BIOAVAILABILITY STUDIES OF ANDROGRAPHOLIDE IN RATS

CHIA LEE HUANG

UNIVERSITI SAINS MALAYSIA

2016

BIOAVAILABILITY STUDIES OF ANDROGRAPHOLIDE IN RATS

by

CHIA LEE HUANG

Thesis submitted in fulfillment of the requirements for the degree of

Master of Science

June 2016

ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest gratitude to my supervisor Prof. Yuen Kah Hay for his useful guidance, patience, caring and providing me with an excellent atmosphere for doing research. His patience and support helped me overcome many crisis situations and finish this project.

I wish to express my sincere gratitude to Dr. Lim Sheau Chin for her aspiring guidance, invaluably constructive criticism and insightful comments during the project work. I am sincerely grateful to her for sharing her truthful and illuminating views on a number of issues related to the project.

My sincere thanks also go to Dr. Wong Chia Woei who gave access to the laboratory and research facilities. I thank my fellow lab mates, namely Song Thai, Gan, Phaik Chin, Fung Wai Yee, Mei Mei, Ai Boey, Siew Siew, Belle, Sharon, Ying Yu, Luen Hui, Mr.Wan, Erena, Kamaliah, June, Vincent, Goldie, Mei Ching, Li Ying, Yanti, Choon Wai Yee for the stimulating discussions and for all the fun we have had. Without their tremendous support and encouragement it would not be possible to conduct and complete this research.

I am also indebted to Dr. Isma and Ms Melati from Animal Research and Service Centre of USM for their enormous support. Last but not least, I thank my family members, especially my mother for supporting me throughout all my studies at University.

TABLES OF CONTENTS

Title Page	i
Acknowledgement	ii
Tables of Contents	iii
List of Appendices	viii
List of Tables	xii
List of Figures	xiv
List of Symbols, Abbreviations or Nomenclature	xvi
Abstrak	xix
Abstract	xxi

CHAPTER 1: INTRODUCTION

1.1 Bioavailability and oral drug absorption	1
1.2 Transport of drug molecules across the gastrointestinal membrane	2
1.2.1 Paracellular pathway	3
1.2.2 Transcellular pathway	3
1.2.2(a) Passive diffusion	3

1.2.2(b) Carrier-mediated transport 4

1.2.2(c) Vesicular transport 5

1.3 Factors affect drug absorption 5

1.3.1 Physiological factors 6

1.3.2 Physiochemical properties of a drug 8

1.3.3 Formulation factor 10

1.4 *Andrographis paniculata* 10

1.4.1 Andrographolide 13

1.4.2 Medicinal benefits of andrographolide 14

1.5 Pharmacokinetic studies of andrographolide 16

1.6 Scope of study 17

CHAPTER 2: DEVELOPMENT OF SIMPLE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR ANALYSIS OF ANDROGRAPHOLIDE IN HUMAN AND RAT PLASMA

2.1 Introduction 18

2.2 Materials and reagents 19

2.3 Instrumentation and chromatographic conditions 19

2.4 Preparation of stock and working solution 20

2.5 Standard solutions and calibration curves 21

2.6 Assay validation 21

2.7 Stability 22

2.8 Results 23

2.9 Discussion 30

2.10 Conclusion 32

CHAPTER 3: PHARMACOKINETIC AND BIOAVAILABILITY STUDIES OF ANDROGRAPHOLIDE, WITH AND WITHOUT PHYSICAL MIXING WITH POLYVINYLPYRROLIDONE K25 IN RATS

3.1 Introduction 33

3.2 Materials 34

3.2.1 Materials 34

3.2.2 Animals 34

3.3 Methods 34

3.3.1 Saturation solubility study 34

3.3.2 Stability study of andrographolide-PVP K25 complex of weight ratio 1:5 35

3.3.3 Pharmacokinetic profiles of pure andrographolide 35

3.3.4 Oral bioavailability of pure andrographolide, with and without polyvinylpyrrolidone (PVP) K25 37

3.3.5 Oral bioavailability of pure andrographolide with different weight ratios of polyvinylpyrrolidone K25 38
3.3.6 Pharmacokinetics and statistical analysis 39
3.4 Results 41
3.4.1 Saturation solubility study 41
3.4.2 Stability study of andrographolide-PVP K25 complex of weight ratio 1:5 42
3.4.3 Pharmacokinetic profiles of pure andrographolide 43
3.4.4 Oral bioavailability of pure andrographolide, with and without polyvinylpyrrolidone (PVP) K25 45
3.4.5 Oral bioavailability of pure andrographolide with different weight ratios of polyvinylpyrrolidone K25 48
3.5 Discussion 51
3.6 Conclusion 54

CHAPTER 4: EFFECT OF CYTOCHROME P450 ENZYME (CYP1A2) ON ANDROGRAPHOLIDE ABSORPTION IN RATS

4.1 Introduction 55
4.2 Materials 56
4.2.1 Materials 56

4.2.2 Animals 57
4.3 Methods 57
4.3.1 Oral bioavailability of andrographolide with different doses, andrographolide-PVP K25 weight ratio 1:5 57
4.3.2 Oral administration of andrographolide with and without co-administration of caffeine 58
4.3.3 Pharmacokinetics 59
4.3.4 Statistical analysis 60
4.4 Results 60
4.4.1 Oral bioavailability of andrographolide with different doses, andrographolide-PVP K25 weight ratio 1:5 60
4.4.2 Oral administration of andrographolide, with and without co-administration of caffeine 64
4.5 Discussion 67
4.6 Conclusion 69

CHAPTER 5: SUMMARY 70

CHAPTER 6: FUTURE STUDIES

6.1 Pharmacokinetics and bioavailability study of andrographolide in human subjects 71

6.2 Formulation with novel drug delivery system 71

6.3 Influence of food matrices on bioavailability of andrographolide and andrographolide extract 72

REFERENCES 73

APPENDICES

Appendix 3.1 Approval from Animal Ethics Committee (AEC) of USM on the study of bioavailability of andrographolide in rat 82

Appendix 3.1(a) Plasma andrographolide concentration values of individual rats after IV administration of 2mg/kg of andrographolide per rat 83

Appendix 3.1(b) Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide per rat 84

Appendix 3.2	Approval letter from Animal Ethics Committee (AEC) of USM on the study of bioavailability of andrographolide in rat, with and without addition of PVP K25	85
Appendix 3.2(a)	Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide per rat	86
Appendix 3.2(b)	Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide and 250mg/kg of PVP K25 per rat	87
Appendix 3.3	Approval letter from Animal Ethics Committee (AEC) of USM on the study of bioavailability of andrographolide in rat, with fixed dose of andrographolide but different weight ratios of PVP K25	88
Appendix 3.3(a)	Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide and 50mg/kg of PVP K25 per rat	89

Appendix 3.3(b)	Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide and 250mg/kg of PVP K25 per rat	90
Appendix 3.3(c)	Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide and 500mg/kg of PVP K25 per rat	91
Appendix 4.1	Approval letter from Animal Ethics Committee (AEC) of USM on the study of bioavailability of different doses of andrographolide in rat, andrographolide-PVP K25 Weight Ratio 1:5	92
Appendix 4.1(a)	Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide and 250mg/kg of PVP K25 per rat	93
Appendix 4.1(b)	Plasma andrographolide concentration values of individual rats after oral administration of 100mg/kg of andrographolide and 500mg/kg of PVP K25 per rat	94

Appendix 4.1(c) Plasma andrographolide concentration values of individual rats after oral administration of 200mg/kg of andrographolide and 1000mg/kg of PVP K25 per rat 95

Appendix 4.2 Approval letter from Animal Ethics Committee (AEC) of USM on the study of effect of metabolising enzyme inhibition on andrographolide absorption in rats 96

Appendix 4.2(a) Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide, 250mg/kg of PVP K25 and 8mg/kg caffeine per rat 97

Appendix 4.2(b) Plasma andrographolide concentration values of individual rats after oral administration of 50mg/kg of andrographolide and 250mg/kg of PVP K25 per rat 98

CERTIFICATE OF ACKNOWLEDGEMENT 99

LISTS OF TABLES

		Page
Table 2.1	Regression parameters and statistics for calibration curves (n=6) of plasma andrographolide assay method	26
Table 2.2	Recovery, within-day and between-day accuracy and precision values (n=6) for plasma andrographolide assay method	28
Table 2.3	Stability studies of andrographolide in human plasma and solution	29
Table 3.1	Intravenous and oral administration following a parallel study design	37
Table 3.2	Oral administration following a parallel study design	38
Table 3.3	Mode of administration following a parallel study design	39
Table 3.4	Solubility of andrographolide with different amount of PVP K25	41
Table 3.5	Individual numerical values of $AUC_{0\rightarrow 4h}$after iv administration of 2mg/kg andrographolide	44
Table 3.6	Individual numerical values of C_{max}, T_{max} and $AUC_{0\rightarrow 6h}$ after oral administration of 50mg/kg of andrographolide, with 250mg/kg of PVP K25	47
Table 3.7	Individual numerical values of C_{max}, T_{max} and $AUC_{0\rightarrow 6h}$ after oral administration of 50mg/kg of andrographolide with different weight ratios of PVP K25	50

Table 4.1 Mode of administration following a parallel study design 57

Table 4.2 Mode of administration following a parallel study design 59

Table 4.3 Individual numerical values of C_{max}, T_{max} and $AUC_{0\rightarrow 6h}$ after oral administration of 50mg/kg, 100mg/kg and 200mg/kg of andrographolide with 1:5 weight ratios of PVP K25 63

Table 4.4 Individual numerical values of C_{max}, T_{max} and $AUC_{0\rightarrow 6h}$ after oral administration of 50mg/kg of andrographolide, with and without co-administration of caffeine 66

LISTS OF FIGURES

		Page
Figure 1.1	Mechanisms of drug transport across an epithelial membrane	2
Figure 1.2	*Andrographis paniculata* and flowers, scale approximately 0.8x	12
Figure 1.3	Chemical structure of andrographolide (Source: Sigma Aldrich)	13
Figure 2.1A	Blank human plasma	24
Figure 2.1B	Human plasma spiked with (1) 1µg/mL andrographolide and (2) 2.5µg/mL carbamazepine	24
Figure 2.1C	Blank rat plasma	25
Figure 2.1D	Rat plasma (3) 10 minutes after intravenous administration of 2µg/mL andrographolide and (4) spiked with 2.5µg/mL carbamazepine	25
Figure 2.2	Calibration curve of andrographolide in human spiked plasma (n=6)	27
Figure 3.1	Stability profile of andrographolide-polyvinylpyrrolidone K25 (1:5) complex at pH1.2, 4.5 and 7.0	42
Figure 3.2	Mean plasma concentrations versus time profiles (Mean ± SEM., n=6) of andrographolide after IV administration of 2mg/kg andrographolide and oral administration of 50mg/kg andrographolide	44

Figure 3.3	Mean plasma concentrations versus time profiles (Mean ± SEM., n=6) of andrographolide after oral administration of 50mg/kg andrographolide, with and without 250mg/kg of PVP K25	46
Figure 3.4	Mean plasma concentrations versus time profiles (Mean ± SEM., n=6) of andrographolide after oral administration of 50mg/kg andrographolide, with different weigh ratios of PVP K25	49
Figure 4.1	Mean plasma concentrations versus time profiles (Mean ± SEM., n=6) of andrographolide after oral administration of 50mg/kg, 100mg/kg and 200mg/kg andrographolide, with 1:5 ratios of PVP K25	62
Figure 4.2	Mean plasma concentrations versus time profiles (Mean ± SEM., n=6) of andrographolide after oral administration of 50mg/kg andrographolide, with and without co-administration of caffeine	65

LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

ACN	Acetonitrile
AND	Andrographolide
ANOVA	Analysis of variance
$AUC_{0\rightarrow t}$	Area under the plasma concentration-time curve from time zero to the last sampling time, t
$AUC_{0\rightarrow 6h}$	Area under the plasma concentration-time curve from time zero to the last sampling time, 6 hours after dosing
$AUC_{0\rightarrow \infty}$	Area under the plasma concentration-time curve from time zero to the infinity
C_{max}	Peak plasma concentrations
C.V.	Coefficient of variation
CYP	Cytochrome P450 enzyme
CYP1	Cytochrome P450 subfamily 1
CYP1A2	Cytochrome P450 subfamily 1A2
CYP3	Cytochrome P450 subfamily 3A
CYP3A4	Cytochrome P450 subfamily 3A4

DMSO	Dimethyl sulfoxide
GI	Gastrointestinal
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
IFN-2	Interferon 2
IL-2	Interleukin 2
iNOS	inducible nitric oxide synthase
k_e	Elimination rate constant
LCMS-MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MMC	Migrating motor complex
MTBE	Methyl-tert-butyl-ether
NF-kβ	Nuclear factor kappa-B
NPM	N-methyl-2-pyrrolidone

PO	Peroral administration
PVDF	Polyvinylidene fluoride
PVP	Polyvinylpyrrolidone
S.E.M.	Standard error of mean
T_{max}	Time to reach peak plasma concentration
TNF-α	Tumour necrosis factor alpha
UV/Vis	Ultraviolet-viscible
v/v	Volume to volume

KAJIAN BIOPEROLEHAN ANDROGRAPHOLIDE DALAM TIKUS

ABSTRAK

Satu kaedah analisis kromatografi cecair prestasi tinggi yang mudah, sensitif and khusus telah dibangunkan dan disahkan untuk pengesanan dan kuantifikasi andrographolide di dalam plasma. Kaedah ini adalah linear dari 31.3ng/mL hingga 2000ng/mL. Nilai koefisi variasi untuk dalam hari dengan antara hari pengesahan adalah kurang daripada 12%. Nilai kejituan untuk dalam hari dengan masing-masing antara hari adalah di antara 90.3% - 103.3% dan 93.5% - 103.9%. Pemulihan mutlak untuk andrographolide adalah lebih daripada 67%. Andrographolide stabil dalam larutan sehingga 6 bulan. Walau bagaimanapun, ia hanya stabil dalam plasma sehingga 2 jam, mungkin kerana terikat kepada protein. Andrographolide tulen adalah kurang larut dalam air. Apabila dicampur dengan polimer polyvinylpyrrolidone (PVP) K25, kelarutan andrographolide dalam air meningkat. Penyelidikan *in vitro* menunjukkan bahawa kebolehlarutan andrographolide meningkat apabila jumlah PVP K25 ditambah. Kompleks andrographolide-PVP K25 adalah stabil pada pH1.2, 4.5 dan 7.0 sehingga 8 jam, dengan lebih daripada 90% kekal dalam larutan. Penyelidikan *in vivo* dalam tikus menunjukkan bahawa andrographolide tulen tidak dapat dikesan dalam plasma walaupun diberi dos setinggi 50mg/kg. Walau bagaimanapun, dengan mencampurkannya dengan PVP K25, sejenis polimer yang larut dalam air, bioperolehan oral andrographolide dalam tikus meningkat dengan ketara. Oleh itu, bioperolehan oral andrographolide rendah terutamanya disebabkan oleh keterlarutan air yang rendah.

Bioperolehan andrographolide yang rendah mungkin juga disebabkan oleh metabolisme laluan pertama.

BIOAVAILABILITY STUDIES OF ANDROGRAPHOLIDE IN RATS

ABSTRACT

A simple, specific and sensitive high performance liquid chromatography (HPLC-UV) analytical method has been developed and validated for detection and quantification of andrographolide level in the plasma. The standard curve was linear from 31.3ng/mL to 2000ng/mL. The coefficient of variation (C.V.) values of this method was less than 12% for both within- and between-day validation. The accuracy for both within- and between-day validation ranged from 90.3% to 103.3% and 93.5% to 103.9% respectively. The absolute recovery for andrographolide was more than 67.3%. Andrographolide in working solution was stable up to 6 months. Nevertheless, it was only stable in plasma up to 2 hours, probably due to high protein-binding. Pure andrographolide was poorly soluble in water. Its aqueous solubility increased when physically mixed with polymer polyvinylpyrrolidone (PVP) K25. *In vitro* studies showed that the solubility of andrographolide increased when increased amount of PVP K25 was added. Andrographolide-PVP K25 complex was stable in pH1.2, 4.5 and 7.0 up to 8 hours, with more than 90% remaining undegraded. *In vivo* rat studies showed that pure andrographolide was barely detected in plasma although given at high dose of 50mg/kg. However, by mixing it with water-soluble polymer PVP K25, the bioavailability of andrographolide in rats increased significantly. Therefore, poor bioavailability of andrographolide is mainly due to its poor aqueous solubility. The poor bioavailability of andrographolide may also be to a lesser extent, due to first pass metabolism.

CHAPTER 1: INTRODUCTION

1.1 Bioavailability and oral drug absorption

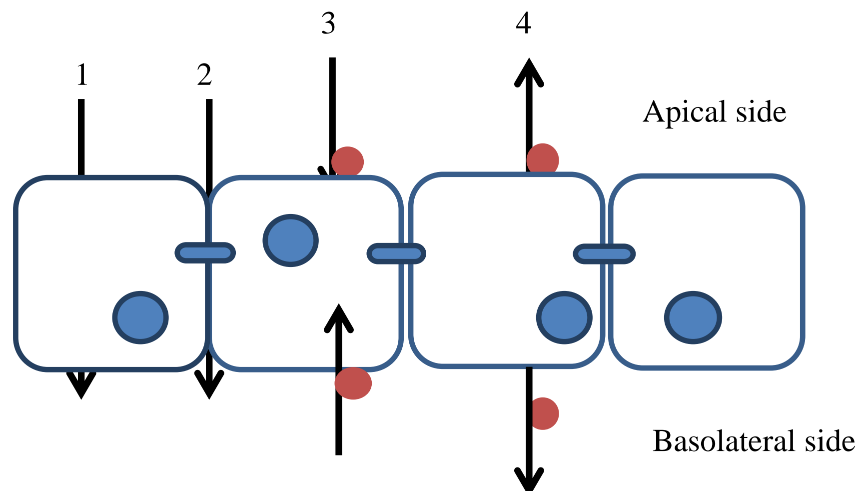
Bioavailability is defined as the rate and extent at which the active moiety is absorbed from a drug and becomes available at the site of action (FDA, 2003). A drug which is administered by intravenous route is considered 100% bioavailable, as it is directly introduced into the systemic circulation. Extravascular administration includes all routes of administration in which the drug is not delivered directly to the bloodstream. Any drug administered extravascularly must first be absorbed from its site of administration to subsequently enter the systemic circulation. The bioavailability of any drug given by extravascular route can range from zero to 100 percent, depending on the fraction of dose that reaches the systemic circulation unchanged. Therefore, the therapeutic efficacy of a drug administered by extravascular route is directly related to its bioavailability.

Absorption is the process of transferring chemical substances from the gastrointestinal tract through its wall into the bloodstream and lymphatic system (Liu, Wang & Hu, 2008). For absorption to occur, a drug must undergo dissolution in the gastrointestinal fluid, and must remain soluble and stable throughout the gastrointestinal tract. The most frequently used and preferred mode of drug administration is the oral route, as it is less invasive, convenient and widely accepted by patient. Following oral administration, a drug in the dosage form must dissolve before it crosses the gastrointestinal wall and subsequently enters the systemic circulation, and then transported to its site of action (Ashford, 2002a). Therefore, the *in vivo* dissolution and intestinal permeation are two critical elements in the oral absorption of a drug.

1.2 Transport of drug molecules across the gastrointestinal membrane

The main cellular barrier to drug absorption is the gastrointestinal membrane that separates the lumen of the stomach and intestines from the systemic circulation. This barrier is semipermeable in nature which selectively prevents the passage of some molecules. In addition, a number of transporter proteins are found in the membrane. These transporter proteins also play a role in the transportation of materials back and forth across the membrane.

There are two main mechanisms of drug transport across the gastrointestinal epithelium: transcellular and paracellular. The mechanisms of drug transport across the epithelial membrane are shown in Figure 1.1.



1. Transcellular
2. Paracellular
3. Active uptake into the cell
4. Active efflux out of cell

Figure 1.1: Mechanisms of drug transport across an epithelial membrane

1.2.1 Paracellular pathway

The paracellular (between the cell) pathway involves transport of molecules through the aqueous pores between the cells. Small hydrophilic molecules, such as water, urea, sugars, amino acids and calcium, can cross the gastrointestinal epithelium via paracellular pathway. The molecular weight cut-off for this pathway is considered to be 200 Daltons, although passage of some larger molecules is possible (Lennernas, 2007). Drug transport by paracellular route is minimal due to the presence of tight junctions between the cells.

1.2.2 Transcellular pathway

Transcellular pathway involves the passage of drug across the cell. It is the most common route of drug transport across the gastrointestinal membrane. Transcellular pathway can be further divided into passive diffusion, carrier-mediated transport and vesicular transport.

1.2.2(a) Passive diffusion

Passive diffusion is the most important pathway for drug molecules to cross the gut wall. In this process, drug molecules pass through lipoidal membranes from a region of high concentration in the intestinal lumen to a region of lower concentration in the blood.

The mechanism of the passive diffusion follows Fick's Law, whereby the absorption rate is proportional to the drug concentration and the surface area. The passage of drug across a membrane is affected by three major molecular properties: size, lipophilicity and degree of ionization. These properties, together with the nature of the membrane, determine the overall speed of movement of a drug across the

membrane. Generally, the higher the lipophilicity of a drug the greater its permeability. Molecular size has a major impact on movement through membranes. This is probably due to the rigidity of cell membrane lipid bilayer, which impedes drug movement. The larger the molecule, the slower its movement across membrane. Degree of ionization is the third major constraint to transmembrane passage. In general, charged molecules are not as permeable as the corresponding uncharged molecules.

According to the Lipinski's Rule of Five, a compound is more likely to be membrane permeable and easily absorbed if the following criteria are met: its molecular weight is less than 500, the calculated octanol-water partition coefficient (log P) is less than 5, the number of hydrogen donor is less than 5, and less than 10 hydrogen-bond acceptors (Lipinski *et al*, 2012).

1.2.2(b) Carrier-mediated transport

Carrier-mediated transport can be further divided into active transport and facilitated diffusion.

Active transport mechanism requires the presence of carrier proteins to form carrier-drug complex, and carry them through the membrane to the bloodstream. This allows drugs to be transported against the concentration gradient across a cell membrane. This process requires energy input and is also temperature-dependent (Zhou and Qiu, 2009). There are many types of transporters families in the small intestine, which can be present either on the apical or on the basolateral cell membrane. These include amino acid transporters, organic anion transporters, peptide transporters, vitamin transporters and multidrug resistant proteins. Due to limited capacity, these carriers

can be saturated. In addition, carrier-mediated transport can be competitively inhibited by substrate analogs or specific inhibitors.

Facilitated diffusion differs from active transport is that the drug moves along the concentration gradient, and hence does not consume energy. This process is saturable and is subjected to inhibition by competitive inhibitors (Zhou and Qiu, 2009). Facilitated diffusion plays a minor role in drug absorption, except in the case of nucleoside analogs.

1.2.2(c) Vesicular transport

Vesicular transport is a process in which cell absorbs molecules from outside the cell by engulfing it with their cell membrane. This process is subdivided into three categories: pinocytosis, phagocytosis and endocytosis. Vesicular transport plays a minor role in drug absorption.

1.3 Factors affecting drug absorption

For most drugs, the optimum site for drug absorption after oral administration is the upper portion of the small intestine, particularly the duodenum region. The presence of fold-like projections, known as villi and microvilli in the intestinal mucosa further increase the surface area for better absorption. A large network of capillaries and lymphatic vessels perfuse the duodenal region. Once the drug is absorbed from the small intestine, it enters to the hepatic-portal vein and the liver via the mesenteric vessels before reaching the bloodstream (Shargel, 2005).

The systemic absorption of an oral drug depends on three aspects: the physiological factors of the gastrointestinal tract, the physiochemical properties of the drug, and the properties of the dosage form.

1.3.1 Physiological factors

The pH of the gastrointestinal fluids changes along the gastrointestinal tract. For an ionisable drug, its dissolution, chemical stability and absorption may be affected by gastrointestinal pH. Chemical degradation due to pH-dependent hydrolysis can occur in gastrointestinal tract. As a result, incomplete bioavailability may occur, as only a fraction of the administered dose reaches the systemic circulation in the form of intact drug (Ashford, 2002b).

Gastrointestinal motility and transit time also affect oral drug absorption. During the fasted or interdigestive state, the "migrating motor complex" (MMC) acts as a propulsive movement that empties the upper GI tract to the caecum. The stomach first experiences a quiescent phase that lasts approximately one hour. This is followed by irregular contractions, and ends with high amplitude "house keeper contractions" that empties the residual contents further down the alimentary canal. On the other hand, the stomach undergoes irregular contractions in the fed state, where mixing of intestinal contents and advancing of the intestinal stream toward the colon in short segments occur (Shargel, 2005). During this phase, solids larger than 2mm are retained whereas smaller particles and liquids are emptied. The rate of stomach emptying is also influenced by food content. Carbohydrates are emptied more rapidly than proteins and fats. It is also notable that gastrointestinal motility may be affected by drug administered. For example, anticholinergic drugs may reduce the rate of gastric emptying and gastrointestinal motility, thus their transit time would be increased.

The bioavailability of a drug may be reduced by pre-systemic metabolism by the enzymes in the gut wall and liver. The rapid metabolism of an orally administered

drug before reaching the general circulation is known as first-pass effect. The cytochrome P450s (CYPs) constitute a superfamily of heme-thiolate proteins that catalyse the biotransformation of xenobiotics, including drugs and toxins. In humans, approximately 50% of the overall elimination of commonly used drugs can be attributed to P450 enzymes. The major forms are CYP3A (about 30% of total P450) and CYP2C (about 20%) isoenzymes (Shimada *et al*, 1994). One example of drug with high pre-systemic metabolism is nitroglycerin. To overcome first-pass effect, nitroglycerin is given sublingually, topically or by nasal route due to very poor oral bioavailability (Shargel, 2005).

The presence of food may affect the bioavailability of a drug. Generally, meals that are high in total calories and fat content are more likely to affect gastrointestinal physiology and hence the bioavailability of a drug. Some drugs are capable of forming insoluble complex with diet component, thereby reducing the drug absorption. For example, tetracycline forms non-absorbable complexes with calcium and ions. Therefore, patients are advised not to take milk, antacids or iron preparation at the same time of day as tetracycline (Shargel, 2005).

Diseases that cause changes in gastrointestinal pH, blood flow, motility, gut wall permeability, alteration of GI flora, digestive enzyme secretion or bile secretion may affect oral drug absorption. For example in diarrhoea, inadequate absorption of a drug may happen due to decrease in transit time (Shargel, 2005).

1.3.2 Physiochemical properties of a drug

To achieve a pharmacological activity, a drug molecule must exhibit certain solubility in physiological intestinal fluids to be present in the dissolved state at the site of absorption.

When solid particles are in the gastrointestinal tract, a saturated layer of drug solution is formed at the surface area of the solid particle. This stagnant layer between the solid particle and bulk solution is known as diffusion layer. Thus, the concentration at the solid surface is assumed to be its solubility and a concentration gradient is formed traversing the stagnant layer. According to Noyes-Whitney equation:

$$\frac{dC}{dt} = \frac{KDS}{h}(Cs - C) \quad \text{--------------} \quad \text{Equation 1.1}$$

$\frac{dC}{dt}$ = Dissolution rate; K = Dissolution rate constant; D = Dissolution coefficient of a drug; S = Surface area of the undissolved drug; h = Thickness of the diffusion layer; Cs = Equilibrium solubility of the drug in the GI fluid; C = Drug concentration

This equation shows that the dissolution of drug in the GI tract is governed by two variables: solubility and surface area of the drug. For the same dose strength, smaller particles lead to a larger surface area and faster dissolution rate. A reduction in particle size increases the effective surface area of a drug. This enhances water penetration into the drug particles, and increases the dissolution rate. Hence, particle size reduction will increase bioavailability if the drug absorption is dissolution-rate limited.

A drug molecule needs to have sufficient lipophilicity to cross the lipid bilayer of the cell membrane. This is important for drugs that are absorbed via passive diffusion.

Nevertheless, this does not imply that the higher the lipophilicity, the better the absorption. Drug molecule with extremely high lipophilicity will bind too tightly to the cell membrane, making absorption impossible to occur.

In general, uncharged molecules are more permeable compared to the corresponding charged species when the compound is absorbed via passive diffusion. For weak acids and bases, the pH at which the majority of the species remain uncharged should be considered, as the solubility of weak acids or bases in the gastrointestinal tract is greatly affected by the degree of ionization.

Passive absorption is limited by the size of the molecule due to the well-packed structure of the membrane lipid bilayer. According to Lipinski's Rule of Five, the optimal molecular weight that facilitates absorption is less than 500 (Lipinski *et al*, 2012).

Polymorphism is the arrangement of drug in various crystal forms. Although sharing the same chemical structure, polymorphs have different physical properties, such as density, hardness and solubility. A metastable polymorph usually exhibits greater dissolution rate compared to the corresponding stable polymorph. The stability of each form is important, as a metastable form may convert to a more stable form over time (Shargel, 2005).

1.3.3 Formulation factor

Drugs are generally given to patients as formulated drug products. The formulated drug products are made up of the active ingredient and the excipients. The type of dosage form, such as tablet, capsule, oral solution, suspension, suppository and the nature of the excipients in the drug product, will affect the dissolution rate and the bioavailability of the drug.

Prior to dissolution, the drug product must disintegrate into small particles and release the drug. Therefore, drug in liquid dosage forms, such as oral solutions, emulsions and suspensions are more readily absorbed compared to solid dosage forms like tablets or capsules.

Although they should be pharmacodynamically inert, excipients may amend the functionality of the drug and the bioavailability of the drug from the dosage form (Shargel, 2005). For instance, excessive quantity of magnesium stearate, a hydrophobic lubricant, may repel water and reduce dissolution. On the other hand, low concentrations of surfactants increase the dissolution rate of the drug by decreasing in surface tension. Nevertheless, the drug dissolution rate decreases with higher surfactant concentrations, due to formation of micelles with the drug.

1.4 *Andrographis paniculata*

Andrographis paniculata (Burm.f) Nees is a medicinal herb belonging to family Acanthaceae in plant kingdom. Due to its extremely bitter taste, it is commonly known as “hempedu bumi”, which literally means “bile of the earth” in Malaysia. *Andrographis paniculata* is an annual herb growing to a height of 0.50-1.0m. The stems are dark green, quadranecular and branches profusely. The leaves are green,

lanceolate, simple, opposite, and glabrous with a short petiole. The leaf size is reduced as the plant matures. The flowers are borne on terminal, white in colour with reddish-purple spots on the petals. The flowers are bisexual in nature, about 1-2cm long, without distinct odour and very irregular in shape. The plant bears small seeds which are abundant, linear-oblong in shape and yellowish-brown in colour (Subramanian, 2012).

Andrographis paniculata is commonly found in wastelands and grasslands in countries such as Southeast Asia, China, Taiwan, India and Sri Lanka. The aerial parts and roots have been used for centuries in Asia and Europe as folklore remedy for a wide spectrum of ailments or as herbal supplement. In India, the herb is a predominant constituent of at least 26 Ayurvedic formulations used to treat liver disorders or neoplasms. In traditional Chinese medicine, *Andrographis paniculata* is reported as a cold property herb and is used for latent-heat clearing and to expel toxins (Varma *et al*, 2011). In Scandinavian countries, the Kan Jang capsule, which consists of the extracts of *Andrographis paniculata* and *Eleutherococcus senticosus*, is commonly used to prevent and treat the common cold. In addition, *Andrographis paniculata* has also been included into "WHO monographs on selected medicinal plants, volume 2" (WHO, 2003). The monographs served as a valuable scientific reference for the healthcare authority and scientist, as well as play an important role in promoting safe and proper use of medicinal plants globally.

Figure 1.2: *Andrographis paniculata* and flowers, scale approximately 0.8x

1.4.1 Andrographolide

Andrographis paniculata contains diterpenoids, polyphenols and flavonoids. Up to date there are more than 20 diterpenoids and over ten flavonoids have been reported from *Andrographis paniculata* (Chao and Lin, 2010). Andrographolide is the primary bioactive constituent of the medicinal plant. The molecular formula and weight of andrographolide is $C_{20}H_{30}O_5$ and 350.45g/mol respectively. It is the major diterpenoid in *Andrographis paniculata*. It makes up about 4%, 0.8-1.2%, and 0.5-0.6% in dried whole plant, stem and leaf extracts respectively (Chao and Lin, 2010). Andrographolide is present in all parts of the plant but is abundant in the stems and leaves. It is a colourless, crystalline solid with extremely bitter taste. The systematic name is 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5, 8α-dimethyl-2-methylene-1-naphthalenyl]ethylidine]dihydro-4-hydroxy-2(3H)-furanone (Smith III *et al*, 1982).

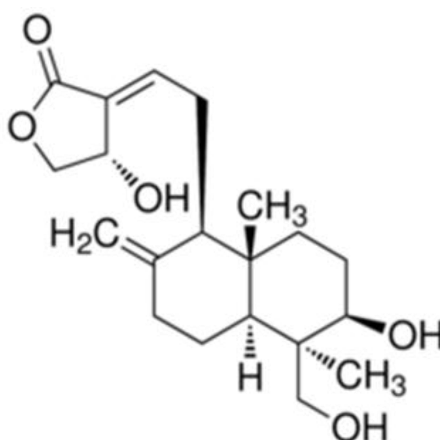


Figure 1.3: Chemical structure of andrographolide (Source: Sigma Aldrich)

1.4.2 Medicinal benefits of andrographolide

Androgapholide, the principal bioactive compound found in *Andrographis paniculata*, is reported to exhibit vast range of biological activities.

In animal studies, andrographolide showed significant hepatoprotective effect against liver damage induced by paracetamol (Handa and Sharma, 1990; Visen *et al*, 1993), galactosamine (Handa and Sharma, 1990), carbon tetrachloride (Kapil *et al*, 1993), or tert-butylhydroperoxide (Kapil *et al*, 1993). On the other hand, Kapir and colleagues (1993) found that other diterpenoids, such as andrographiside and neoandrographolide, exhibited stronger hepatoprotective effect than andrographolide. Hence, the hepatoprotective effect of the plant may not solely be due to andrographolide, but it is the synergistic effect of other phytochemicals present in the plant.

Investigations from all over the world showed that andrographolide possessed antineoplastic activities. *In vitro* studies showed that andrographolide or its extract exhibited anticancer activity by acting both directly and indirectly on the cancer cells. Proliferation of cancer cells was inhibited by induction of cell-cycle arrest, apoptosis or necrosis (Ji *et al*, 2007; Li *et al*, 2007; Manikam and Stanslas, 2009; Shi *et al*, 2009). In addition, andrographolide may exhibit immunomodulatory activity by triggering body's own immune system against cancerous cells. Most of the experiments carried out were on *in vitro* cellular toxicity assays with different concentrations. More studies are needed on animal models of cancer or on human in order to draw a conclusion about the anticancer activity of andrographolide (Varma *et al*, 2011).

Studies revealed that andrographolide exerts its anti-inflammatory effects by reducing the expression of several pro-inflammatory mediators. Andrographolide had been shown to inhibit NF-kβ signalling pathway by binding to deoxyribonucleic acid, thus reducing the expression of pro-inflammatory proteins in neutrophils in many *in vitro* (cell culture) and *in vivo* studies (animals)(Hidalgo *et al*, 2005; Xia *et al*, 2014). NF-kβ is thought to be the regulating genes responsible for both the innate and adaptive immunity. It was reported that andrographolide suppressed the production of other pro-inflammatory proteins like nitric oxide synthetase (iNOS), and TNF-α (Chiou *et al* 1998; Chiou *et al* 2000; Qin *et al*, 2006). In addition, evidence also showed that the anti-inflammatory activities of andrographolide could be due to the interference with intracellular signaling pathway involved in the cytokine expression, such as IL-2, TNF-α, and IFN-2 (Chao and Lin, 2010).

Subramanian and colleagues (2008) demonstrated that andrographolide caused a significant reduction in peak blood glucose and area under the curve in diabetic rats, and suggested that the antidiabetic activity may due to α-glucosidase and α-amylase inhibition. In rat studies conducted by Lin *et al.* (2009), andrographolide was reported to exhibit antioedema and analgesic activities in a concentration dependent manner. However, more *in vivo* studies are needed for studying the antidiabetic, antioedema and analgesic activities of andrographolide.

1.5 Pharmacokinetic studies of andrographolide

To date, there is little information about the pharmacokinetic studies of andrographolide in animals and humans. Panossian and colleagues (2000) conducted pharmacokinetic studies in rats and humans using *Andrographis paniculata* extract and Kan Kang tablets. Wangboonskul and colleagues (2006) conducted pharmacokinetic study of *Andrographis paniculata* tablets in healthy male volunteers. Nevertheless, for human studies conducted by these researches, conclusion could not be drawn due to low number of volunteers involved. Ye and colleagues (2008) studied the oral bioavailability of andrographolide in rats and the reported absolute bioavailability of andrographolide was 2.67%. This finding was in contrast with Panossian *et al.* whereby the reported oral bioavailability of andrographolide was 91% at a dose of 20mg/kg, but reduced to 21.4% when the dose was increased to 200mg/kg.

Maiti *et al.* (2010) compared the oral bioavailability of pure andrographolide and its herbosome complex at a dose of 25mg/kg. The bioavailability of the herbosome complex was higher than the pure form and the complex maintained its effective plasma concentration for a longer period of time.

Chellampillai and Pawar (2011) compared the bioavailability of andrographolide from optimised nanoparticles and pure andrographolide in male albino Wistar rats at a dose of 10mg/kg. There was an almost 2.2 fold increase in oral bioavailability of andrographolide from nanoparticle suspension compared to the pure form. The reported $AUC_{0\rightarrow\infty}$ for pure andrographolide and andrographolide nanoparticles was 2.169µg.h/mL and 4.807µg.h/mL, respectively.

1.6 Scope of study

Several studies suggested that andrographolide possesses beneficial health effects including anticancer and anti-inflammatory activities. The actual beneficial effects to human cannot be fully realised as information regarding its bioavailability is limited. Therefore, the present study was focused on investigation of the bioavailability of andrographolide and to elucidate the possible factors affecting its bioavailability.

The objectives of the present study are:

1. To develop a High Performance Liquid Chromatography (HPLC) method to detect and quantify the amount of andrographolide in human and rat plasma.

2. To study the potential of polyvinylpyrrolidone (PVP) K25 in enhancing the aqueous solubility of andrographolide.

3. To investigate the bioavailability and pharmacokinetics of pure andrographolide and mixture of pure andrographolide with polyvinylpyrrolidone K25 in rats. The term ‘pure’ was used throughout this study in order to differentiate andrographolide with 98% purity from andrographolide extract.

4. To investigate the potential influence of cytochrome P450 and P-glycoprotein in limiting the bioavailability of andrographolide.

CHAPTER 2: DEVELOPMENT OF SIMPLE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD WITH ULTRAVIOLET (UV) DETECTION FOR ANALYSIS OF ANDROGRAPHOLIDE IN HUMAN AND RAT PLASMA

2.1 Introduction

Andrographis paniculata is a traditional plant which is used in Asia for common cold, diarrhea, and fever associated with infectious diseases. The main active component of *Andrographis paniculata* is andrographolide, a colourless, bitter and crystalline diterpene lactone.

Several methods have been developed for the determination of andrographolide in plasma. Determination of andrographolide in rat whole blood, rabbit plasma and chicken plasma by HPLC-UV method has been reported, with a limit of quantification of 0.053μg/mL, 0.05μg/mL, and 0.04μg/mL respectively (Suo, Zhang and Wang, 2007; Chen *et al*, 2007; Liu *et al*, 2009). There are shortcomings of these methods: analysis of compound in rat whole blood is not advisable as the components in blood will cause interference in many assay techniques and this will damage the chromatography column easily. Both rabbit and chicken are not suitable models for pharmacokinetic study as their digestive systems are less complicated compared to humans.

There are also reports of determination of andrographolide in human plasma by liquid-chromatography-electroscopy ionisation tandem mass spectrometry (HPLC-ES/MS), with the limit of quantification of 9.9ng/mL and 1ng/mL respectively (Gu *et al*, 2007, Xu *et al*, 2009). However, the method required use of expensive equipment and the sample preparation is very much laborious and complicated.

Although various analytical methods have been developed to quantify andrographolide in biological samples, HPLC-UV remains the preferred method as it is simple, rapid, specific, sensitive and economical. Therefore, developing a HPLC-UV method that fulfils the criteria mentioned, as well as with high efficiency and reproducibility form the basis of this part of study. The method should be able to detect and quantify andrographolide in both human and rat plasma, and subsequently can be used in the pharmacokinetic studies of andrographolide in rats.

2.2 Materials and reagents

Andrographolide powder of 98% purity was obtained from Sunrise Nutrachem Group (Qingdao, China). The standard of andrographolide (98% purity) was purchased from Sigma Aldrich (St.Louis, MO, USA). Carbamazepine was obtained from Hovid Sdn. Bhd (Ipoh, Perak, Malaysia). Both the andrographolide and carbamazepine were packed in sealed glass bottle, stored at fridge before use. Analytical grade acetonitrile (ACN) and acetic acid were purchased from Merck (Darmstadt, Germany). Human blank plasma was obtained from Penang General Hospital (Pulau Pinang, Malaysia).

2.3 Instrumentation and chromatographic conditions

UV-viscible spectrophotometric analysis was carried out on a Genesys 10S UV-Vis spectrophotometer (Waltham, MA, USA). The maximum absorbance of andrographolide was recorded at 229nm.

The chromatographic system composed of a Jasco PU-980 pump (Jasco, Hachioji City, Tokyo, Japan), a Gilson UV/Vis 151 detector (Middleton, WI, USA) and a

Hitachi D-2500 chromato-integrator (Hitachi, Tokyo, Japan). The detection wavelength was set at 229nm and the sensitivity was set at 0.002 AUFS.

The mobile phase consisted of ACN and distilled water, at a ratio of 30:70, with 0.05% of acetic acid added to the distilled water to provide an acidic environment. The mobile phase was vacuum filtered with 0.45µm HNWP04700 nylon membrane filter paper (Millipore, USA) prior to use. Chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 column (4.6mm x 250mm, 5µm, Agilent, USA), fitted with a refillable guard column (Upchurch Scientific, Oak Harbour, WA, USA), packed with Perisorb RP-18 (30-40µm, pelliular, Upchurch Scientific, Oak Harbour, WA, USA).

The isocratic flow rate was set at 1.0mL/min and the injection volume was 50µL. The column temperature was maintained at room temperature of 25°C throughout the analysis.

2.4 Preparation of stock and working solution

Fifty milligram of andrographolide standard was accurately weighed in a 50mL volumetric flask. The volume was then adjusted to 50mL with methanol to make up a stock solution of 1mg/mL. To prepare a secondary stock solution of 200µg/mL, an aliquot of 5.0mL of the primary stock solution was transferred into a 25mL volumetric flask and the volume was made up to 25mL.

The internal standard carbamazepine was accurately weighed (25mg) in a 50mL volumetric flask. The volume was adjusted to 50mL with methanol to make up a stock solution of 0.5mg/mL. Further dilutions with methanol were performed to

obtain a working solution of 2.5µg/mL. All solutions were kept in amber glass bottle sealed with parafilm and stored at 4°C until assay.

2.5 Standard solutions and calibration curves

In order to construct calibration curves of andrographolide in plasma, 10mL of pooled human blank plasma was spiked with 100µL aliquot of the 200µg/mL stock solution to obtain a concentration of 2000ng/mL. Serial dilutions were performed with pooled human plasma to obtain a series of concentrations of 1000.0, 500.0, 250.0, 125.0, 62.5, 31.3 ng/mL.

A hundred microliter of plasma sample, 50µL of carbamazepine 2.5µg/mL and 5mL of chloroform were pipetted into a centrifuge tube with a screw cap. The mixture was vortex-mixed for 60 seconds, and then centrifuged at 12800g for 15 minutes.

The organic layer was transferred to a reactival and evaporated to dryness at 40°C under the gentle stream of nitrogen. The residue samples were stored at -20°C until analysis. Prior to analysis, the samples were brought to room temperature, reconstituted with 100µL of mobile phase and vortexed for 60 seconds. 50µL of the resulting supernatant was then injected into the HPLC system.

2.6 Assay validation

Blank human plasma was analysed to demonstrate the absence of interfering endogenous peaks. Interference from andrographolide was also investigated by analysing the spiked concentration (0.5µg/mL) in blank human plasma.

Standard calibration curves were constructed by spiking pooled human plasma with a known amount of andrographolide at a concentration range of 31.3—2000ng/mL. These plasma standards were also used to determine the within-day and between-day

precision and accuracy six times on the same day and continuously for six days (n=6). Quantification of samples was performed by using peak height ratio of the andrographolide over the internal standard (carbamazepine). The mean recoveries of andrographolide from plasma were calculated by comparing the response of andrographolide and internal standard obtained after extraction, with mean peak areas of the same amounts of unextracted solutions.

The limit of detection (LOD) was defined as the lowest concentration with detectable response. The limit of quantification was defined as the lowest quantifiable concentration with satisfactory between-day and within-day precision and accuracy of less than 20% for both coefficient of variation and percentage of error.

2.7 Stability

Andrographolide stock solution of 2000ng/mL was assessed for short-term and long term stability. The stock solutions were kept in 4°C refrigerator. The concentrations of the stability samples were determined by comparing the concentrations of stability test samples to those of corresponding freshly prepared samples. A replicate of n=3 was conducted for each sample. Data were presented in mean percentage.

To assess the stability of andrographolide in spiked human plasma, three aliquots of low (31.3ng/mL), medium (250ng/mL) and high (2000ng/mL) concentrations of andrographolide plasma samples were used. The samples were stored in a freezer (-20°C). The concentrations of the stability samples were determined by comparing the concentrations of stability test samples to those of corresponding freshly prepared samples. A replicate of n=3 was conducted for each sample and data was presented in mean percentage.

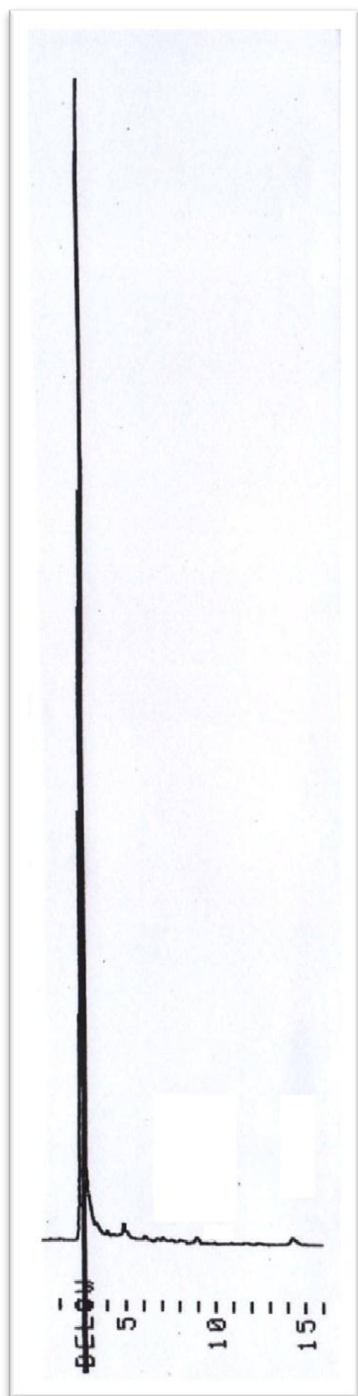
2.8 Results

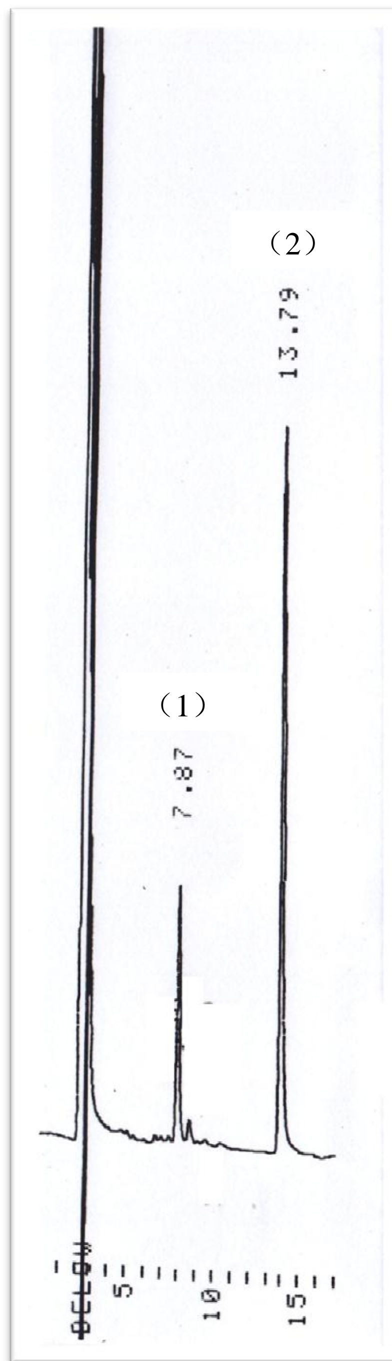
Figures 2.1(a) and 2.1(b) show the chromatograms of the blank human plasma and human plasma spiked with 0.5μg/mL of andrographolide. It can be seen that andrographolide is well separated under these chromatographic conditions and free of interference from endogenous compounds in human plasma. The mean retention times for andrographolide and carbamazepine were 7.9±0.1 and 13.8±0.1 respectively. The total run time for each injection was approximately 15 minutes.

The extraction recovery, within-day and between-day accuracy and precision values for andrographolide are shown in Table 2.1. The accuracy of the assay method at all the concentrations evaluated was between 90.3% and 105.9%. The precision, which is expressed as the coefficient of variation (C.V) were between 3.1% and 12.0%. The mean extraction recovery for andrographolide at the concentrations studied were between 67.3% and 87.6%, whereas carbamazepine was between 88.0% and 105.3%.

The limit of detection (LOD) of the present method was 15.7ng/mL, whereas the limit of quantification (LOQ) was 31.3ng/mL. The standard calibration curve (n=7) was found to be linear over the concentration range of 31.3 – 2000ng/mL. The equation of calibration curve was y=0.0005x.

Andrographolide stock solution was stable up to 6 months at 4°C. Nevertheless, when it was spiked in human plasma, andrographolide was stable up to 2 hours at minus 20°C. Concentration of andrographolide in plasma was found to drop 15% of its original concentrations at 24 hours although this was not observed in its stock solutions.





2.1A

2.1 B

Figure 2.1A: Blank human plasma

Figure 2.1B: Human plasma spiked with (1) 1μg/mL andrographolide and (2) 2.5μg/mL carbamazepine.