studies show that elevated plasma levels of total cholesterol (total-C), LDL-cholesterol (LDL-C), and apolipoprotein B (apo B) promote human atherosclerosis and are risk factors for developing cardiovascular disease, while increased levels of HDL-C are associated with a decreased cardiovascular risk. In animal models, lowers plasma cholesterol and lipoprotein levels by inhibiting HMG-CoA reductase and cholesterol synthesis in the liver and by increasing the number of hepatic LDL receptors on the cell-surface to enhance uptake and catabolism of LDL; atorvastatin also reduces LDL production and the number of LDL particles. Atorvastatin reduces LDL-C in some patients with homozygous familial hypercholesterolemia (FH), a population that rarely responds to other lipid-lowering medication (7).

2.3 Clinical applications

For the clinical in patients who have not met the low-density lipoprotein cholesterol (LDL-C) goal as recommended by the National Cholesterol Education Program Adult Treatment Panel II guidelines, atorvastatin 10-80 mg/d may be used as monotherapy or as an adjunct to other lipid-lowering agents and dietary modifications. In placebo-controlled clinical trials, atorvastatin 10-80 mg/d lowered LDL-C by 35-61% and triglyceride (TG) concentrations by 14-45%. In comparative trials, atorvastatin 10-80 mg/d showed a greater reduction of serum total cholesterol (TC), LDL-C, TG concentrations, and apolipoprotein B-100 (apo B) compared with pravastatin, simvastatin, or lovastatin. In comparison, currently available HMG-CoA reductase inhibitors (lovastatin, simvastatin, pravastatin, fluvastatin, cerivastatin) lower LDL-C concentrations by approximately 20-40% and TG concentrations by approximately 10-30%. In pooled placebo-controlled clinical trials of up to a duration of 52 weeks, atorvastatin in dosages up to 80 mg/d appeared to be well tolerated. The most common adverse effect of atorvastatin was gastrointestinal upset. The incidence of elevated serum hepatic transaminases may be greater at higher dosages of atorvastatin. The risk of myopathy and/or rhabdomyolysis is increased when an HMG-CoA reductase inhibitor is taken concomitantly with cyclosporine, gemfibrozil,

niacin, erythromycin, or azole antifungals. In the result showed that Atorvastatin appears to reduce TC, LDL-C, TG concentrations, and apo B to a greater extent than do currently available HMG-CoA reductase inhibitors. Atorvastatin may be preferred in patients requiring greater than a 30% reduction in LDL-C or in patients with both elevated LDL-C and TG concentrations, which may obviate the need for combination lipid-lowering therapy. Adverse effects of atorvastatin appear to be similar to those of other HMG-CoA reductase inhibitors and should be routinely monitored. Long-term safety data (>1 year) on atorvastatin compared with other HMG-CoA reductase inhibitors are still needed. Cost-effectiveness studies comparing atorvastatin with other HMG-CoA reductase inhibitors remain a subject for further investigation. Published clinical studies evaluating the impact of atorvastatin on cardiovascular morbidity and mortality are still needed. Additionally, clinical studies evaluating the impact of lipid-lowering therapy in a larger number of women, the elderly (> 70 years), and patients with diabetes for treatment of primary and secondary prevention of coronary heart disease are needed (8-10).

In 2005, the PROVE IT-TIMI 22 trial compared 40mg/day pravastatin with 80mg/day atorvastatin (11). Taken directly from the results of the trial: "Standard treatment (statin) with a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (pravastatin 40 mg/day) resulted in a 22% reduction in LDL cholesterol levels at 30 days compared with a 51% reduction with intensive therapy (atorvastatin 80 mg/day). At 2 years, a relative risk reduction of 16% (95% confidence interval, 5-26%; P=0.005) in the primary end point rate (death, myocardial infarction, documented unstable angina requiring hospitalization, coronary revascularization, or stroke) was seen in patients receiving intensive statin treatment compared with standard statin therapy. The benefit of intensive treatment was apparent as early as 30 days and was consistent over time. The PROVE IT-TIMI 22 data indicate that patients recently hospitalized for an ACS benefit from early and continued lowering of LDL cholesterol to levels substantially below current guideline recommendations.

3. Pharmacokinetics and Pharmacodynamics (10, 12, 13)

3.1 Pharmacodynamics

Atorvastatin as well as some of its metabolites are pharmacologically active in humans. The liver is the primary site of action and the principal site of cholesterol synthesis and LDL clearance. Drug dosage rather than systemic drug concentration correlates better with LDL-C reduction. Individualization of drug dosage should be based on therapeutic response.

3.2 Pharmacokinetics

3.2.1 Absorption

Atorvastatin is rapidly absorbed after oral administration; maximum plasma concentrations occur within 1 to 2 hours. Extent of absorption increases in proportion to atorvastatin dose. The absolute bioavailability of atorvastatin (parent drug) is approximately 14% and the systemic availability of HMG-CoA reductase inhibitory activity is approximately 30%. The low systemic availability is attributed to pre-systemic clearance in gastrointestinal mucosa and/or hepatic first-pass metabolism. Although food decreases the rate and extent of drug absorption by approximately 25% and 9%, respectively, as assessed by C max and AUC, LDL-C reduction is similar whether atorvastatin is given with or without food. Plasma of atorvastatin concentrations are lower (approximately 30% for C_{max} and AUC) following evening drug administration compared with morning. However, LDL-C reduction is the same regardless of the time of day of drug administration (13).

3.2.2 Distribution

Mean volume of distribution of atorvastatin is approximately 381 liters. Atorvastatin is ≥ 98% bound to plasma proteins. A blood/plasma ratio of approximately 0.25 indicates poor drug penetration into red blood cells. Based on observations in rats, atorvastatin is likely to be secreted in human milk Pregnancy and Lactation.

3.2.3 Metabolism

Atorvastatin is extensively metabolized to ortho- and parahydroxylated derivatives and various beta-oxidation products. *In vitro* inhibition of HMG-CoA

reductase by ortho and parahydroxylated metabolites is equivalent to that of atorvastatin. Approximately 70% of circulating inhibitory activity for HMG-CoA reductase is attributed to active metabolites. *In vitro* studies suggest the importance of atorvastatin metabolism by cytochrome P450 3A4, consistent with increased plasma concentrations of atorvastatin in humans following co-administration with erythromycin, a known inhibitor of this isozyme. In animals, the ortho-hydroxy metabolite undergoes further glucuronidation (14).

3.2.4 Excretion

Atorvastatin and its metabolites are eliminated primarily in bile following hepatic and/or extra-hepatic metabolism; however, the drug does not appear to undergo enterohepatic recirculation. Mean plasma elimination half-life of atorvastatin in humans is approximately 14 hours, but the half-life of inhibitory activity for HMG-CoA reductase is 20 to 30 hours due to the contribution of active metabolites. Less than 2% of a dose of atorvastatin is recovered in urine following oral administration.

4. Bioanalytical assay methods overview of Atorvastatin

Various methods have been published for the analysis of atorvastatin in biological fluids. These include HPLC, LC-MS/MS and GC methods using variety extraction procedures (15, 16). A summary of these methods in chronological order is given below and Table 1.

4.1 ***Bahrami, G. et al*, HPLC –UV detector (2)**.

High-performance liquid chromatographic method was validated and described for determination of atorvastatin in human serum. Following liquid–liquid extraction of the drug and an internal standard (sodium diclofenac), chromatographic separation was accomplished using C18 analytical column with a mobile phase consisting of sodium phosphate buffer (0.05 M, pH 4.0) and methanol (33:67, v/v). Atorvastatin and the internal standard were detected by ultraviolet absorbance at 247 nm. The average recoveries of the drug and internal standard were 95% and 80%, respectively. The lower limits of detection and quantification were 1 and 4 ng/ml, respectively, and

the calibration curves were linear over a concentration range of 4–256 ng/ml of atorvastatin in human serum.

4.2 ***Hermann, M. et al,*** **HPLC MS/MS** (17).

To develop a chromatographic method for the analysis of atorvastatin, o- and p-hydroxyatorvastatin (acid and lactone forms) in human plasma after administration of atorvastatin at the lowest registered dose (10 mg) in clinical studies. Sample preparation was performed by solid-phase extraction and was followed by separation of the analytes on an HPLC system with a linear gradient and a mobile phase consisting of acetonitrile, water and formic acid. Detection was achieved by tandem mass spectrometry operated in the electrospray positive ion mode. Validation of the method for the compounds for which reference compounds were available (acid forms of atorvastatin, o- and p-hydroxyatorvastatin) showed linearity within the concentration range (0.2-30 ng/ml for atorvastatin acid and p-hydroxyatorvastatin acid, and 0.5-30 ng/ml for o-hydroxyatorvastatin acid) (r^2 > or = 0.99, n = 5 for all analytes). Accuracy and precision (evaluated at 0.5, 3 and 30 ng/ml for atorvastatin, p-hydroxyatorvastatin and 1, 3 and 30 ng/ml for o-hydroxyatorvastatin) were both satisfactory. The detection limit was 0.06 ng/ml for atorvastatin and p-hydroxyatorvastatin, and 0.15 ng/ml for o-hydroxyatorvastatin.

4.3 ***Borek-Dohalsky, V et al,*** **HPLC MS/MS** (16).

In this publication a validated, highly sensitive, and selective isocratic HPLC method is reported for quantitative determination of the major statin drug atorvastatin and its metabolite 2-hydroxyatorvastatin. Detection was performed with an electrospray ionization triple-quadrupole mass spectrometer equipped with an ESI interface operating in positive-ionization mode. Multiple reactions monitoring (MRM) was used for MS-MS detection. The calibration plot was linear in the concentration range 0.10-40.00 ng/mL for both ATV and HATV. Inter-day and intra-day precision and accuracy of the proposed method were characterized by measurement of relative standard deviation (RSD) and percentage deviation, respectively; both were less than 8% for both analytes. The limit of quantitation was 0.02 ng/ml for and 0.07 ng/ml for 2-hydroxyatorvastatin. The method was used for pharmacokinetic study of atorvastatin and 2-hydroxyatorvastatin.

4.4 *Al-Rawithi.et al.*, HPLC fluorescence detector (18)**.**

High performance liquid chromatographic assay for the determination of fluvastatin (FV) using atorvastatin as the internal standard (IS). After a simple deproteinization of 1.0 mL of plasma with acetonitrile, the drug and IS were extracted with tert-methyl butyl ether (TMBE). An efficient separation was performed using an 8 mm x 10 cm Nova Pak $C_{(18)}$ 4-microm particle size radial compression cartridge. The mobile phase consisted of an aqueous solution containing 20 mmol/l dibasic sodium dihydrogen phosphate with 1 mmol/l sodium lauryl sulfate adjusted to pH 7 with phosphoric acid and acetonitrile (70:30 v/v) delivered at a flow rate of 1.0 ml/min. The compounds of interest were detected using a fluorescence detector with the excitation wavelength set at 305 nm and the emission at 380 nm.

5. Bioavailability and Bioequivalence

5.1 Bioavailability

Bioavailability means the rate and extent to which the active ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of drug action (19).

Rate is defined in terms of a description of a concentration time profile. The usual approach is to estimate the time of maximum concentration, the time of the sample with the highest measured concentration (20).

The extent can be computed from the integral of the concentrations predicted in the compartment representing the systemic circulation of the area under the curve or AUC approach (20).

Absolute bioavailability indicated that the bioavailability is determined by comparing the rate and extent of absorption of the drug from its administered dosage.

Table 1. Chromatography–mass spectrometry methods for the quantification of atorvasatin and their metabolites in biological matrix.

Drug, Metabolites	Matrix	Column	Mobile phase (v/v)	Sample preparation	GC or LC	Internal standard(IS)	LOQ (ng/ml)	Ref.
AV=Atorvastatin, 2-OH=2-hydroxyatorvatatin, 4-OH=4-hydroxyatorvastatin								
AV, 2-OH-AV, 4-OH-AV	Plasma	Waters symmetry C_{18}	0.03% Formic acid/acetonitrile (30:70)	LLE	LC	RV	0.1	(21)
AV, 2-OH-AV, 4-OH-AV	Plasma	YMC J'Sphere H80, C_{18}	Acetonitrile/0.1% acetic acid (70:30)	LLE	LC	d_5-AV; d_5-4-OH-AV	0.25	(15)
AV, 2-OH-AV, 4-OH-AV, AV-LC	Serum	YMC Basic, C_{18}	Gradient (A) water/methanol/formic acid (95:5:0.0043) (B) acetonitrile/methanol/formic acid (95:5:0.0043)	LLE	LC	A deuterium labeled analog	0.5	(22)
AV, 2-OH-AV	Plasma	Atlantis dC_{18}	Acetonitrile/0.1% acetic acid (70:30)	LLE	LC	Clindamycin	0.1	(16)
AV, 2-OH-AV, 4-OH-AV	Plasma	Omnisphere C_{18}	Gradient (A) acetonitrile/formic acid (1 mM; 30:70) (B) acetonitrile/formic acid (1 mM; 70:30)	SPE	LC	Methaqualone	0.2 for AV, 2-OH-AV; 0.5 for 4-OH-AV	(2)
AV	Plasma	–	Acetonitrile/0.1% acetic acid (70:30)	-	–	Losartan	0.4	(16)

from to the data obtained following intravenous administration of the drug, as a reference preparation. Absolute bioavailability is expressed on a scale of 0-100%.

Relative bioavailability is a term that refers to the bioavailability of one drug product as compared to another standard dosage formulation with the same drug chemical entity, or to other established standards (23). If an intravenous solution cannot be administered, an intramuscular injection may be allowed as a reference standard. Also, in certain cases where parenteral administration is no advisable, the reference drug preparation is administered as an oral solution.

5.2 Bioequivalence

Bioequivalence is a relative term which indicates that the drug substance in two or more similar dosage forms reaches the systemic circulation at the same relative rate and extent (24).

5.3 Determination of Bioavailability

Bioavailability of drug products in human provides the most reliable method available for determining bioequivalence. The testing is normally performed in young healthy male adult volunteers under restricted dietary conditions and fixed activity levels. Effect of sex and age of the volunteers on bioavailability are considered when there is a specific concern that they may affect drug safety or efficacy. In conducting bioavailability studies, drug in blood levels or cumulative urine excretion data following the administration of a standard or reference preparation. The best reference is an intravenous dose of the drug where bioavailability is assured to be 100%. In this case, absolute bioavailability is determined. The relative bioavailability is used in establishing bioequivalence between same generic drug products from multiple source and the innovator's (original formulation) usually used as the reference preparation due to its approved clinical efficacy (23).

The bioavailability studies should be conducted with sensitivity and regarded for the moral and ethical issues involved in using human volunteers for the experiments. The possibility of adverse effect and the hazards in various blood sample collection must be highlighted. Special populations, such as children and pregnancy or breast-feeding women, must not be included for humanitarian reasons. Also,

individuals with certain enzyme deficiencies or abnormal metabolism should be avoided, as should any individual requiring certain medications on a regular basis, to minimize any bias or source of variability. The Guidelines for Biopharmaceutical Studies in Man, published by the Academy of Pharmaceutical Sciences recommends that test volunteers should be normal healthy adult males, expect where is not applicable, such as in case of antifertility drugs where female volunteers have to be used. Generally, the test volunteers range in age from 20-40 years and in weight within a narrow range (10%) of their ideal weight. Individuals with any past history of gastrointestinal tract, liver or kidney malfunctions must be excluded. Also, those with significant organ abnormality or diseases should be avoided. All participating volunteers should be subjected to a through physical examination and the following hematological and clinical chemistry tests. Participants for oral studies are required to fast overnight prior to and for at least four hours after dosing. Also, they should obtain from any medications for a minimum of one week and should have taken no enzyme-inducing drug for one month prior to dosing. A standard diet can be specified as well as the type and volume of fluid intake. Strenuous physical activities and demanding sports should be avoided during the prior of the study (23).

Regarding the number of subject needed, there is no specific recommendation, as it depends on several factors. These may include the inherent subject-to-subject variability for the drug under study, the expected magnitude of the difference between the test dosage forms and the particular statistical design of the study. However, the AphA guidelines suggest general rules for deciding the appropriate pharmacokinetic parameters for which comparisons are conducted, the magnitude of differences among the test preparations that must be detected should be specified to the statistician. The design of the experiment, partial cross over, total cross over, Latin square, etc., should be specified and, accordingly, the statistician will be able to recommend the appropriate number of participants. A well-designed protocol is a flexible one that assesses the overall variability of the results sequentially during the progress of the study and modifies the experimental plan accordingly. Study conditions should be adhered to as rigorously as possible since they are a major source for variability. As a rule of thumb, a minimum number of twelve healthy subjects may be employed in a crossover bioequivalence study, provided that the testing conditions are strictly

standardized and the assay methology utilized has been thoroughly validated to generate sufficiently accurate and reproducible results. The number should be increased when the patients and/or the parallel are used (23).

Study of bioavailability can be determined in blood, urine, saliva, sweat and feces sample. Of these, blood and urine samples are mostly measured for drug concentrations, then calculated for total under the plasma concentration and time curve and the total amount of drug excreted in the urine, respectively.

Urinary excretion of the unchanged drug is, in most cases, directly proportional to the plasma concentration of total drug (bound and unbound form). Therefore, if a drug is excreted, even partially, in the urine, it is possible to determine its bioavailability from the cumulative urinary excretion data. In practice, determination of bioavailability using urinary excretion data can be conducted only if at least 20% of the dose is excreted in the urine after an intravenous dose. The fraction of drug entering the blood stream and being excreted by the kidney is assumed to remain constant. The collection of the urine must be continued until the drug has been excreted completely. This may require about five biological half-lives of drug for excretion of greater than 95% of the drug. Also, at each sample collection, total emptying of the bladder is essential or else the residual amount of urine will be erroneously added to the next points to establish a reliable cumulative urinary excretion curve that reflects the blood level profile. The rate of absorption is determined from the plot of excretion rate versus time. Frequent sampling, especially at the beginning, is essential for the accurate description of the excretion curve and the precise determination of the peak time. The utility of urinary excretion data for determining the rate of bioavailability is most applicable for drugs that are predominantly unchanged via kidney. It is also convenient for comparing the rate of absorption of drug in a standard form with other dosage forms (23).

In assessing bioavailability for blood data; three parameters usually measured in order to estimate bioequivalence as following:

1. Maximum concentration (C_{max}) represents the highest drug concentration in the systemic circulation. The peak height is related to the intensity of the biologic response and should always be above the minimum effective level or not more than the minimum toxic level of the drug.

2. Time to peak concentration (t_{max}) represents the length of time required to achieve the maximum concentration of the drug in the systemic circulation. The parameter describes the onset of the peak level of the biological response and can be utilized as a rough estimation for the rate of absorption. Its value is critical in evaluation the performance of drugs used for the treatment of acute conditions such as analgesics and antispasmodics.

3. Area under the curve (AUC) represents the total integrate area under the concentration/time curve. When comparing a test formulation to a reference, this parameter describes the extent of bioavailability and can be used as a rough estimation of the amount of drug absorbed.

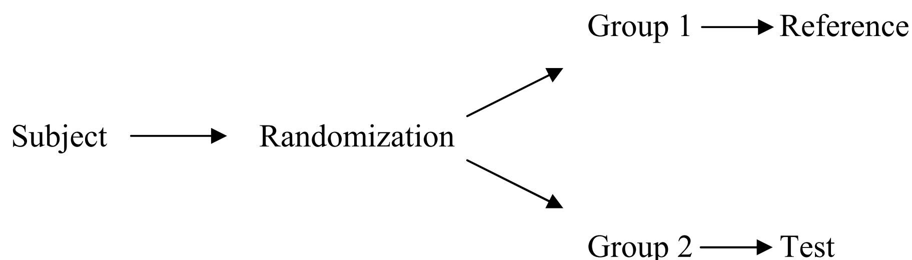
5.4 Factor Affecting Bioavailability

The bioavailability of a drug can be affected by various factors such as dosage form, administration route and site, food and drug interaction. Physicochemical equivalence in drug products does not necessarily assure biological and clinical equivalency. Also, the chemical of a drug from dosage form can be affected by various factors. Therefore, the variation of the chemical may led to failure of therapy of development of intoxication. In the present day, the marketing authorization requirement is based on the following consideration; generic product need, for the assurance of equivalent efficacy and safety, to have not only the same standard of quality but also the same chemical as the innovator's pharmaceutical product.

5.5 Design of Bioequivalence Trials

There are two general types of designs that can be used in a bioequivalence study. There are models are the parallel and the crossover design (25-27).

5.5.1 Parallel design



In the parallel-groups trial, an even number of subjects is divided randomly into two equal groups, one group receiving one formulation of the drug and the other group the second formulation. In most bioequivalence trials, one formulation will be considered as the "reference" and the other the "test" formulation; and the objective of the trial is to determine whether the test formulation is bioequivalent to the reference. The parallel-group concept can be readily generalized to more than two groups, and in this case one formulation will be the reference generally and several test formulations will be compared with it.

This type of design is not the one of choice, but may be the only alternative in certain situations with the crossover design cannot be used. For instance, if a drug with a long half-life is to be studies, the washout period needed may be too long for the crossover to be effective. The ANOVA model the parallel design is

$$X = \mu + FORM + \epsilon$$

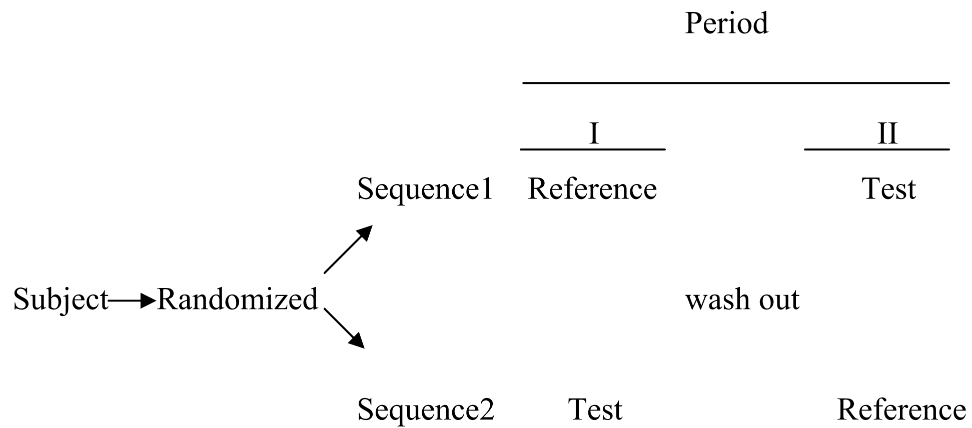
Where:

X	=	parameter of interest
μ	=	overall mean
Form	=	formulation effect
ϵ	=	between and within subject error

One of most striking features of bioavailability data is the enormous differences that can occur among human subjects (inter-subject variability). Not only in their size, weight and presumable blood volume, but also in the way they metabolize a drug. Consequently, in a parallel design, the error sum of squares is

likely to be large and the test for a difference between formulations will be insensitive. This fact has led to a strong preference for the crossover design in bioequivalence trial.

5.5.2 Crossover design



A crossover design is made up of a set of sequences that describe the order in which all or some of the formulations are to be the subjects in the periods. Subjects are randomized to one of sequences and the formulations are randomized by a different formulation (the second period) in the same number of times. For example, in sequence 1, reference (the first period) is followed by test (the second period). The periods are separated by an adequate washout time which should be equal to at least ten elimination half-lives of the drug.

The basic principle behind a crossover design in that subjects generally differ less within themselves when a particular trait is measured repeatedly than they do with other subjects. The immediate consequence is that comparisons of formulations are made within subject. Recall that the intra-subject variance is usually a small component of the variance from the parallel design only. The ANOVA model for a crossover design is

$$X = \mu + SEQ + SUB\ (SEQ) + PER + FORM + \epsilon$$

Where X = parameter of interest (AUC, C_{max}, T_{max})

μ = overall mean

SEQ	=	sequence effect
SUB (SEQ)	=	subject effect nested within sequence effect (or between subject error)
PER	=	period effect
FORM	=	formulation effect
ϵ	=	within subject error

This model assumes no interaction between the main effect of sequence, period, treatment and that the sample variances are homogenous (24).

For assessment of bioequivalence, a typical approach is to employ the standard two-sequence, two-period (2x2) crossover design. Subjects are randomly assigned to receive either sequence of RT or sequence of TR, where T and R are test product and reference product, respectively. Subjects within sequence RT receive product R during the first dosing period and product T during the second dosing period. Also, subjects within sequence TR receive product T during the first dosing period and product R during the second dosing period. Usually, dosing periods are separated by a washout of sufficient length for the drug received in the first period to be completely eliminated from the body. Note that for convenience sake, we the standard 2x2 crossover design by RT and TR.

Under the standard 2x2 crossover design, bioequivalence can be assessed using Schuirmann's two one-sided tests procedure or the method of confidence interval. In addition, a two sized tests procedure for assessment of bioequivalence in variability of bioavailability. As a result, under the standard 2x2 crossover design, average bioequivalence can be assessed.

One of major disadvantages for of using the standard 2x2 crossover design is that the sequence effect is confounded with carry-over effect which cannot be separated and estimated. As a result, if we observe a statistically significant sequence effect, it means that there is a true sequence effect, or there is true carry-over effect, or there is true formulation-by-period interaction, or there is failure of randomization. In this case, the FDA recommends that the assessment of bioequivalence (average bioequivalence) should be carefully examined. For example,

the FDA guidance indicates that bioequivalence can still be claimed when there is a statistically significant sequence effect under certain circumstances.

As indicated earlier, it is recognized that average bioequivalence does not guarantee drug interchangeability under current regulatory requirement because the assessment of average bioequivalence ignores intrasubject variabilities and subject-by-formulation interaction. Under the standard 2x2 crossover design, however, statistical assessment for average bioequivalence by the confidence interval approach or Schuirmann's two one-sided tests procedure is still valid even in the presence of subject-by-formulation interaction and unequal intrasubject variabilities between the test and reference formulation. Since each subject only receives each formulation once, the standard 2x2 crossover design can neither provide independent estimates of intrasubject variability nor give a test for the presence of the subject-by-formulation interaction. Estimation of intrasubject variabilities and the subject-by-formulation interaction provide useful information for assessment of population bioequivalence for drug prescribability and for assessment of individual bioequivalence for drug switchability. As a result, this information is necessary for establishment of drug interchangeability. To provide independent estimation of intrasubject variability and/or study the subject-by-formulation interaction, it is recommended that each formulation should be administered at least twice to each subject.

CHAPTER III
MATERIALS AND METHODS

Materials

1. Atorvastatin (TO chemical, Ltd., THAILAND)
2. Fluvastatin (TO chemical, Ltd., THAILAND)
3. Acetonitrile (AR grade, J.T.Baker, USA)
4. Acetonitrial (HPLC grade, J.T.baker, USA)
5. Methanol (AR grade, J.T.Baker, USA)
6. Water (HPLC grade, Merck, Germany)
7. Ammonium acetate (Merck, Germany)
8. Acetic acid (Analar R, England)

Equipments

1. Vortex mixer (Jurabo Lab., German)
2. API-4000 LC-MS/MS system (MDS SCIEX, Canada)
3. Column Higgins C_{18} 10*3.0 cm 5micron
4. Centrifuge machine (Mikrp, Germany)
5. pH meter (Orion, USA)
6. pH electrode (Orion, USA)
7. Ultrasonicator (Sornicor®, USA)
8. Electronic analytical balance (Metter Electronic® Corp., USA)
9. Micropipette (50-200 µl) (Glison, France)
10. Micropipette (200-1000 µl) (Glison, France)
11. Pipette tips (1-200 µl) (Axygen, USA)
12. Pipette tips (200-1000 µl) (Axygen, USA)
13. Polypropylene microtube 2.0 ml (Axygen, USA)
14. Nylon membrane filter 0.45 µm (National Scientific, UK)
15. Parafilm (American National Can™, USA)
16. Aluminium foil (Reynold Metals Company, USA)

17. Volumetric flask (10, 25, 100, 250, 1000 ml)
18. Transfer pipette (1, 2, 3, 4, 5, 10, 15, 20,25 ml)
19. Cylinder (10, 100, 250, 1000 ml)
20. Evaporator (Rapid vap., USA)

Methods

1. Method for bioanalysis of atorvastatin

1.1 Preparation of standard solution

1.1.1 Atorvastatin stock standard solution

Stock solution of atorvastatin is prepared by dissolving 20 mg of atorvastatin in 200 ml of 50% acetonitrial in water. Separated solutions are prepared for calibration curve and quality control sample. Furthermore, solutions are obtained by serial dilution of stock solution with 50% acetonitrial in water. All stock solutions are stored at 2 to 8 °C.

1.1.2 Fluvastatin stock internal standard solution

A stock solution of Fluvastatin is prepared by dissolving 12 mg of compound in 100 ml 50% acetonitrial in water. The solution of Fluvastatin is diluted 1:10 (v/v) with 50% acetonitrial in water and this solution is used as the internal standard.

1.2 Sample preparation and Solid-phase extraction

Preparation of sample from healthy volunteers and plasma samples spiked with aliquots of the analytes are preformed by SPE, and the samples are kept frozen at -80 °C at all times until measure. The plasma samples 250 µl are spiked with 6 ng/ml of internal standard, and 200 µl of 0.1 mM acetic acid (pH=3) are added after which they are vortexed and centrifuged at 18000 rpm, 10 min. The 100 µl of supernatant is subsequently transferred to SPME.

SPE extraction procedure

1. Sorbent Conditioning
   a. Pipette 100 μl 100% methanol, repeat
   b. Pipette 100 μl 0.1 mM acetic acid pH ~ 3, repeat
2. Sample Application: pipette 100 μl prepared sample as shown in the previous section and slowly expel the sample out of the tip
3. Rinse Step: pipette 100 μl 5% aqueous methanol and expel the solution
4. Drying Step: quickly pipette air through the tip 2 to 4 times to dry the sorbent bed
5. Elution: pipette and elute with 100 μl of 95:5 methanol:10 mM aqueous formic acid, repeat for total elution volume of 500 μl
6. Evaporation to dryness under a stream of nitrogen.
7. The residual are reconstituted in 100 μl acetonitril:water (50:50 v/v) and centrifuged at 18,000 rpm., 10 min at 4 °C, prior to transferal to HPLC vial and injection of 10 μl in the HPLC system

1.3. LC-MS/MS condition for the analysis of atorvastatin follows:

1.3.1 Chromatographic condition

The liquid chromatograph system consisted of algilent serial 1100 LC binary pump and auto-sampler. The analytes is separate through Higgins C18 10x3.0 cm 5micron at a flow rate 0.6 ml/min under 30 °C. Mobile phase is optimized at 60/40 ammonium acetate (10 mM)/acetonitril. The retention time is about 4.5 min for atorvastatin acid and fluvastatin.

1.3.2 MS/MS conditions

A PE Sciex API 4000 triple quadrupole mass spectrometer interfaced with the liquid chromatograph via a turbo ion spray source is used for mass analysis and detection. The ESI temperature is at 350 °C. Quantitation is performed using multiple reaction monitoring (MRM) precursor-product ion transitions at *m/z* 559.2 and 440.1 for atorvastatin acid and *m/z* 412.2 and 224.2 for internal standard, respectively (Table 2).

Table 2. Tandem mass-spectrometer main working parameters

Parameter	Value
Source temperature (°C)	400
Dwell time per transition (ms)	200
Ion source gas (gas 1) (psi)	20
Ion source gas (gas 2) (psi)	40
Curtain gas (psi)	25
Collision gas (psi)	5
Ion spray voltage (V)	5500
Entrance potential (V)	10
Declustering potential (DP) (V)	98 (analyte) and 80 (IS)
Collision energy (V)	50 (analyte) and 30 (IS)
Collision cell exit potential (V)	30 (analyte) and 13 (IS)
Mode of analysis	Positive
Ion transition for atorvastatin (*m*/*z*)	559.2/440.1
Ion transition for fluvastatin (*m*/*z*)	414.2/224.2

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring. A turbo electrospray interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 2 Data processing was performed on Analyst 1.4 software package (SCIEX).

l.4 Method validation (5)

1.4.1 Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyze in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least 5 six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ). Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyze, each analyze should be tested to ensure that there is no interference**.**

1.4.2 Precision

The precision of a bioanalytical method is a measure of random error and is defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage coefficient of variance (% CV) or relative standard deviation (R.S.D.) of the replicate measurements.

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

SD = standard deviation

$\bar{X}$ = mean value of analyzed drug concentration in spiked sample

1.4.2.1 Intra-Assay Precision

This is also known as repeatability i.e. the ability to repeat the same procedure with the same analyst, using the same reagent and equipment in a short interval of time, e.g. within a day and obtaining similar results.

1.4.2.2 Inter-Assay Precision

The ability to repeat the same method under different conditions, e.g. change of analyst, reagent, or equipment; or on subsequent occasions, e.g. over several weeks or months, is covered by the between batch precision or reproducibility, also known as inter assay precision. The reproducibility of a method is of prime interest to the analyst since this will give a better representation of the precision during routine use as it includes the variability from a greater number of sources.

A minimum of three concentrations in the range of expected concentrations is recommended. The %CV determined at each concentration level, should not exceed 15% except for the LLOQ, where it should not exceed 20%.

1.4.3 Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy. The accuracy is calculated as follows.

$$\%\text{Deviation} = \frac{\text{measure concentration} - \text{added concentration}}{\text{added concentration}} \times 100$$

1.4.4 Recovery

Recovery of assay procedure is evaluated by comparing the peak area ratio of peak area for extracts blank plasma spiked with each standard respect to the

peak area of the bioequivalent unextracted standard solution. The percentage of recovery was calculated by the equation:

$$\% \text{ Extraction recovery} = \frac{\text{Peak area ratio of drug to IS in plasma after extraction sample}}{\text{Peak area ratio of drug to IS in unextracted sample}} \times 100$$

1.4.5. Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyst in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analyses during sample collection and handling, after long-term (frozen at the 7 intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyze stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of analyze in the appropriate analyze-free, interference-free biological matrix. Stock solutions of analyze for stability evaluation should be prepared in an appropriate solvent at known concentrations.

1.4.5.1 Freeze and thaw stability

Analyze stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle should be repeated two more times, and then analyzed on the third cycle. If an analyst is unstable at the intended storage temperature, the stability sample should be frozen at -80 °C during the three freeze and thaw cycles.

1.4.5.2 Short-term temperature stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

1.4.5.3 Long-term stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

1.4.5.4 Stock solution stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

1.4.5.5 Post-preparative stability

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of an analyze stability in a biological matrix can be used. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

2 Pharmacokinetics and bioequivalence study

2.1 Crossover design for bioequivalence study

A crossover design is made up a set of sequences which describe the order in which all or some of the formulations are to be the subjects in the periods. Subjects are randomized to one of sequences and the formulations are randomized to the letters defining the group of sequences. The randomization of atorvastatin products were show in Table 1. Each formulation is followed by a different formulation (the second period) in the same number of times. For example, in sequence 1, reference (the first period) is followed by test (the second period). The periods are separated by an adequate washout time which should be equal to at least ten elimination half-life of the drug.

2.2 Subjects

Twenty-four healthy volunteers are recruited in the study based on the follow criteria.

Inclusion criteria

1. Healthy, confirmed by physician through physical examination, medical history and laboratory test.
2. Male and female subjects between the age of 18 and 45 years with the body weight within $\pm$10% of ideal body weight or BMI between 18-24 kg/m^2.
3. Do not take any medication during a period of at least 2 weeks before and throughout the study.
4. Do not take any drugs that induce the matabolizing enzyme (e.g. phenytoin, phynobartital, rifampicin) and/or inhibit the metabolizing enzyme (e.g. cimetidine, ketoconazole, erythromycin) and/or nonsedating H_1-receptor antagonists (e.g. loratadine, fexofenadine) within 90 days before and throughout the study.
5. Do not drink alcohol and do not smoke cigarette.
6. Agree with the study and sign the written inform consent.

Table 3. Randomization table of Atorvastatin products of each subject

Subject number	First time	Second time
1	R	T
2	R	T
3	T	R
4	R	T
5	T	R
6	T	R
7	R	T
8	R	T
9	T	R
10	R	T
11	T	R
12	R	T
13	T	R
14	R	T
15	T	R
16	R	T
17	T	R
18	T	R
19	T	R
20	R	T
21	T	R
22	T	R
23	R	T
24	T	R

Note: T = Test product, R = Reference product

Exclusion criteria

1. History of gastrointestinal, cardiovascular, hepatic and renal diseases.
2. History of smoking.
3. History of alcoholism and/or drug abuse.
4. History of hypersensitivity of atorvastatin.

2.3 Drug administration

After fasting overnight or not less than 8 hours, each subject is administered orally a single dose of atorvastatin tablet 40 mg with 240 ml of drinking water. Food is withheld until 4 hours after dosing. Drinking coffee, tea, other caffeine-containing beverages, alcohol, fruit juice (e.g. grapefruit juice) and milk are not allowed 24 hours prior and during the study.

2.4 Collection plasma sample

Five milliliters of blood are withdrawn from heparinized catheter prior to drug administration and at 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 48 hours after dosing and then centrifuge at 5000 rpm for 10 minutes at room temperature. The plasma is separated and kept at stable temperature (-80 $^{\circ}$C) until assay.

2.5 Bioequivalence data analysis

2.5.1 Noncompartmental analysis

The WinNonlin professional edition version 3.1 is used for noncompartmental and bioequivalence analysis. The plasma concentration time profile in each subject is fitted using noncompartmental model of extravascular input. The λ_z is calculated according to the log-linear portion of terminal phase of plasma concentration time course. The time range for determining λ_z is selected to get log-linearity, then AUC_{last} is calculated. The T_{max} and C_{max} are observed directly from the plasma concentration time curve. The average of these parameters for test and reference are calculated.

2.5.2 ANOVA analysis

The ANOVA model for two way crossover design is as following:

$$X = \mu + SEQ + SUB(SEQ) + PER + FORM + \varepsilon$$

Where

X is the each noncompartmental parameters, μ is overall mean, SEQ is the sequence effect, SUB(SEQ) is subject effect nested within sequence effect, PER is period effect, FORM is formulation effect and ε is residual error.

The WinNonlin program is used to perform the ANOVA analysis. The F-test for the formulations is conducted to test the hypothesis of the equality of least square means of each test parameters and those of each reference parameter. The bioequivalence is based on the criteria that the percent ratio of test parameters to reference parameters is within the range of 80-125% with the 90% ($\alpha = 0.05$) level of confidence. For AUC(0-24), AUC(0-INF) and C_{max}, logarithm transformation is conducted whereas for T_{max}, the normal value was used. The useful consequence of the logarithmic transformation is that confidence intervals constructed following ANOVA was on the ratio of mean rather than difference of means.

CHAPTER IV
RESULTS AND DISCUSSION

1. Mass spectrometry

In order to develop a method with the desired sensitivity (< 1 ng/ml), it was necessary to use MS/MS detection, as the compound did not posses the UV absorbance or fluorescence properties needed to achieve this limit. The inherent selectivity of MS-MS detection was also expected to be beneficial in developing a selectivity and sensitive method. The positive ion turboIonspray Q1 mass spectrum and product ion mass spectrum of atorvastatin are shown in figure2 and figure 3 respectively. The $[M + H]^+$ was the predominant ion in the Q1 spectrum, and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from *m/z* 559.2 to 440.1 for atorvastatin and *m/z* 414.2 to 224.2 for fluvastatin (IS).

The ion spray voltage, declusturing potential, entrance potential, collision energy and collision cell exit potential were optimized to deliver effective fragmentation of the $[M + H]^+$ without excessive fragmentation, which would have reduced sensitivity. The parameters presented in Table 2 are the result of this optimization. The positive ion TurboIonspray Q1 mass spectrum and product ion mass spectrum of internal standard are shown in Figure 4 and Figure 5, respectively.

2. Performance of LC

The HPLC conditions were optimized such that the retention time was kept short at about 4.4 min in order to assure high throughput. The total runtime for each sample was 10 min by using mixture of 50 mM ammonium acetate buffer/acetonitrile (10:90). The higgen C18 HPLC column (3 µm, 100 mm × 3 mm) was chosen based on peak shape and the best response for atorvastatin and fluvastatin with acceptable analysis time**.** Internal standard substance should match the chromatographic retention, recovery and ionization properties with the matrix of atorvastatin.

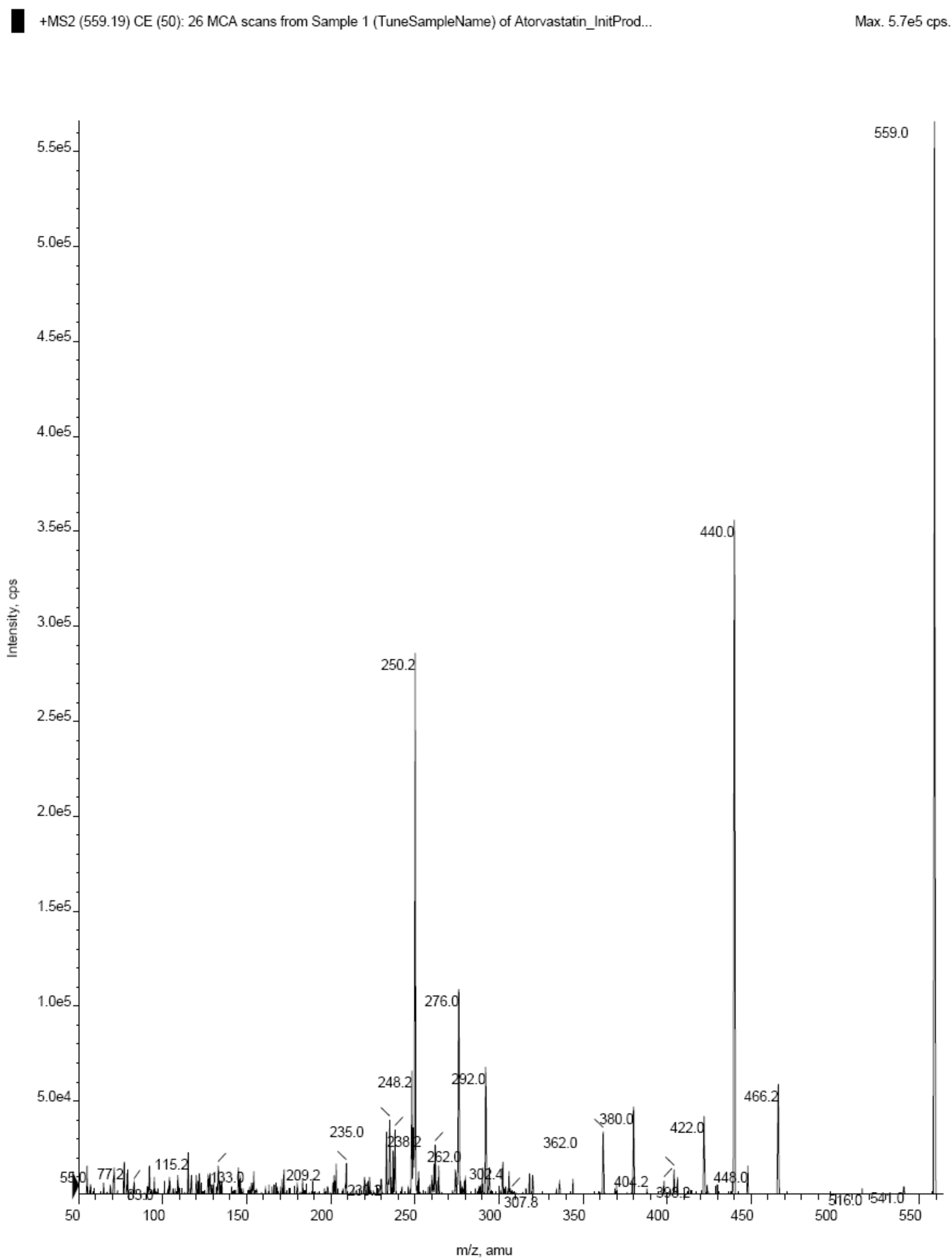


Figure 2. Full scan positive ion turboIonspray Q1 mass spectra of Atorvastatin.

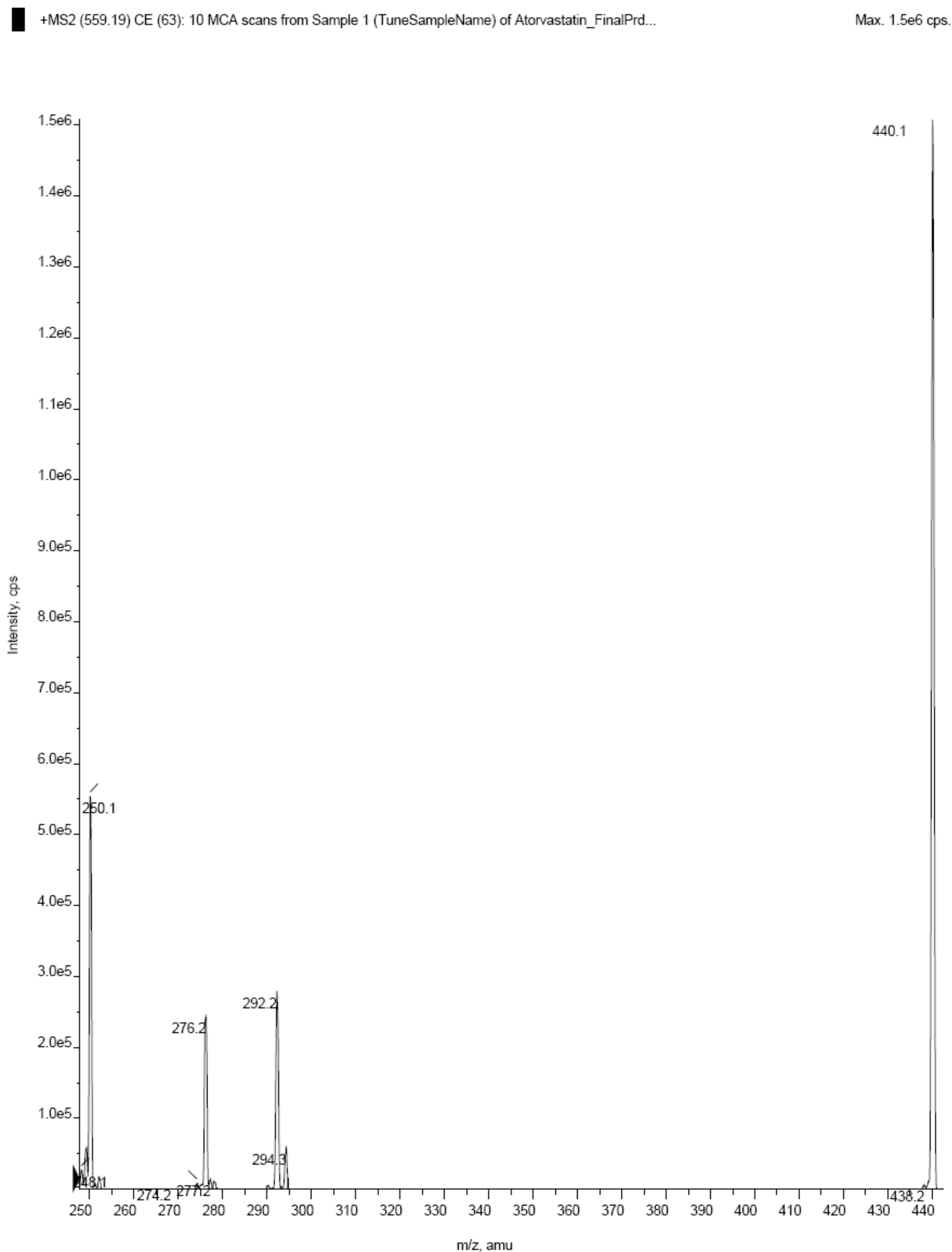


Figure 3. Full scan positive ion TurboIonspray product ion mass spectra of Atorvastatin.

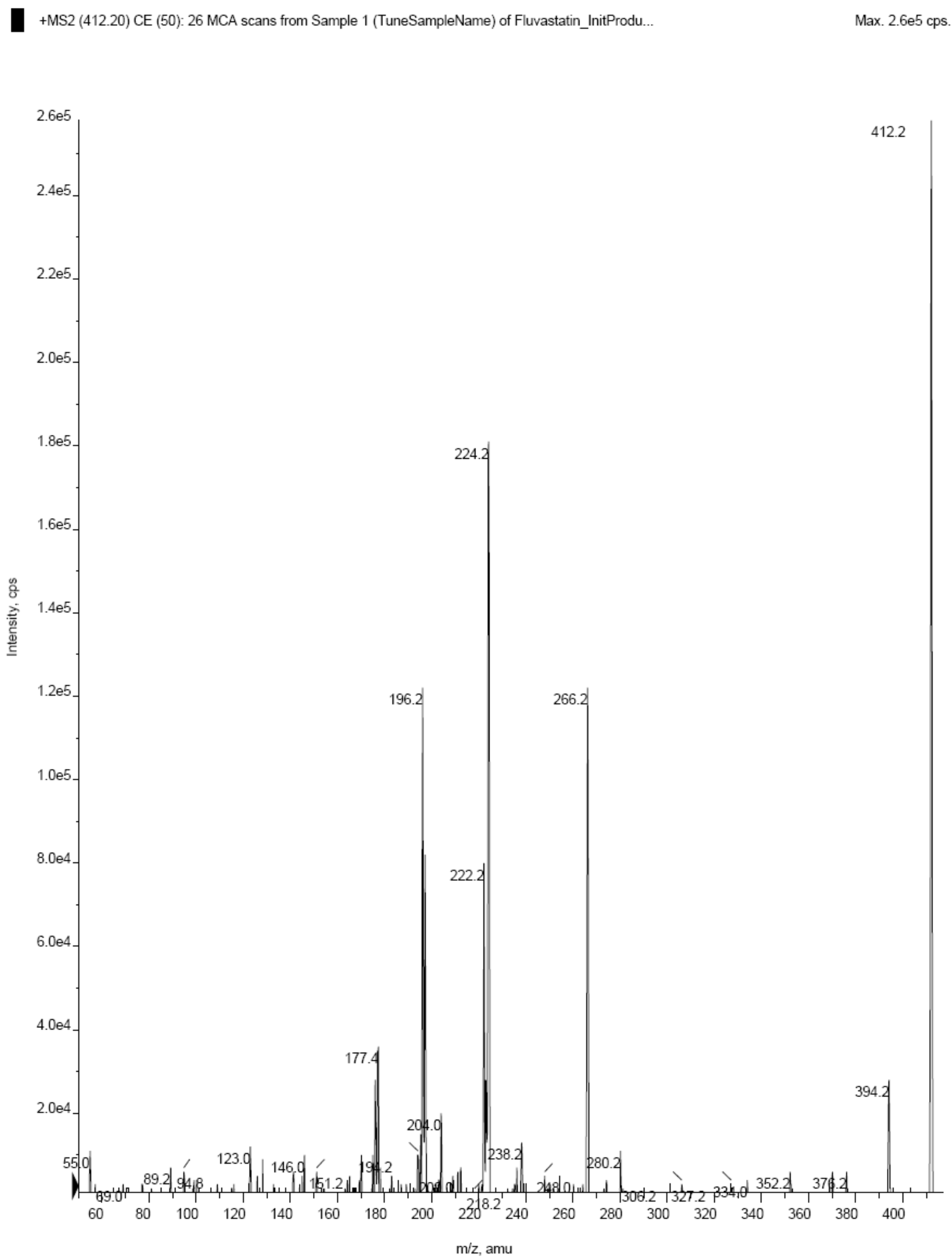


Figure 4. Full scan positive ion TurboIonspray Q1 mass spectra of internal standard (fluvastatin).

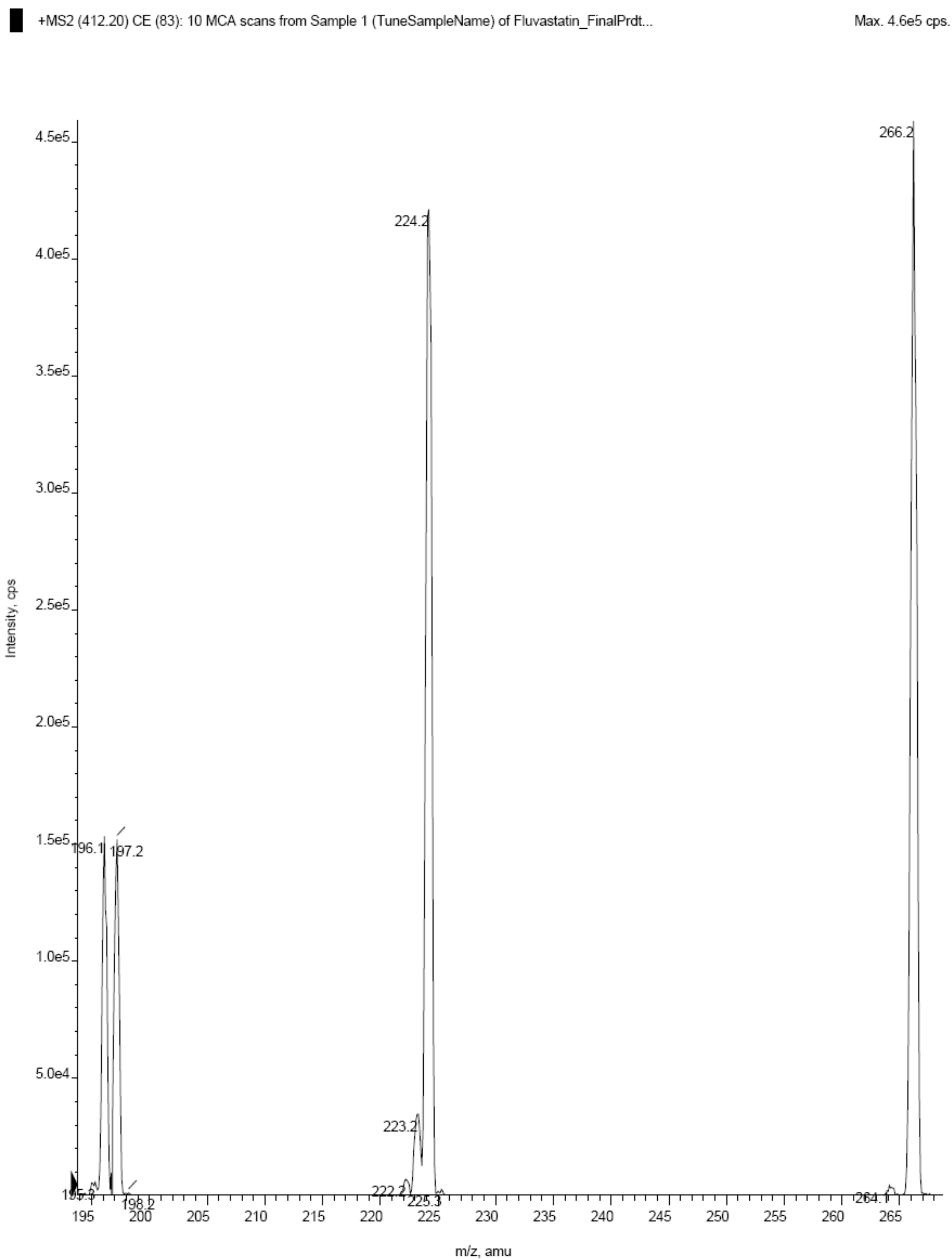


Figure 5. Full scan positive ion TurboIonspray product mass spectra of internal standard (Fluvastatin).

Fluvastatin was found to fulfill these criteria sufficiently. The matrix effects were similar to the matrix effects of atorvastatin. Hence fluvastatin has been chosen as internal standard in the quantitative assay for atorvastatin from plasma.

3. Bioanalytical method validation

3.1 Specificity and selectivity

The specificity/selectivity of the method was investigated by analyzing six blank human plasma extract (n=6) (Figure 6-11), extract spiked drug only (Figure 12) and extract spiked internal standard only (zero sample) (Figure 13), no significant interference in the blank plasma traces was seen from endogenous substances in drug free human plasma at the retention time of the analyte.

Under the chromatographic conditions described, atorvastatin and the internal standard were eluted with nearly same retention time.

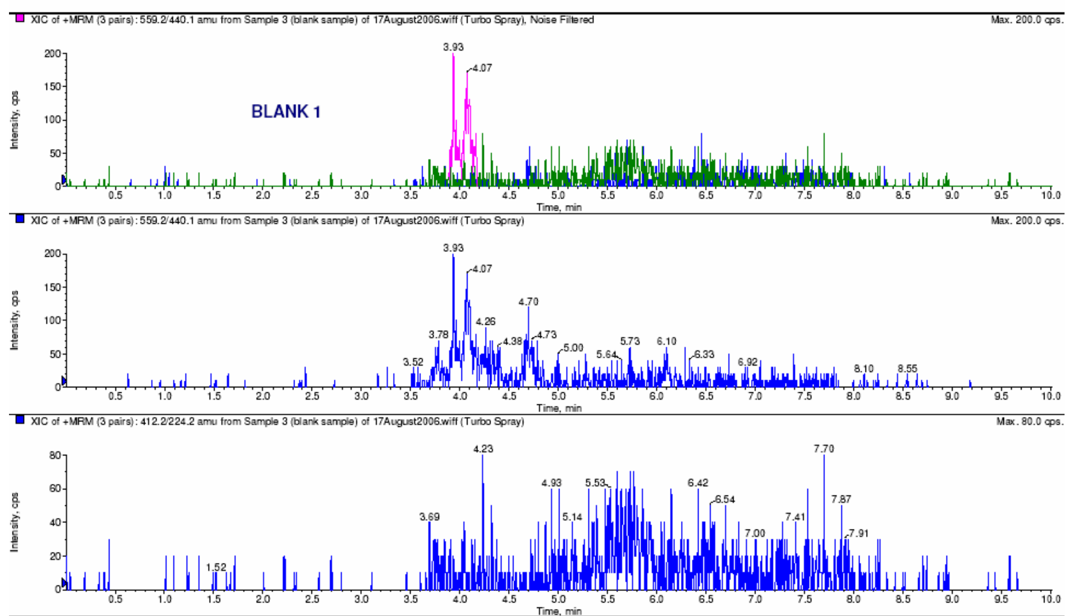


Figure 6. MRM ion-chromatograms resulting from the analysis of blank No.1 (drug and internal standard free) human plasma for atorvastatin and Internal standard.

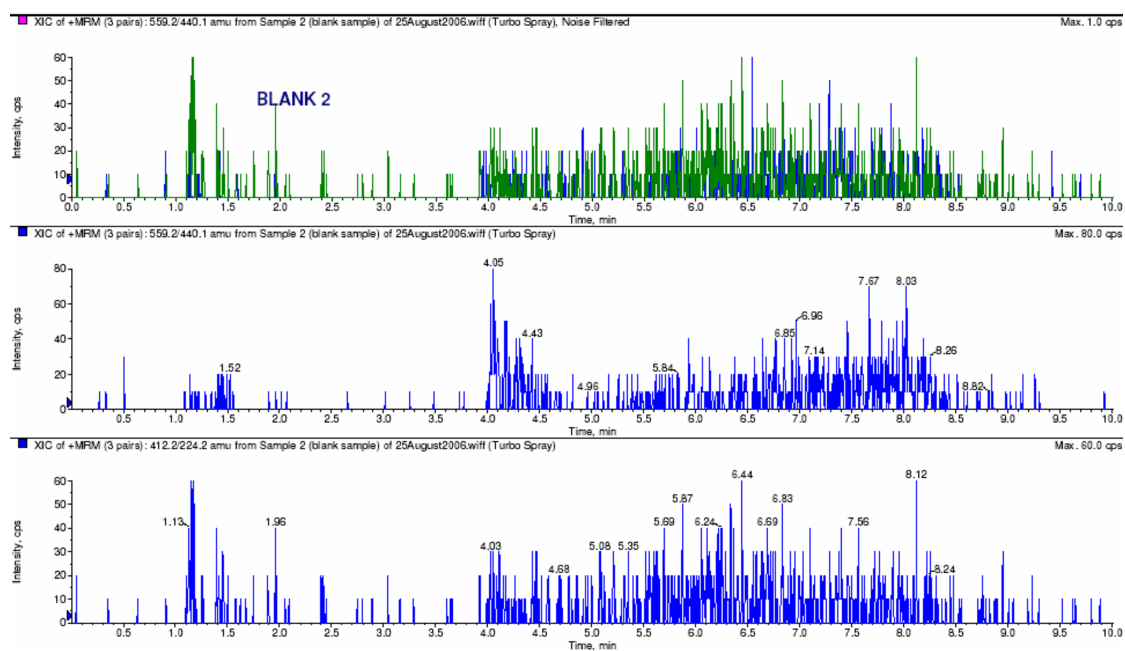


Figure 7. MRM ion-chromatograms resulting from the analysis of blank No.2 (drug and internal standard free) human plasma for atorvastatin and internal standard.

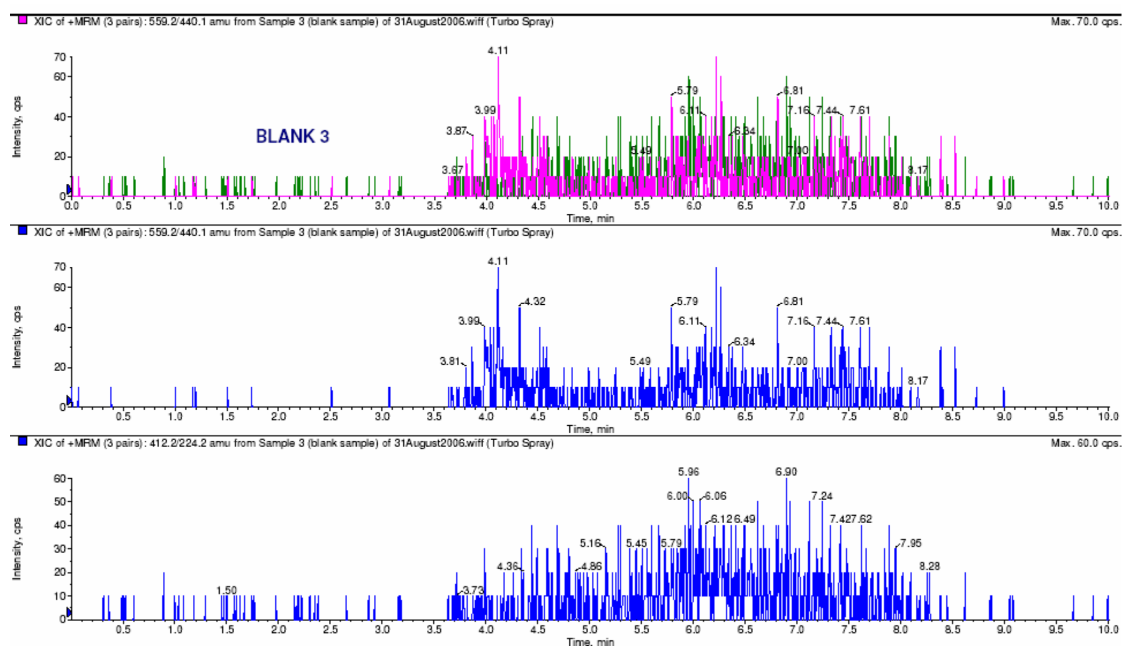


Figure 8. MRM ion-chromatograms resulting from the analysis of blank No.3 (drug and internal standard free) human plasma for atorvastatin and internal standard.

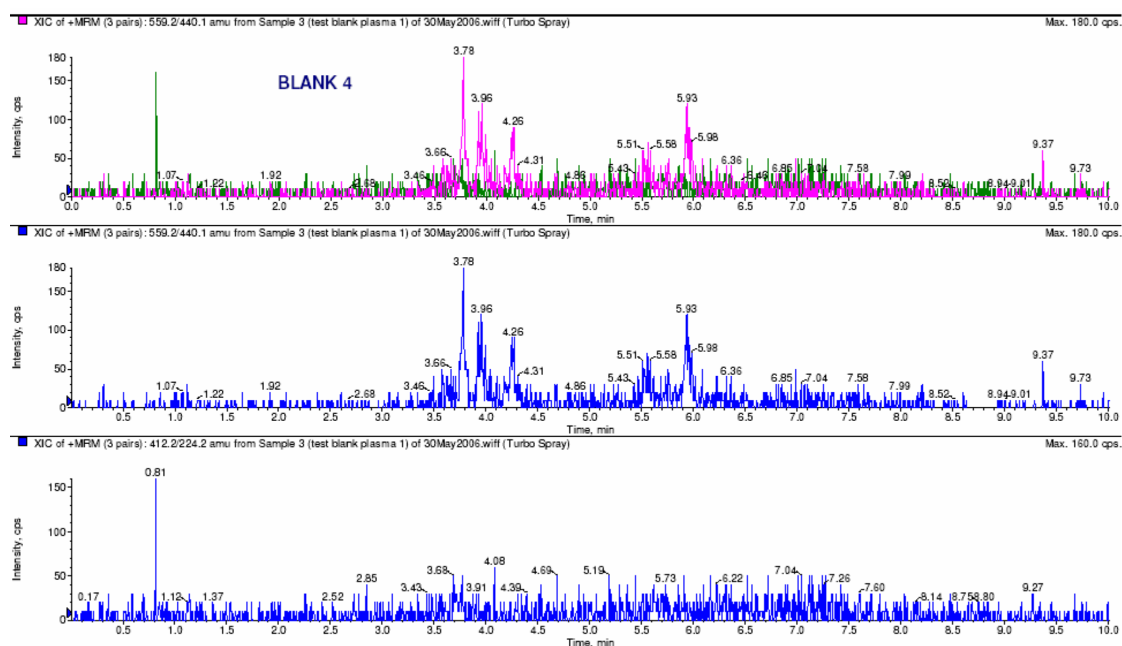


Figure 9. MRM ion-chromatograms resulting from the analysis of blank No.4 (drug and internal standard free) human plasma for atorvastatin and internal standard.

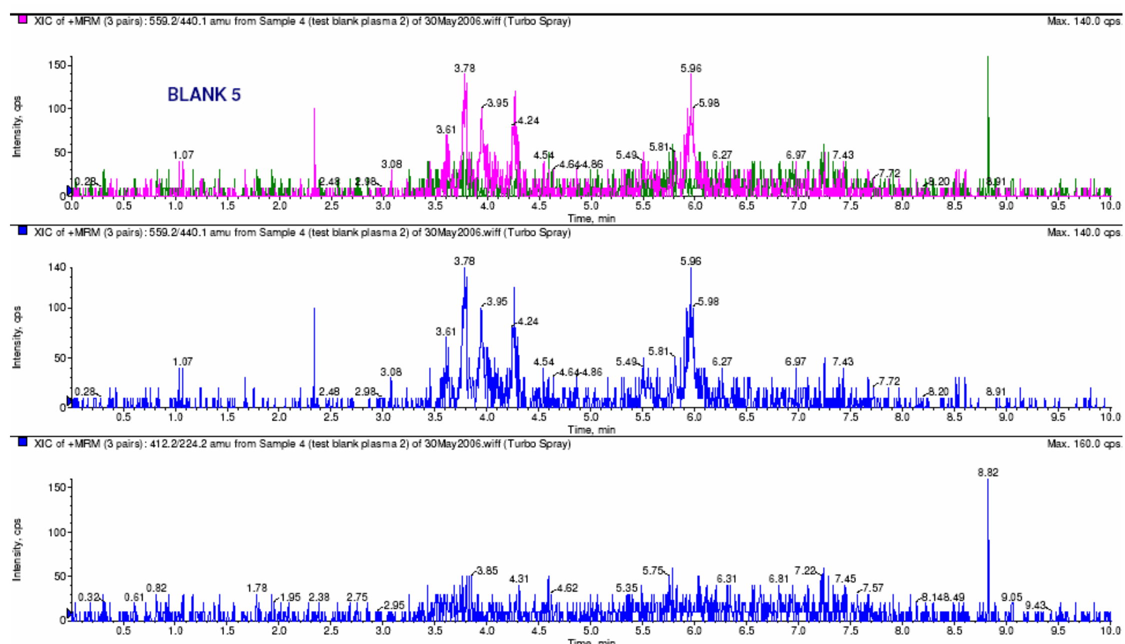


Figure 10. MRM ion-chromatograms resulting from the analysis of blank No.5 (drug and internal standard free) human plasma for atorvastatin and internal standard.

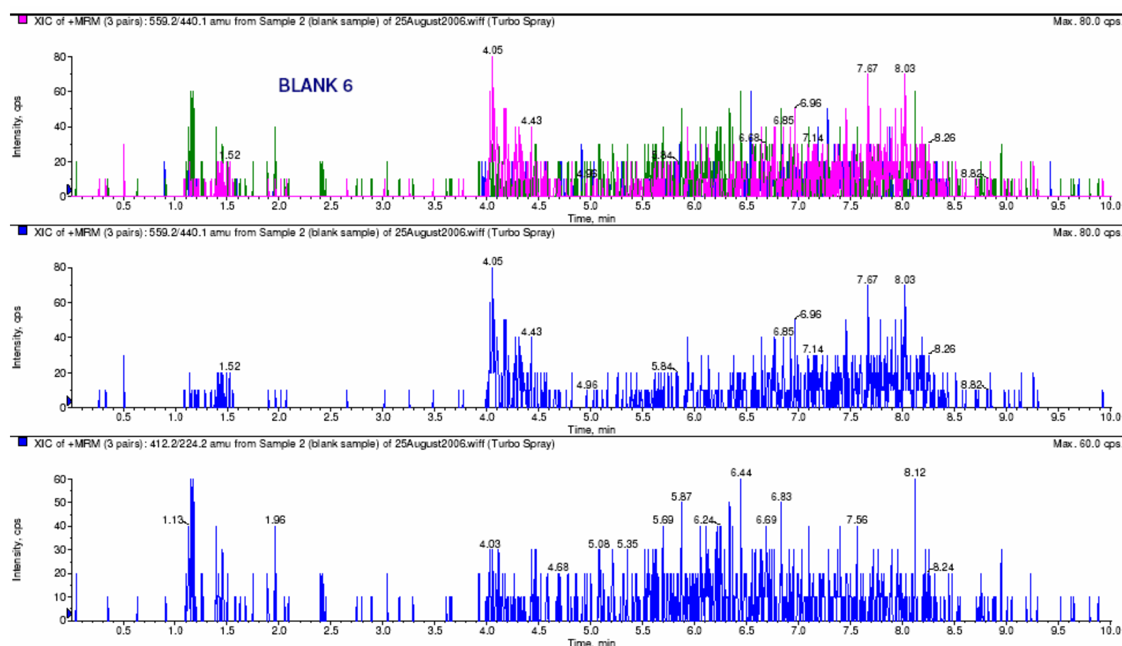


Figure 11. MRM ion-chromatograms resulting from the analysis of blank No.6 (drug and internal standard free) human plasma for atorvastatin and internal standard.

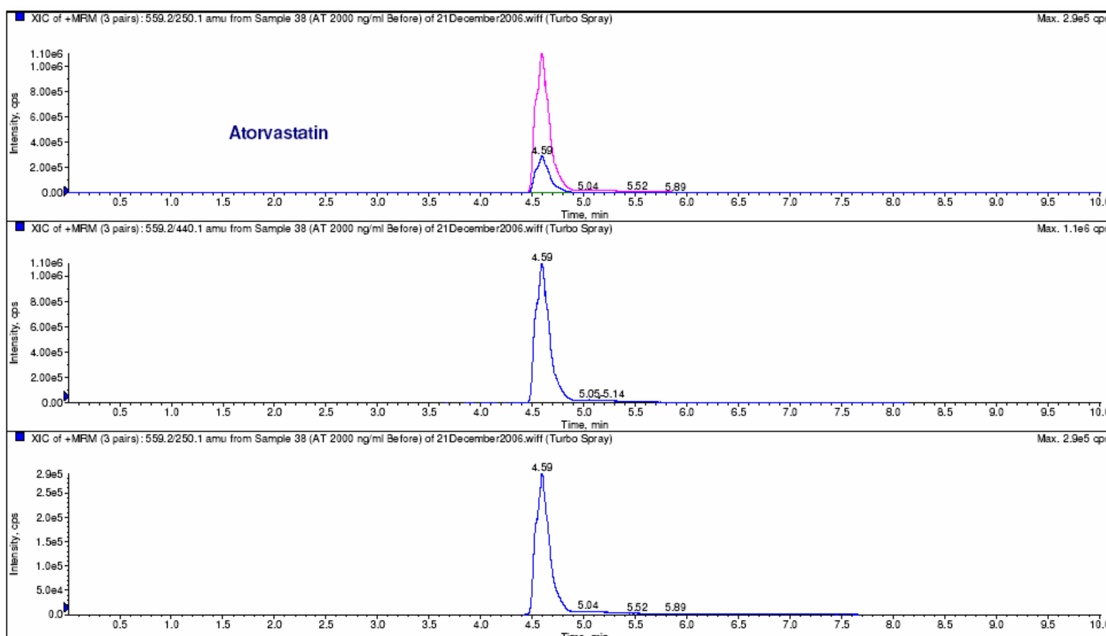


Figure 12. MRM ion chromatograms resulting from the analysis of blank plasma spiked with drug only.

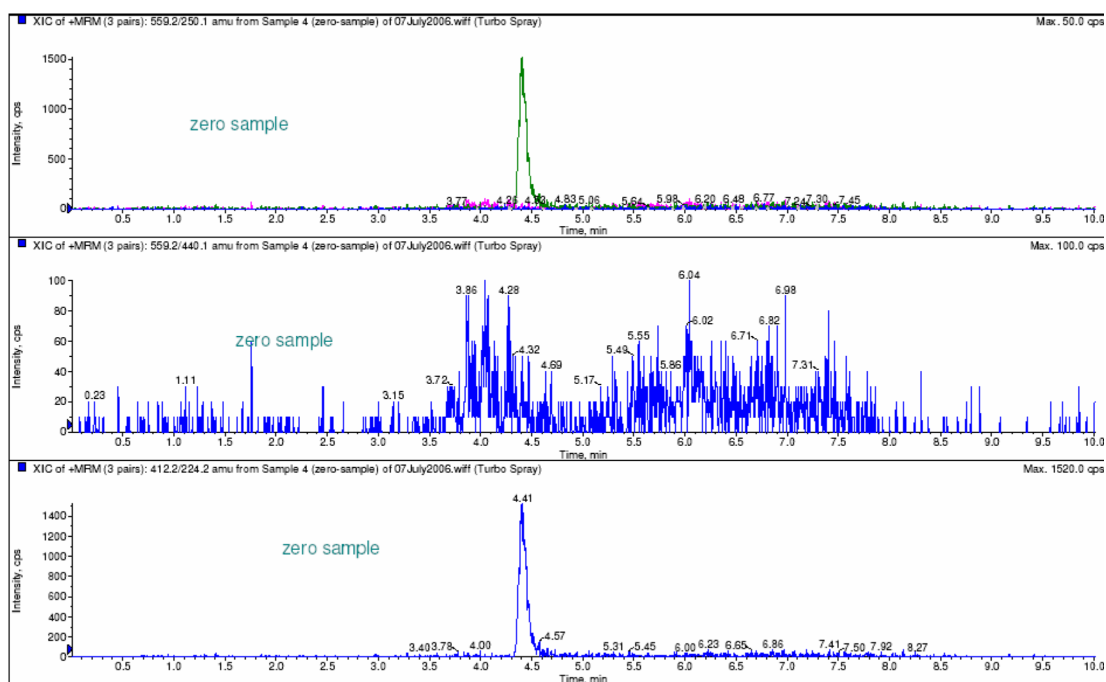


Figure 13. MRM ion chromatogeams resulting from the analysis of blank plasma spiked with internal standard only (Zero sample).

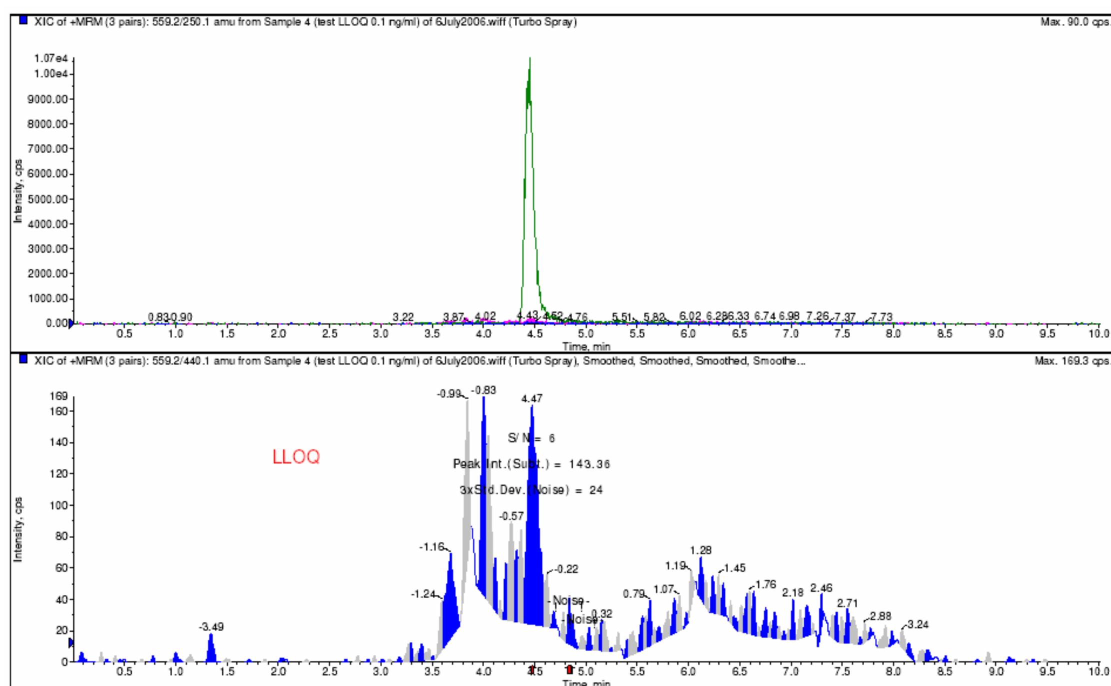


Figure 14. Representative MRM ion chromatograms resulting from the analysis 0.1 ng/ml (LLOQ) of atorvastatin.

3.2. Extraction recovery

The average recoveries for atorvastatin at three different concentrations (low, medium and high QC samples) are show in Table 4.

The recovery of the internal standard was 84.16% at the concentration used in the assay (24 ng/ml)

3.3. Calibration curve

Calibration curve was linear over the concentration range of 0.2-80 ng/ml for analyte. The calibration curve was fitted to a $1/x^2$ weighted linear regression (where x was the concentration of the analyte) as this was judged to be the weighting, which made the assay most robust. The mean linear regression equation of calibration curve for the analyte was y=0.0312 (±0.0286) + 1.8250 (±0.2668)x, where y was the peak area ratio of the analyte to I.S. and x was the concentration of the analyte. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.9965 for the analyte. Table 5 summarizes the calibration curve results for the analyte.

Table 4. The recoveries of atorvastatin

Sample concentration (ng/ml)	Recovery (%) (mean ± S.D., n=6)
0.25 (QC A)	84.16 ± 7.99
10 (QC B)	96.82 ± 6.14
40 (QC C)	102.84 ± 6.73

Table 5. Precision and accuracy data of back-calculated concentrations of calibration samples for atorvastatin in human plasma.

Concentration added (ng/mL)	Concentration found (mean ± S.D. *n* = 10) (ng/mL)	CV (%)	Deviation (%) (inaccuracy)
0.2	0.209 ± 0.023	9.94	4.85
0.5	0.470 ± 0.033	8.86	-6.08
2	1.865 ± 0.150	8.09	-6.75
5	4.963 ± 0.594	5.73	−0.74
15	15.72± 1.429	4.63	4.8
20	21.17 ± 1.601	3.87	5.85
50	51.25 ± 3.212	7.57	2.5
80	77.23 ± 4.372	4.02	−3.46

3.4. Limit of detection and quantitation

The lower limit of detection (LOD), estimated as the amount of atorvastatin furnishing a signal three time greater than the noise (S/N=3:1), was showed to be 0.05 ng/ml (Figure 15).

The lower limit of quantitation (LLOQ) was defined as the lowest concentration that can be measured with acceptable accuracy and precision, and found to be 0.1ng/ml in human plasma (Table6). The mean response for analyte peak at the assay sensitivity limit (0.1ng/ml) was about 6 fold greater than the mean response for peak in six blank human plasma samples at the retention time of the analyte.

The between-batch precision at the LLOQ was 3.475% and between-batch accuracy was 99.18% (Table7). The within-batch precision was 3.48-9.28% and within-batch accuracy was 99.18-115% (Table6).

The LLOQ was =0.1ng/mL but Hermann et al.2005 methods, in which LLOQ was 0.2ng/mL reported with solid phase extraction.

3.5. Accuracy and precision

Intraday and interday assay were performed to evaluate precision (%CV) and accuracy.

The coefficients of variation for intraday was between 0.72-8.87 % and the accuracy values were found to be between 99.18-113.67% in human plasma as present in Table6.

Interday accuracy was between 96.32-106.8% with coefficient of variation of 2.348-6.226% Table7.

The result suggested that within run and between run experiments the precision and accuracy for the analyte met the acceptance criteria.

Table 6. Between-run precision and accuracy of the method for determining atorvastatin concentrations in plasma samples.

Accuracy and precision	QC sample	Concentration added (ng/ml)	Concentration found (mean ± S.D. *n* = 6) (ng/ml)	CV (%)	Accuracy (%)
Intra day	LLOQ	0.1	0.099±0.003	3.47	99.18
	Low	0.25	0.250±0.022	8.87	99.95
	Mid	10	11.37±0.082	0.72	113.67
	High	40	45.18±0.646	1.43	113

Table 7. Within-run precision and accuracy of the method for determining atorvastatin concentrations in plasma samples.

Accuracy and precision	QC sample	Concentration added (ng/ml)	Concentration found (mean ± S.D. *n* = 5) (ng/ml)	CV (%)	Accuracy (%)
Interday	LLOQ	0.1	0.1068±0.006	6.226	106.8
	Low	0.25	0.2408±0.062	5.579	96.32
	Mid	10	8.169±0.116	2.348	99.11
	High	40	40.474±1.29	3.235	101.19

3.6 Stability

3.6.1. Freeze-thaw stability

The results showed that the analyte was stable in human plasma through three freeze-thaw cycle (Table8). The precision was <10% and accuracy was<±10%. The results demonstrated that human plasma sample could be thawed and refrozen without compromising the integrity of the sample. The stability data were used to support repeat analysis.

3.6.2. Long-term storage

The precision and accuracy for the analyte was < 3% and <± 10%, respectively (Table8). The sample long-term storage stability at -20 °C was acceptable for storage for subject samples.

3.6.3. Auto sample stability

The stability of QC sample kept in the autosample for 24 hours was also assessed. The results indicate that solution of atorvastatin and fluvastatin can remain in the autosample for at least 24 hours without showing significant loss in the quantified values, indicating that samples should be processed within this period of time (Table8).

3.6.4. Short-term stability

The precision and accuracy for analyte was <10% and <±10%, respectively (Table8). The results indicate that the analyte was stable in neat plasma for up to 24 hours at room temperature.

3.6.5 Stock solutions stability

The stock solutions were stable for at least 1 month when stored at 4°C by express the %variation <±2 %.(Table9).

Table 8. Stability of atorvastatin in human plasma

Sample concentration (ng/ml) (*n*=5)	Concentration found (mean ± S.D. *n* = 5) (ng/ml)	Precision (CV %)	Accuracy (%)
Short-term stability for 24 h in plasma			
0.25	0.27±0.023	8.377	109.12
40	38.24±1.940	5.058	95.91
Three freeze–thaw cycles			
0.25	0.25±0.006	2.212	100.11
40	36.44±0.935	2.566	91.12
Auto sampler stability for 24 h			
0.25	0.27±0.015	5.34	109.07
40	44.30±1	2.26	110.75
Long-term stability for 3 month in plasma (-20 °C)			
0.25	0.26±0.026	10.04	105.28
40	38.37±1.99	5.18	95.95

Table 9. Stock solution stability of atorvastatin and fluvastatin (internal standard)

	Peak areas			
	Stock solution of Atorvastatin (2000 ng/ml)		Stock solution of Fluvastatin (300 ng/ml)	
	Fresh	After 30 days	Fresh	After 30 days
	10135	9599	615910	615120
	10146	9895	615400	602050
	9780	9899	604320	594280
	9868	10003	619530	600880
	9642	9582	599590	587245
	10000	9695	593740	587829
Mean	9928.500	9778.833	608081.667	597900.667
SD	201.423	176.803	10371.568	10494.663
% CV	2.029	1.808	1.706	1.755
% Variation	1.507		1.674	

3. Pharmacokinetics of atorvastatin in healthy volunteers

Mean values of pharmacokinetic parameters of atorvastatin obtained from the plasma-concentration time curve of each product from each subject in the study. The atorvastatin plasma concentration-time profile received from the study would be used in pharmacokinetic analysis by WinNonlin professional edition version 3.1 and noncompartmental model.

Four parameters were studied C_{max}, $AUC_{0\text{-}last}$, AUC0-$_{inf\ (observe)}$ and T_{max}. These parameters were commonly used in bioequivalence analysis.

The plasma concentrations of atorvastatin at specified sampling time obtained from 24 healthy Thai subject following oral administration of 40 mg dose are presented in Table 10 to 32. The plasma-concentration time profiles of individual subject after single oral administration of 40 mg atorvastatin tablet of reference and test product are show in Figure 15 to 38. The mean plasma-concentration time profile with SD bar is shown in Figure 38. Spaghetti plot for plasma concentration time profile of reference product and test product for all subjects are shown in Figure 40-41. The mean and SD value of reference and test product at specified sampling time obtained from 24 healthy Thai subjects are presented in Table38. For most subjects, atorvastatin was rapidly absorbed. It reaches to maximum concentration within 1-2 hour. The highest maximum plasma concentration (36 ng/ml) was found from reference product.

Data of maximum plasma concentration (C_{max}), area under the plasma-concentration time curve from time zero to last time ($AUC_{0\text{-}last}$), area under the plasma-concentration time curve from time zero to the infinity time from observed ($AUC_{0\text{-}inf\ (observed)}$) and time to reach maximum plasma concentration (T_{max}) of atorvastatin after single dose oral administration of 40 mg atorvastatin tablet of the reference and test product were compare as following (28):

1. Maximum plasma concentration (C_{max})

Mean ± SEM values of maximum plasma concentration of atorvastatin were 17.3 ± 1.42 and 16.8 ± 1.51 ng/ml for the reference and the test product, respectively.

2. Area under the plasma-concentration time curve from time zero to the last time ($AUC_{0\text{-}last}$)

Mean ± SEM values of area under the plasma-concentration time curve from time zero to the last time of atorvastatin were 4700 ± 437 and 4660 ± 368 (min x ng/ml) for the reference and the test product, respectively.

3. Area under the plasma-concentration time curve from time zero to the infinity time from observed ($AUC_{0\text{-inf(observed)}}$)

Mean ± SEM values of area under the plasma-concentration time curve from time zero to the infinity time from observed of atorvastatin were 4950 ± 2310 and 4820 ± 1950 (min x ng/ml) for the reference product and the test product, respectively.

4. Time to reach maximum plasma concentration (T_{max})

Median (Min ± Max) values of time to reach maximum plasma concentration of atorvastatin were 75 (15 ± 360) and 120 (30 ± 240) min. for the reference product and the test product, respectively.

For maximum plasma concentration (C_{max}), area under the plasma-concentration time curve from time zero to the last time ($AUC_{0\text{-last}}$), area under the plasma-concentration time curve from time zero to the infinity time from observed ($AUC_{0\text{-inf(observed)}}$), logarithm transformation value was used in analysis. Whereas normal value was used for analyzing time reach to maximum plasma concentration (T_{max}).

The basic pharmacokinetic characteristics of atorvastatin obtained in our study, and those published earlier, are summarized in Table40.

The geometric mean values of area under the plasma-concentration time curve from time zero to the last time ($AUC_{0\text{-inf}}$) for atorvastatin, between 33.5-149.3 ng x ml/hour, reflect the high variability of the pharmacokinetics of atorvastatin acid may occur from pharmacogenetic variation (6, 29-31). The mean values are approximately same the values obtained by HPLC MS/MS after administration of single dose of atorvastatin (9, 16). In contrast, result obtained by HPLC UV methods is more difference values (32).

The comparative dose proportionality of the pharmacokinetics of atorvastatin, the peak concentration (C_{max}) of atorvastatin is good agreement with reported data –it is third measured after a 40 mg dose (32).

The observed median values and range of atorvastatin (T_{max}) were 1.25 hours and 0.2-6 hours, respectively. The median is not difference with the previous reported

values, but the range observed is difference with 0.2-6 hours. Because foods considerably reduce the rate of atorvastatin absorption, the difference may also be because of the longer fasting period in this study, at least 8 hours rather than the 1 to 2 hours report by Kantola et al. and Lilja et al., respectively.

The median elimination half-life was found to be 7.9 hours (range 1.2 to 21 hours), found that it the same values with several reports.

5. The bioequivalence study

T_{max} has nonparametric distribution. A non-parametric test (distribution-free) used to compare observations repeated on the same subjects. This is also called a non-parametric randomized black analysis of variance. The test statistic for the Friedman's test is a Chi-square with k-1 degrees of freedom, where k is the number of repeated measures. The H0 for the test is no difference in mean ranks for repeated measures. When the p-value for this test is small ($p < 0.05$) it has evidence to reject the null hypothesis. Thus, in this cross over design study, the Friedman test in Kinetica 2000 software was used. There are no statistical differences of T_{max} for both formulations. The printout of the result from Kinetica 2000 was illustrated Table 43.

All 24 subjects were completed the study. However due to randomization error, the number of subject in sequence RT was not similar to TR. Thus, the study is unbalanced. There were 11 subjects who were administered in the sequence RT and the other 13 subjects were administered in sequence TR. Thus, the ANOVA type III for unbalanced data was applied for this study. The 90% confidence interval of the logarithmic transformed AUC and C_{max} were both contained in 80-125%. Due to error in randomization, the period and sequence effects were significant. However, there was no treatment effect which also confirmed 90% confidence interval of the logarithmic transformed AUC and C_{max} results. The subject nested in sequence effect is very common to be observed in bioequivalence trial due to the small subject number of subjects. Nonparametric, Friedman's test for T_{max} was also demonstrated the no significantly different between both formulation. Thus, based on the 90% CI criteria, the bioequivalence can be claimed.

Table 10. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.1

Time (min.)	Test	Reference
0	0	0
15	4.51	2.05
30	8.21	13.6
45	22.1	13.8
60	18.1	9.14
90	9.72	5.81
120	5.13	5.39
180	5.04	4.44
240	5.81	3.01
360	4.1	3.03
480	3.08	2.22
720	1.73	1.08
1140	0.379	0.374
2880	0.124	0.713
1140	0.379	0.374
2880	0.124	0.713

Table 11. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.2

Time (min.)	Test	Reference
0	0	0
15	2.41	0.0702
30	7.82	0.179
45	26.6	0.828
60	15	1.54
90	14.8	2.04
120	10	1.91
180	7.49	12.5
240	8.22	9.16
360	5.99	3.74
480	4.58	2.47
720	2.47	1.19
1140	0.616	0.318
2880	0.238	0.045

Table 12. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.3

Time (min.)	Test	Reference
0	0.00	0.00
15	0.81	0.23
30	23.80	0.32
45	21.00	0.80
60	16.50	1.22
90	12.00	2.16
120	10.70	4.93
180	8.42	23.80
240	7.53	17.90
360	5.05	8.90
480	3.88	4.93
720	1.60	2.10
1140	0.44	0.57
2880	0.11	0.22

Table 13. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.4

Time (min.)	Test	Reference
0	0.00	0.00
15	0.10	0.78
30	0.65	10.85
45	1.11	7.89
60	8.22	8.22
90	11.10	11.10
120	11.90	21.75
180	17.40	7.18
240	10.00	3.64
360	5.81	2.30
480	3.98	1.01
720	1.97	1.03
1140	0.74	0.20
2880	0.15	0.00

Table 14. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.5

Time (min.)	Test	Reference
0	0	0
15	2.05	4.51
30	13.8	8.21
45	13.6	22.1
60	9.14	18.1
90	5.81	9.72
120	5.39	5.13
180	4.44	5.04
240	3.01	5.81
360	3.03	4.1
480	2.22	3.08
720	1.08	1.73
1140	0.374	0.379
2880	0.713	0.124

Table 15. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.6

Time (min.)	Test	Reference
0	0	0
15	0.0151	0.994
30	0.446	5.5
45	0.691	3.91
60	0.387	2.39
90	2.87	13.7
120	3.33	12.5
180	6.96	5.62
240	4.97	4.3
360	3.47	3.64
480	2.88	2.4
720	1.33	2.27
1140	0.538	0.616
2880	0.077	0

Table 16. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.7

Time (min.)	Test	Reference
0	0	0
15	0.326	0.776
30	0.536	3.48
45	0.667	11.2
60	0.637	7.74
90	1.16	6.58
120	22.1	4.2
180	4.51	2.81
240	2.74	4.43
360	2.79	3.35
480	1.35	1.71
720	0.905	0.929
1140	0.3	0.341
2880	0.112	0.251

Table 17. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.8

Time (min.)	Test	Reference
0	0.00	0
15	0.05	0.621
30	0.43	8.68
45	0.73	6.13
60	1.87	4.76
90	1.52	3.65
120	1.51	17.4
180	2.99	5.74
240	11.20	2.9
360	3.80	1.84
480	2.58	0.81
720	1.29	0.823
1140	0.47	0.161
2880	0.09	0.026

Table 18. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.9

Time (min.)	Test	Reference
0	0	0
15	N/A	0
30	5.71	0.55
45	7.37	0.95
60	7.7	2.43
90	20.88	1.97
120	10.5	6.8
180	6.88	10.4
240	3.48	12
360	2.21	22.5
480	0.97	3.3
720	0.98	1.67
1140	0.19	0.6
2880	0.31	0.11

Table 19. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.10

Time (min.)	Test	Reference
0	0.00	0.00
15	0.09	0.87
30	4.00	4.00
45	6.20	6.00
60	8.00	8.80
90	21.80	12.10
120	13.00	25.10
180	13.90	8.06
240	10.00	4.06
360	7.22	2.57
480	4.92	1.13
720	2.45	1.16
1140	0.89	0.22
2880	0.17	0.00

Table 20. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.11

Time (min.)	Test	Reference
0	0	0
15	0.058	0.0792
30	0.772	3.29
45	0.268	7.38
60	0.946	36
90	1.51	18.1
120	4.36	18.7
180	26.4	11.2
240	14.7	9.48
360	11.7	8.08
480	6.41	6.23
720	4.53	5.98
1140	1.92	2.48
2880	0.21	0.821

Table 21. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.12

Time (min.)	Test	Reference
0	0	0
15	0.0292	9.75
30	0.308	16.4
45	0.516	11.6
60	0.454	11
90	8.2	11.7
120	15.8	8.1
180	18.7	6.41
240	14.8	5.65
360	7.05	3.39
480	3.28	2.43
720	0.281	1.43
1140	0.613	0.72
2880	0	0.27

Table 22. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.13

Time (min.)	Test	Reference
0	0	0
15	0.39	12.6
30	1.57	9.11
45	2.39	6.19
60	3.65	6.76
90	3.34	5.27
120	4.59	5.9
180	6.02	6.42
240	4.47	3.6
360	2.76	3.36
480	2.71	2.25
720	1.37	1.78
1140	0.597	0.899
2880	0.168	0.281

Table 23. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.14

Time (min.)	Test	Reference
0	0	0
15	0.387	0.902
30	8.43	13.7
45	10.6	16.7
60	12.7	12.3
90	9.89	6.48
120	7.36	4.19
180	4.76	3.42
240	4.16	2.43
360	2.55	2.65
480	2.02	1.73
720	0.892	0.501
1140	0.0894	0.314
2880	0.0677	0.0488

Table 24. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.15

Time (min.)	Test	Reference
0	0	0
15	0.0366	0.244
30	0.531	1.26
45	0.845	1.83
60	0.424	2.16
90	0.876	2.45
120	4.48	2.8
180	3.55	7.27
240	3.95	2.35
360	3.43	3.7
480	2.11	4.25
720	0.589	1.95
1140	0.368	0.79
2880	0.0351	0.434

Table 25. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.16

Time (min.)	Test	Reference
0	0.00	0.00
15	0.00	0.33
30	2.85	6.41
45	3.00	12.98
60	6.80	10.16
90	2.31	9.06
120	15.00	7.32
180	12.54	7.67
240	7.23	5.72
360	4.19	4.70
480	2.82	2.54
720	2.31	1.28
1140	0.49	0.69
2880	0.24	0.77

Table 26. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.17

Time (min.)	Test	Reference
0	0.00	0.00
15	0.07	0.00
30	8.54	3.96
45	16.50	5.33
60	12.44	3.56
90	11.93	7.89
120	8.72	16.35
180	8.67	16.80
240	7.46	9.68
360	6.42	6.24
480	3.30	3.81
720	1.39	2.37
1140	0.72	0.30
2880	0.60	0.18

Table 27. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.18

Time (min.)	Test	Reference
0	0	0
15	0.168	3.47
30	0.759	6.46
45	2.08	5.42
60	1.19	5.88
90	2.03	5.31
120	3.04	3.37
180	6.34	2.69
240	3.28	1.66
360	2.09	1.65
480	1.48	0.935
720	0.777	0.887
1140	0.182	0.211
2880	0.132	0.0223

Table 28. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.19

Time (min.)	Test	Reference
0	0	0
15	0.222	13.4
30	0.881	22.7
45	1.1	21.2
60	0.648	19.6
90	0.92	17.7
120	4.68	24.4
180	10.6	15.7
240	7.54	9.46
360	5.81	6.14
480	2.95	2.6
720	2.08	1.32
1140	0.56	0.272
2880	0	0.153

Table 29. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.20

Time (min.)	Test	Reference
0	0	0
15	0.438	1.36
30	1.85	6.01
45	1.73	5.94
60	6.35	4.93
90	11.1	4.64
120	16.5	3.59
180	6.37	4.33
240	4.71	5.8
360	2.52	3.33
480	2.3	2.1
720	0.987	0.979
1140	0.373	0.33
2880	0.182	0.212

Table 30. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.21

Time (min.)	Test	Reference
0	0	0
15	1.11	0.664
30	1.06	11.5
45	6.6	19.4
60	20.1	13.3
90	21.7	12.4
120	12.2	8.45
180	14.1	7.28
240	16.2	7.1
360	11	8.11
480	6.01	3.4
720	2.13	3.05
1140	1.06	0.816
2880	0.0788	0

Table 31. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.22

Time (min.)	Test	Reference
0	0	0
15	5.3	0.826
30	18.8	1.54
45	15.8	1.48
60	10.2	1.72
90	5.17	3.86
120	7.24	3.15
180	9.48	22.9
240	10.8	9.46
360	11.8	11.2
480	7.22	4.78
720	4.05	3.05
1140	0.873	3.62
2880	0.331	0.418

Table 32. Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.23

Time (min.)	Test	Reference
0	0	0
15	4.22	1.67
30	15.7	10.4
45	23.4	20.8
60	33.5	19.5
90	26.5	19.5
120	22.3	17.2
180	15.5	8.34
240	9.18	6.66
360	2.49	3.36
480	3.95	1.24
720	0.41	0.541
1140	0.172	0.0752
2880	0.0341	0

Table 33 Plasma atorvastatin concentrations (ng/ml) at various times after single dose administration of 40 mg atorvastatin table in subject No.24

Time (min.)	Test	Reference
0	0.00	0.0
15	1.77	0.6
30	5.63	3.4
45	5.22	5.0
60	7.91	3.9
90	9.00	12.5
120	9.99	12.1
180	8.49	8.9
240	7.52	9.7
360	4.25	9.0
480	3.71	4.4
720	1.49	3.2
1140	0.99	0.8
2880	0.27	0.6

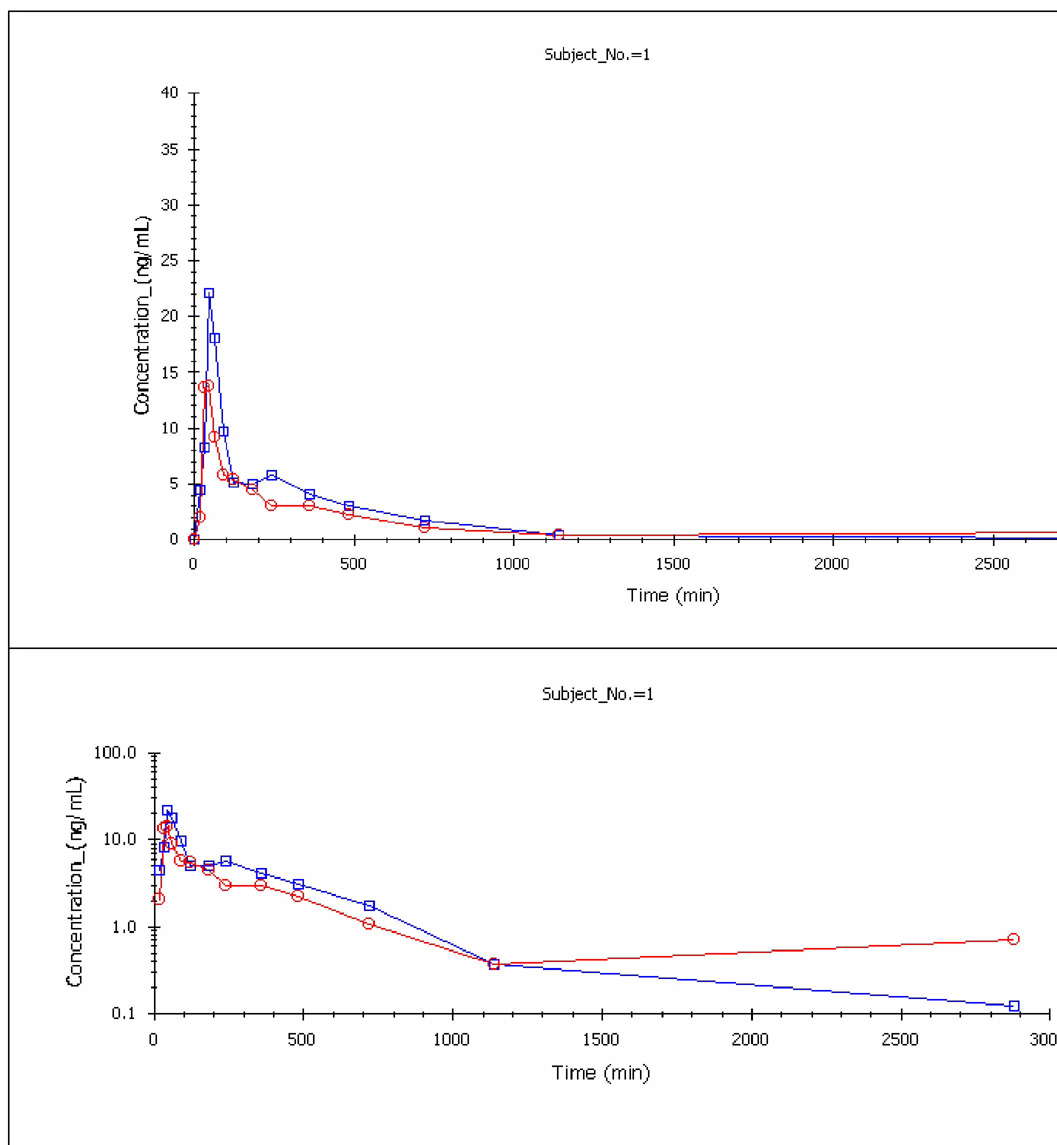


Figure 15. Plasma Concentration Time Profile of Atorvastatin in Subject 1 Normal Plot (Above) and Semilog Plot (Below)

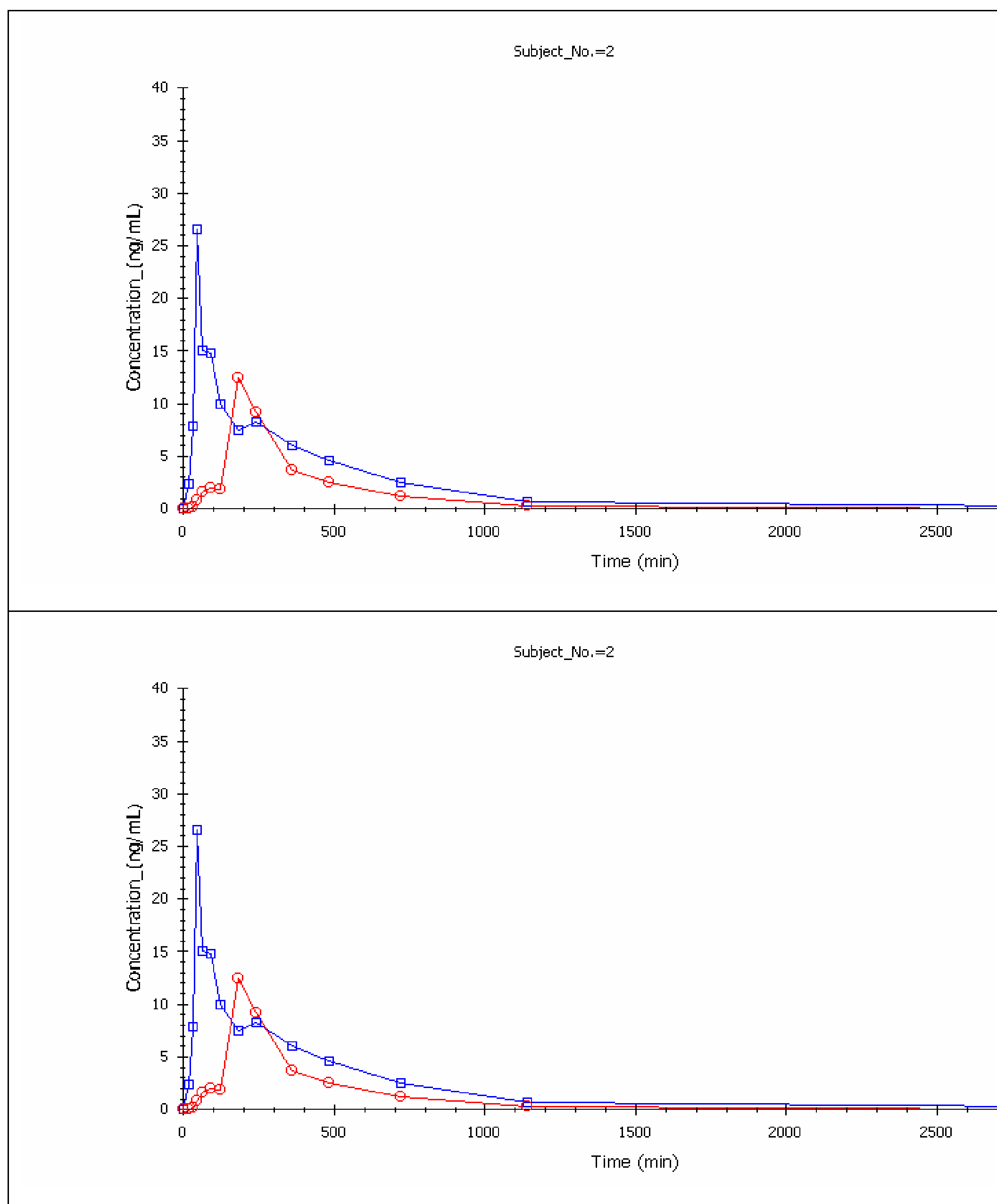


Figure 16. Plasma Concentration Time Profile of Atorvastatin in Subject 2 Normal Plot (Above) and Semilog Plot (Below)

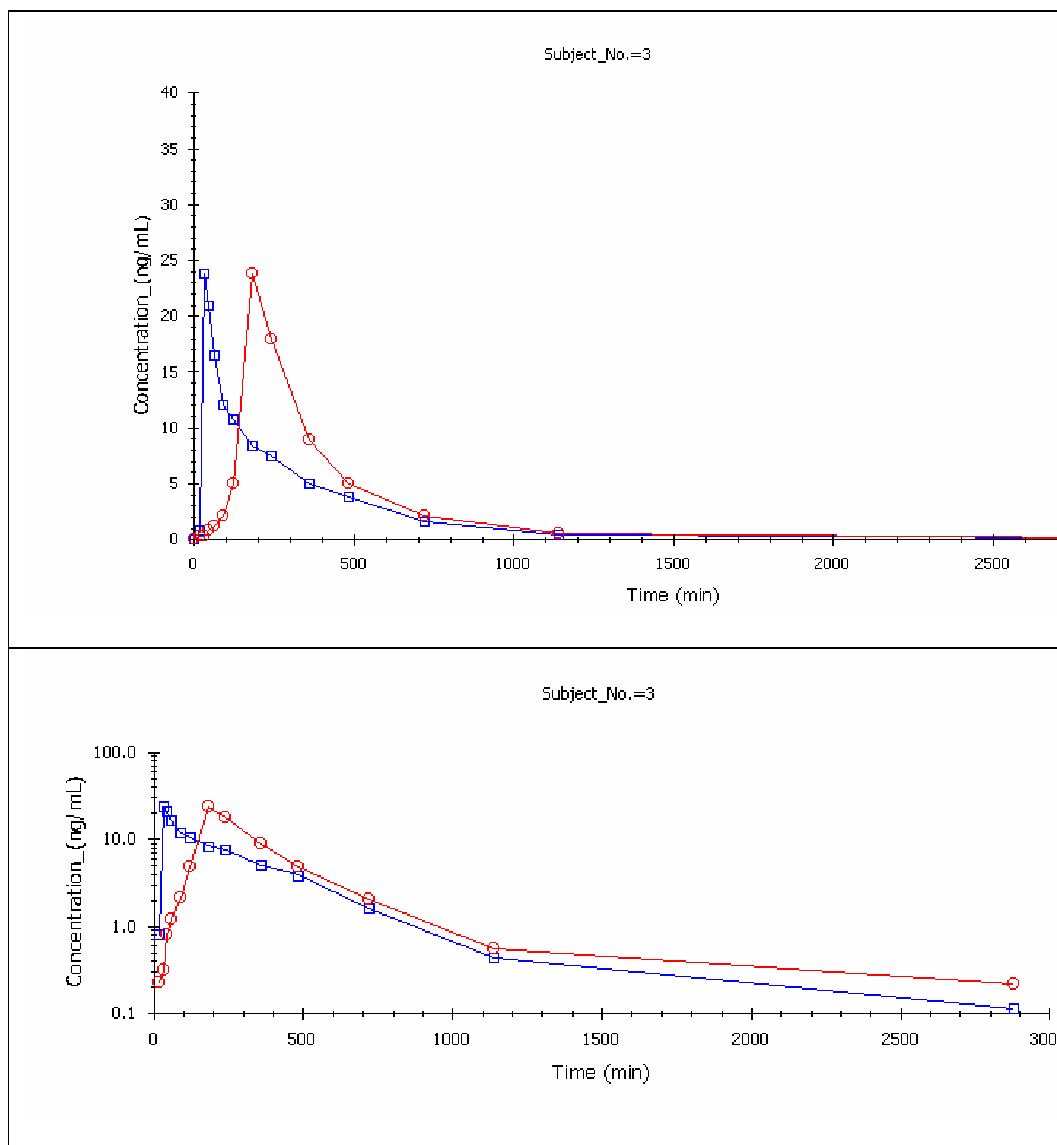


Figure 17. Plasma Concentration Time Profile of Atorvastatin in Subject 3 Normal Plot (Above) and Semilog Plot (Below)

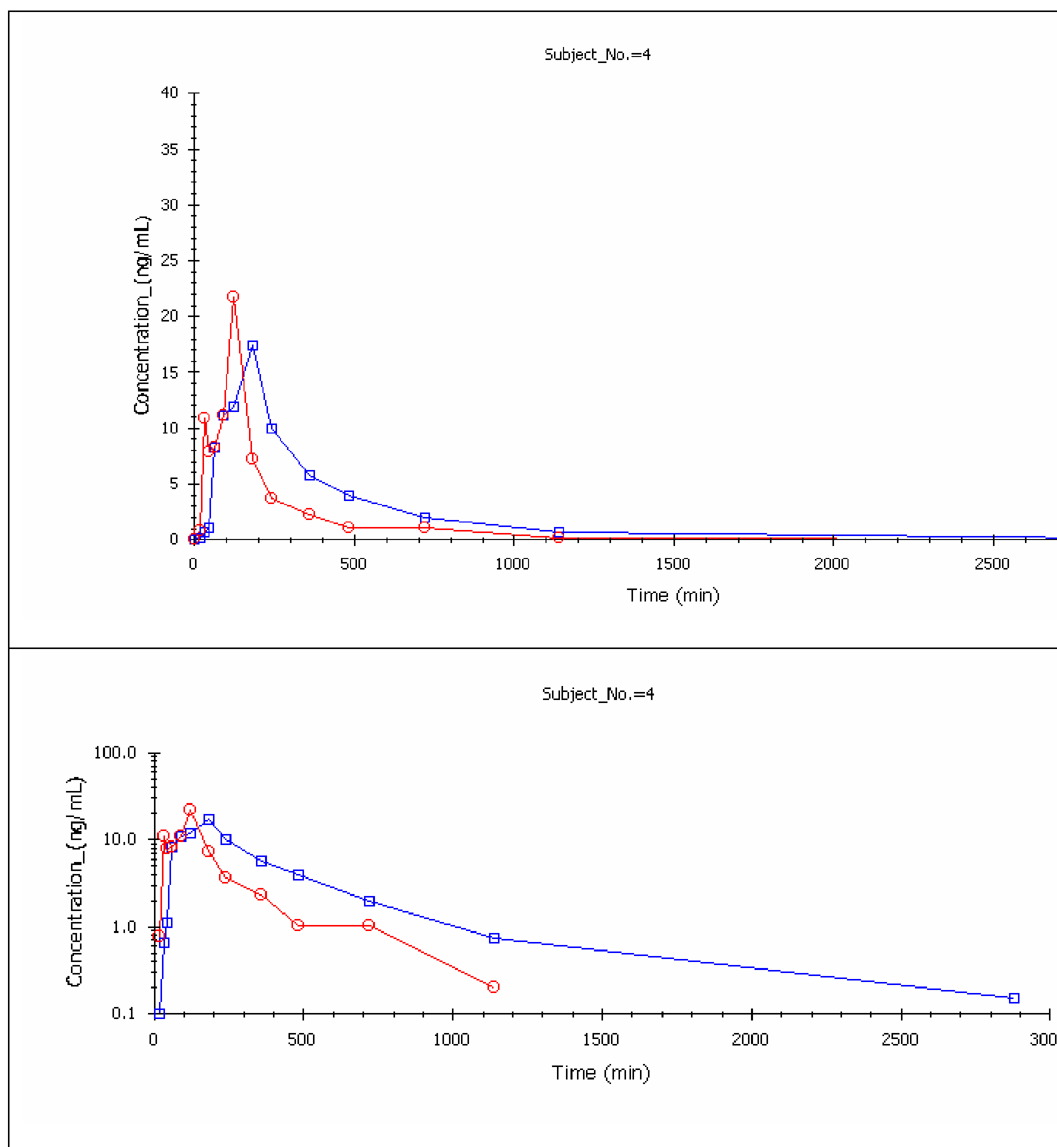


Figure 18. Plasma Concentration Time Profile of Atorvastatin in Subject 4 Normal Plot (Above) and Semilog Plot (Below)

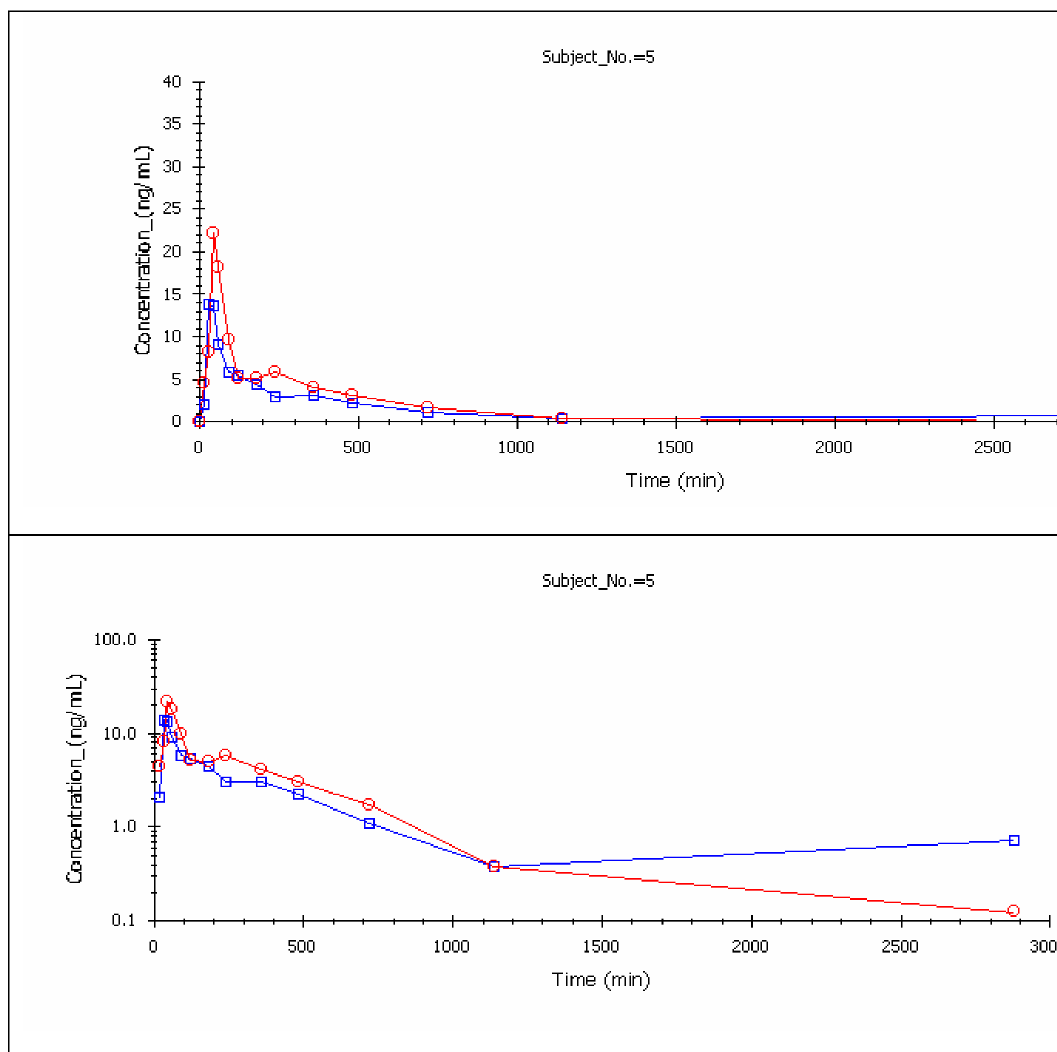


Figure 19. Plasma Concentration Time Profile of Atorvastatin in Subject 5 Normal Plot (Above) and Semilog Plot (Below)

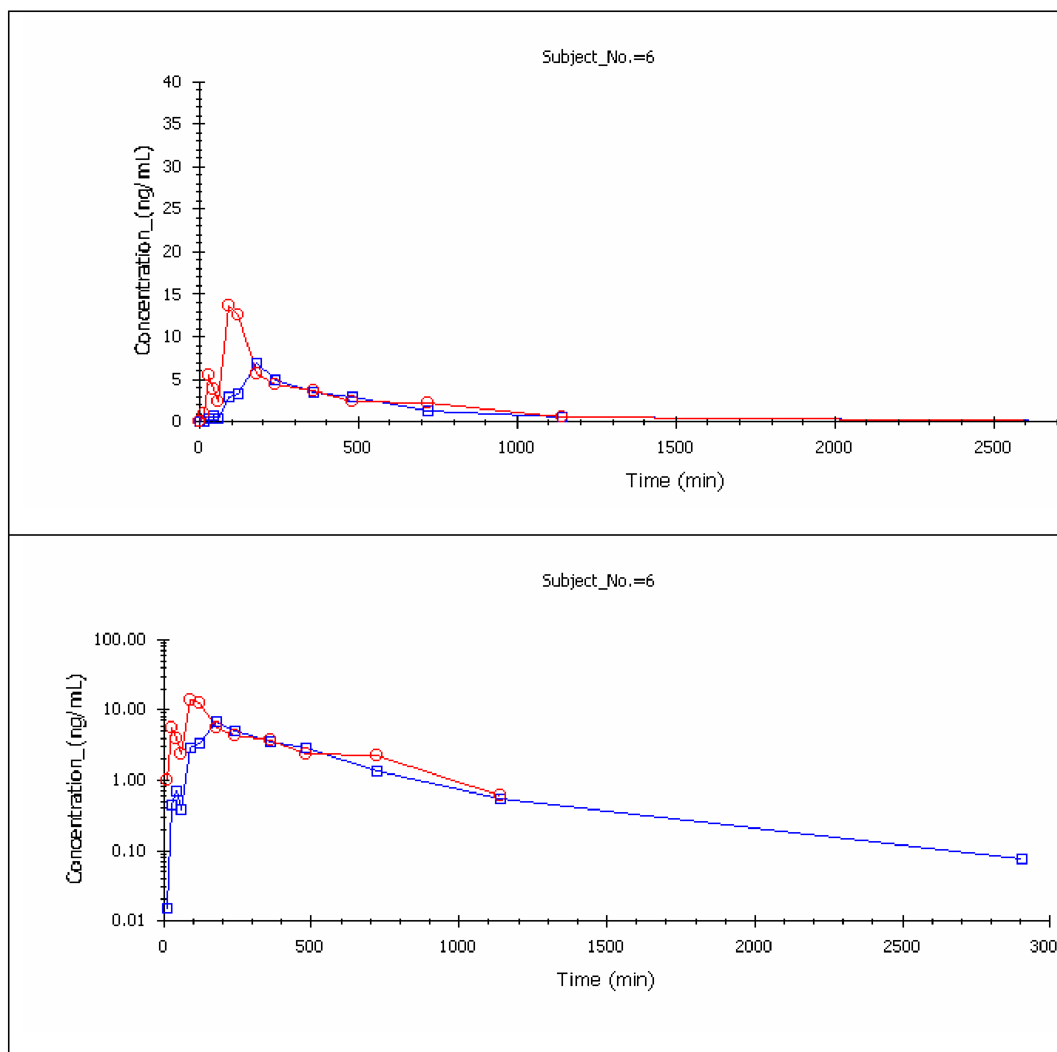


Figure 20. Plasma Concentration Time Profile of Atorvastatin in Subject 6 Normal Plot (Above) and Semilog Plot (Below)

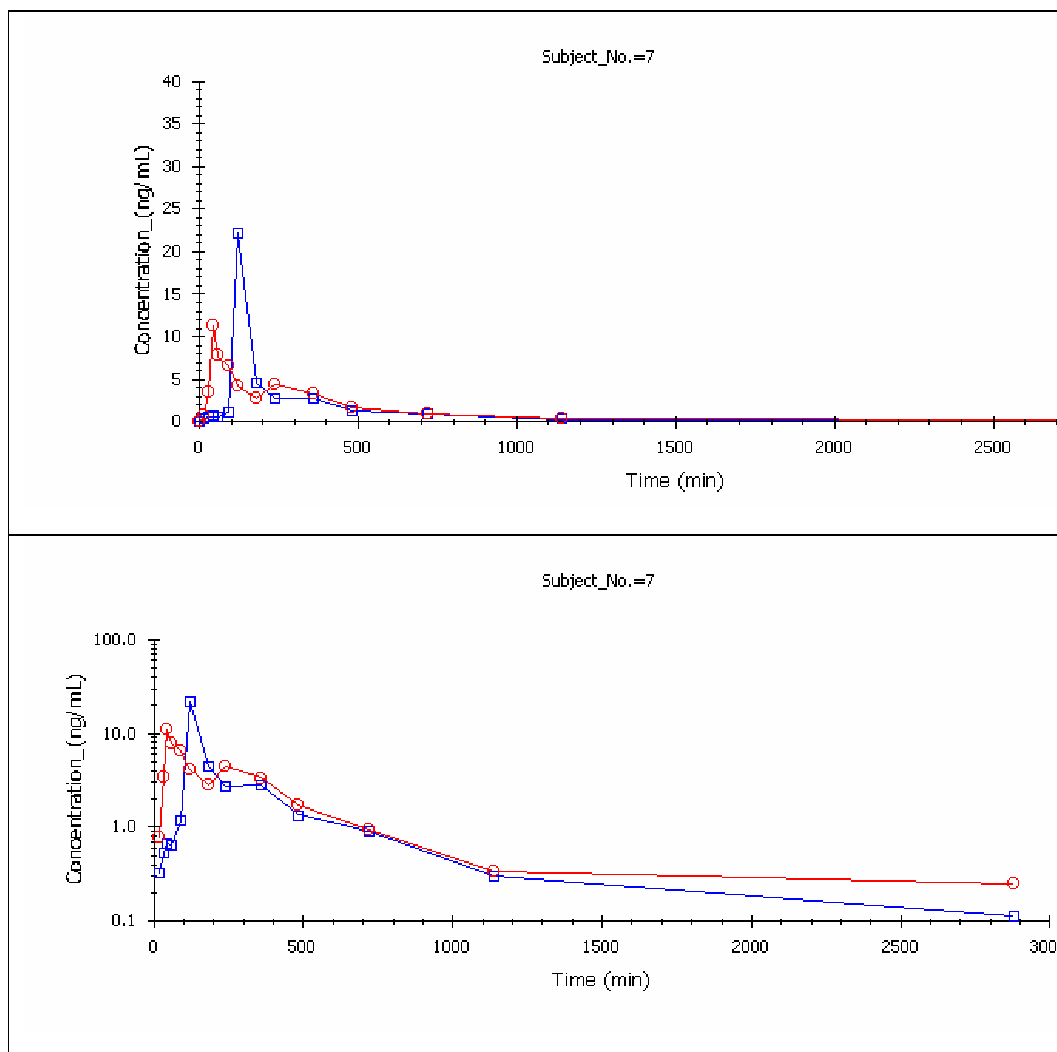


Figure 21. Plasma Concentration Time Profile of Atorvastatin in Subject 7 Normal Plot (Above) and Semilog Plot (Below)

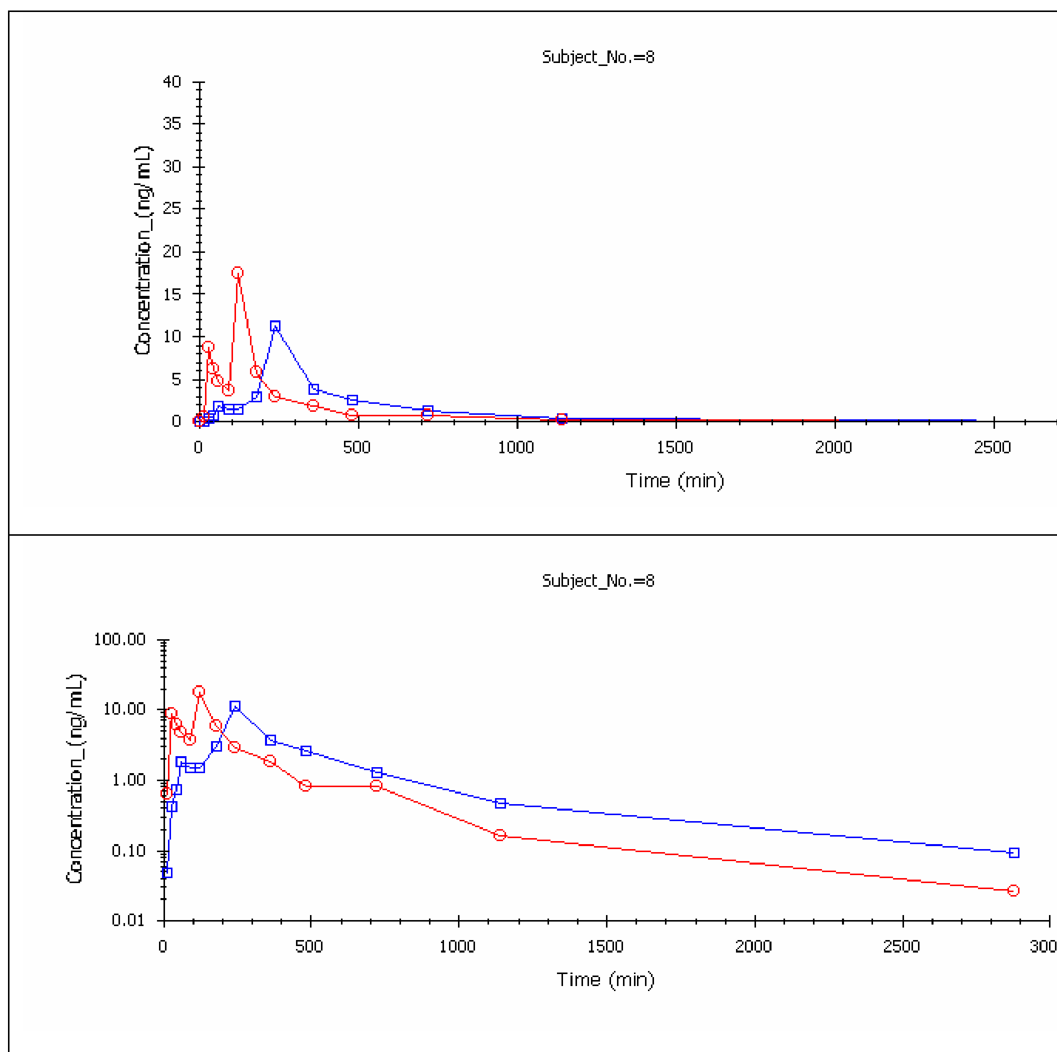


Figure 22. Plasma Concentration Time Profile of Atorvastatin in Subject 8 Normal Plot (Above) and Semilog Plot (Below)

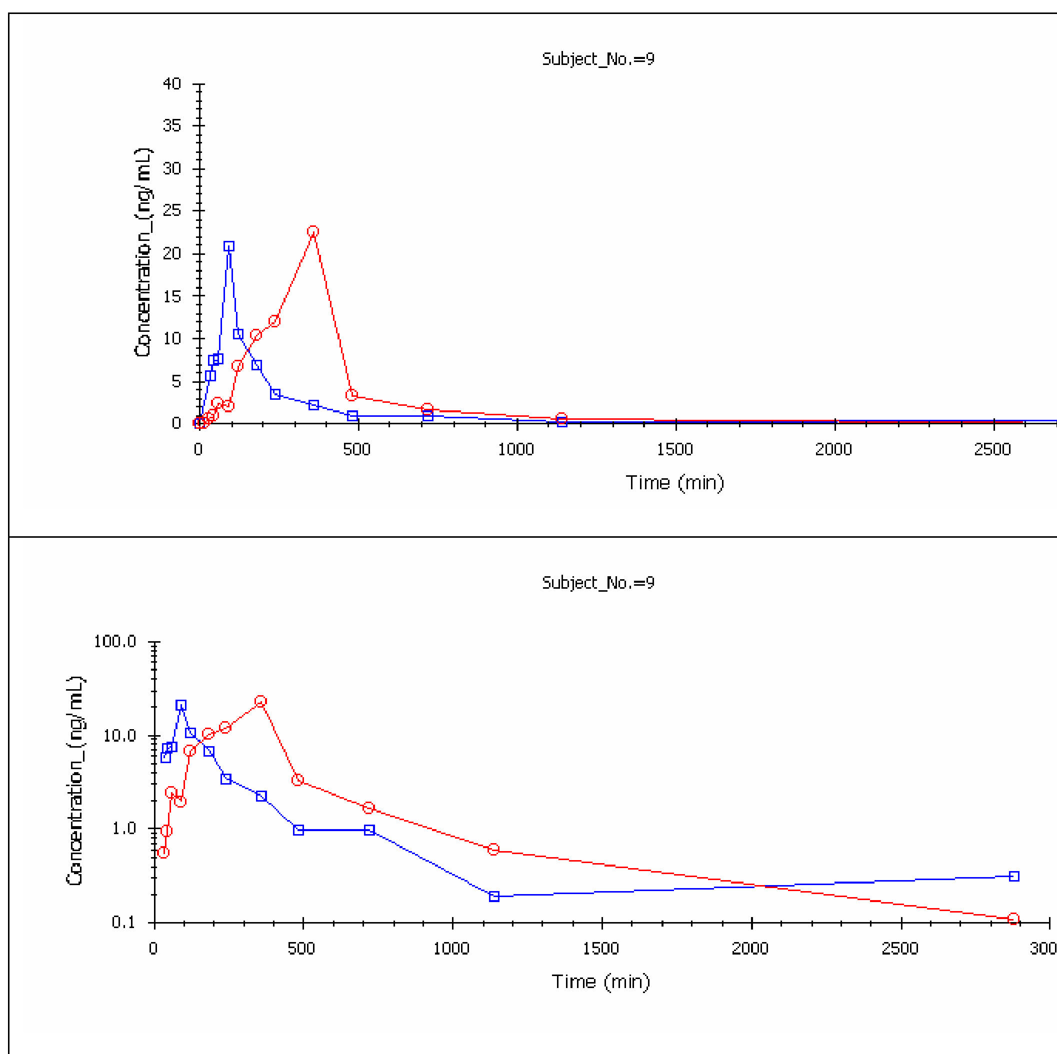


Figure 23. Plasma Concentration Time Profile of Atorvastatin in Subject 9 Normal Plot (Above) and Semilog Plot (Below)

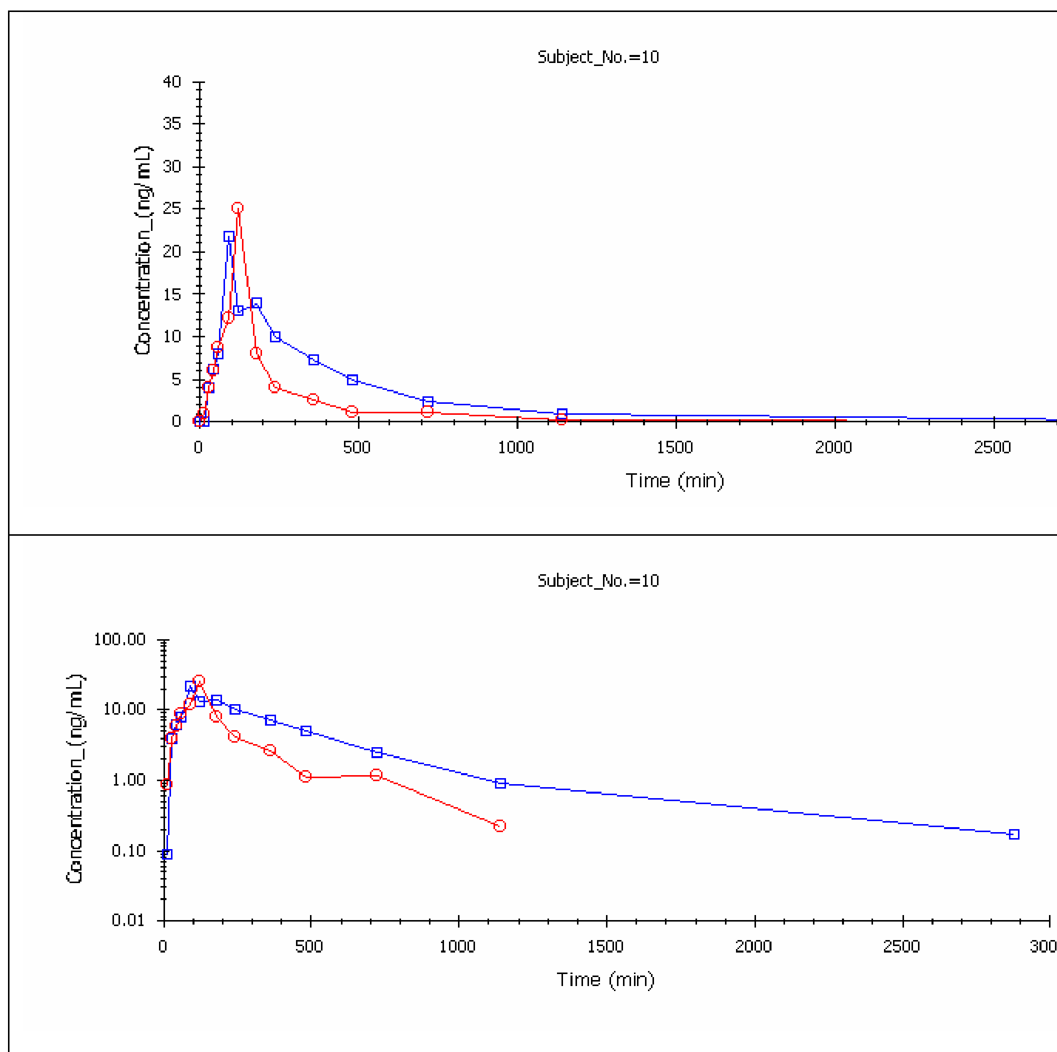


Figure 24. Plasma Concentration Time Profile of Atorvastatin in Subject 10 Normal Plot (Above) and Semilog Plot (Below)

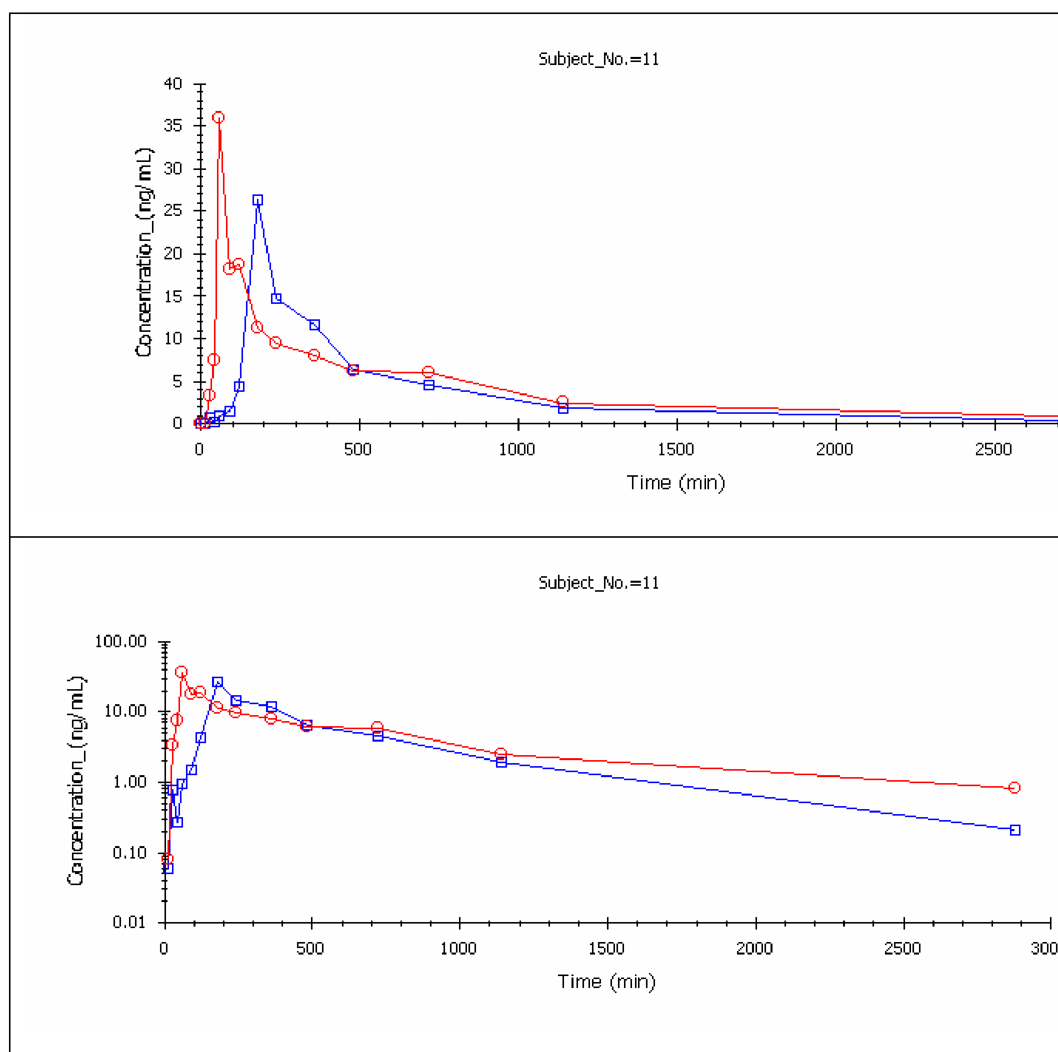


Figure 25. Plasma Concentration Time Profile of Atorvastatin in Subject 11 Normal Plot (Above) and Semilog Plot (Below)

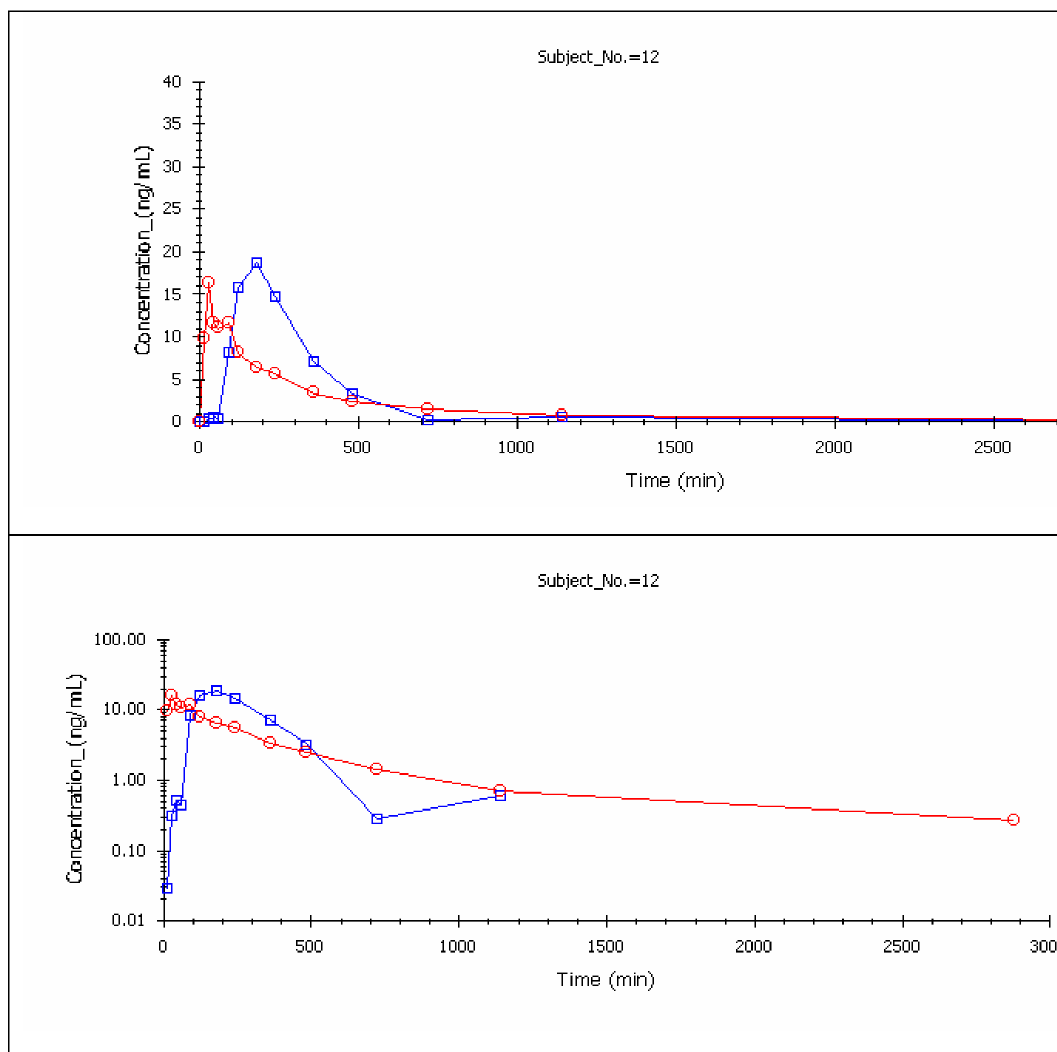


Figure 26. Plasma Concentration Time Profile of Atorvastatin in Subject 12 Normal Plot (Above) and Semilog Plot (Below)

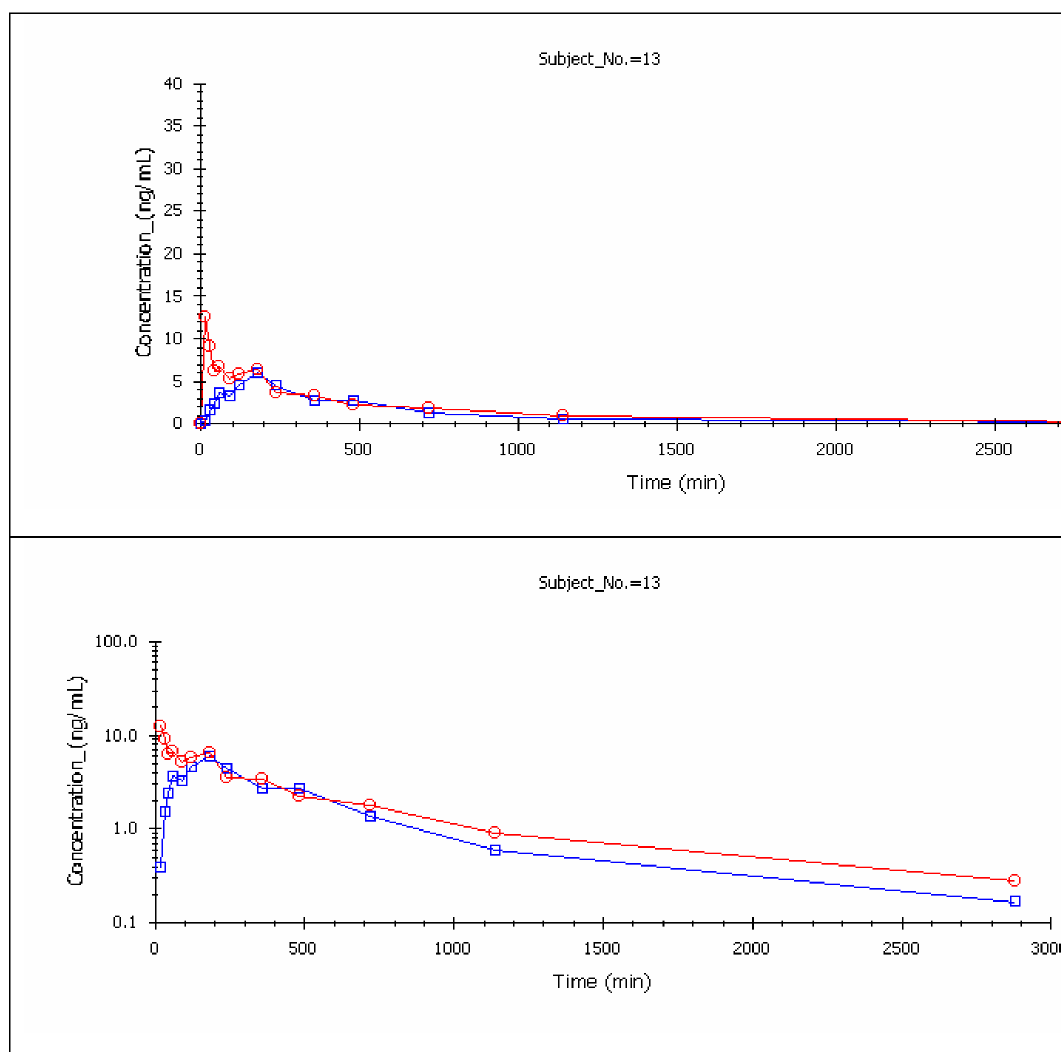


Figure 27. Plasma Concentration Time Profile of Atorvastatin in Subject 13 Normal Plot (Above) and Semilog Plot (Below)

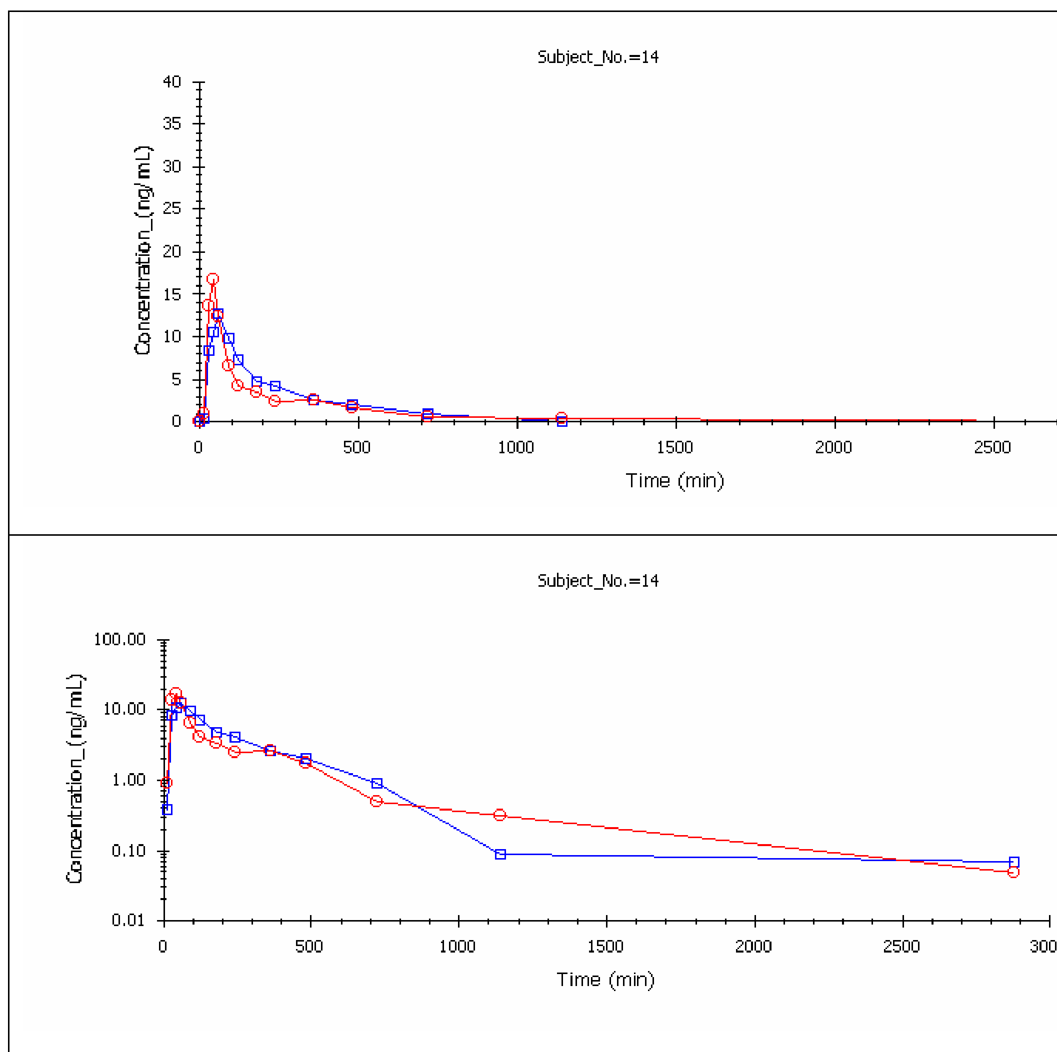


Figure 28. Plasma Concentration Time Profile of Atorvastatin in Subject 14 Normal Plot (Above) and Semilog Plot (Below)

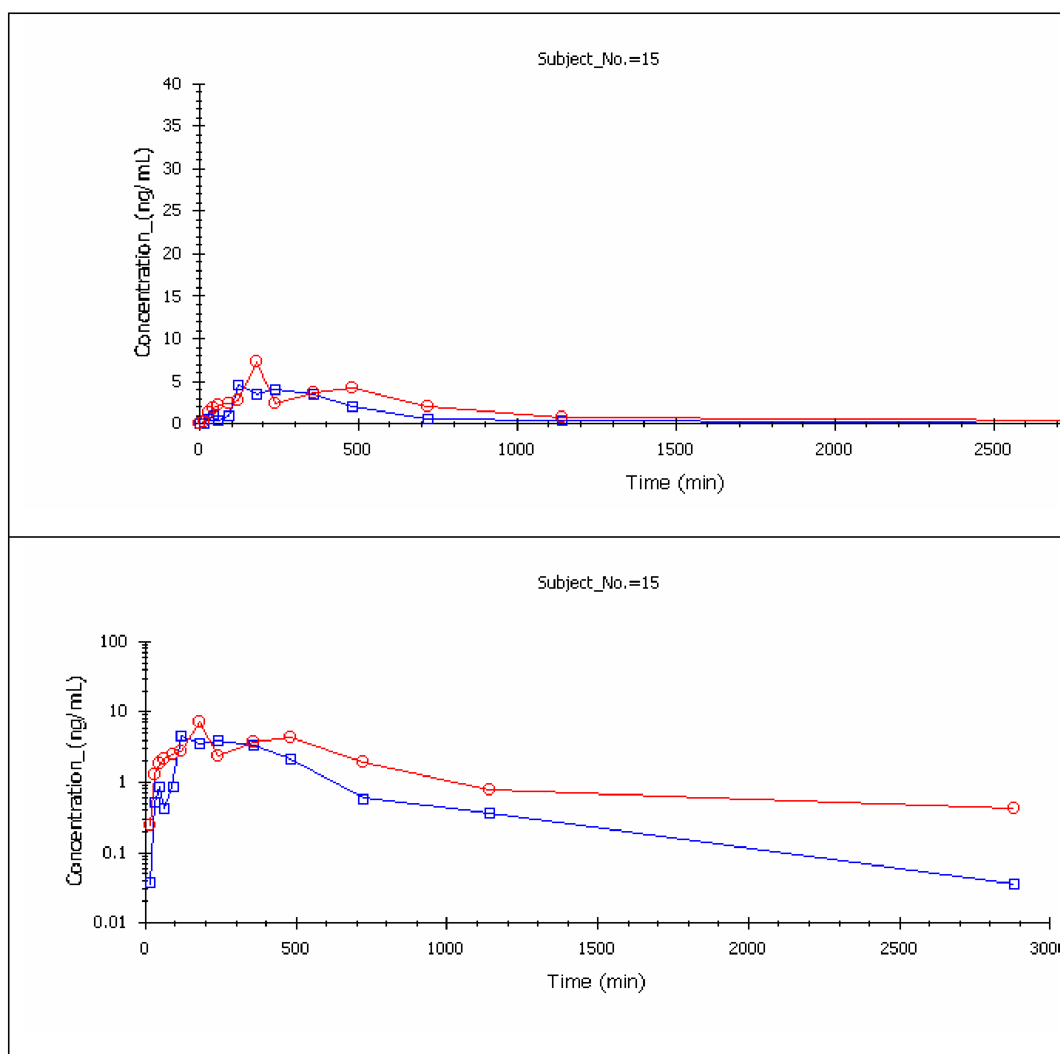


Figure 29. Plasma Concentration Time Profile of Atorvastatin in Subject 15 Normal Plot (Above) and Semilog Plot (Below)

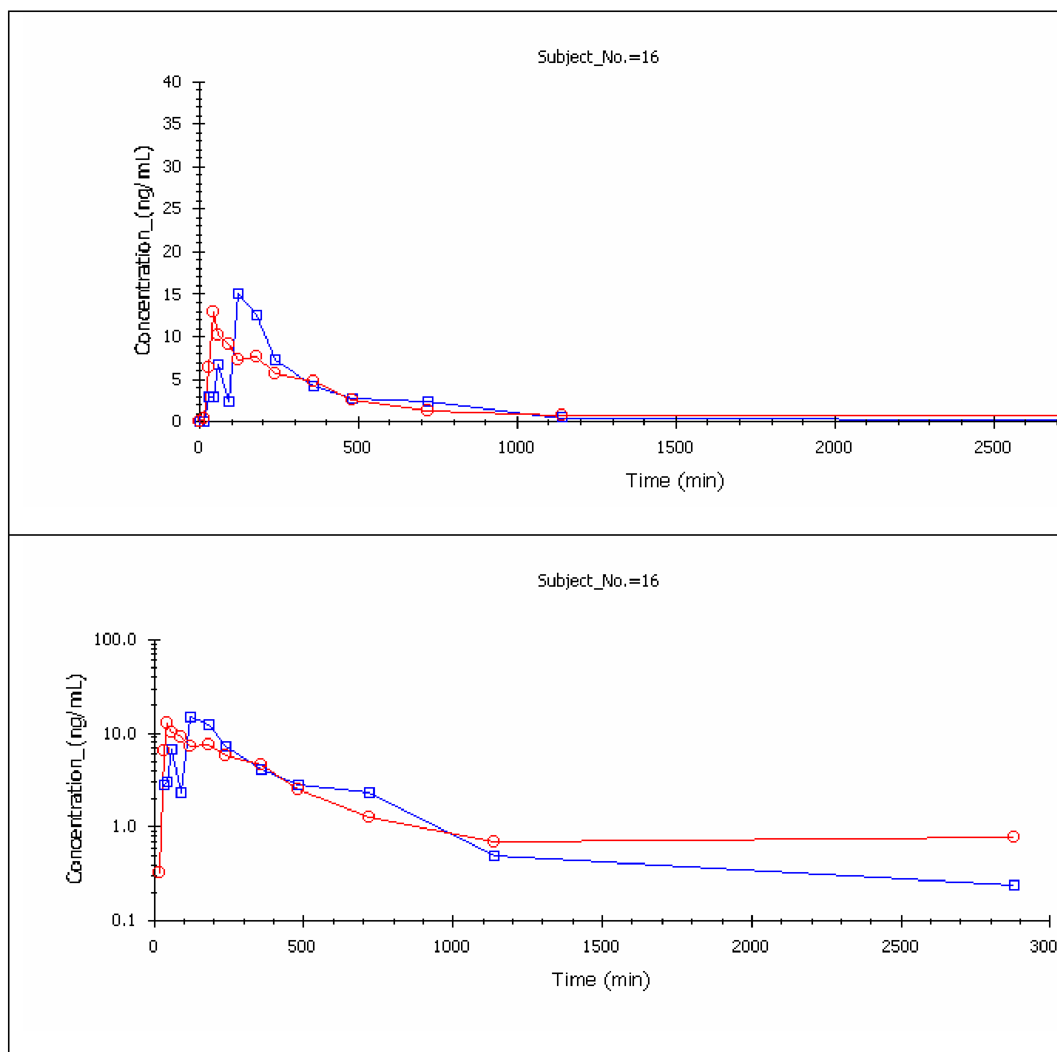


Figure 30. Plasma Concentration Time Profile of Atorvastatin in Subject 16 Normal Plot (Above) and Semilog Plot (Below)

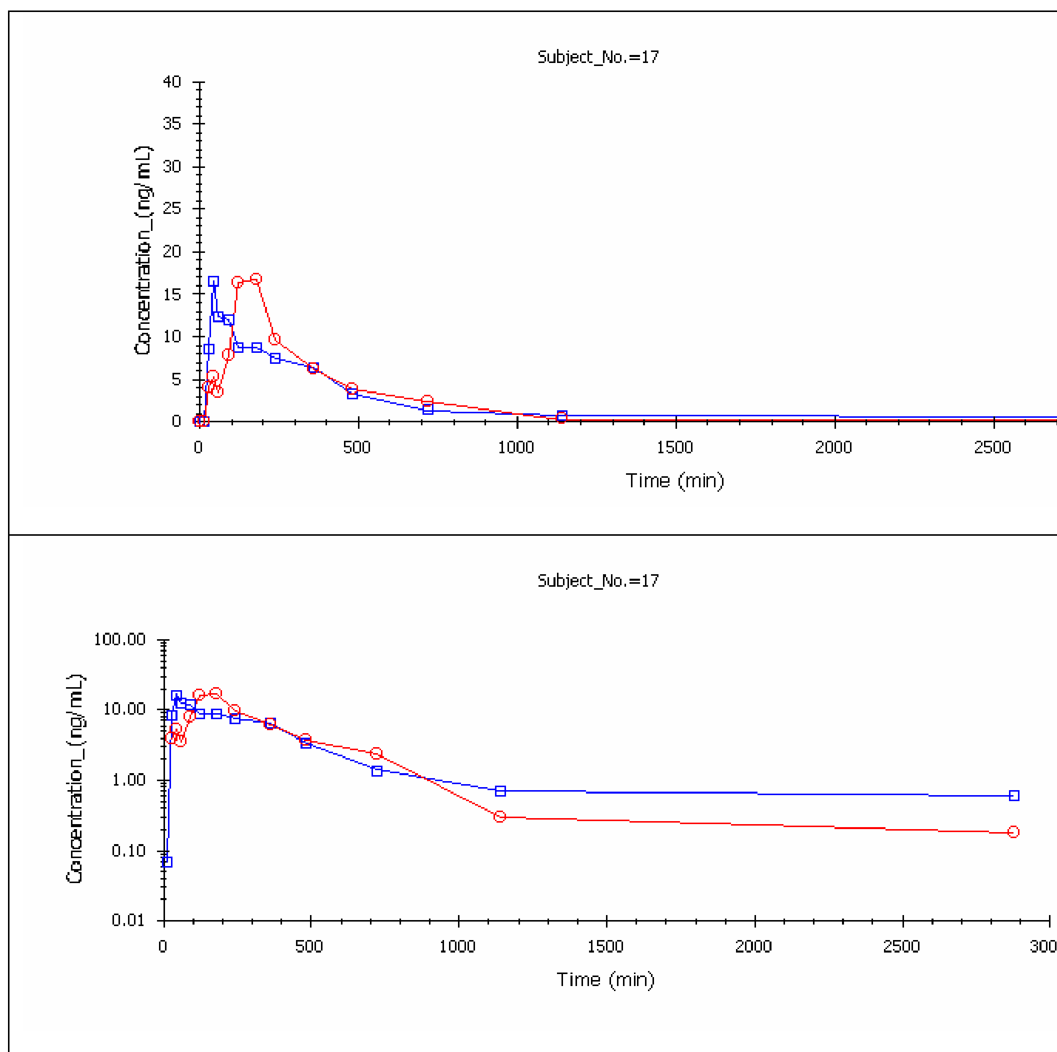


Figure 31. Plasma Concentration Time Profile of Atorvastatin in Subject 17 Normal Plot (Above) and Semilog Plot (Below)

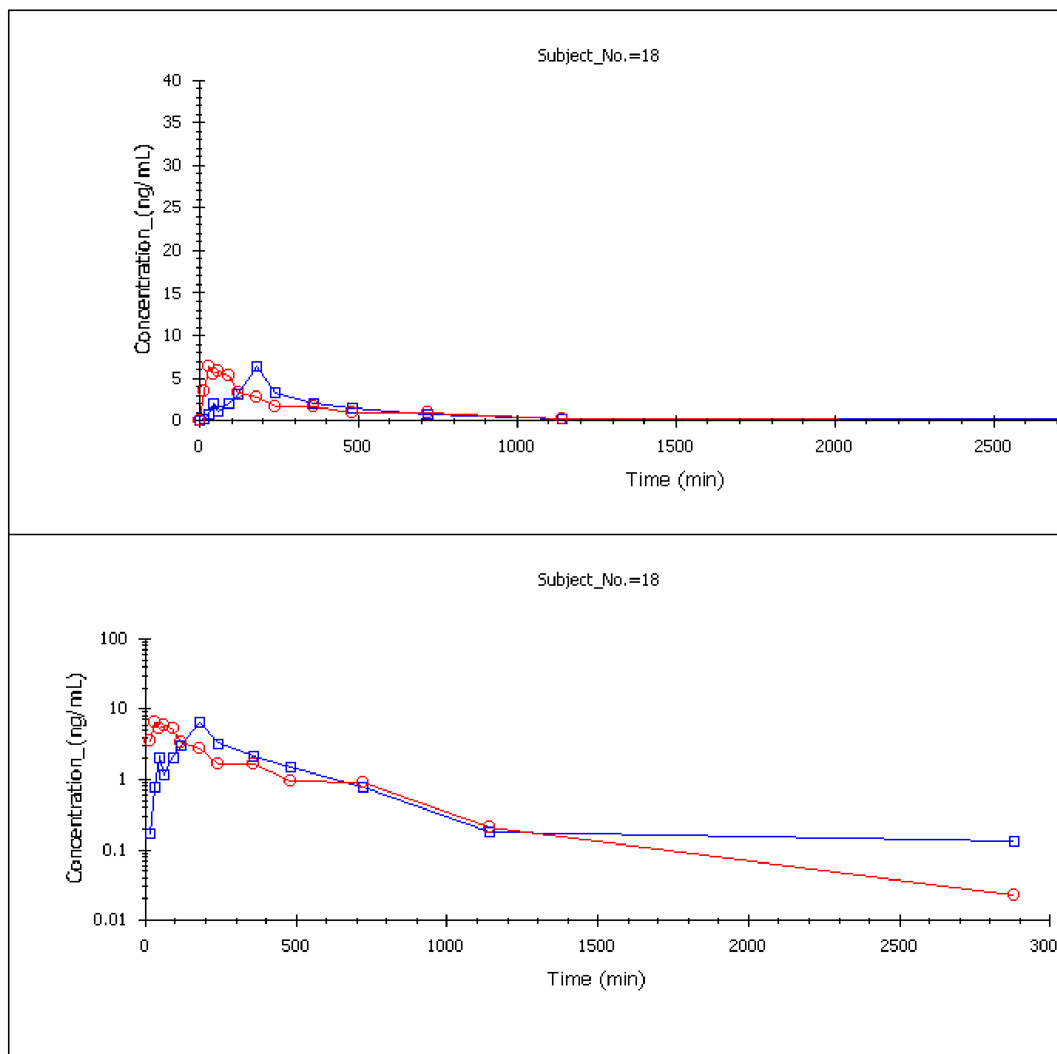


Figure 32. Plasma Concentration Time Profile of Atorvastatin in Subject 18 Normal Plot (Above) and Semilog Plot (Below)

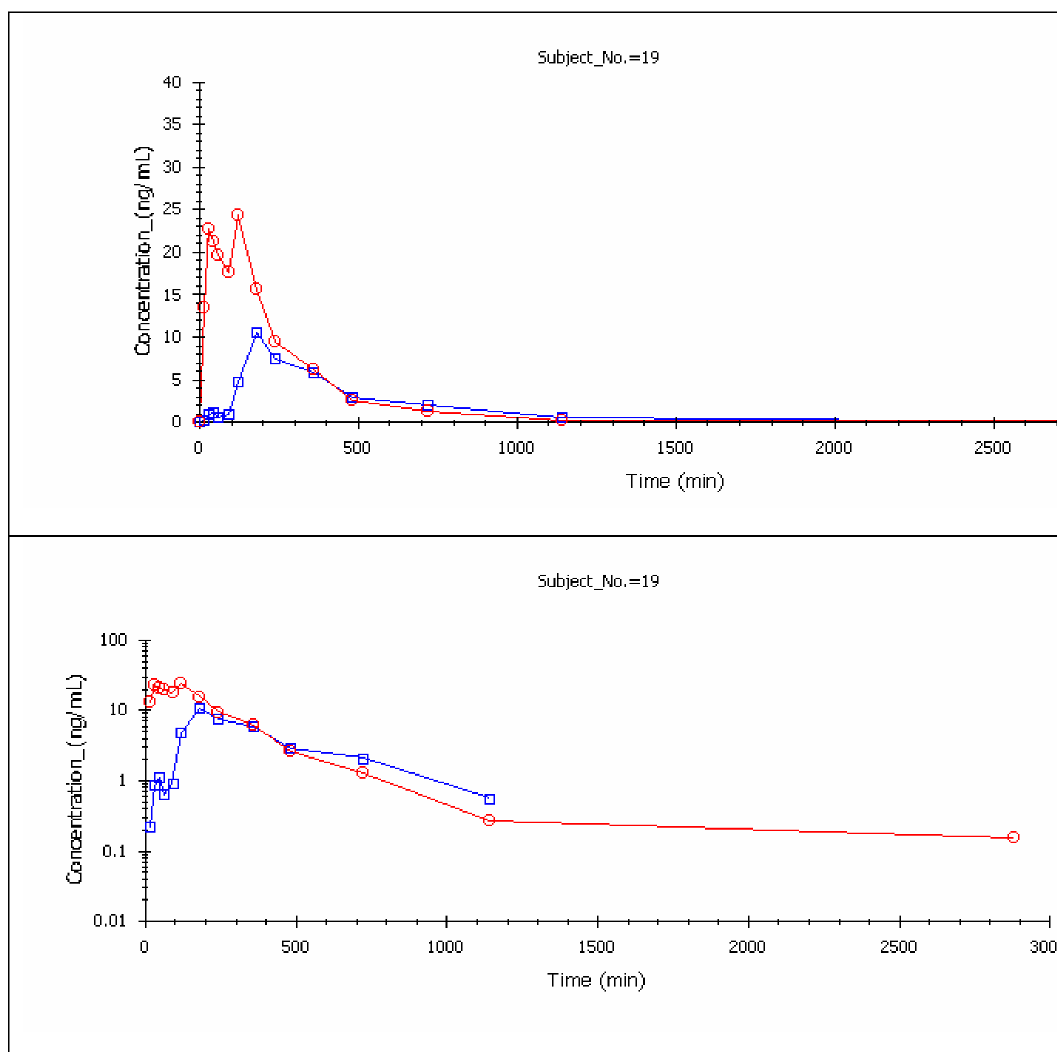


Figure 33. Plasma Concentration Time Profile of Atorvastatin in Subject 19 Normal Plot (Above) and Semilog Plot (Below)

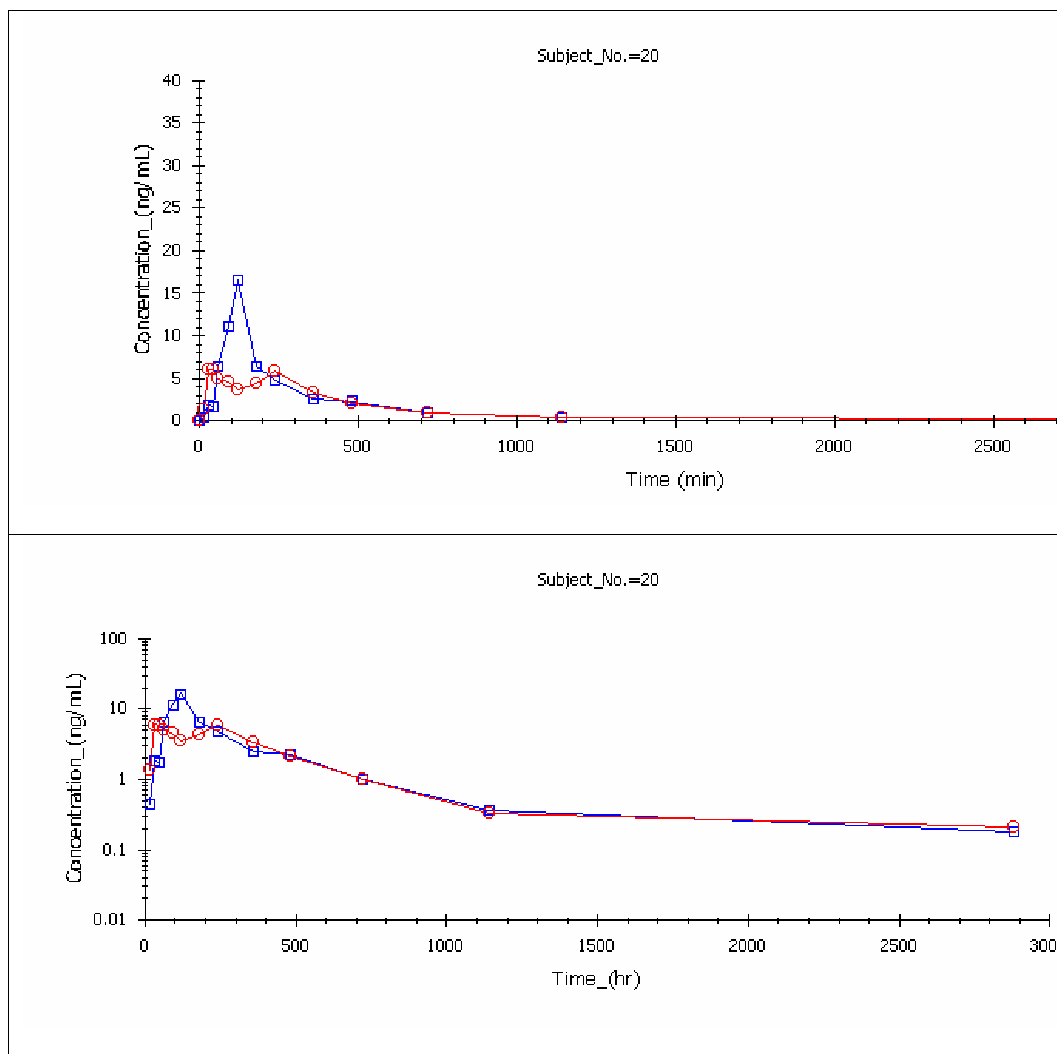


Figure 34. Plasma Concentration Time Profile of Atorvastatin in Subject 20 Normal Plot (Above) and Semilog Plot (Below)

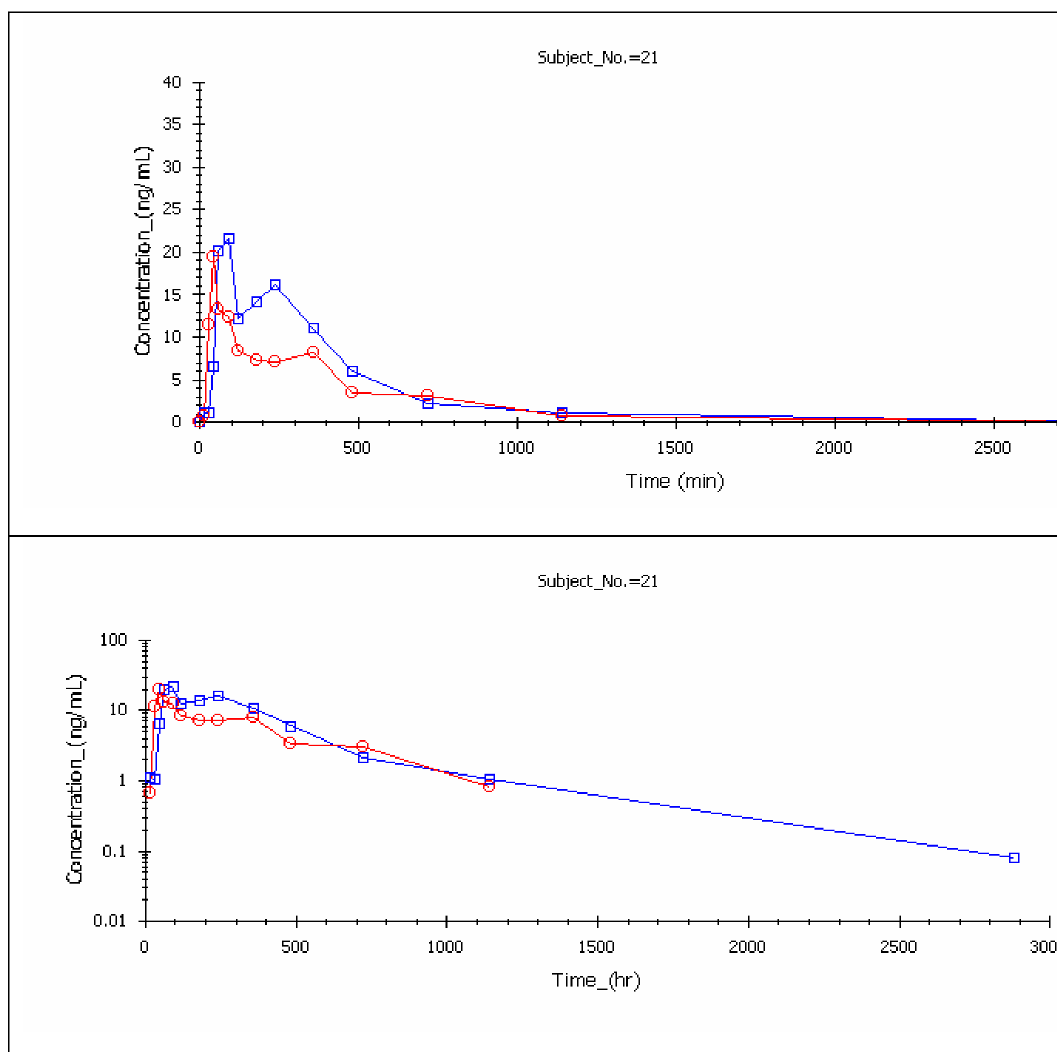


Figure 35. Plasma Concentration Time Profile of Atorvastatin in Subject 21 Normal Plot (Above) and Semilog Plot (Below)

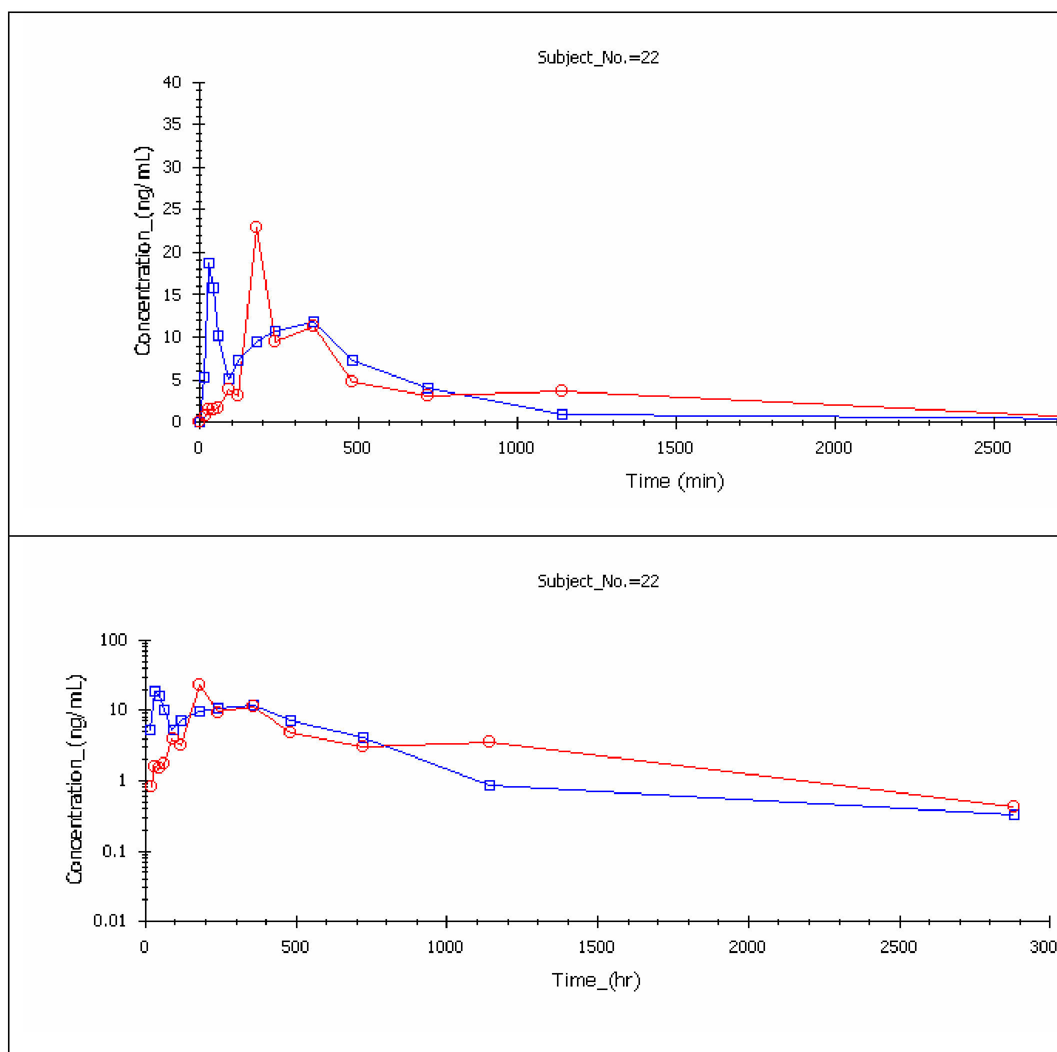


Figure 36. Plasma Concentration Time Profile of Atorvastatin in Subject 22 Normal Plot (Above) and Semilog Plot (Below)

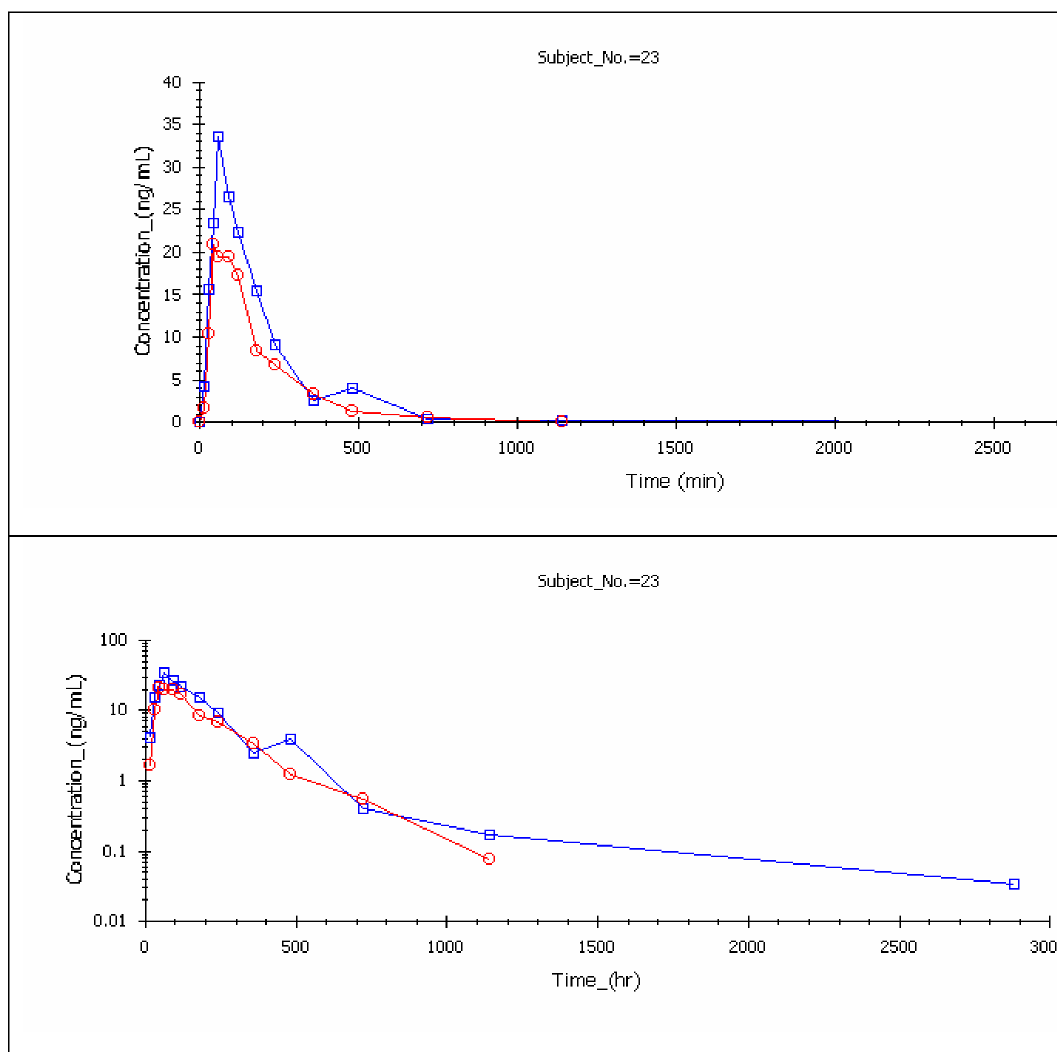


Figure 37. Plasma Concentration Time Profile of Atorvastatin in Subject 23 Normal Plot (Above) and Semilog Plot (Below)

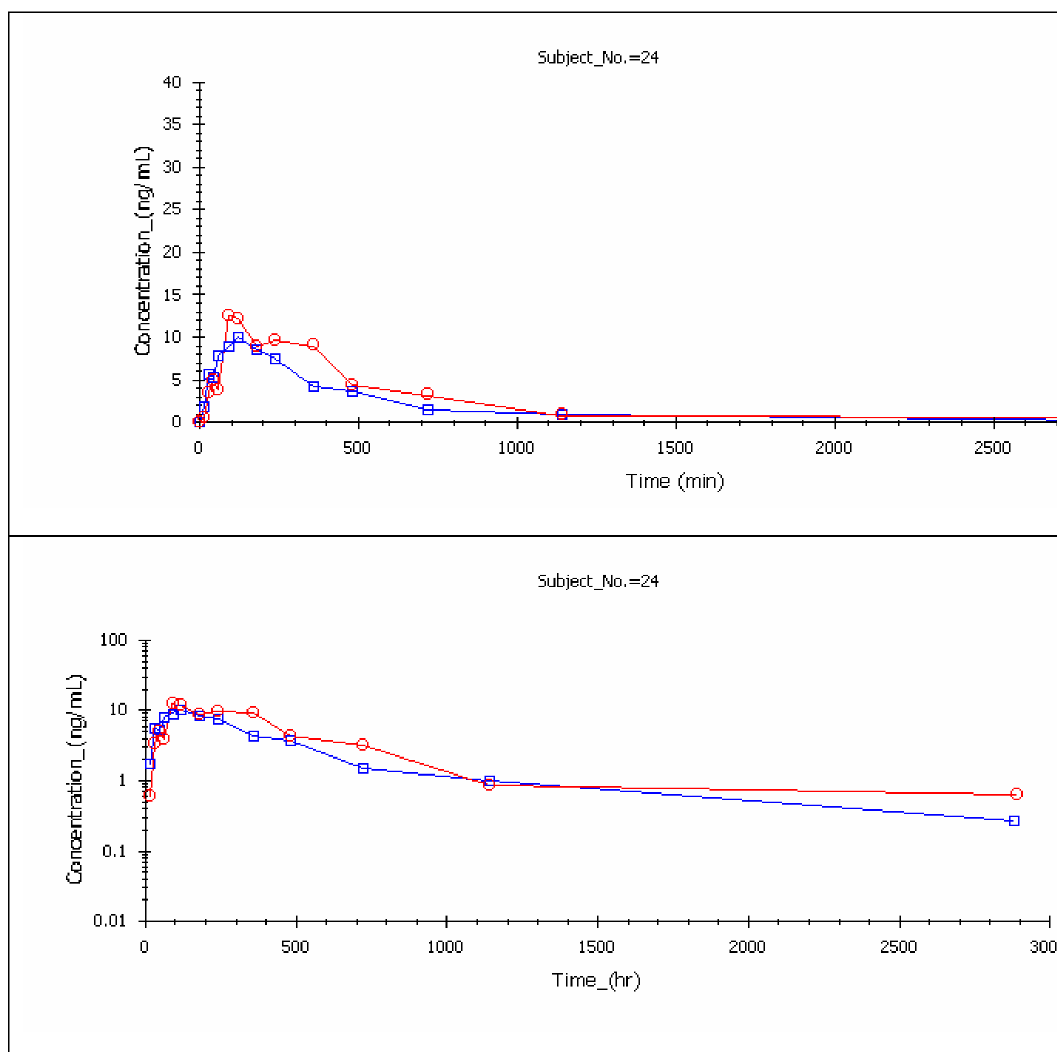


Figure 38. Plasma Concentration Time Profile of Atorvastatin in Subject 24 Normal Plot (Above) and Semilog Plot (Below)

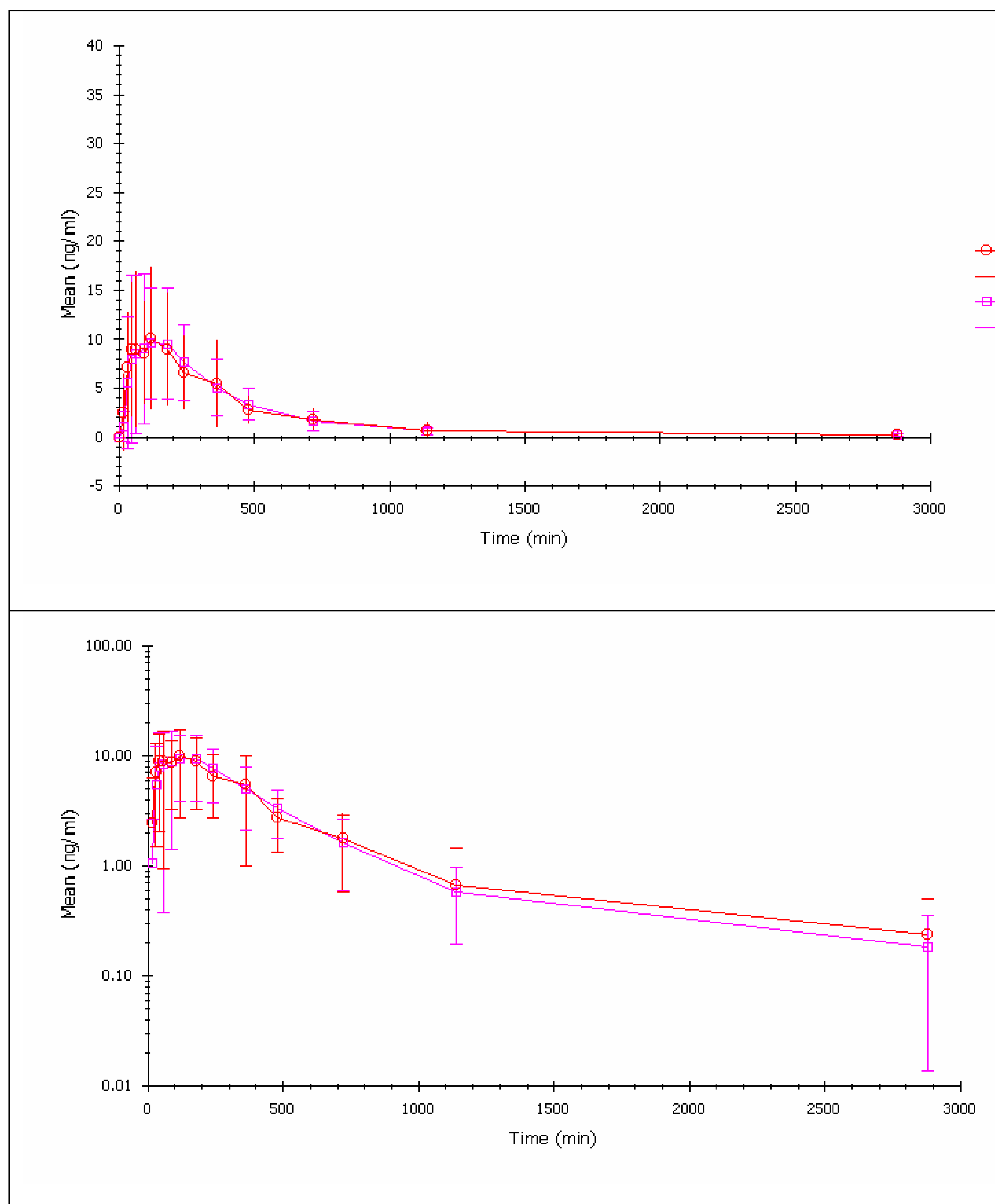


Figure 39. Average + S.D. of Plasma Concentration Time Profile of Atorvastatin in the Study Normal Plot (Above) and Semilog Plot (Below) for all subjects (n=24)

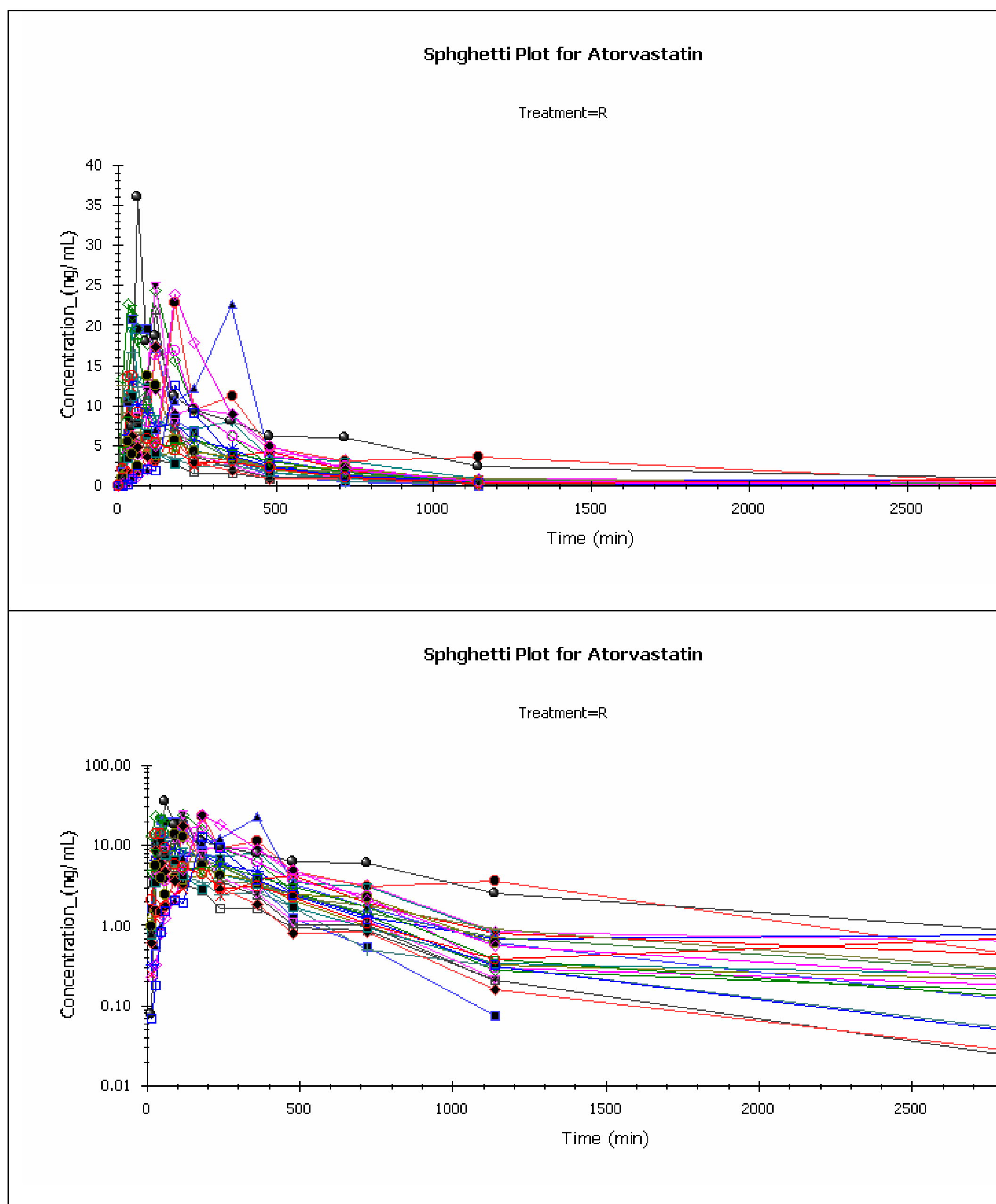


Figure 40. Spaghetti Plot for Plasma Concentration Time Profile of Reference Treatment Atorvastatin for All Subjects Normal Plot (Above) and Semilog Plot (Below) (n=24)

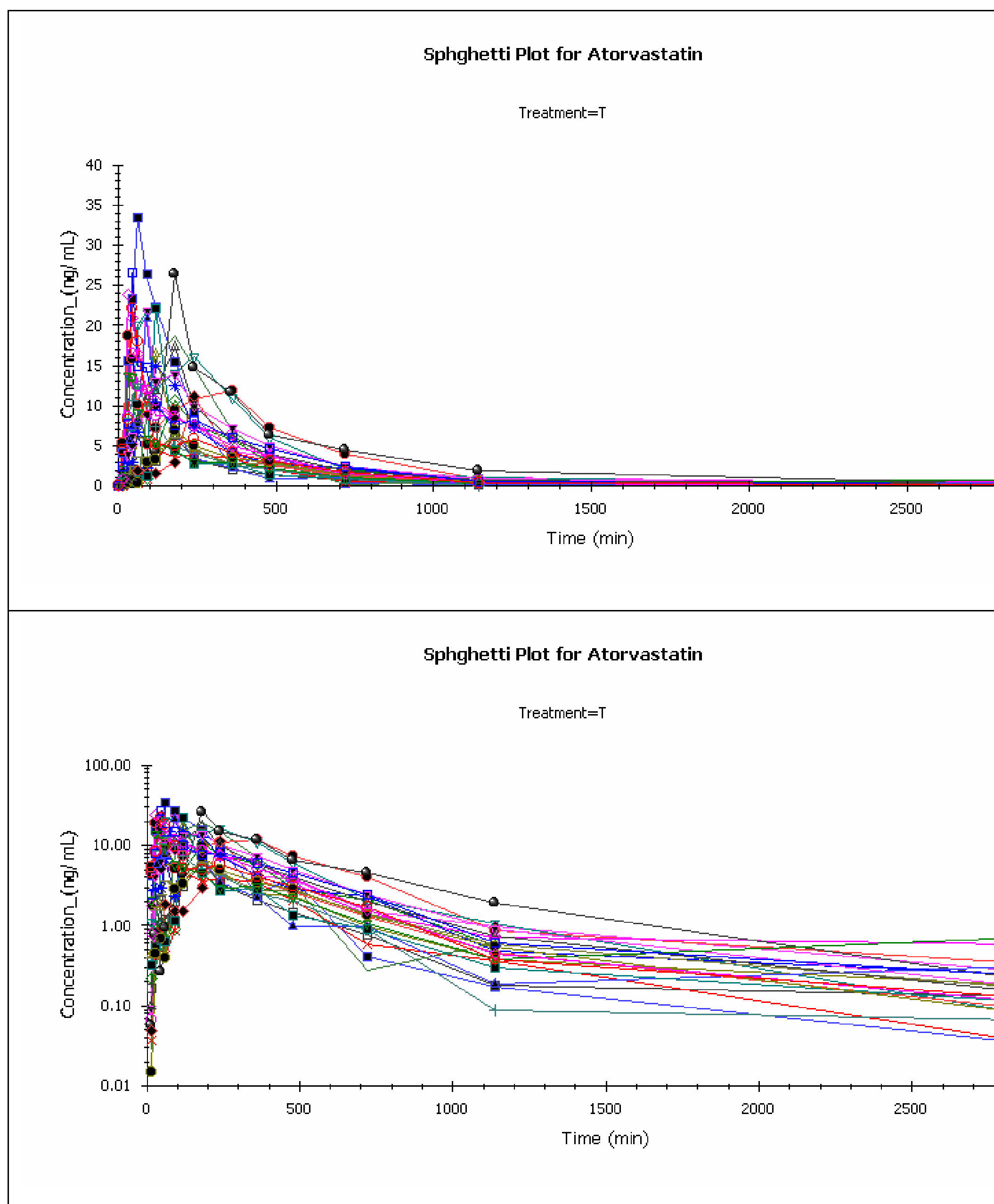


Figure 41. Spaghetti Plot for Plasma Concentration Time Profile of Test Treatment Atorvastatin for All Subjects Normal Plot (Above) and Semilog Plot (Below) (n=24)

Table 34. Maximum concentration (C_{max}) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24)

Subject No.	C_{max} (ng/ml)	
	Test	Reference
1	22.1	13.8
2	26.6	12.5
3	23.8	23.8
4	17.4	21.8
5	13.8	22.1
6	6.96	13.7
7	22.1	11.2
8	11.2	17.4
9	20.9	22.5
10	21.8	25.1
11	26.4	36.0
12	18.7	16.4
13	6.02	12.6
14	12.7	16.7
15	4.48	7.3
16	15.0	13.0
17	16.5	16.8
18	6.34	6.5
19	10.6	24.4
20	16.5	6.0
21	21.7	19.4
22	18.8	22.9
23	33.5	20.8
24	9.99	12.5

Table 35. Area under the plasma-concentration curve ($AUC_{0\text{-last}}$) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24)

Subject No.	$AUC_{0\text{-last}}$ (min x ng/ml)	
	Test	Reference
1	4260	3660
2	6010	3250
3	5310	6570
4	5770	3230
5	3660	4260
6	2970	3550
7	2680	2900
8	3080	2510
9	3350	6210
10	6690	3460
11	8820	10900
12	4860	4640
13	3150	4170
14	2890	2610
15	2090	3890
16	4740	4900
17	5640	5570
18	1970	1790
19	3537	6630
20	3560	2960
21	7900	5210
22	7820	9000
23	6010	4090
24	5050	6770

Table 36. Area under the plasma-concentration curve ($AUC_{0\text{-inf (observe)}}$) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24)

Subject No.	$AUC_{o\text{-inf (observed)}}$ (min x ng/ml)	
	Test	Reference
1	4300	3920
2	6090	3280
3	5350	6730
4	5090	3300
5	3920	4360
6	3020	3830
7	2790	3190
8	3150	2530
9	3450	6310
10	6800	3540
11	8970	11190
12	4930	4960
13	3310	4520
14	2900	2650
15	2110	4620
16	5020	5230
17	6720	5720
18	2010	1800
19	3730	6670
20	3760	3040
21	7950	5520
22	8150	9410
23	6010	4110
24	5390	7620

Table 37. Time to maximum concentration (T_{max}) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24)

Subject No.	T_{MAX} (min)	
	Test	Reference
1	45	45
2	45	180
3	30	180
4	180	120
5	30	45
6	180	90
7	120	45
8	240	120
9	90	360
10	90	120
11	180	60
12	180	30
13	180	15
14	60	45
15	120	180
16	120	45
17	45	180
18	180	30
19	180	120
20	120	30
21	90	45
22	30	180
23	60	45
24	120	90

Table 38. AUC $_{0\text{-last}}$/AUC0-$_{\text{inf (observe)}}$) of atorvastatin after single oral administration of 40 mg atorvastatin tablet of reference and test product to healthy volunteers (n=24)

Subject No.	AUC $_{0\text{-last}}$ /AUC $_{0\text{-inf(observe)}}$	
	Test	Reference
1	0.99	0.93
2	0.99	0.99
3	0.99	0.98
4	0.98	0.98
5	0.93	0.98
6	0.98	0.93
7	0.96	0.91
8	0.98	0.99
9	0.97	0.98
10	0.98	0.98
11	0.98	0.97
12	0.99	0.94
13	0.95	0.92
14	1.00	0.98
15	0.99	0.84
16	0.94	0.94
17	0.84	0.97
18	0.98	0.99
19	0.95	0.99
20	0.95	0.97
21	0.99	0.94
22	0.96	0.96
23	1.00	1.00
24	0.94	0.89

Table 39. Descriptive statistical of pharmacokinetics parameter

Descriptive statistical value	Pharmacokinetic parameter							
	C_{max} (ng/ml)		$AUC_{0\text{-}last}$ (min)		$AUC_{0\text{-}inf\ (predict)}$ (min x ng/ml)		T_{max} (min)	
	Reference	Test	Reference	Test	Reference	Test	Reference	Test
Mean	17.3	16.8	4700	4660	4950	4820	100	113
SD	6.97	7.42	2140	1890	2310	1950	79.7	62.4
SEM	1.42	1.51	437	386	472	397	16.3	12.7
Variance	48.6	55.0	4580000	3580000	5340000	3790000	6350	3890
Min	6.01	4.48	1790	1970	1800	2010	15	30
Median	16.8	17.0	4130	4500	4440	4610	75	120
Max	36.0	33.5	10900	8820	11900	8970	360	240
%CV	40.3	44.1	45.6	40.6	46.7	40.3	79.7	55.2
Geometric mean	15.9	15	4300	4300	4510	4450	75.1	94.2

Table 40. Result from pharmacokinetic study of atorvastatin acid

Characteristic	AUC_{0-last} (hour x ng/ml) ± SEM	AUC_{0-inf} (hour x ng/ml) ± SEM	C_{max} (ng/ml) ± SEM	T_{max} (hour)	$T_{1/2}$ (hour) ± SEM
Obtained for reference product Dose 40 mg	71.66±35.7	75.17±38.5	15.9±6.97	2(0.5-4)	7.9±4.8
Kantola et al Dose 40 mg (9)	54.2±24.2	-	13.4±9.5	1(0.5-3)	7.0±3.7
Lilja et al Dose 40 mg (16)	61.4±36.2	-	12.7±7.8	1.5(1-3)	9.7±2.8
Bahrami et al Dose 40 mg (2)	787.7±548.5	1349.2±694.5	50.1±30.7	3.2(-)	35.7±15.1
Borek-Dohalsky et al Dose 80 mg (16)	91.9±42.9	95.1±43.8	23.2±10.4	0.5 (0.25-3)	12.4±7.0

Table 41. BE Statistical Result (ANOVA) Type III for all subjects (N=24) (balanced dataset)

Dependent	Effect	SS	df	MS	F	Probability
Ln(Cmax)	Sequence	0.291188	1	0.291188	3.73	0.0665
Ln(Cmax)	Subject_No.(Sequence)	8.045711	22	0.365714	4.68	0.0003
Ln(Cmax)	Period	0.862953	1	0.862953	11.05	0.0031
Ln(Cmax)	Treatment	0.013485	1	0.013485	0.17	0.6818
Ln(Cmax)	Error	1.718162	22	0.078098		
Ln(AUClast)	Sequence	0.469378	1	0.469378	11.72	0.0024
Ln(AUClast)	Subject_No.(Sequence)	6.050697	22	0.275032	6.86	0.0001
Ln(AUClast)	Period	0.662051	1	0.662051	16.52	0.0005
Ln(AUClast)	Treatment	0.004425	1	0.004425	0.11	0.7428
Ln(AUClast)	Error	0.881415	22	0.040064		
Ln(AUCINF(observed))	Sequence	0.571411	1	0.571411	12.52	0.0018
Ln(AUCINF(observed))	Subject_No.(Sequence)	6.099108	22	0.277232	6.07	0.0001
Ln(AUCINF(observed))	Period	0.662609	1	0.662609	14.51	0.0010
Ln(AUCINF(observed))	Treatment	0.000465	1	0.000465	0.01	0.9206
Ln(AUCINF(observed))	Error	1.004400	22	0.045655		

Table 42. BE Statistical Result 90% Westlake Confidence Interval of Parameters for all subjects (N=24)

Dependent	Ratio	CL_90_Lower	CL_90_Upper	WL_90_Lower	WL_90_Upper	Power
Ln(Cmax)	96.6920	84.1405	111.1160	85.6796	114.3204	0.7489
Ln(AUClast)	101.9456	92.2821	112.6209	89.2695	110.7305	0.9551
Ln(AUCINF(observed))	100.6263	90.4778	111.9132	89.2287	110.7713	0.9300

Table 43. BE Statistical Result of T_{max} Using Nonparametric Friedman's Test for all Subjects

Friedman Design for Data, Subject, Treatment

For Atorvastatin TO

K=2; n=24

Si[1]=32.5
Si[2[=39.5

L=2.0417

Chi Square (0.95-1) =3.841

The difference between is not significant.

CHAPTER V
CONCLUSION

The bioanalytical methodology described in this report was specific, sensitive accurate and precise enough to be successfully applied to bioequivalence study. The method employed sample preparation by solid phase micro-extraction (SPME) with adequate recovery and less time consuming, followed by Gradient HPLC coupled with tandem mass spectrometric detection (LC MS/MS). The LC MS/MS method was determination of atorvastatin in human plasma, in the concentration range 0.2-80 ng/ml. There were no interferences from endogenous plasma component or other sources. The need of plasma vloume less than 250 μl for each sample made it possible to decrease volume of sample preparation per time. The method was successfully validated and proved appropriate for the analysis of atorvastatin in human plasma and can be applied for pharmacokinetics, bioavailability and bioequivalence studies. The analytical method presented here has proved to be useful for investigation of the characteristics of atorvastatin in human plasma in pharmacokinetic studies.

The statistical analysis of pharmacokinetics parameters confirms that the test product of atorvastatin, when compare with the reference product were equivalent in term of rate and extent of absorption found that the 90% confidence interval of the logarithmic transformed AUC and C_{max} were both contained in 80-125%. Due to error in randomization, the period and sequence effects were significant. However, there was no treatment effect which also confirmed 90% confidence interval of the logarithmic transformed AUC and C_{max} results. The subject nested in sequence effect is very common to be observed in bioequivalence trial due to the small subject number of subjects. Nonparametric, Friedman's test for T_{max} was also demonstrated the no significantly different between both formulation. Thus, based on the 90% CI criteria, the bioequivalence can be claimed.

REFERENCES

1. Schachter M. Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. Fundamental & clinical pharmacology. 2005 Feb;19(1):117-25.
2. Bahrami G, Mohammadi B, Mirzaeei S, Kiani A. Determination of atorvastatin in human serum by reversed-phase high-performance liquid chromatography with UV detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2005 Nov 5;826(1-2):41-5.
3. Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, et al. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Conference report. European journal of drug metabolism and pharmacokinetics. 1991 Oct-Dec;16(4):249-55.
4. James CA, Breda M, Frigerio E. Bioanalytical method validation: a risk-based approach? J Pharm Biomed Anal. 2004 Jun 29;35(4):887-93.
5. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). Guidance for industry: Available from: http://www.fda.gov/cder/guidance/4252fnl.htm [Accessed 10 October, 2006].
6. Shitara Y, Sugiyama Y. Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: drug-drug interactions and interindividual differences in transporter and metabolic enzyme functions. Pharmacology & therapeutics. 2006 Oct;112(1):71-105.
7. Available from: http://www.rxlist.com/cgi/generic/atorvastatin_cp.htm [Accessed October 20, 2006].

8. Yee HS, Fong NT. Atorvastatin in the treatment of primary hypercholesterolemia and mixed dyslipidemias. The Annals of pharmacotherapy. 1998 Oct;32(10):1030-43.
9. Kantola T, Kivisto KT, Neuvonen PJ. Effect of itraconazole on the pharmacokinetics of atorvastatin. Clinical pharmacology and therapeutics. 1998 Jul;64(1):58-65.
10. Malhotra HS, Goa KL. Atorvastatin: an updated review of its pharmacological properties and use in dyslipidaemia. Drugs. 2001;61(12):1835-81.
11. Rouleau J. Improved outcome after acute coronary syndromes with an intensive versus standard lipid-lowering regimen: results from the Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 (PROVE IT-TIMI 22) trial. The American journal of medicine. 2005 Dec;118 Suppl 12A:28-35.
12. Corsini A, Bellosta S, Baetta R, Fumagalli R, Paoletti R, Bernini F. New insights into the pharmacodynamic and pharmacokinetic properties of statins. Pharmacology & therapeutics. 1999 Dec;84(3):413-28.
13. Kovarik JM, Hartmann S, Hubert M, Berthier S, Schneider W, Rosenkranz B, et al. Pharmacokinetic and pharmacodynamic assessments of HMG-CoA reductase inhibitors when coadministered with everolimus. J Clin Pharmacol. 2002 Feb;42(2):222-8.
14. Goosen TC, Bauman JN, Davis JA, Yu C, Hurst SI, Williams JA, et al. Atorvastatin Glucuronidation is Minimally and Non-Selectively Inhibited by the Fibrates Gemfibrozil, Fenofibrate and Fenofibric Acid. Drug Metab Dispos. 2007 Apr 30.
15. Bullen WW, Miller RA, Hayes RN. Development and validation of a high-performance liquid chromatography tandem mass spectrometry assay for atorvastatin, ortho-hydroxy atorvastatin, and para-hydroxy atorvastatin in human, dog, and rat plasma. J Am Soc Mass Spectrom. 1999 Jan;10(1):55-66.
16. Borek-Dohalsky V, Huclova J, Barrett B, Nemec B, Ulc I, Jelinek I. Validated HPLC-MS-MS method for simultaneous determination of atorvastatin

and 2-hydroxyatorvastatin in human plasma-pharmacokinetic study. Anal Bioanal Chem. 2006 Sep;386(2):275-85.

17. Hermann M, Christensen H, Reubsaet JL. Determination of atorvastatin and metabolites in human plasma with solid-phase extraction followed by LC-tandem MS. Anal Bioanal Chem. 2005 Jul;382(5):1242-9.
18. Al-Rawithi S, Hussein RF, Alzahrani A. Sensitive assay for the determination of fluvastatin in plasma utilizing high-performance liquid chromatography with fluorescence detection. Ther Drug Monit. 2003 Feb;25(1):88-92.
19. Benet LZ. Bioavailability and bioequivalence: definition and difficulties in acceptance criteria. In: Midha KK, blume HH, editors. bio-international bioavailability, bioequivalence and pharmacokinetics; 1993; Stuttgart: Medpharm GmbH scientific; 1993.
20. Holford NGH. Pharmacodynamic and bioequivalence. In: Jackson AJ. Generics and bioequivalence. Florida: CRC Press; 1994.
21. Nirogi RV, Kandikere VN, Shukla M, Mudigonda K, Maurya S, Boosi R, et al. Simultaneous quantification of atorvastatin and active metabolites in human plasma by liquid chromatography-tandem mass spectrometry using rosuvastatin as internal standard. Biomed Chromatogr. 2006 Sep;20(9):924-36.
22. Jemal M, Ouyang Z, Chen BC, Teitz D. Quantitation of the acid and lactone forms of atorvastatin and its biotransformation products in human serum by high-performance liquid chromatography with electrospray tandem mass spectrometry. Rapid Commun Mass Spectrom. 1999;13(11):1003-15.
23. Abdou H. Dissolution, bioavailability & bioequivalence. Pennsylvania: Mark Plublish Company 1989.
24. Wastlake WJ. Bioavailability and bioequivalence of pharmaceutical formulations. In: Peace KE. Biopharmaceutical statistics for drug development, New York: Marcel dekker; 1998.
25. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). Guidance for industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies

for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System. Available from: http://www.fda.gov/cder/guidance/index.htm [Accessed 10 October, 2006]

26. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). Guidance for industry: Guidance for Industry Statistical Approaches to Establishing. Bioequivalence. Available from: http://www.fda.gov/cder/guidance/3616fnl.htm [Accessed 10 January 2007].
27. Chen ML, Lesko LJ. Individual bioequivalence revisited. Clinical pharmacokinetics. 2001;40(10):701-6.
28. Koytchev R, Ozalp Y, Erenmemisoglu A, van der Meer MJ, Alpan RS. Bioequivalence study of atorvastatin tablets. Arzneimittelforschung. 2004 Sep;54(9a):573-7.
29. Kameyama Y, Yamashita K, Kobayashi K, Hosokawa M, Chiba K. Functional characterization of SLCO1B1 (OATP-C) variants, SLCO1B1*5, SLCO1B1*15 and SLCO1B1*15+C1007G, by using transient expression systems of HeLa and HEK293 cells. Pharmacogenet Genomics. 2005 Jul;15(7):513-22.
30. Thompson JF, Man M, Johnson KJ, Wood LS, Lira ME, Lloyd DB, et al. An association study of 43 SNPs in 16 candidate genes with atorvastatin response. The pharmacogenomics journal. 2005;5(6):352-8.
31. Kajinami K, Brousseau ME, Ordovas JM, Schaefer EJ. Interactions between common genetic polymorphisms in ABCG5/G8 and CYP7A1 on LDL cholesterol-lowering response to atorvastatin. Atherosclerosis. 2004 Aug;175(2):287-93.
32. Nirogi R, Mudigonda K, Kandikere V. Chromatography-mass spectrometry methods for the quantitation of statins in biological samples. J Pharm Biomed Anal. 2007 Feb 13.

APPENDIX

APPENDIX

EXPERIMENTAL DATA

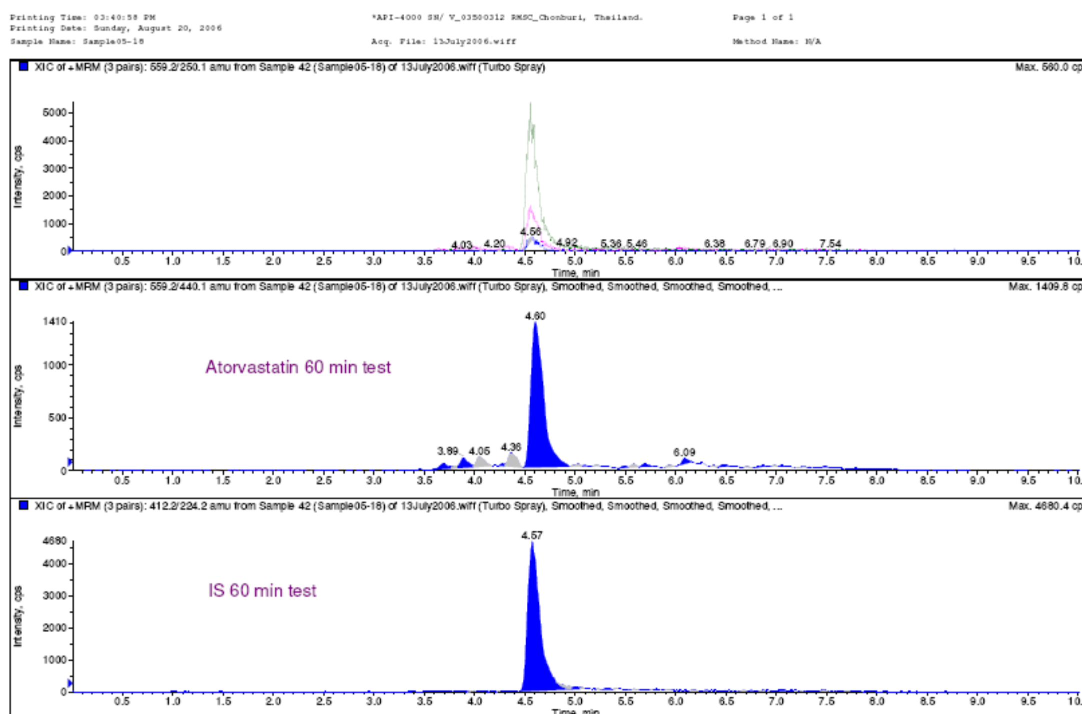


Figure 42. MRM ion-chromatograms resulting from the analysis of subject No.5 at 60 minutes for test product in human plasma for atorvastatin and internal standard.

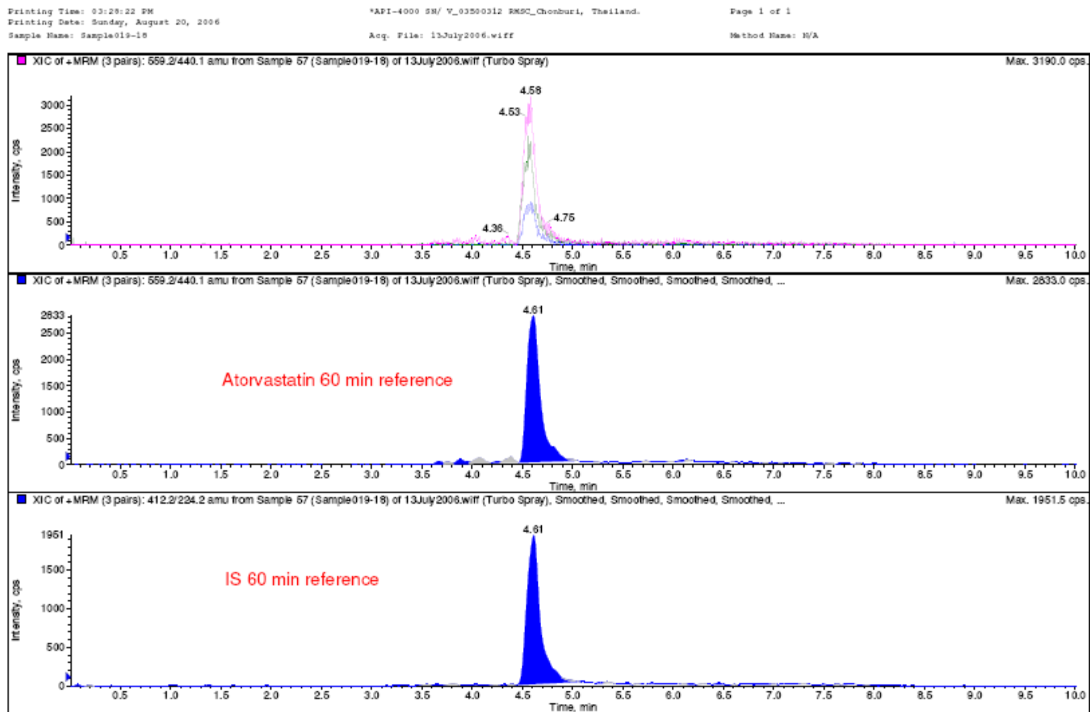


Figure 43. MRM ion-chromatograms resulting from the analysis of subject No.5 at 60 minutes for reference product in human plasma for atorvastatin and internal standard.

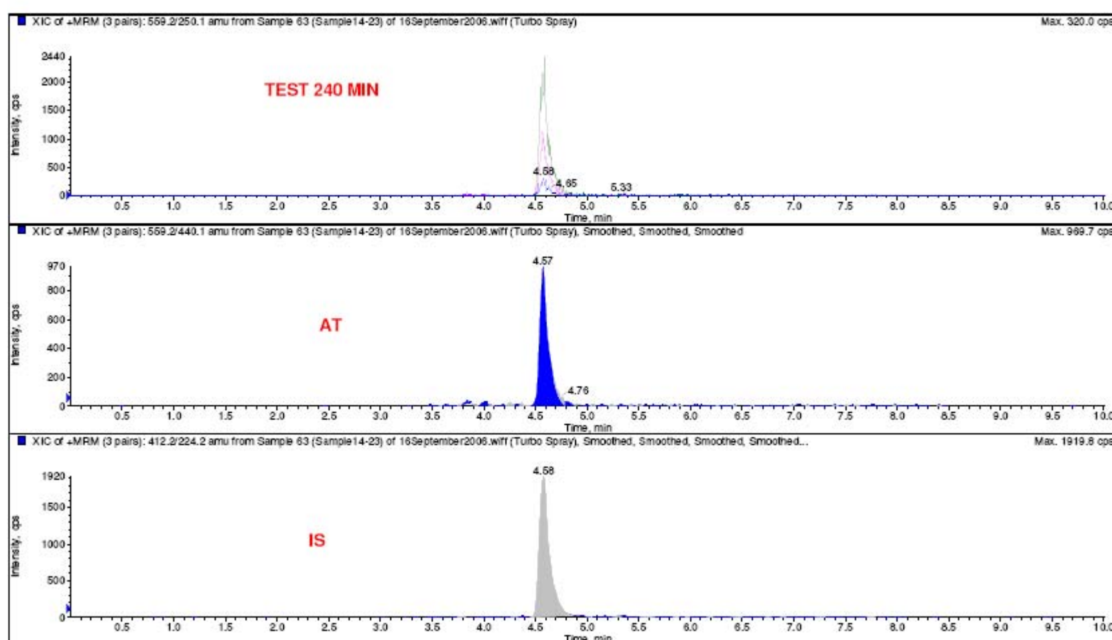


Figure 44. MRM ion-chromatograms resulting from the analysis of subject No.14 at 240 minutes for test product in human plasma for atorvastatin and internal standard.

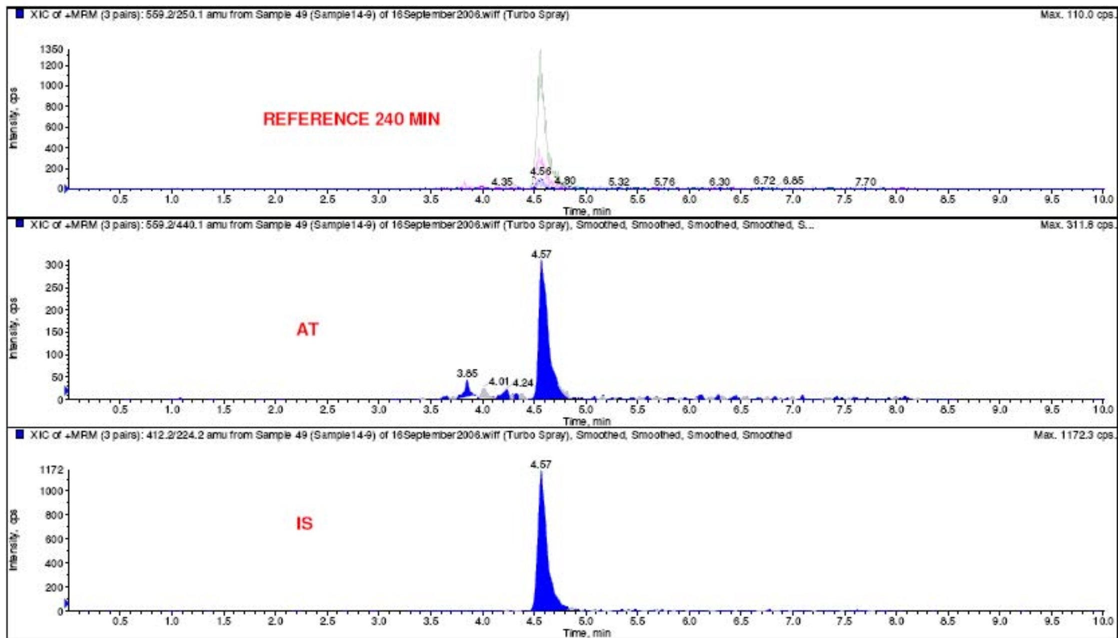


Figure 45. MRM ion-chromatograms resulting from the analysis of subject No.14 at 240 minutes for reference product in human plasma for atorvastatin and internal standard.

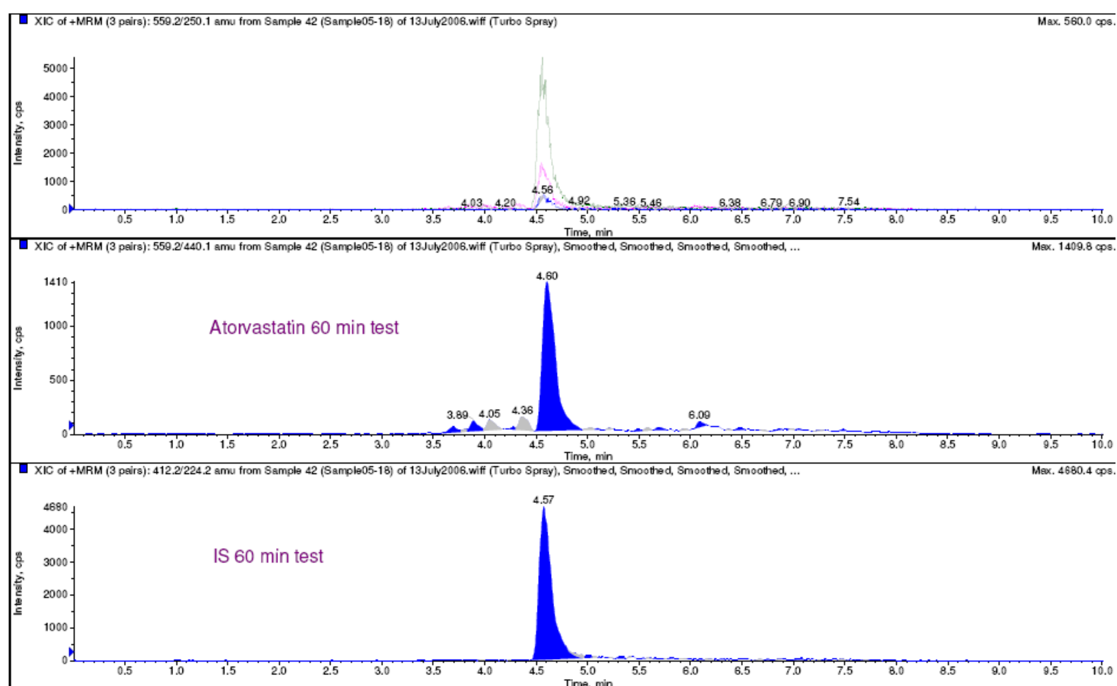


Figure 46. MRM ion-chromatograms resulting from the analysis of subject No.18 at 60 minutes for test product in human plasma for atorvastatin and internal standard.

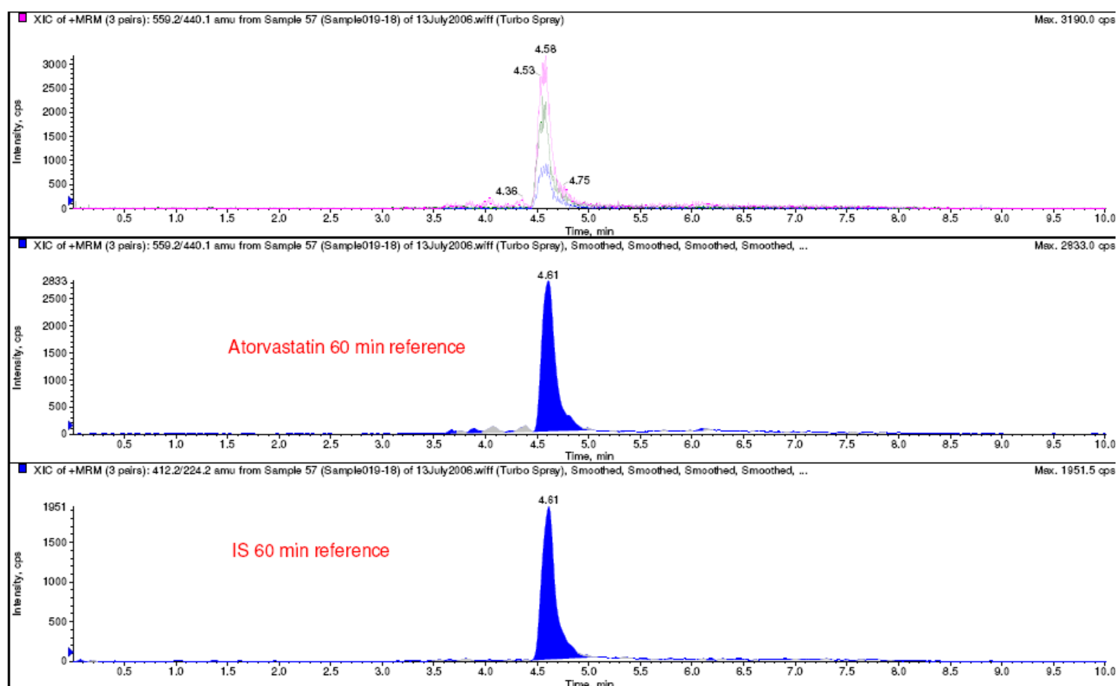


Figure 47. MRM ion-chromatograms resulting from the analysis of subject No.18 at 60 minutes for reference product in human plasma for atorvastatin and internal standard.

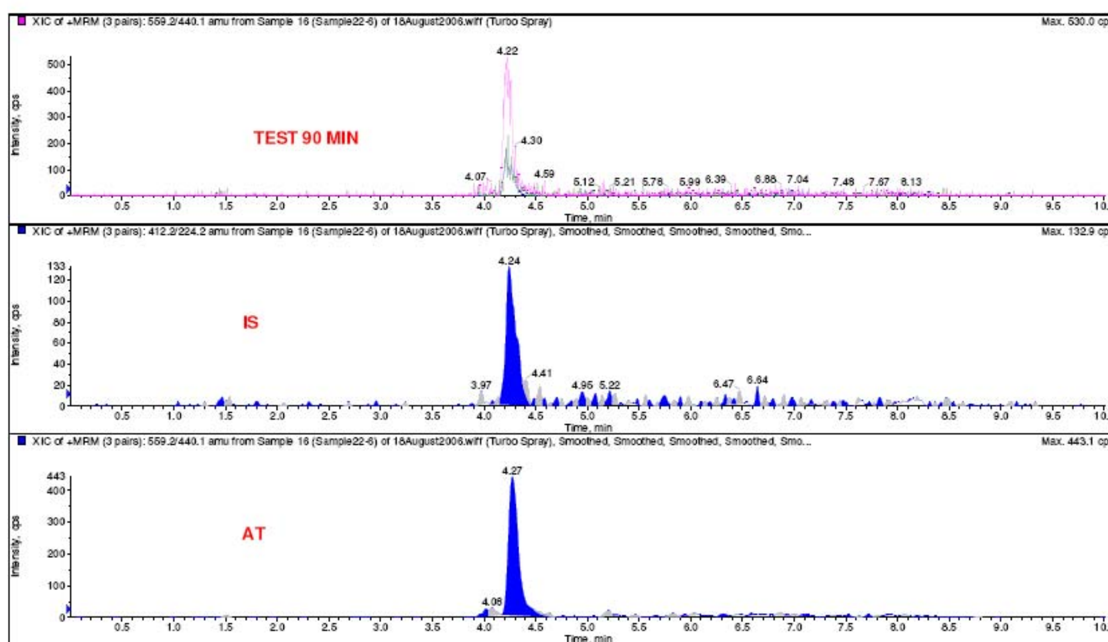


Figure 48. MRM ion-chromatograms resulting from the analysis of subject No.22 at 90 minutes for test product in human plasma for atorvastatin and internal standard.

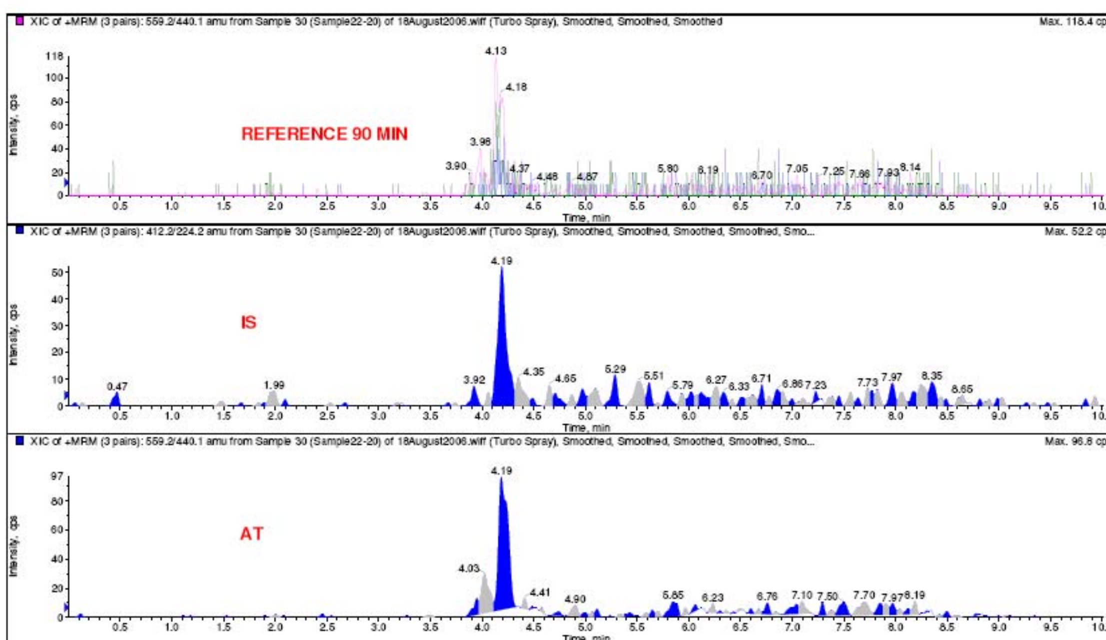


Figure 49. MRM ion-chromatograms resulting from the analysis of subject No.22 at 90 minutes for reference product in human plasma for atorvastatin and internal standard.

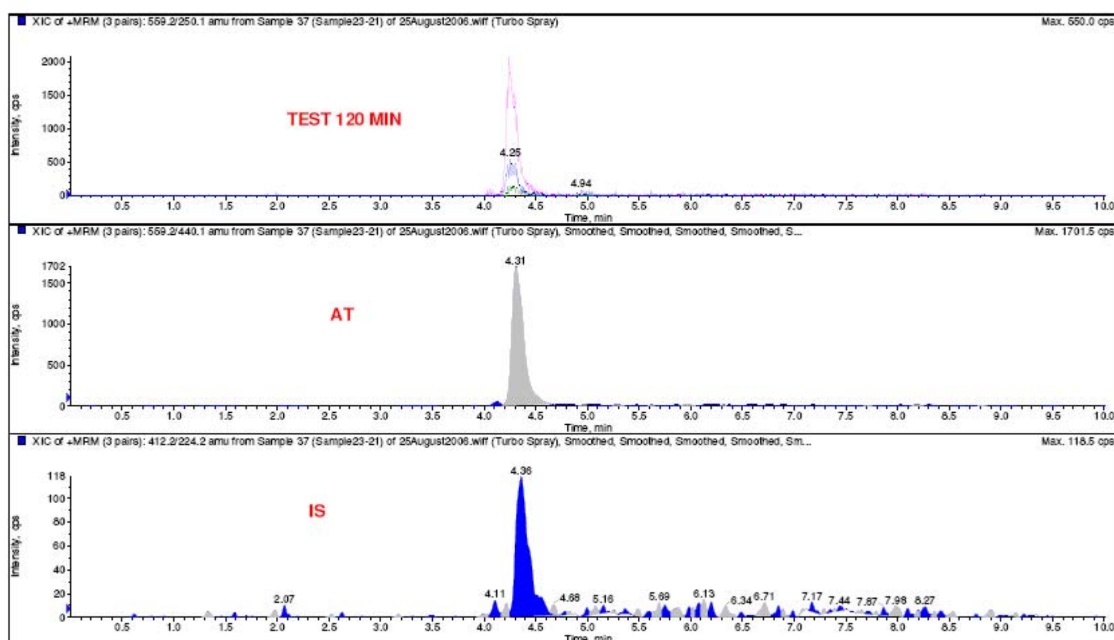


Figure 50. MRM ion-chromatograms resulting from the analysis of subject No.23 at 120 minutes for test product in human plasma for atorvastatin and internal standard.

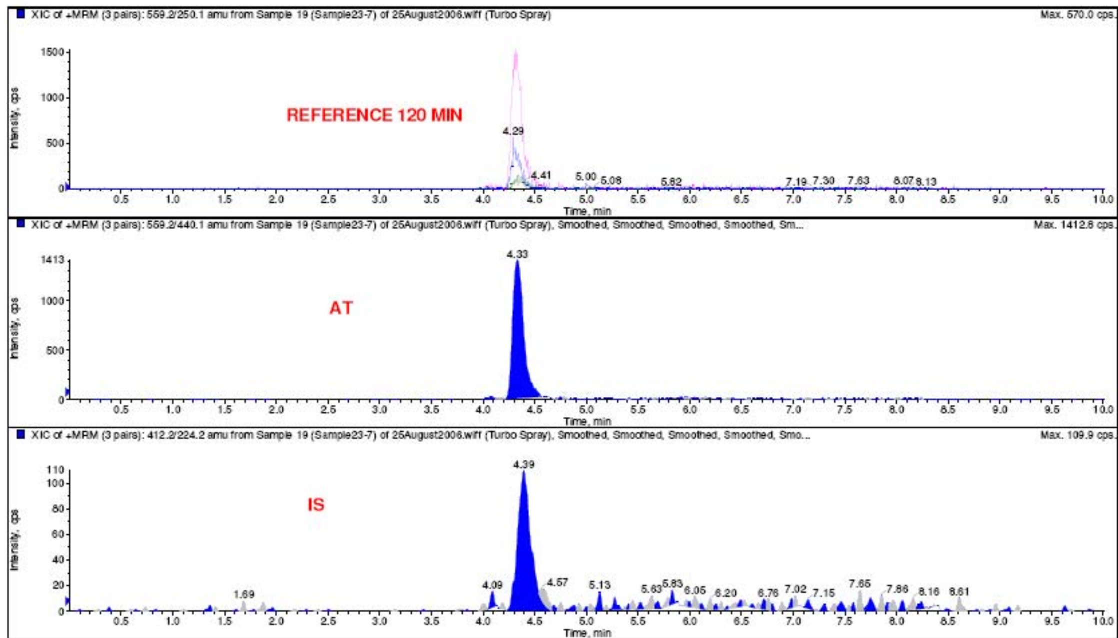


Figure 51. MRM ion-chromatograms resulting from the analysis of subject No.23 at 120 minutes for reference product in human plasma for atorvastatin and internal standard.

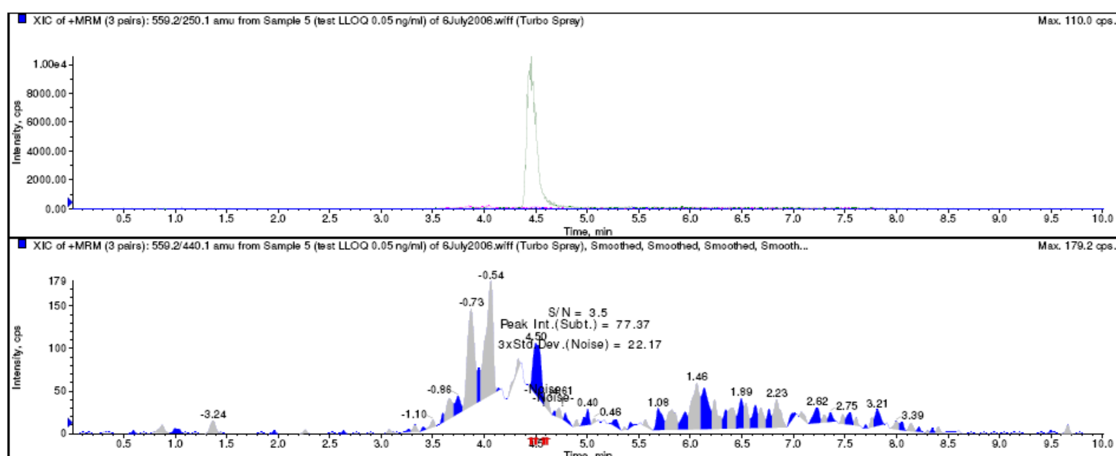


Figure 52. MRM ion-chromatograms resulting from the analysis of lower limit of detection (LOD) in human plasma for atorvastatin and internal standard.

Table 44. Report of sequence data after LC MS/MS.

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Peak Height (cps)	Analyte Concentration (ng/mL)	IS Peak Area (counts)	IS Peak Height (cps)	Calculated Concentration (ng/mL)	Accuracy (%)
1	blank sample	July2006\26July200	0.00e+000	0.00e+000	0.00	1.32e+002	3.91e+001	N/A	N/A
2	zero-sample	July2006\26July200	3.36e+002	8.93e+001	0.00	7.57e+003	1.07e+003	N/A	N/A
3	std AT 0.2	July2006\26July200	1.57e+003	2.57e+002	0.200	8.57e+003	1.25e+003	0.225	113.
4	std AT0.5	July2006\26July200	3.62e+003	5.90e+002	0.500	9.89e+003	1.23e+003	0.459	91.9
5	std AT2	July2006\26July200	1.82e+004	2.94e+003	2.00	1.08e+004	1.58e+003	2.16	108.
6	std AT5	July2006\26July200	3.65e+004	4.91e+003	5.00	9.76e+003	1.17e+003	4.78	95.6
7	std AT15	July2006\26July200	1.36e+005	1.78e+004	15.0	1.14e+004	1.34e+003	15.3	102.
8	std AT20	July2006\26July200	2.22e+005	2.55e+004	20.0	1.60e+004	1.94e+003	17.8	88.9
9	std50	July2006\26July200	1.45e+005	1.83e+004	50.0	3.85e+003	4.95e+002	48.4	96.7
10	std80	July2006\26July200	1.65e+005	2.05e+004	80.0	2.53e+003	3.02e+002	83.7	105.
11	methanol	July2006\26July200	2.80e+002	8.11e+001	N/A	0.00e+000	0.00e+000	0.00	N/A
12	Sample19-01	July2006\26July200	0.00e+000	0.00e+000	N/A	7.85e+003	7.58e+002	No Peak	N/A
13	Sample19-02	July2006\26July200	1.46e+003	1.56e+002	N/A	8.12e+003	7.88e+002	0.220	N/A
14	Sample19-03	July2006\26July200	5.91e+003	7.02e+002	N/A	8.59e+003	9.10e+002	0.872	N/A
15	Sample19-04	July2006\26July200	1.13e+004	1.02e+003	N/A	1.33e+004	1.34e+003	1.09	N/A
16	Sample19-05	July2006\26July200	4.60e+003	4.55e+002	N/A	9.05e+003	8.21e+002	0.641	N/A
17	Sample19-06	July2006\26July200	8.28e+003	8.07e+002	N/A	1.15e+004	1.05e+003	0.911	N/A
18	Sample19-07	July2006\26July200	3.68e+004	3.62e+003	N/A	1.01e+004	9.57e+002	4.64	N/A
19	Sample19-08	July2006\26July200	6.07e+004	5.95e+003	N/A	7.38e+003	6.90e+002	10.5	N/A
20	Sample19-09	July2006\26July200	2.94e+004	3.01e+003	N/A	5.03e+003	5.06e+002	7.47	N/A
21	Sample19-10	July2006\26July200	3.62e+004	3.36e+003	N/A	8.05e+003	8.08e+002	5.75	N/A
22	methanol	July2006\26July200	0.00e+000	0.00e+000	N/A	6.77e+001	2.01e+001	No Peak	N/A
23	QC A1	July2006\26July200	1.84e+003	2.30e+002	0.250	8.79e+003	8.34e+002	0.258	103.
24	methanol	July2006\26July200	0.00e+000	0.00e+000	N/A	0.00e+000	0.00e+000	No Peak	N/A
25	Sample19-11	July2006\26July200	1.29e+004	1.28e+003	N/A	5.62e+003	5.81e+002	2.92	N/A
26	Sample19-12	July2006\26July200	1.52e+004	1.41e+003	N/A	9.43e+003	9.25e+002	2.06	N/A
27	Sample19-13	July2006\26July200	2.21e+003	2.40e+002	N/A	5.02e+003	5.02e+002	0.555	N/A
28	Sample19-14	July2006\26July200	0.00e+000	0.00e+000	N/A	5.04e+003	5.31e+002	No Peak	N/A
29	Sample19-15	July2006\26July200	0.00e+000	0.00e+000	N/A	5.64e+003	5.64e+002	No Peak	N/A
30	Sample19-16	July2006\26July200	9.92e+004	1.04e+004	N/A	9.56e+003	1.05e+003	13.3	N/A
31	Sample19-17	July2006\26July200	2.04e+005	2.10e+004	N/A	1.16e+004	1.18e+003	22.5	N/A
32	Sample19-18	July2006\26July200	1.16e+005	1.25e+004	N/A	7.09e+003	9.01e+002	21.0	N/A
33	Sample19-19	July2006\26July200	1.17e+005	1.29e+004	N/A	7.73e+003	8.14e+002	19.4	N/A
34	Sample19-20	July2006\26July200	1.54e+005	1.50e+004	N/A	1.13e+004	1.11e+003	17.5	N/A
35	methanol	July2006\26July200	0.00e+000	0.00e+000	N/A	5.41e+001	2.68e+001	No Peak	N/A
36	QC B1	July2006\26July200	6.57e+004	6.22e+003	10.0	8.64e+003	8.70e+002	9.72	97.2
37	methanol	July2006\26July200	0.00e+000	0.00e+000	N/A	2.44e+002	3.35e+001	No Peak	N/A
38	Sample19-21	July2006\26July200	2.24e+005	2.31e+004	N/A	1.19e+004	1.23e+003	24.2	N/A
39	Sample19-22	July2006\26July200	1.04e+005	1.12e+004	N/A	8.53e+003	9.18e+002	15.6	N/A
40	Sample19-23	July2006\26July200	5.38e+004	5.64e+003	N/A	7.35e+003	7.68e+002	9.37	N/A
41	Sample19-24	July2006\26July200	3.79e+004	3.79e+003	N/A	7.97e+003	7.51e+002	6.08	N/A
42	Sample19-25	July2006\26July200	6.76e+003	7.51e+002	N/A	3.34e+003	3.26e+002	2.58	N/A

BIOGRAPHY

NAME	Mr. Pattarawit Rukthong
DATE OF BIRTH	3 January 1979
PLACE OF BIRTH	Nakhonsrithamaraj, Thailand
INSTITUTIONS ATTENDED	Srinakharinwirot University, 1996-2001: Bachelor of Science in Pharmacy Mahidol University, 2003-2007: Master of Science in Pharmacy (pharmaceutics)
RESERCH GRANT	Commission of higher Education Staff Development Project, Ministry of University Affairs. Thailand (2003-2004)
POSITION & OFFICE	2001-present: Department of Pharmaceutical technology, Faculty of pharmaceutical science, Srinakharinwirot University. Nakronayok, Thailand Position: Lecturer
HOME ADDRESS	120/ 49 rangsit-phatom Rd. Tumbon banglaung, Ampur Muang, Phatomthani, Thailand 12600 E-mail: pattarawitr@gmail.com