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IMPROVING THE ORAL BIOAVAILABILITY OF DRUGS THROUGH THE DESIGN OF MODELED PRE-SYSTEMIC CYTOCHROME P450 INHIBITORS

Pius Sedowhe Fasinu

A dissertation submitted to the Faculty of Health Sciences,
University of the Witwatersrand, in fulfillment of the requirements for the degree of
Master of Science in Medicine



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Johannesburg, 2010

DECLARATION

I, Pius Sedowhe Fasinu declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the field of Pharmaceutics in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.

……

Signature

……

Date

RESEARCH PUBLICATION AND PRESENTATIONS

PUBLICATIONS

Review: Advances in the Approaches for Enhancement of Oral Drug Bioavailability.
Pius Fasinu, Viness Pillay, Valence Ndesendo, Lisa du Toit and Yahya Choonara.
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A Study of the Novel Computationally Modeled Cytochrome P450 3A4 Inhibitors.
Pius Fasinu, Viness Pillay, Yahya Choonara, Lisa du Toit and Riaz Khan.
To be submitted

Investigation of the Oral Bioavailability-Enhancing Property of 8-arm-poly(ethylene glycol), a Novel Computationally Modeled Cytochrome P450 3A4 Inhibitor
Pius Fasinu, Viness Pillay, Yahya Choonara, Lisa du Toit and Leith Meyer.
To be submitted

PODIUM PRESENTATIONS

A Novel Application of Computational Modeling in Biomodulation: Pharmaceutical Grade Polymers Inhibits *In Vitro* CYP3A4-Catalysed Felodipine Metabolism.
Pius Fasinu, Viness Pillay, Yahya Choonara and Lisa du Toit
The 2nd Annual Cross Faculty Postgraduate Research Symposium, University of the Witwatersrand, Johannesburg, South Africa, 20th -21st October, 2009

An *In Vitro* Investigation of the Effects of CYP3A4 Inhibiting Flavonoids on CYP1A2-catalysed Phenacetin *O*-deethylation Employing Human Liver Microsomes
Pius Fasinu, Viness Pillay, Yahya Choonara and Lisa du Toit
The Postgraduate Research Day, School of Therapeutic Sciences, University of the Witwatersrand, Johannesburg, South Africa, 12th August, 2009.

POSTER PRESENTATIONS

Optimization of the *In Vitro* Metabolism of Dihydropyridines by Human Liver Microsomal Enzymes.

Pius Fasinu, Viness Pillay, Yahya Choonara and Lisa du Toit.

The American Association of Pharmaceutical Scientists' (AAPS) Annual Meeting and Exposition, 7th-12th Novermber, 2009.

CYP1A2-Mediated *O*-deethylation of Phenacetin to Produce Paracetamol: *In Vitro* Optimization and Determination of Kinetic Parameters.

Pius Fasinu, Viness Pillay, Yahya Choonara and Lisa du Toit.

The 5th International Conference on Pharmaceutical and Pharmacological Sciences, North-west University, Potchefstroom, South Africa, 23th – 26th September, 2009.

In vitro Investigation of the Effects of Quercetin, Naringin and Naringenin on the Metabolism of Felodipine by Human Liver Microsomes.

Pius Fasinu, Viness Pillay, Yahya Choonara and Lisa du Toit.

The 5th International Conference on Pharmaceutical and Pharmacological Sciences, North-west University, Potchefstroom, South Africa, 23th – 26th September, 2009.

PATENTS

Improving the Oral Bioavailability of Drugs through the Design of Modeled Pre-Systemic Cytochrome P450 Inhibitors.

Pius Fasinu, Viness Pillay, Yahya Choonara and Lisa du Toit, South Africa, Patent Application filed, awaiting priority date.

ABSTRACT

The poor bioavailability of drugs has been identified as the single most important challenge in oral drug delivery. Prominent among the factors responsible for this are the metabolic activity of the intestinal and hepatic cytochrome P450 enzyme family. This dissertation presents novel cytochrome P450 inhibitors generated through the application of computational modeling of known cytochrome P450-substrate and cytochrome P450-inhibitor interactions. In vitro metabolism of felodipine by cytochrome P450 3A4-expressed human liver microsomes was optimized yielding a typical Michaelis-Menten plot through the application of Enzyme Kinetic Module software from where the enzyme kinetic parameters were determined. Quercetin, naringin and naringenin which are the major phytochemical component of grapefruit juice, a well known cytochrome P450 3A4 inhibitor, were separately incubated in human liver microsomes together with felodipine at concentration equivalent to the determined Michaelis-Menten Constant (K_m) value. Compared to verapamil, a known competitive inhibitor of cytochrome P450 3A4, all three flavonoids inhibited felodipine metabolism with IC_{50} values of 208.65, 177.81 and 121.97μM respectively confirming earlier suggestions that the flavonoid contents of grapefruit juice are responsible for known grapefruit-drug interactions. Following a detailed study of the quantitative structure-activity relationship of these flavonoids and verapamil, their binding properties with cytochrome P3A4, the amino acid sequence and binding affinity of cytochrome P3A4, computational modeling software on a non-silicon graphic system was employed to generate pharmaceutical grade and commercially available polymers based on activity prediction aided by computational biomimetism and simulations. Thus grapefruit-felodipine interaction (a typical cytochrome P3A4 inhibitor-substrate interaction) served as the basis for the computational modeling where several modeled compounds including 8-arm-poly(ethylene glycol), o-(2-aminoethyl)-o-methyl poly(ethylene glycol), 4-arm-poly(ethylene glycol) (M_W=10000g/mol and 20000g/mol) and poly (L-lysine) were generated and investigated for inhibitory activity against felodipine metabolism by human liver microsomes and human intestinal microsomes where 8-arm-poly(ethylene glycol) demonstrated the highest inhibitory potency with an IC_{50} value of 7.22μM. An ex vivo method employing freshly excised pig intestinal tissue was developed and validated to investigate the inhibition of cytochrome P450-induced drug metabolism in living tissues. Both naringenin and 8-arm-poly(ethylene glycol) exhibited significant inhibitory effects against felodipine metabolism in pig intestinal tissues. The ex vivo studies yielding IC_{50} values of 179.88 and 487.75μM for naringenin and 8-arm-poly(ethylene glycol) respectively demonstrated a promising in vivo inhibitory activity against intestinal cytochrome P450 3A4. The potential utility of 8-arm-poly(ethylene glycol) in oral drug delivery was investigated by assessing its influence on the formulation and behavior of tablet matrices. Results showed that 8-arm-poly(ethylene glycol) possessed satisfactory compressional, binding and friability characteristics with acceptable drug release profiles. In vivo studies of the effects of 8-arm-poly(ethylene glycol) on the oral bioavailability of felodipine were performed on the Large White pig model. Compared to controls, a >100% increase in plasma felodipine levels was observed. The outcome of this research presents 8-arm-poly(ethelyne glycol) as a promising oral bioavailability enhancer.

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And finally, unto the King eternal, immortal, invisible, the only wise God, be honor and glory for ever and ever.

DEDICATION

In memory of my late Dad and Brothers: David, Joseph and Daniel Fasinu on whose wings I first learn to soar

ANIMAL ETHICS DECLARATION

I, Pius Sedowhe Fasinu hereby confirm that the study entitled ‘Improving the oral Bioavailabilty of Drugs through the Design of Modeled Pre-Systemic Cytochrome P450 Inhibitors’ received the approval from the Animal Ethics Committee of the University of the Witwatersrand with Ethics Clearance Number 2009/01/05 (Appendix).

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CHAPTER ONE

INTRODUCTION AND MOTIVATION FOR STUDY

1.1. Background of this Study

The desired site of drug action, systemic or localized, is a major factor for consideration in deciding the route of drug administration. Systemic routes of drug administration are traditionally classified into enteral and parenteral routes (Hughes et al., 2005). Enteral routes are those involving the gastrointestinal tract (GIT) which include oral ingestion, sublingual, buccal and rectal administration while parenteral drug delivery routes include intra-arterial, intravenous, intrathecal, intramuscular, intracardiac, cutaneous and subcutaneous injections, surgical site-specific implantations, and inhalational routes (Yadav and Prakashan, 2008). Drugs intended for local actions are administered through topical application on the skin and mucosal membranes of the eye, ear, rectum and the anus. The choice of route of drug administration is decided by a number of factors including the physicochemical properties of the drug, biopsychosocial condition of the patient, the desired site and onset of drug action, dosage frequency as well as ease and convenience of administration (Strang et al., 2006). For toxicological considerations, the possibility of reverse administration like gastric lavage and stimulation of emesis are extreme considerations in the choice of route of drug administration (Rathbone et al., 1994). The choice of route of drug administration has a significant influence on patient compliance, therapeutic efficacy and manifestation of side-effects (Liu et al., 1997; Sterling et al., 1997; Schwartzman and Morgan, 2004).

Oral drug delivery remains the most favorable and preferred route of drug administration both by patient and physicians (Wong et al., 2006). Oral administration is generally accepted, easy and convenient; and offers the patient the possibility of self administration requiring no expertise (De Jong et al., 2007). Compared to injectables and implants, pharmaceutical products intended for oral administration are cheaper and currently >60% of drugs are marketed as oral products (Pretorius and Bouic, 2009). Researchers and pharmaceutical companies are increasingly aware of the need for earlier assessment of new drug entities for their potential as oral candidates (Davis and Wilding, 2001). The merits of new drugs are measured in their delivery convenience in addition to their

therapeutic efficacy (Breimer, 1999). The possibility of self-administration, patient compliance and less risk of irreversible side-effects has placed the oral route as a standard in drug delivery (Sastry et al., 2000; Tong, 2007).

1.1.1. Barriers to oral drug administration

The most significant limitation to oral drug administration is the resulting poor systemic bioavailability of various drugs as a result of first-pass metabolism (DeMario and Ratain, 1998; Veber et al., 2002). In numerous instances, significant allowances are made for pre-systemic drug loss in the design of the dosage regimen and the drug delivery system. Previous studies on structurally diverse drugs have also revealed that subject variableness in bioavailability was indirectly proportional to the extent of bioavailability which implies higher subject variability for poorer bioavailable drugs (Hellriegel et al., 1996; Gidal et al., 2000; Ezzet et al., 2005). This variation and the resulting poor control of plasma drug concentrations would particularly be of concern for drugs that have a narrow therapeutic window or a precipitous dose-effect profile (Aungst, 2000). Prominent among impediments to oral absorption are intestinal efflux proteins (Chan et al., 2004; Takano et al., 2006; Yamagata et al., 2007), physicochemical stability of the drug in the various compartments of the gastrointestinal tract (Tong, 2007), insufficient contact time in transit (Severijnen et al., 2004), poor permeability across the gastrointestinal mucosa (Thanou et al., 2001; Wu and Benet, 2005) and digestive and metabolic enzyme activity (Jeong et al., 2005; Cao et al., 2006). The extremely poor solubility of certain drugs such as the bisphosphonates can make their oral delivery difficult. This challenge is even greater when the required dose is high (Veber et al., 2002; Hu et al., 2004).

In addition to digesting and absorbing nutrients, the GIT wall forms a physiological barrier against the invasion of foreign substances including pathogens, antigens, toxins and poisons (Brenchley and Douek, 2008). This barrier comprises the cell membranes, tight junctions between adjacent epithelial cells, the mucus layer, catabolic enzymes and the efflux proteins that propel molecules back into the GIT lumen after oral administration (Wang et al., 2005). Drug molecules are often recognized as foreign substances and therefore, the absorption of drug is also inhibited (Hamman et al., 2007). Overcoming these challenges in oral drug delivery has been one of the most challenging endeavors facing the pharmaceutical industry for decades.

When drugs are administered orally, apart from their exposure to possible physical degradation, chemical inactivation or microbial biotransformation, the anatomical

proximity of the liver to the GIT necessitates the passage of absorbed drug through the liver where drugs are metabolized to varying degrees by a process known as the first-pass effect (Gibaldi et al., 1971; Kwan, 1997; Back and Rogers, 2007). The most important of all the factors responsible for poor oral drug bioavailability is the cytochrome P450 (CYP)-mediated first-pass metabolism. In a few cases, >90% of administered drug is lost to pre-systemic metabolism (Ghilzai, 2004; Hamman et al., 2005; Leonard et al., 2006; Majumdar and Mitra, 2006). The human CYP enzyme system present in the intestines and liver is responsible for the metabolism of a wider range of drugs (Fang and Xiao-yin, 2005). A sub-family of this enzyme system CYP 3A is responsible for the metabolism of >50% of marketed drugs to a large extent (Rendic, 2002). The ability of CYP3A to metabolize numerous structurally unrelated compounds apart from being responsible for the poor oral bioavailability of numerous drugs is responsible for the large number of documented drug-drug and drug-food interactions (Quintieri et al., 2008). Successful inhibition of the metabolic activity of these enzymes on orally administered drug may enhance the drug oral bioavailability.

Various strategies have been employed to improve the systemic availability of orally administered drugs (Gomez-Orellana, 2005). The principles are generally based on the modification of the physicochemical properties of the drug (Delie and Blanco-Prieto, 2005), addition of novel functionality to the molecular structure of the drug to enhance intestinal wall penetration (Hajduk and Greer, 2007) and modification of pharmaceutical formulation technology by the use of novel drug delivery carrier systems such as microparticles (Muhrer et al., 2006; Ozeki et al., 2005), nanoparticles (Moharanj and Chen, 2006; Bawarski et al., 2008) and dry emulsions (Morishita and Peppas, 2006).

Due to changes in the composition and thickness of the GIT mucus layer, the GIT regional pH as well as the surface area and enzyme activity, certain drugs undergo site-specific absorption (Hamman et al. 2005). The most significant site of GIT drug absorption is the small intestine (Lacombe et al. 2004, Masaoka et al., 2006). Although the small surface area and short residence time of most drugs in the stomach limits gastric absorption, gastro-retentive drug delivery systems have been used to enhance the local action of drugs in the stomach such as anti-diarrheals (Connor et al., 2001), antacids (Fabregas et al., 1994), anti-ulcer agents like misoprostol (Oth et al., 2004), and to facilitate absorption of furosemide in the stomach and the upper small intestine (Streubel et al. 2006). Gastroretentive drug delivery systems are also crucial for drugs such as captopril and ranitidine that are unstable in the intestine and colon (Drummer and Jarrott, 2006), and diazepam that exhibits low solubility at high pH values (Castrol et al. 1999).

Site specificity in drug absorption also involves the use of enteric coating to delay the release of drug until it reaches the small intestine. Various polymers which dissolve at a pH range of 5-7 have been used to target the delivery of drug to the intestines while those that degrade above a pH value of 7 have been used to target colonic drug delivery in diseases such as colitis (Rubinstein, 2005). Colonic absorption may be enhanced due to a reduced concentration of enzymes such as peptidases that degrade peptide drugs, long residence times, high sensitivity to absorption enhancers, natural absorptive characteristics and the abundance of lymphoid tissue follicles in the colon (Yang et al., 2002).

Most of these various approaches have been successfully employed to improve oral drug delivery which has often translated in enhanced drug absorption. The challenges of poor oral bioavailability are still prominent despite these breakthroughs. This is because CYP-induced metabolism is the single most important of all the factors responsible for poor oral bioavailability of drugs (Benet and Cummins, 2001) and to date, no commercially available formulation is known to inhibit CYP-induced first-pass metabolism.

1.1.2. Specific cytochrome P450 enzymes involved in drug metabolism

The CYP superfamily is generally involved in oxidative, peroxidative and reductive biotransformation of xenobiotics and endogenous compounds (Nebert and Russell, 2002). It is conventionally divided into families and subfamilies based on nucleotide sequence homology (Zeldin and Seubert, 2008). There is a high degree of substrate specificity among the various families. CYP belonging to the families 1, 2 and 3 are principally involved in xenobiotic metabolism while others play a major role in the formation and elimination of endogenous compounds such as hormones, bile acids and fatty acids (Hedlund et al., 2001; Norlin and Wikvall, 2007). The most important CYP subfamilies which are majorly responsible for drug metabolism in humans are 1A2, 2A6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 (Ono et al., 1996).

CYP1A1 and 1A2 are the two major members of the human CYP1A subfamily. CYP 1A1 is mainly expressed in extra-hepatic tissues such as the kidney, the intestines and the lungs while CYP1A2 constitutes about 15% of total hepatic CYP. CYP2B6 is involved in drug metabolism while most other members of the CYP2B subfamily play less significant metabolic roles. The subfamily 2C is the second most abundant CYP after 3A representing over 20% of the total CYP present in the human liver. It comprises three

active members: 2C8, 2C9 and 2C19 all of which are also involved in the metabolism of some endogenous compounds including retinol and retinoic acid.

Few clinically relevant drugs including paracetamol, chlorzoxazone and enflurane are metabolized by CYP2E1, the most active of the 2E subfamily. CYP3A subfamily constitutes over 30% of the total CYP in the human body (although the levels may vary 40-fold among individuals) with CYP3A4 being the most abundant of all isoforms highly expressed in the liver and the intestines and participates in the metabolism of about half of drugs in use today (Taavitsainen, 2001; Nelson et al., 2009). The specificity and selectivity of substrates and inhibitors for these enzymes are particularly useful in pharmacokinetic and toxicological studies.

1.1.3. Known cytochrome P450 inhibitors

As seen in Table 1.1, various drugs are able to inhibit the activities of the different CYP subfamilies. This explains the reason for many clinically known drug-drug interactions following competitive substrate binding and inhibitory actions of drugs on CYP enzymes. Other chemical compounds have been documented to exert inhibitory actions on CYP.

Table 1.1: A list of common substrates and inhibitors of major CYP isoforms (Flockhart, 2009).

1A2		2C9		2D6		3A4	
Substrates	**Inhibitors**	**Substrates**	**Inhibitors**	**Substrates**	**Inhibitors**	**Substrates**	**Inhibitors**
Amitriptyline	Mibefradil	Diclofenac	Fluconazole	Carvedilol	Quinidine	Quinidine	Indinavir
Caffeine	Cimetidine	Ibuprofen	Amiodarone	Metoprolol	Paroxetine	Midazolam	Nelfinavir
Clomipramine	Interferon	Naproxen	Fenofibrate	Timolol	Bupropion	Felodipine	Ritonavir
Clozapine	Furafylline	Tolbutamide	Fluvastatin	Desipramine	Duloxetine	Nifedipine	Itraconazole
Olanzepine	Ciprofloxacin	Glipizide	Isoniazid	Imipramine	Amiodarone	Ritonavir	Ketoconazole
Estradiol	Fluvoxamine	Losartan	Lovastatin	Paroxetine	Cimetidine	Indinavir	Saquinavir
Fluvoxamine	Amiodarone	Irbesartan	Probenecid	Haloperidol	Sertraline	Tacrolimus	Aprepitant
Haloperidol	Methoxsalen	S-warfarin	Sertraline	Thioridazine	Celecoxib	Cisapride	Verapamil
Imipramine	Enoxacin	Torsemide	Teniposide	Fluoxetine	Cinacalcet	Astemizole	Diltiazem
Mexiletine	Caffeine	Tamoxifen	Zafirlucast	Flecainide	Citalopram	Verapamil	Cimetidine
Naproxen	Echinacea	Rosiglitazone	Voriconazole	Alprenolol	Clemastine	Simvastatin	Amiodarone
Olanzapine	Verapamil	Glyburide	Flavonoids	Lidocaine	Cocaine	Amlodipine	Fluvoxamine
Ondasetron	Zileuton	Fluvastatin	Flavones	Bufuralol	Doxepin	Quinine	Gestodine
Phenacetin	Flavonoids	Fluoxetin	Fenofibrates	Atomoxetine	Hydroxyzine	Estradiol	Imatinib
Propranolol	Flavones	Celecoxib	Fluvastatins	Codeine	Ritonavir	Lovastatin	Milbefradil
Riluxole		Amitriptyline	Isonizid	Tramadol	Ranitidine	Alfentanyl	Mifepristone
Ropivacaine		Phenytoin	Sertraline	Tamoxifen	Methadone	Cafergot	Norfloxacin
Tacrine		Meloxicam	teniposide	Sparteine	Midodrine	Buspirone	Voriconazole
Theophylline		Suprofen		Propanolol	Ticlopidine	Cocaine	Quercetin
Tizanidine		Glimepiride		Phenformin	Haloperidol	Dapsone	Bergapten
Verapamil		Glibenclamide		Oxychodone		Fentanyl	Piperine
Warfarin		Glimepiride		Ondasetron		Docetaxel	Star fruit
Zileuton		Lornoxicam		Nebivolol		codeine	Efavirenz

Bailey and co-workers (1998) reported an unexpected increase in plasma felodipine levels in a felodipine-alcohol interaction studies. Subjects had been given grapefruit juice to obscure the obnoxious taste of ethanol. This observation, on a further investigation was

absent with other common fruit juices. The pharmacokinetic profile of parenteral felodipine was not altered following concomitant consumption of grapefruit juice. It was thus concluded that certain phytochemical contents of grapefruit juice could have inhibited the CYP3A4-dependent pre-systemic metabolism of the dihydropyridine. Grapefruit juice produces similar effects with other CYP3A4 substrates including midazolam, testosterone, statins, nisoldipine, cisapride, cyclosporine, triazolam and nifedipine. It has been suggested also that the flavonoid and the furanocoumarins found in grapefruit juice are capable of modulating cytochrome-dependent metabolism (Hertog and Feskens, et al., 1993; Ho, P. and Saville, D. 2001; Bailey et al., 1998; Kolars et al., 1991; Lown et al., 1997 and Paine et al., 2008).

Rotenone, a naturally occurring phytochemical used in insecticide formulations is known to inhibit CYP activity by interfering with the electron transfer of the heme iron (Sanderson et al., 2004). Photo-products of nitrobenzene, which are substrates to CYP3A4, have also been reported to competitively inhibit the metabolism of other substrates (Paula and Douglas, 1996). Other CYP3A4 inhibitors are resveratrol, a natural polymer, and tryptophan, an amino acid (Chun et al., 1999; Rannug et al., 2006).

1.2. Rationale and Motivation for this Study

The ability of administered drug to elicit the desired pharmacologic response and reverse or modify a disease condition is the ultimate goal of drug therapy (Chan and Holford, 2001). Serum drug concentration determines the availability of drug molecules at their receptor site (Hammett-Stabler et al., 2002). Thus, the rate and extent of drug absorption (bioavailability) from the GIT is an important factor that determines the plasma concentrations of orally administered drug. With a general preference for the oral route of drug administration, first-pass drug metabolism has become one of the most singularly important considerations in drug delivery. Drug loss due to poor absorption and/or pre-systemic metabolism is significant in certain cases such that oral delivery is practically impossible (Majumdar and Mitra, 2006). This has left researchers with two options: a search for new drugs capable of bypassing impediments of oral delivery or development of novel delivery systems to enhance the oral bioavailability of poorly bioavailable drugs.

The search for new drug candidates is a lengthy, complex and usually an expensive adventure. Much success is reported for most new drug candidates in the processes of identification, synthesis, characterization, screening and assaying for therapeutic efficacy (Ulrich and Friend, 2002; Angell, 2005). These successes are however short-lived in most

cases as the drug development stage often reveals poor drug delivery properties, the same challenges with older drugs that prompted such researches. At this stage of new drug molecule development, an average expenditure of between US$802 million and US$1.9billion is incurred, lasting 10-15 years (Dimasi et al., 2003; Adams and Brantner, 2006; Woodcock and Woosley, 2008).

The development of novel drug delivery systems typified by recent breakthroughs in drug delivery (Kondoh et al., 2006; Liu et al., 2007) has further strengthened the belief that new drug candidates, most of which end up with similar delivery challenges might not offer all the desired solutions to the challenges encountered with the old ones (Lichtenberg, 2001). Research attention therefore, in recent times is geared towards developing old drugs with emphasis on improvement on their delivery challenges (Gabizon, 2001).

With regard to CYP3A4 substrates, the abundance of CYP3A4 in the intestines and the liver makes the oral bioavailability of its substrates extremely poor. An average of 80% of administered CYP3A4 substrates including dihydropyridines and statins are lost to first-pass metabolism (Cockcroft and Wikinson, 2000). The choice of parenteral route for these drugs is more challenging especially in chronic use. In the particular case of felodipine, 85% of the administered dose is lost to CYP3A4 mediated pre-systemic metabolism (Joseph, et al., 2003). Thus, successful inhibition of their pre-systemic activity presents the potential for improved oral bioavailability of drugs. In addition, the required oral dosage may be greatly reduced, thus enhancing patient convenience and compliance. This would translate in the reduction of production costs to manufacturers and a transferred economic and further medical benefit to the patient in terms of affordability and a lower manifestation of side-effects with more predictable drug outcomes and pharmacokinetic properties.

Studies in this area have shown that certain chemical compounds including therapeutic agents, herbal extracts and phytochemicals are capable of inhibiting the metabolic activity of CYP3A4 (Table 1.1). This has been useful for understanding food-drug and drug-drug interactions. The ability of CYP inhibitors to also exert pharmacological action, however, limits their application in drug formulations for the sole aim of inhibiting CYP3A4. Grapefruit juice and its flavonoid contents are herbal products with non-uniform standards. Though commercially available, they exhibit various physiological actions including their antioxidant activity and are not approved by the United States Food and Drug Administration (FDA) for use in pharmaceutical formulations (Pietta, 2000).

Therefore, the novelty of this study is its intention to model pharmaceutical grade compounds on the chemical structure, structure-activity relationship and molecular binding properties of grapefruit flavonoids taking into consideration the amino acid sequence and binding properties of CYP3A4, with the aid of HyperChem 7.5 software (HyperCube Inc. Gainesville, FL, USA). The focus is to generate commercially available, USFDA-approved chemical substances which are physiologically inactive for possible inclusion in oral formulations with the aim of inhibiting the metabolism of CYP3A4 substrates (Figure 1.1). The success of this study promises a breakthrough in improving the oral bioavailability of drug substrates of CYP3A4.

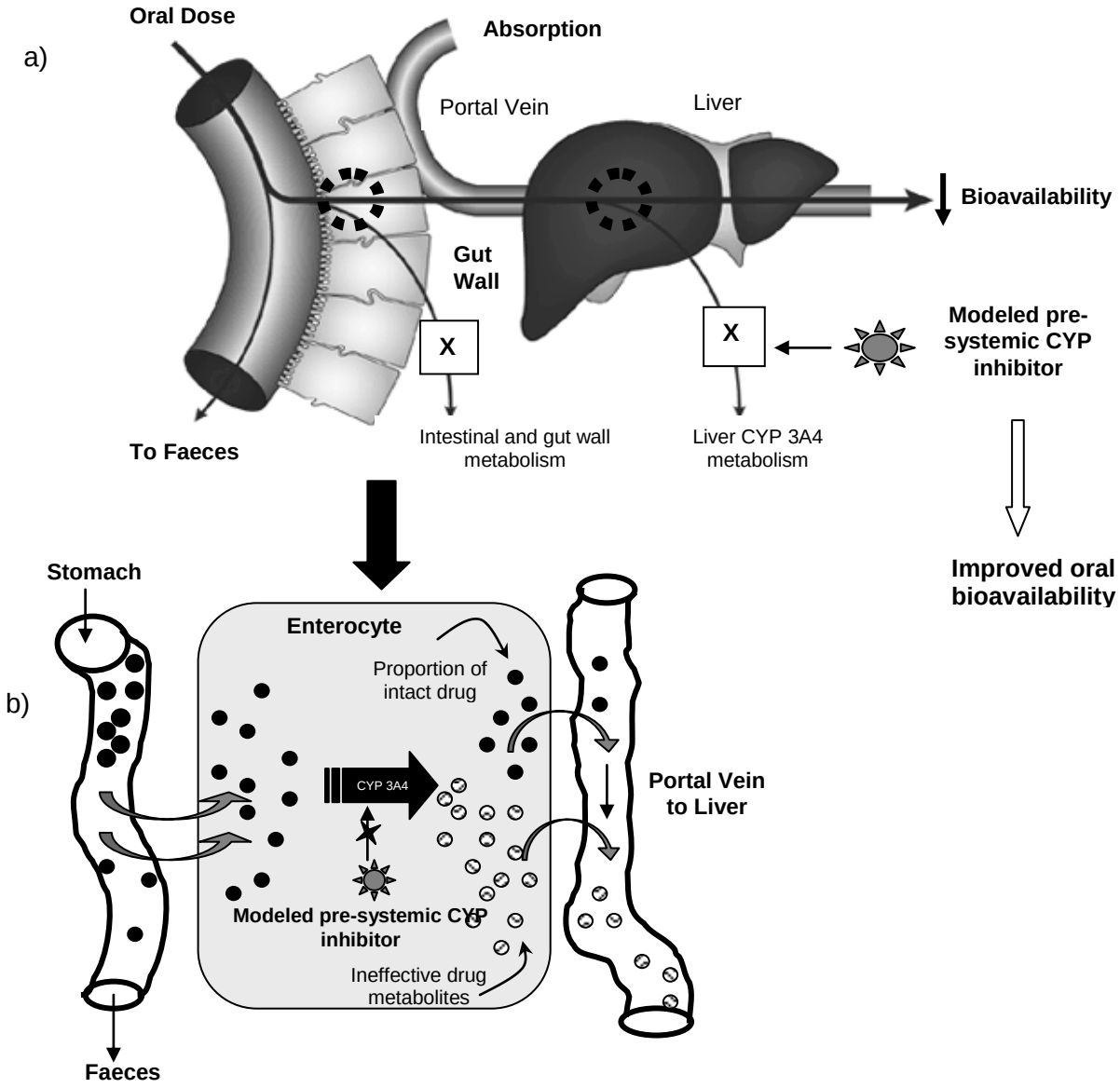


Figure 1.1: Schematic illustrating a) the pre-systemic fate of drug administered via the oral route and b) an expanded intestinal view, with both showing the proposed sites of action of the modeled pre-systemic CYP inhibitors.

1.3. Aim and Objectives of this Study

The overall aim of this study is to generate Modeled Pre-systemic CYP Inhibitors (MPCI) that are biocompatible, biodegradable and can be combined with various drugs in a single drug delivery system in order to improve the oral bioavailability of the drug. With a detailed study of the structure and the Qualitative Structure-Activity Relationship (QSAR) of flavonoids and verapamil, pharmaceutical grade and USFDA-approved compounds are intended to be computationally modeled through computational simulation and biomimetism, and screened for inhibitory activity against CYP3A4. This study considers the possibility of designing a commercializable drug-MPCI combination that will deliver drug to the systemic circulation with increased bioavailability. In order to achieve this aim, the specific objectives of this study would include:

1. To optimize and validate an *in vitro* method of metabolism of CYP-specific substrates such as felodipine (CYP3A4-dependent) and phenacetin (CYP 1A2-dependent) employing Human Liver Microsomes (HLM) and to determine their enzymatic kinetic parameters.

2. To verify the *in vitro* inhibitory activity of quercetin, naringin and naringenin on the metabolism of CYP-specific substrates such as felodipine and phenacetin by HLM in comparison to that of verapamil.

3. To model pharmaceutical entities (MPCI) with the potential of inhibiting the pre-systemic metabolic activity of CYP3A enzymes.

4. To investigate the ability of the MPCI to inhibit the *in vitro* CYP3A4-dependent felodipine metabolism employing CYP3A4 expressed HLM and Human Intestinal Microsomes (HIM).

5. To formulate an oral tablet matrix comprising felodipine and an MPCI and assess the influence of the MPCI on the formulation properties including felodipine release profiles.

6. To perform *in vivo* animal studies using an oral tablet matrix developed in 5 above in a Large White Pig model in order to assess the influence on improving bioavailability.

1.4. Technology Applied in this Study

1.4.1. Ultra Performance Liquid Chromatography (UPLC)

UPLC is a highly sensitive quantitative and qualitative analytical technique that allowed samples to be detected and analysed with the shortest run-time. It functioned on the principles of High Performance Liquid Chromatography (HPLC) with in-built software for inter-method conversions compatible with modern auto-analytical systems that demonstrated high sensitivity. It employed porous sub-2µm particles at high linear velocities for analyte separation (Waters, 2008). Analytical determinations were achieved with optimized methods that usually involved standard parameter settings including pH, flow-rate, column type, column length and the choice of mobile phase. Its sensitivity allowed the identification and quantification of a wide range of non-volatile compounds at extremely low (nano-range) concentrations.

A Waters® Acquity UPLC™ System (Milford, MA, USA) was used in this study and it possessed the following features:

- Comprehensive system components, including an Acquity UPLC® Sample Organizer, Column Manager and Column Heater/Cooler, Binary Solvent Manager and Sample Manager, for customized throughput and condition requirements
- HPLC to UPLC method conversion with the Acquity UPLC console calculator
- Support for existing HPLC methods
- Predictive system support with Connections Insight® remote intelligent services
- Chemistries and formats to suit specific analytical tasks, including Acquity UPLC 1.7µm columns, VanGuard pre-columns or Acquity UPLC HSS and HSS T3 1.8µm columns
- Extended detection capabilities with Acquity UPLC Fluorescence (FLR), Tunable UV (TUV), Photodiode Array (PDA), and Evaporative Light Scattering (ELS) detectors to single, tandem, and time-of-flight mass spectrometers
- Compatible with third-party MS solutions

The UPLC was coupled with a Photodiode Array (PDA) detector that was responsible for filtering samples through wavelengths of absorption. It identified the wavelength (λ_{max}) where maximum light absorption or transmission occurred. It allowed for the detection and quantification of lower concentrations of sample analytes and compared spectra across

wavelengths and broad concentration ranges. Data rates of up to 80 Hz, noise specifications of 10 µAU, and an extended linear range was used. It provided flexibility for simultaneous 2D and 3D analysis (Waters, 2008). The PDA detector (Waters® Acquity UPLC PDA Detector, Milford, MA, USA) used in this study possessed the following features:

- The ability to quantitate trace impurities at levels as low as 0.004% with low signal-to-noise ratios, high optical and digital resolution, and library matching which helped in accurate determinations of analytes
- The ability to achieve UPLC/PDA separations with flexible parameter control and sampling rates for accurate, reproducible integration
- Efficient light transmission for maximum sensitivity
- Provided simultaneous 2D and 3D operations for more reliable determinations
- Wavelength range of 190-500 nm and up to 2.0 *Area Under the Curve* (AU) without compromising linearity;

    - 1.3% deviation at 2.0 AU
    - 5.0% deviation at 2.8 AU

    which was employed in determining λ_{max} and confirming complete separation of analyte without interference.

1.4.3. Computational modeling of pre-systemic CYP3A4 inhibitors

A detailed study of the structure of the amino acid sequence of CYP3A4 was performed simultaneously with the QSAR of its known inhibitors. This involved computational modeling employing Hyperchem 7.5 modeling software on a non-silicon graphics system (HyperCube Inc. Gainesville, FL, USA). The templates derived from the known substrate (felodipine) was step-wise modified as a single variant within the CYP3A4-felodipine complex taking into consideration the overall electronegativity/total charge density, dipole moment, bond length, bond angle, stereo-orientation and effective geometry. The most stable form of the resulting compounds was determined by estimation of the hydration energy and the energy of conformation. This approach generated a number of potential pre-systemic CYP3A4 inhibitors.

1.4.4. Tissue incubation and drug metabolism studies

Felodipine was incubated in simulated intestinal fluids along with freshly excised intestinal tissues employing a modified method of de Kanter and co-workers (2005) in order to assess the drug-metabolizing ability of freshly excised intestinal tissue and the influence of MPCI on such metabolic activity. This was applied to ascertain a closer extrapolation of the *in vitro* results with the MPCI. The employment of Galaxy® R Series CO^2 incubator (Galaxy Incubators, RS Biotech Laboratory Equipment (Pty) Ltd. Ayrshire, Scotland, UK) enhanced tissue viability.

The Galaxy incubator created a precise environment for tissue propagation and cellular metabolic activity. It offered a temperature range of 25-50°C, a CO_2 range of 0.3-19.9% and an O_2 control of 1-95%. A refrigerated centrifuge (Xiang Yi L-535R™ Centrifuge, Changsha, China) was employed to precipitate denatured enzyme proteins. The temperature-regulator feature of the centrifuge makes it suitable for final termination of enzyme activity. Refrigerated centrifugation not only terminated enzyme action, but also facilitated the separation of the components of the incubation mixture preparatory to further analysis.

1.5. Potential Benefits of this Study

The novel MPCI intended developed in this research has the potential of solving the CYP3A4-dependent oral bioavailability related problems. The successful incorporation of MPCI into oral formulations of drugs which normally undergoes significant CYP3A4-induced pre-systemic intestinal and hepatic metabolism may significantly enhance the oral bioavailability of the CYP3A4-substrates. The resulting enhanced oral bioavailability may help in reducing the required doses and frequency of dosing of such drugs, thus saving cost and enhancing compliance. Such MPCI may also make oral administration possible for a wide range of drugs that are traditionally administered parenterally due to CYP3A4-induced poor oral bioavailability.

1.6. Overview of this Dissertation

Chapter One provides a background to this study. The factors influencing oral drug bioavailability including GIT barriers, CYP-induced pre-systemic drug metabolism are discussed along with the need to enhance oral drug bioavailability. It outlines the

rationale, motivation, aim and objectives of the study, the technology used and the potential benefits of the study.

Chapter Two reviews relevant literature on various approaches applied to enhance oral drug bioavailability. It explains preferences for oral drug delivery and the attendant challenges involved. Manipulation of intestinal physiology including the inhibition of pre-systemic activity of CYP and efflux proteins is discussed. Site-specific drug delivery for regional drug absorption including gastroretentive systems and enteric coating for small intestinal drug targeting as an option for improved oral drug delivery and enhanced bioavailability is explored. Other methods for improving oral drug bioavailability discussed include modification of formulation techniques including solid dispersion systems, lipid suspensions, solutions, emulsions and nanotechnology.

In Chapter Three, an optimized method for *in vitro* metabolism of CYP substrates namely felodipine and phenacetin are described. The effects of grapefruit juice flavonoids; naringin, naringenin and quercetin on CYP3A4 and CYP1A2 are also reported and discussed. UPLC analytical methods developed, validated and applied for the quantification of both substrates are also presented.

Chapter Four focuses on the application of computational modeling to generate MPCIs. It reports the effects of co-incubation of felodipine with the various MPCI on the rate and extent of metabolism of the latter.

Chapter Five presents the detailed study of 8-arm-PEG and its ability to enhance oral bioavailability of felodipine. An *ex vivo* study of the metabolism of felodipine by freshly excised pig intestinal tissue supplemented with a biomimetic environment is presented. The influence of 8-arm-PEG on the intestinal tissue metabolism of felodipine is also presented. Felodipine tablet matrices incorporating varying concentration of 8-arm-PEG were administered to Large White Pigs and the observed changes in plasma felodipine levels attributable to the presence of 8-arm-PEG is discussed.

Chapter Six provides the conclusions and recommendations for further study in this field. A list of references used in this study is finally presented.

CHAPTER TWO

ADVANCES IN THE APPROACHES FOR ENHANCEMENT OF ORAL DRUG BIOAVAILABILITY: A LITERATURE SURVEY

2.1. Introduction

In conscious and co-operating patients, oral drug delivery remains the preferable route of drug administration. However, not all drugs possess the desirable physicochemical and pharmacokinetic properties which favor oral administration resulting in poor bioavailability. This has in some cases led to the choice of other routes of administration, which may compromise the patient comfort and convenience, with increasing risk of non-compliance. Poor bioavailability has necessitated the administration of higher than normally required oral doses which often leads to economic wastages, risk of toxicity, erratic and unpredictable responses. The challenge over the years has been to design techniques that will allow oral administration of most drugs, irrespective of their properties and yet achieve a therapeutic systemic availability. This will be a worthy achievement since over 90% of therapeutic compounds are known to possess oral bioavailability limitations.

In this chapter therefore, an attempt is made to survey the literature on the various approaches that have been explored in the recent years to improve oral drug bioavailability, including physical and chemical means. Design of pro-drugs to bypass metabolism or enhance solubility as well as modification of formulation techniques such as the use of additives, permeation enhancers, solubilizers, emulsifiers and non aqueous vehicles are discussed. Pharmaceutical application of nanotechnology, which is an emerging area in drug delivery, has also been addressed. This review sought to assess each method aimed at enhancing the oral bioavailability of drugs in terms of the purpose, scientific basis, limitations, commercial application as well as the areas for further research improvement.

2.1.1. Factors that influence gastrointestinal drug absorption and oral bioavailability

The fate of orally administered drugs is determined by a number of factors. These include chemical degradation, physical inactivation through binding or complexation,

biotransformation induced by GIT microflora and idiosyncratic behavior among others. The fraction of the dose that survives these initial impediments is available for absorption. Of this fraction, some may be metabolized in transit through the GIT wall while unchanged fraction that reaches the hepatic portal vessels may undergo further extraction through CYP-catalyzed metabolism or hepatobiliary excretion (Figure 2.1). Thus, the fraction (*F)* of administered dose that reached the general circulation unchanged (bioavailability) is the product of the fraction (*Fx)* of drugs absorbed, the unmetabolized fraction (*Fg)* after a single passage through the gut wall and the fraction (*FH)* that survives first passage through the liver as represented by Equation 2.1.

$F = F_x F_g F_H$ *Equation 2.1*

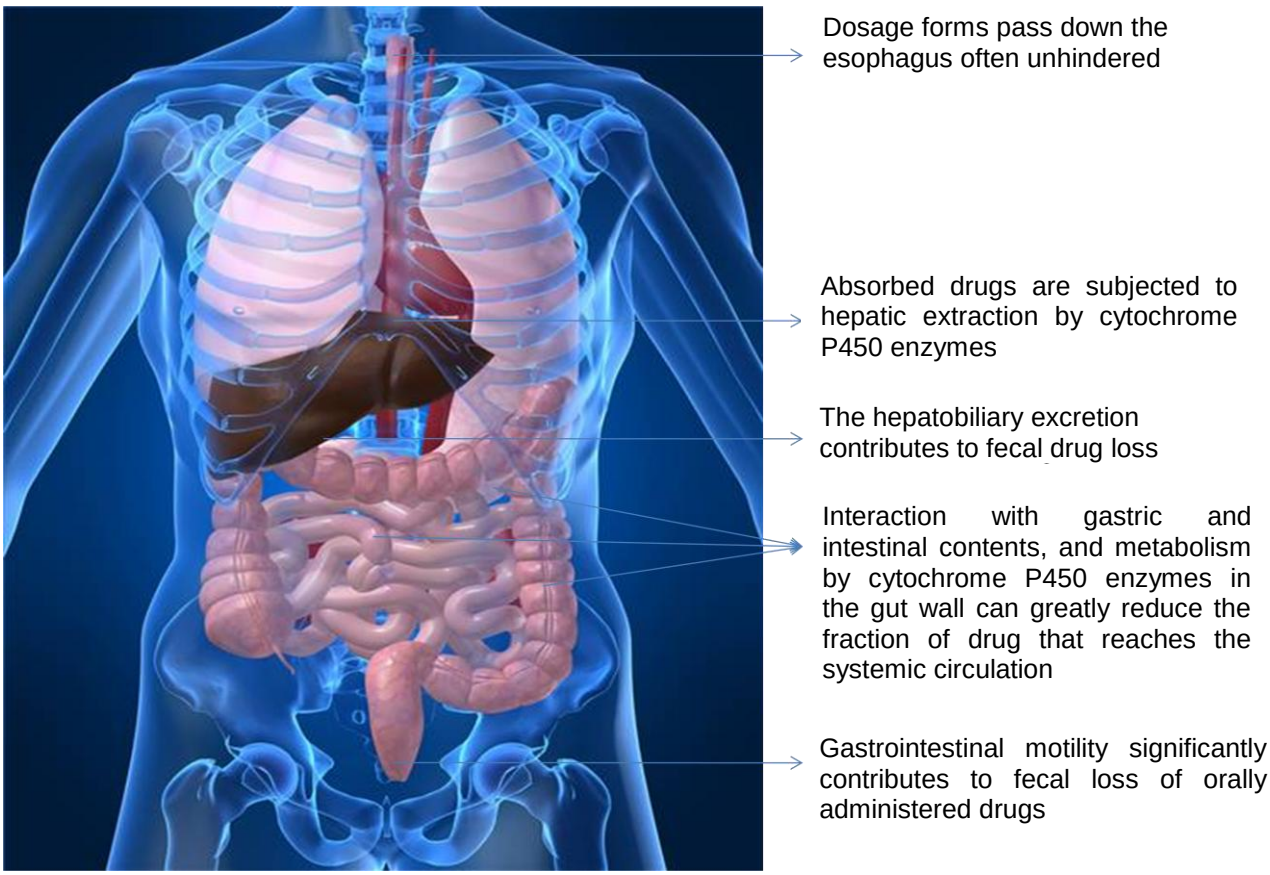


Figure 2.1: Factors influencing the oral bioavailability of drugs (Source: Gobin et al., 1985).

These factors that influence oral bioavailability can thus be broadly classified into three categories. These include features due to 1) the drug 2) the patient and 3) the physiology of the gastrointestinal tract (GIT). Drug properties include the dosage form, solubility, acid-base characteristics, partition and aqueous ionization potentials. The dosage form of

an active ingredient can have a great effect on its solubility and permeability, thereby influencing its absorption and bioavailability. The Biopharmaceutics Classification System classifies drugs into four groups: Class 1: high permeability, high solubility; Class 2: high permeability, low solubility; Class 3: low permeability, high solubility; and Class 4: low permeability, low solubility (van de Waterbeemd, 2000). Low bioavailability is often associated with oral dosage forms of Class 2-4 drugs, i.e., drugs with low solubility, low permeability, or both.

While the GIT forms a barrier to absorption, the presence of metabolizing enzymes and efflux proteins in the GIT lumen, the physicochemical properties of the GIT fluids and the irreversible removal by first-pass organs including the intestine, liver and lungs, are additional factors influencing oral bioavailability (Pang, 2003). The necessary passage of orally administered drugs through various tissues and organs, as illustrated in Figure 2.1, contributes greatly to the resulting poor oral bioavailability. Although most of these factors affect the oral bioavailability of different drugs to a varying extent, the single most important cause of poor oral bioavailability is the CYP-induced first pass metabolism. Table 2.1 illustrates typical examples of extremely poor bioavailable drugs and factors responsible for this feature.

In order to improve oral bioavailability therefore, a strategy that will achieve any of the following must be adopted: 1) the modification and manipulation of the physicochemical properties of the drug molecule, 2) the addition of a novel functionality (e.g. receptor recognition or cell permeability and 3) design of novel drug delivery systems that can bypass the impediments or interfere with these physiological barriers. This is a more promising approach to pharmaceutical research in pharmacotherapy than development of new drug entities as most new drug candidates face similar problems (Morishita and Peppas, 2006).

Table 2.1: Some poorly bioavailable (≤ 20%) drugs and drug classes in accordance with BCS classification.

Drugs	Bioavailability (%)	Reported Reasons	Reference
Allendronate	0.59-0.78	Poor solubility, poor absorption	Cremers et al., 20005
Atorvaststin	14	CYP450 and P-gp activity	Reinoso et al., 2002
Bromocriptine	5-10	Extensive first pass effect	Turner et al., 2003
Clodronate	1	Poor solubility, poor absorption	Lambrinoudaki, et al., 2006
Cytarabine	20	Intestinal and hepatic first pass	Joseph and Sharma, 2007
Domperidone	15	First pass effect, liver and gut wall	Ahmad et al., 2006
Doxorubicin	5	Intestinal and hepatic metabolism	Sturgill et al., 2000
Etidronate	5	Poor solubility, poor absorption	Cremers et al., 2005
Felodipine	15	CYP450 and P-gp activities	Dorne et al., 2003
Isradipine	15	CYP450 and P-gp activities	Tse and Jaffe, 1987
Nimodipine	13	CYP450 and P-gp activities	Choi and Burm, 2006
Fluvastatin	20	CYP450 and P-gp activities	Garcia et al., 2003
Hyoscine	20	Hepatic metabolism	Bennett et al., 2002
Ketamine	20	Intestinal and hepatic metabolism	Kharasch and Labroo, 1992
Lovastatin	<5	CYP450 and P-gp activities	Buse, 2003
Morphine	20-33	liver and gut first pass	Manoir et al., 2006
Pyridostigmine	14	Poor absorption	Aquilonius et al., 1980
Naloxone	2-10	90%absorption, extensive first pass	Kleiman-Wexler et al., 1989
Naltrexone	5-40	Enterohepatic recycling, first pass	Buse, 2003
Pamidronate	1	Poor solubility, poor absorption	Cremers et al., 2005
Pravastatin	17-34	CYP450 and P-gp activities	Reinoso et al., 2002
Prochlorperazine	20	Intestinal and hepatic first pass	Finn et al., 2005
Risedronate	<1	Poor solubility, poor absorption	Cremers et al., 2005
Selegiline	20	Extensive first pass	Clarke and Jankovic 2006
Simvastatin	5-48	CYP450 and P-gp activities	Buse, 2003
Sumatriptan	20	Hepatic first pass effect	Cossons and Fusseau, 1999
Tacrine	10-30	Hepatic first pass effect	Jann et al., 2002
Terbutaline	9-21	Poor absorption, first pass	Nakhat et al., 2007
Tiludronate	6	Poor solubility, poor absorption	Cremers et al., 2005
Lidocaine	3	Hepatic first pass effect	Isohanni et al., 2006
Budesonide	11[2]	Hepatic first pass effect	Dorne et al., 2003
Zoledronic acid	0.9-1.8	Poor solubility, poor absorption	Lambrinoudaki, et al., 2006
Class II compounds		High permeability, low solubility	van de Waterbeemd, 2000
amiodarone HCl, atazanavir sulfate, atorvastatin, azithromycin, benazepril HCl, bicalutamide, candesartan cilexetil, carbamazepine, carisoprodol, carvedilol, celecoxib, clarithromycin, diazepam, divalproex sodium, docetaxel, donepezil HCL, efavirenz, etodolac, ezetimibe, fenofibrate, finasteride, gemfibrozil, glimepiride, glyburide, ibuprofen, indapamide, indomethacin, irbesartan, ketoconazole, lansoprazole, loratadine, lovastatin, meclizine HCL, metaxalone, moxifloxacin HCl, mycophenolate mofetil, nabumetone, nelfinavir mesylate, olmesartan medoxomil, pioglitazone HCl, prednisone, raloxifene HCl, risperidone, ritonavir, rofecoxib, simvastatin, spironolactone, tacrolimus, temazepam, valdecoxib, valsartan, ziprasidone HCl.			
Class III compounds		Low permeability, high solubility	van de Waterbeemd, 2000
albuterol, alendronate sodium, amlodipine besylate, amoxicillin, atenolol, baclofen, buspirone HCl, captopril, carboplatin, ceftriaxone, ciprofloxacin, ciprofloxacin HCl, colchicine, fluconazole, folic acid, gabapentin, gemcitabine HCl, granisetron HCl, hydrochlorothiazide, hyoscyamine sulfate, lamivudine, lamotrigine, levetiracetam, levofloxacin, lisinopril, metformin HCl, metronidazole, minocycline HCl, morphine sulfate, niacin, oxaliplatin, oxycodone HCl, oxycontin, penicillin VK, progesterone, ranitidine, risedronate sodium, rosiglitazone, sumatriptan, terazosin HCl, thalidomide, timolol maleate, topiramate, valacyclovir HCl, zoledronic acid, zolpidem.			
Class IV compounds		low permeability, low solubility	van de Waterbeemd, 2000
acyclovir, allopurinol, aspirin, cefdinir, cefprozil, cephalexin, clindamycin HCl, doxycycline hyclate, famotidine, felodipine, furosemide, glipizide, linezolid, meloxicam, mesalamine, methocarbamol, methotrexate, nifedipine, nitrofurantoin, olanzapine, oxcarbazepine, phenobarbital, sildenafil citrate, tadalafil, temozolomide, tetracycline, theophylline.			

2.2. Manipulation of Gastrointestinal Physiology

2.2.1. Inhibition of pre-systemic metabolic enzymes

Cytochrome is the main oxidative drug metabolizing enzyme family (Guéraud et al., 1999; Glue and Clement, 2004). It is highly expressed in the liver and intestines and as a result of this, it is responsible for most pre-systemic metabolism of orally administered drugs, in humans (Glue and Clement, 2004). By anatomical design, blood circulation of the intestine is unique due to the fact that the intestine is the portal tissue that regulates the flow of substrates to the liver. The majority of blood supply to the liver, about 75%, is from intestinal venous pool (Pang, 2003). Thus, all absorbed drugs are subjected to passage through the hepatic system (Figure 2.1).

Cytochrome consists of approximately 12 families and 17 subfamilies (Nelson et al., 1993), but it is only a small number of these enzyme families that is responsible for the majority of drug oxidation (Quintieri et al., 2008). This is the human cytochrome P450 (CYP3A) subfamily which is responsible for the metabolism of more than 50% of currently marketed drugs (Quintieri et al., 2008). It is also known from the literature that there is a large inter-individual variability in cytochrome P450 (CYP) 3A expression as already determined with the erythromycin breath test (Guengerich, 1998). CYP3A4 is the major isoform, largely expressed in human liver and occupy more than 70% of gastrointestinal cytochrome expression and catalyzes the oxidative metabolism of most clinically important drugs including statins, and dihydropyridines (Figure 2.2). CYP3A5 is a polymorphic isoform, present in significant amounts in 20-60% of human livers (Guengerich, 1998). When present however, it accounts for at least 50% of total CYP3A. CYP3A enzymes metabolize numerous structurally unrelated compounds, a property that is said to be responsible for the large number of documented drug-drug and drug-food interactions (Quintieri et al., 2008).

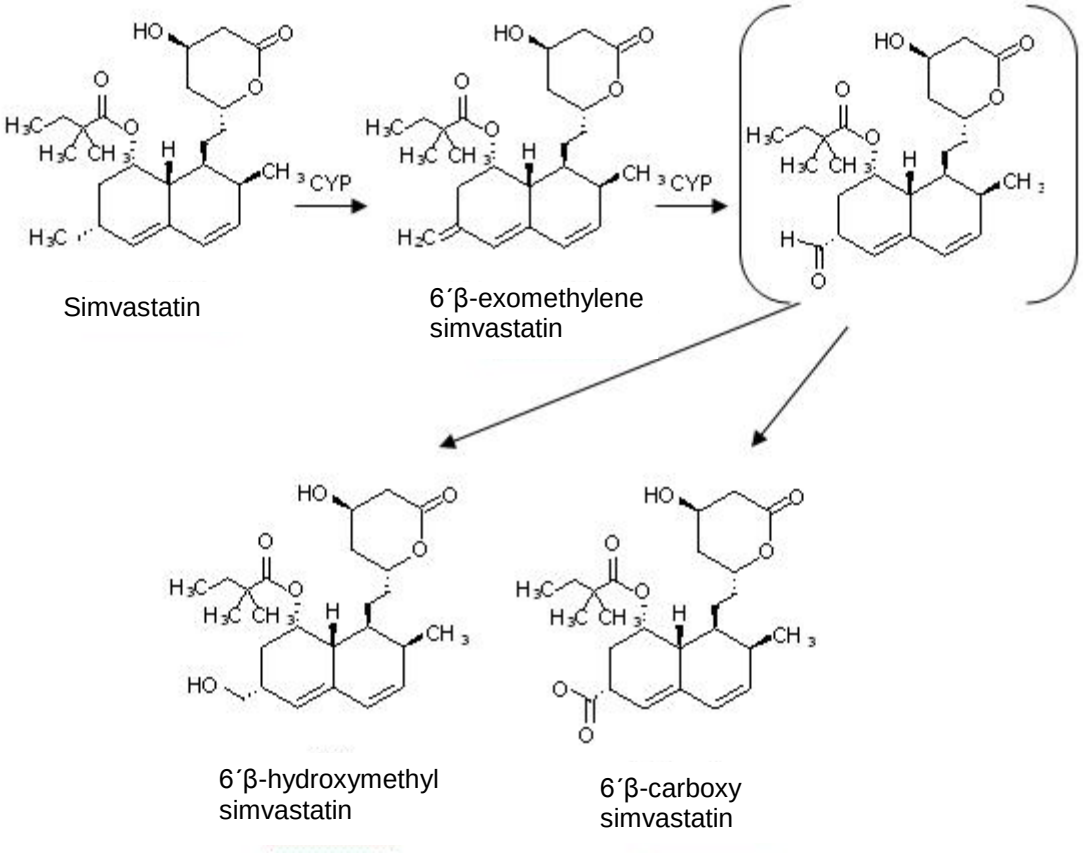


Figure 2.2: A schematic showing the structural characteristics and the mechanism of action involved in the pre-systemic metabolism of Simvastatin by cytochrome P450 (Source: Reinoso et al., 2002).

Interactions between grapefruit juice and clinically used drugs have been reported in recent years (Bailey et al., 1998). The drugs include cyclosporine, midazolam, triazolam and calcium channel blockers such as felodipine and nisoldipine (Bailey et al., 1998). All of these drugs are substrates for CY3A4 and undergo extensive metabolism by intestinal CYP3A4 (Kolars et al., 1991; Lown et al., 1997; Paine et al., 2008). Various researchers have suggested that flavonoid contents of grapefruit juices could be responsible for the inhibition of the cytochrome enzymes (Hertog et al., 1993; Ho and Saville, 2001; Paine et al., 2008). Flavonoids are polyphenolic compounds with antioxidant properties and are widely distributed in foods of plant origin such as vegetables, fruit, tea and wine (Hertog et al., 1993). When administered orally, natural flavonoids such as flavone, tangeretin and nobiletin can activate rat and human benzopyrene hydroxylase and some other CYP activity (Ho and Saville, 2001). Flavonoids are believed to exert various other physiological actions. This limits their commercial use in pharmaceutical preparations in enhancing oral bioavailability. In this regard, no commercialized technique of inhibiting pre-systemic metabolism as a way of enhancing oral bioavailability has been reported. Further exploitation of this approach is anticipated.

2.2.2. Inhibition of efflux pumps to enhance drug bioavailability

Efflux transporters such as P-glycoprotein (P-gp) and the multidrug resistance related protein (MRP), which have been known for decades to be over-expressed in tumor cells, are also widely distributed throughout normal tissues in humans, including the liver, kidney, intestinal mucosa and endothelial cells of the brain (Gupta, 1995; Ambudkar et al., 1999; Hugger et al., 2002a). Tumor cells often become resistant not only to the drugs which have been used during the treatment but also to other drugs which are structurally and functionally unrelated. This is called multidrug 'resistance' (MDR). MDR is frequently associated with decreased drug accumulation resulting from enhanced drug efflux. This is correlated with the presence of a membrane protein, P-glycoprotein, which pumps a wide variety of drugs out of cells (Borrel et al., 1994).

P-gp is a type of ATPase, and an energy-dependent transmembrane drug efflux pump which belongs to members of ATPase Binding Cassette (ABC) transporters (Juliano and Ling, 1976). It is a 1280 long amino acid glycoprotein with a molecular weight of 170kDa, expressed as a single chain containing two homologous portions of equal length, each containing six membrane domains and two ATP binding regions separated by a flexible linker polypeptide region between the Walker A and B motifs (Schinkel et al., 1993; Pokharkar et al., 2006). Immunohistochemical analysis using monoclonal antibodies provided evidence for localization of P-gp in a wide range of tissues, particularly in columnar epithelial cells of the lower GIT, capillary endothelial cells of brain and testis, canalicular surface of hepatocytes and apical surface of proximal tubule in the kidney (Thiebut et al., 1987). Due to selective distribution at the port of drug entry and exit, P-gp has been speculated to play a major physiological role in absorption, distribution and excretion of xenobiotics. Drug efflux pumps like P-gp have been identified recently to play a major role in altering the pharmacokinetics of various drugs and are particularly associated with poor bioavailability in co-ordination with gut wall metabolism. Though much research has been carried out in the process of establishing the role of P-gp in multidrug resistance in cancer cells, it is only recently that it is gaining importance in absorption enhancement due to its selective distribution at the sites of drug absorption as depicted in Table 2.2 (Varma et al., 2003; Varma et al., 2006).

Table 2.2: Distribution of metabolic enzymes and transporters in the liver and small intestine.

Enzymes and transporters	Duodenum	Jejunum	Ileum	Liver	Reference
Cytochrome P-450 (rat, humans)	Higher	Lower	Lowest	Highest	Bergheim et al., 2005; Pang et al., 2007
UDP-Glucuronosyltransferase (rat)	Higher		Lowest	Highest	Li et al., 2002
Sulfotransferase (guinea pig)	Higher		Lowest	Highest	Heydel et al., 2001
Glutathione S-transferase (rat)	Highest		Lowest		Dooley, 1998
Sulfatase (rat)	Highest		Lowest		Hayes et al., 1989
Oligopeptide transporter 1 - PEPT1 (rabbit)	Highest				Miki et al., 2002; Ziegler et al., 2002
Apical sodium-dependent bile acid transport - ASBT (hamster, rat)	Lowest		Highest		Botka et al., 2000
Monocarboxylic acid transporter 1 - MCT1 (rat)	Lower	Highest	Lowest		Shneider et al., 1995
Organic anion transporting polypeptide 3 - Oatp3 (rat)	Lower	Higher	Lower		Cong et al., 2001
Multidrug resistance protein MDR1 or Pgp (rat)		Lowest	Higher	Highest	Chen and Pang, 2005
Multidrug resistance-associated protein 2 - MRP2 (rat)	High	Highest	Lowest		Liu et al., 2006
Multidrug resistance-associated protein 3 - MRP3 (rat)	Lowest	Higher	Highest		Mottino et al., 2000; Gotoh et al., 2000

The motivation for the search and use of modulators of P-gp and other efflux proteins in bioavailability enhancement and in improving pharmacokinetic properties include the following (Varma et al., 2006):

(i) Products of histochemical researches have given evidences of the presence of high concentrations of P-gp in the absorbing surfaces of the gastrointestinal tract.

(ii) P-gp acts as barrier to intestinal absorption of a number of clinically significant drugs. Further, these drugs induce P-gp expression, which may lead to inefficient therapy on chronic use.

(iii) Concert role of P-gp and CYP further limits bioavailability of orally administered drugs due to a great overlap between substrate specificity and intestinal distribution of both proteins (Table 2.2).

(iv) Distribution of P-gp extends to the major excretory organs like kidney and liver leading to change in distribution and elimination kinetics of administered drugs (Table 2.2)

Studies have identified a number of clinically important drugs as P-gp substrates, which include anthracyclins (doxorubicin, daunorubicin), alkaloids (reserpine, vincristine, vinblastine), specific peptides (valinomycin, cyclosporine), steroid hormones (aldosterone, hydrocortisone) and local anesthetics (dibucaine) (Schuetz et al., 1996; Fromm, 2002; Hu, 2004a). Even dye molecules (Rhodamine123) and some pharmaceutical excipients have been found to exhibit P-gp substrate activity (Lin et al., 2007). The identification of few substrates of P-gp as inhibitors set off an opportunity in multidrug resistance reversal (Johnson, 2002). Improved clinical efficacy of various drugs observed by P-gp inhibition, especially conditions subjected to multidrug resistance, led to the design and development of modulators, which specifically block P-gp efflux and improve the toxicity profiles (Johnson, 2002). P-gp inhibitors are gaining recognition to improve bioavailability by inhibiting P-gp in intestine, brain, liver and kidneys as has been observed by many researchers in recent years (Eytan et al., 1996; van Asperen et al., 1997; Breedveld et al., 2006).

Borrel and co-workers (1994) reported that mobile ionophores such as valinomycin, nonactin, nigericin, monencin, calcimycin, and lasalocid inhibit the efflux of anthracycline by P-gp whereas channel forming ionophores such as gramicidin do not. Cyclosporine which is also a strong calcium chelating agent was reported to inhibit the P-gp-mediated efflux of anthracycline. P-gp inhibitors are traditionally classified into three generations based on specificity and affinity. First generation inhibitors are pharmacological actives, which are clinically used for other indications but have been shown to inhibit P-gp. These include calcium channel blockers (e.g. verapamil) and immunosuppresants (e.g. cyclosporine A); alkaloids (e.g. reserpine; quinidine, yohimbine) and antiestrogens (e.g. tamoxifen and toremifen). The ability of risperidone and its major active metabolite, 9-hydroxy-risperidone (paliperidone) to inhibit P-gp activity *in vitro* where both compounds significantly increases the intracellular accumulation of Rhodamine 123 and Doxorubicin in a concentration-dependent manner, has also been reported (Zhu et al., 2007). The usage of these compounds is limited by their toxicity due to the high serum concentrations achieved with the dose that is required to inhibit P-gp. Further research directed at

improving the toxicity profile resulted in second and third-generation inhibitors that specifically modulate P-gp (Breedveld et al., 2006). Second-generation modulators are agents that lack the pharmacological activity of the first generation compounds and usually posses a higher P-gp affinity. However, inhibition of two or more ABC transporters leads to complicated drug-drug interactions by this class of compounds, which include non-immunosuppressive analogues of cyclosporine A, PSC 833; D-isomer of verapamil, and dexverapamil; and others such as biricolar (VX-170), elacridar (GF120918), zosuquidar (LY335979), tariquidar (XR9576) and MS-209 (Zhu et al., 2007). On the other hand, several other novel third-generation P-gp blockers are under development primarily aimed at improving the treatment of multidrug resistant tumours and to inhibit P-gp with high specificity. Modulators such as LY335979, OC144093 and XR9576 have been identified to be highly potent and selective inhibitors of P-gp with a potency of about 10-fold more than the first- and second-generation inhibitors (Breedveld et al., 2006).

Generally, P-gp can be inhibited by: 1) blocking drug binding site either competitively, non-competitively or allosterically; 2) interfering with ATP hydrolysis; or 3) altering the integrity of cell membrane lipids. Although most of the drugs inhibit P-gp function by blocking drug binding sites, presence of multiple binding sites complicate understanding as well as hinder developing a true, conclusive structure-activity relationship for substrates or inhibitors. However the mode of handling of substances and inhibitors is the same for P-gp if the protein transport and/or inhibition are mediated only through binding sites. The point of great concern which therefore needs to be addressed is establishing why, down to the molecular level, substrates and inhibitors are discriminated. In this regard, Eytan and co-workers (1996), proposed a plausible explanation that the modulator or inhibitor ‘flipped’ by P-gp can ‘flop’ back into the inner leaflet of the membrane, for further transport, which rapidly creates a big difference between the rate of efflux of the substrate and inhibitor. Thus the P-gp modulator is cycled repeatedly, preventing efflux of substrates, which depends on the hydrophobicity of the compound. This concept has been proved from the drug delivery point of view that the absorption of high affinity drugs to proteins need not necessarily be limited by P-gp (e.g. verapamil), if they are highly permeable whereas less permeable drugs (though may be weak substrates) may undergo a substantial extrusion mediated by P-gp, e.g. tanilol (Doppenschmitt et al., 1999). Compounds that inhibit ATP hydrolysis could serve as better inhibitors since they are unlikely to be transported by P-gp. These kinds of agents will require a low dose which is desirable for local use in the gut lumen. Quercetin is a naturally occurring flavonoid which has been proposed to block the P-gp function by an unknown mechanism, possibly by interfering with ATPase activity (Shapiro and Ling, 1997). Since none of the substrates

have been found to interact with the nucleotide binding site thereby interfering P-gp ATPase catalytic cycle, further research to explore the detailed mechanism of inhibition of ATP hydrolysis would provide new and better inhibitors with potent and specific activity.

2.2.2.1. Pharmaceutical surfactants used as P-gp inhibitors

The commonly used pharmaceutical surfactants are emerging as a different class of P-gp inhibitors, which act by altering integrity of membrane lipids (Hugger et al., 2002b). The change in secondary and tertiary structure is found to be the reason for loss of P-gp function due to disturbance in hydrophobic environment by surfactants (Hugger et al., 2002a). In a series of studies by Hugger and co-workers (2002a), it was observed that excipients such as poly(ethylene) glycol (PEG)-300, Cremophor® EL, and Tween® 80 inhibit P-gp activity in epithelial colorectal adenocarcinoma (CaCO-2) cell monolayers. Surfactants seem to be a better choice since they have already been approved for routine use in pharmaceutical formulations. However, they have only been tested at the *in vitro* level to date, which therefore necessitates a further evaluation in animal or human subjects. Likewise, the clinical hypothesis that P-gp inhibitors interfere with ATP hydrolysis and catalytic cycle is hampered by lack of understanding of the exact mode of inhibition which therefore posses to be another potential area for further research.

2.3. Application of Site-Specific Drug Delivery

2.3.1. The significance of regional drug absorption

The differences in the composition and thickness of the mucus layer, pH, surface area and enzyme activity of the GIT is responsible for the possibility of site-specific absorption (Hamman et al., 2005). The most important site for GIT drug absorption is the small intestine due to the large absorptive surface area as a result of the presence of villi and microvilli (Lacombe et al., 2004). Drug absorption is generally believed to be higher in the upper region of the small intestine compared to the lower parts due to higher permeability (Masaoka et al., 2006). Esophageal absorption is usually negligible due to short transit time of the passing drug. The stomach is responsible for initial digestion. The small surface area and short residence time in the stomach limits gastric absorption.

2.3.2. Gastroretentive systems for the enhancement of site-specific absorption

Gastroretentive systems have been designed to enhance local action in the stomach for drugs such as antacids, misoprostol, certain antidotes and antibiotics (Ali et al., 2006).

Furthermore, gastroretentive systems have been employed to improve the bioavailability of drugs such as captopril and ranitidine which have been found to be unstable in the intestine and the colon (Drummer and Jarrott, 2006). Furosemide is absorbed mainly in the stomach and upper small intestine (Streubel et al., 2006) due to its weak acidic properties (pKa 3.9). This narrow absorption window of furosemide is the major cause of its low oral bioavailability (Klausner et al., 2003). The feasibility of widening the absorption window for furosemide has been demonstrated by controlling the pH in the distal portions of the GIT through the co-administration of Eudragit® L100-55 (Terao et al., 2001). Drugs like diazepam, which exhibit low solubility at high pH are best absorbed in the stomach (Castrol et al., 1999).

Gastroretentive systems employed in pharmaceutical formulations for drugs that are preferentially absorbed in the stomach may be bioadhesive (Rosa et al., 1994), swelling and expanding (Deshpande et al., 1996; 1997), floatable (Menon et al., 1994; Whitehead et al., 1998), delayed gastric emptying (Singh and Kim, 2000; Chawla and Bansal, 2003), simplex lattice (Patel et al., 2001), high density (Shine and More, 2008), modified shape (Garg and Sharma, 2003), super porous hydrogels, or magnetic systems (Bardonnet et al., 2005).

It is, however, pertinent to note that an effective gastroretentive drug delivery system must be able to withstand the peristaltic forces, constrictions, grinding and churning mechanisms in order to resist premature gastric emptying. Novel gastroretentive drug delivery systems combined with a timed release mechanism holds the promise of a future once-a-day regimen for a wide range of drugs. However, as promising as gastroretentive drug delivery system appears, it must be pointed out that it is not suitable for drugs that may cause gastric irritation or those unstable in acidic medium. Examples of commercially available gastroretentive formulations are Valrelease®, a diazepam floating capsule; Madopar®, a combination formulation of benserazide and levodopa; Topalkan®, an aluminium-magnesium antacid, liquid Gaviscon®; a floating liquid alginate preparation; and Almagate Flot-Coat®, an antacid preparation (Shinde and More, 2008).

2.3.3. Enteric coating technology for small intestinal drug targeting

The technology of enteric coating has been used for many years effectively to delay the release of active ingredients in drug formulation until it reaches the small intestine (Marvola et al., 1999; Ho and Saville, 2001; Atyabi et al., 2005). Targeting drug release in the small intestine by employing a pH-dependent coating is required principally to prevent

drug destruction by gastric enzymes or gastric fluids, as well as to prevent nausea and vomiting caused by the irritation of the gastric mucosa. However, absorption from enteric coated tablets is erratic and to a high degree dependent on the gastric emptying time (Zeitoun et al., 2006). For example, erythromycin is administered either in an enteric coated dosage form or in the form of a less soluble salt or as a pro-drug since it is thought that it has low bioavailability due to the hydrolysis caused by the acidic environment of the stomach (Somogyi et al., 1995). Specific bioadhesive drug delivery systems using lectins have been proposed to improve the oral bioavailability of poorly absorbed drugs such as peptides and proteins through prolonged and/or intensified contact with the intestinal mucosa (Lehr et al., 1992). Various polymers have been used as enteric coatings which breakdown to release the active ingredient within the pH range of 5-7 (Aungst, 2000; Cremers et al., 2005). Thus synthesizing polymers that are soluble only at the intestinal pH may be a useful approach to improve the oral bioavailability of drugs that are not stable at the gastric pH.

Drugs are generally absorbed to a smaller extent in the colon than in the small intestine (Atyabi et al., 2005; Chourasia and Jain, 2003; 2004; Laila et al., 2006). Both nisoldipine and dilazep hydrochloride, are known to be preferentially absorbed in the colon (Antonin, 1993). Such drugs have to be protected through coating to delay their release from the formulation matrix until they reach their site of absorption. In certain pathological instances, such as in the treatment of inflammation of the colon where local action is desired, it is expedient to target drug release in the colon. This helps to prevent the absorption of the drug before it reaches the colon. In addition, for drugs such as peptides, which are metabolized by the endogenous enzymes of the small intestine, colonic targeting for absorption becomes favorable since the colon exhibits a relatively low digestive enzyme activity. It should also be noted that drugs generally have relatively long contact times with the absorbing surface of the colon (Rouge et al., 1996). Several physiological factors such as the regional pH, gastrointestinal transit time and colonic microflora are the major considerations in targeting the release of orally administered drugs to the colon. Reduced concentration of enzymes such as peptidases that degrade peptide drugs in the colon, long residence time, enhanced sensitivity to absorption enhancers, demonstration of natural absorptive characteristics and the abundance of lymphoid tissue follicles are some of the factors that favor drug absorption in the colon (Hamman et al., 2007). Some delayed-release units with a gastroresistant film use the relatively constant small intestine transit time.

Gazzaniga and co-workers (1994), described a system consisting of a drug-containing core coated with two or three polymeric layers. The outer layer dissolves at a pH greater than 5 when the system has left the stomach. The intestinal layer which is made of hydrophilic swellable polymers is the one responsible for the lag phase that favors the protection of the core drug during the transit through the small intestine. An inner layer composed of an enteric film soluble at a pH greater than 6.5-7 may be added as a further control element. Polymers such as USP methacrylic acid copolymer type B (Eudragit® S, Rohm and Haas, Philadelphia, USA) that degrade above a pH of 7 have been used for colonic drug delivery in diseases such as colitis (Gibaldi, 1984).

The number of microorganisms is significantly higher in the colon (Wahlqvist and Ball, 2002). According to Ashford and Fell, (Ashford et al., 1994) two main classes of enzymes that are produced by the microbial population in the colon, namely azoreductase and polysaccharidases, are considered reproducible enough to be exploited in drug targeting. The use of pro-drugs such as azo, glucuronide and dextran conjugate compounds has been proposed to target drugs to the colon (McLeod et al., 1994). This approach seems, however, to be limited to only specific drugs. Thus, it is considered that a more promising approach would be to coat the dosage form with agents resistant to gastric and intestinal fluid, but susceptible to bacterial degradation such as azo-linked coatings or lauric acid dextran esters (Gazzaniga et al., 1994; Kesslhut and Bauer, 1994; Rouge et al., 1996). Matrices made of polysaccharides comply with these biodegradation requirements, but they are soluble in water and therefore have to be transformed into water-insoluble products. This has been achieved by pectin (by forming the calcium salt or by selecting a high methoxypectin) or by chondroitin sulphate (by cross-linking) (Rouge et al., 1996). It should be noted that the drug has to be released by erosion and simple diffusion must be prevented. Therefore, these matrices are only valuable for insoluble drugs (Rouge et al., 1996). Thus, it can be concluded that targeting specific regions of the GIT through coating techniques, may render it possible for drugs to be delivered to sites where their absorptions is most favored thereby leading to the improvement of their bioavailability.

2.4. Modification of Formulation Techniques for Oral Bioavailability Enhancement

Drug dosage forms and formulation design can be modified to improve oral bioavailability of drugs. Conventional approaches to enhancing the solubility properties and oral bioavailability of hydrophobic drugs include the following (van den Mooter et al., 1994):

- Synthesis of molecular species such as salts to facilitate dissolution e.g. addition of hydrochloride, sulphate or phosphate moieties.
- Drug particle size reduction by physical grinding and milling to improve the surface area and improve membrane permeability as often employed in formulating griseofulvin, a poorly water-soluble drug.
- Use of amorphous crystal formation for production of solid/resin dispersions: typically melt extrusion technology, in which spectra-melt temperatures are used to produce a more soluble amorphous dispersion of chemical drug molecules in a polymer diluent such as polyethylene glycol (PEG).
- Use of surfactants/emulsifiers, for example in self-emulsifying/micro-emulsifying systems: anhydrous lipid-based formulations containing water-insoluble drug dissolved in oil/s, together with surfactants and co-solvents; generally administered in soft gel capsules for spontaneous emulsion formation on contact with gastrointestinal fluids.
- Conjugation/derivation of delivery system including integration of two or more different delivery systems e.g. formulation of effervescent tablets and encapsulation of emulsions.

Two transport routes, namely transcellular and paracellular are responsible for the passage of drugs through the GIT lumen and into the blood stream (Figure 2.2). In the transcellular transport, compounds cross the epithelial cells by crossing the intestinal cell membrane, generally by passive diffusion. In the small intestine, drugs with structures similar to those of nutrients may be taken up by facilitated diffusion or by active transport both of which represent carrier-mediated transport (Figure 2.2). The absorption of drugs which are poorly absorbed because of their hydrophilic character can be influenced either by manipulating the drug, the dosage form or the intestinal membrane. Therefore, most strategies for absorption enhancement either change the permeability properties of the enterocytes or alter the physico-chemical properties of the compound (Ezra and Golomb, 1999).

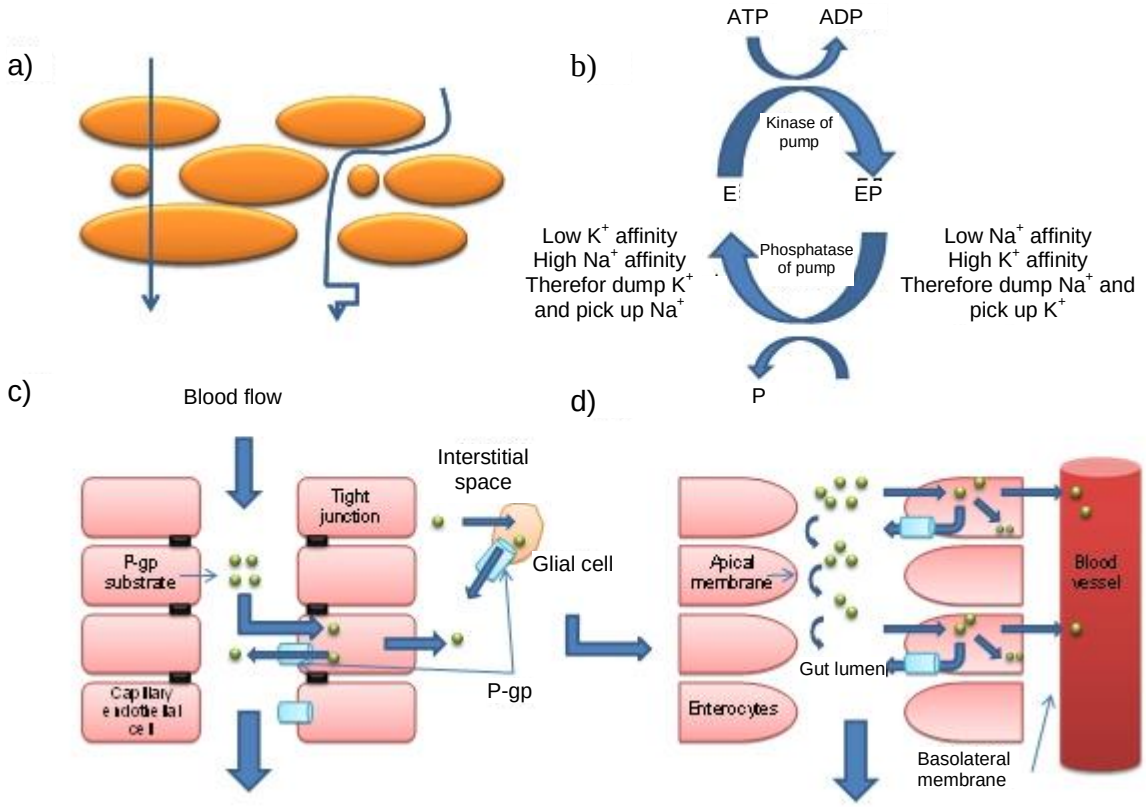


Figure 2.3: A schematic depicting the a) transcellular and paracellular transport routes b) active transport c) passive transport and d) facilitated transport of compounds across the GIT lumen (Adapted from: Matheny et al., 2009).

In order to lower the physical barrier function of structural elements of the intestinal mucosa towards poorly absorbed drugs, the potentials of co-administration of absorption enhancing agents have been extensively investigated (van Hoogdalem et al., 1989; Takatsuka et al., 2006). The absorption enhancers used belong to widely differing chemical entities and it appears that the only common characteristic they share is their ability to promote absorption. Desired effect for an absorption enhancing formulation includes the absence of toxic manifestation, the potential of which must be evaluated. Unfortunately, toxicity is probably often closely related to the mechanism of intestinal permeation enhancement. Some mechanisms may inherently have lower potential to cause toxicity than other mechanisms just as a transient opening of tight junctions would seem less damaging than a disruption of cell membrane structure.

The main classes of enhancers of intestinal drug absorption are surfactants, fatty acids, medium chain glycerides, steroidal detergents, acyl carnitine and alkanoyl-cholines, N-acetylated non-α-amino acids, chitosan and other mucoadhesive polymers, bile salts and its analogues, chelating agents, salicylates and phosphonate derivatives (Kawahara et al., 2000). According to an investigation undertaken by Janner and co-workers (1991), the

absorption of Alendronate which is a bisphosphonate without EDTA was at a range of 1-3% while the addition of EDTA increased its absorption about ten-fold at an allendronate dose of 0.6mg/kg and about two-fold at lower doses of 0.1 and 0.3mg/kg (Gibaldi, 1984). EDTA was also reported to increase the absorption of Clodronate (Legen et al., 2006). Similarly, in a screening evaluation of some pharmaceutically acceptable excipients as permeation enhancers for amoxicillin, Legen and co-workers (2006), reported that sodium lauryl sulphate (0.2mg/mL) increased the intestinal permeability of amoxicillin in the mucosa-to-serosal direction. Likewise, Sodium caprate, a medium chain fatty acid, is known to enhance the transport of drugs across the intestinal mucosa in cell culture systems and small animal species (Legen et al., 2006). It has also been demonstrated that it can improve the oral absorption of two chemically modified antisense oligonucleotides ISIS 2503 (phosphorothioate) and ISIS 104838 (methoxyethyl modified phosphorothioate) when studied in an intra-intestinal catheterized pig model (Raoof et al., 2002). However, it is apparent that many, if not most of the compounds examined as membrane permeation enhancers *in vitro* cause cytotoxicity or membrane damage. The dilemma is whether the concentrations associated with membrane permeation enhancement are different from concentrations causing membrane damage. Most studies however suggested that enhancers often have steep concentration versus effect profiles *in vitro,* with relatively small safety margins (Kawahara et al., 2000). Either, it has been demonstrated through *in vitro* investigation that papain which is a proteolytic enzyme can serve as an effective permeation enhancer for orally administered low molecular weight heparin (Grabovac et al., 2007). It has further been argued that most likely papain lacks the toxicity associated with oral absorption enhancers due to its presence in fruit such as papaya, and in over-the-counter products as well as in drugs such as Wobenzym N® (MucosPharma GmbH & Co, Gretsried, Germany) (Raoof et al., 2002).

2.4.1. Modification of drug solubility properties

Another alternative for altering the permeability characteristics of a drug molecule is to modify the solubility properties. In order for a drug to be absorbed into the systemic circulation following oral administration, the drug must be dissolved in the gastrointestinal fluids. For hydrophobic drugs, it is this dissolution process which acts as the rate-controlling step and therefore, determines the rate and extent of absorption. One of the major challenges to drug development today is poor solubility. It has been estimated that 40% of all newly developed drugs are poorly soluble or insoluble in water (Watt and Morrison, 2001; Payghan, 2008). In addition, up to 50% of orally administered drugs suffer from formulation problems related to their high lipophilicity (Naseem et al., 2004;

Gursoy and Benita, 2004). One of the methods that has often been employed in pharmaceutical practice to improve solubility properties, and hence bioavailability is mechanical micronization through milling. However, the major disadvantage of milling is the limited opportunity to control important characteristics of the final particle such as size, shape, morphology, surface properties and electrostatic charge (Ward and Schultz, 1995). Milling also causes disruption in the drugs crystal lattice resulting in the presence of disordered or amorphous regions in the final product which make such particles thermodynamically unstable and render them susceptible to recrystallization on storage (Ward and Schultz, 1995). Alternatives to milling are spray drying, solvent-diffusion and supercritical fluid technology (Quintanar-Guerrero et al., 1998; Hu, 2004a). One of the advantages of these methods is the possibility of designing the formulation with certain beneficial characteristics such as enhancing dissolution rate by inclusion of surfactant or increasing the stability of amorphous material by incorporation of sugars. Poloxamer 407, a hydrophilic surfactant, has been used to improve the particle wetting and dissolution rate, via spray drying so as to improve the oral absorption of griseofulvin which is known to have poor solubility properties (Wong et al., 2006).

2.4.2. Solid dispersion systems for bioavailability enhancement of poorly water-soluble drugs

Solid dispersion is another promising method for improving the oral bioavailability of poorly water-soluble drugs. Reduction of the particle size of a drug to an absolute minimum improves the wettability which may significantly improve the bioavailability. Solid dispersions usually present as amorphous products and are mainly obtained by two different methods, such as melting and solvent evaporation. Recently, surfactants have been included into solid dispersions to avoid recrystallization and also to potentiate their solubility (Vasconcelos et al., 2007). The success that has been recorded with the application of solid dispersions has been largely due to the use of polymeric carriers since they can give rise to amorphous solid dispersions. Polymeric carriers are either synthetic or natural product-based macromolecules. Synthetic polymers include povidone, poly(ethylene glycol) and polymethacrylates. Natural product-based polymers are mostly synthesized from cellulose derivatives, such as hydroxymethylpropylcellulose, ethylcellulose, hydroxypropylcellulose or starch derivatives like cyclodextrins. A higher efficiency is expected from solid dispersions than solubilization and particle size reduction techniques because of the particle size reduction limit of between 2 and 5 microns in the latter methods. This particle size range is frequently not enough to considerably improve the drug solubility or drug release in the small intestine and consequently the

bioavailability (Karavas et al., 2006; Muhrer et al., 2006). However, despite extensive expertise that exists regarding solid dispersions, they are not broadly used in commercial preparations mainly due to the possibility that during processing (mechanical stress) or storage (temperature and humidity stress), the amorphous state may undergo crystallization (Pokharkar et al., 2006). Another drawback associated with solid dispersions is their poor scaling-up possibility for manufacturing purposes (Vasconcelos et al., 2007). An improvement to these problems might be the development of a dry emulsion which does not require any milling or chemical modification as a useful delivery approach. For example, Jang and co-workers (2006), reported an improved photostability and oral bioavailability of amlodipine using a redispersible dry emulsion method.

2.4.3. Lipid suspensions, solutions and emulsion systems for bioavailability enhancement of poorly water-soluble drugs

Recently, there has been an increasing interest in formulations of poorly water-soluble drugs in lipid vehicles as a means of enhancing drug solubilization and absorption in the GIT (Pouton, 2006; Porter et al., 2007) due to some findings which have demonstrated that the oral bioavailability of certain poorly water–soluble drugs, as well as lipophilic drugs may be enhanced when co-administered with a fat-rich meal (Welling, 1996; Sunesen et al., 2005). As a result, lipid suspensions, solutions and emulsions have all been employed to enhance the oral bioavailability of poorly water-soluble drugs and more recently, there has been an increase in the utility of self-emulsifying lipid-based formulations (Sunesen et al., 2005). In formulating lipid solutions however, digestive lipids such as soybean oil, corn oil or olive oil are often preferred. The enhanced drug solubilization in the colloidal species formed by digestion of these lipid vehicles and the resultant intercalation of the lipid digestion products into endogenous bile salts and phosphatidylcholine micellar structures, have been reported to be responsible for the observed improved bioavailability (Porter et al., 2008). However, despite the proven utility of self-emulsifying lipid-based formulations, relatively few lipid-based products have been scaled up and made available commercially. The existing few examples include Neoral® (cyclosporine, Novartis Pharma, Basel, Switzerland), Norvir® (ritonavir, Abbott Laboratories, Abbott Park, Illinois, USA), Fortovase® (saquinavir, Roche Laboratories, Basel, Switzerland), Agenerase® (amprenavir, Glaxosmithkline, Uxbridge, Middlesex, UK), cholecalciferol lipid solution (Vitamin D) and other lipid soluble vitamins. It has often been observed that the formulation is as important as the active drug. Thus, proper manipulation of drug formulation technologies has great potential for improving the oral

bioavailability of drug compounds, and therefore solving the array of drug delivery problems to a degree.

2.5. Nanotechnology Approaches for Bioavailability Enhancement of Poorly Water-Soluble Drugs

Nanotechnology offers a means of providing novel formulations for existing marketed drugs and new drug candidates with poor water solubility. Nanocrystal formulations shrink the drug's particle size thereby increasing the surface area and enhancing dissociation (du Toit et al., 2007) (Table 2.3). Pharmaceutical application of nanoscience seems to have evolved from observed increase in solubility of chemical substances after size reduction. When the size of material is reduced to less than 100nm, the principles inherent in quantum physics increasingly apply and materials begin to demonstrate entirely new properties such as enhanced solubility which is helpful in increasing the oral bioavailability of poorly water-soluble drugs (du Toit et al., 2007). This has therefore led to a number of nano-based drug delivery systems being developed. These systems serve as drug carriers and include micelles, nanoparticles, nanogels, nanoemulsions, liposomes, nanofibres, polymer therapeutics and nanodevices, examples of which are represented in Figure 2.4.

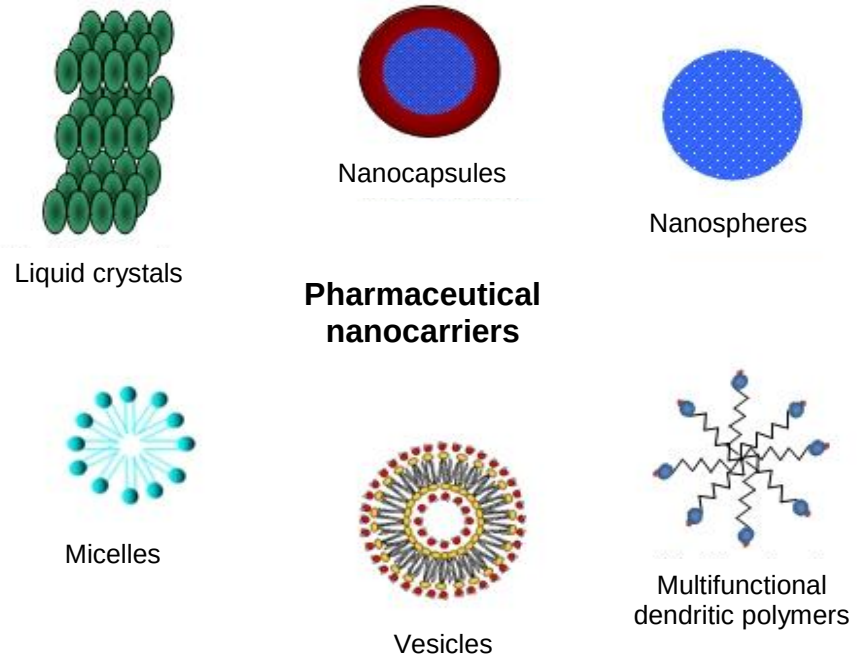
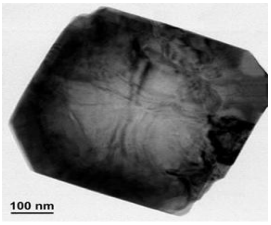
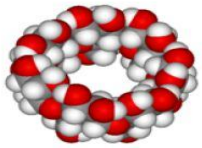
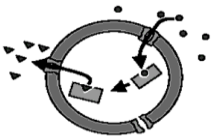
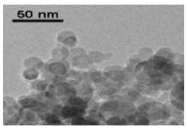
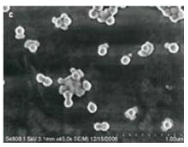
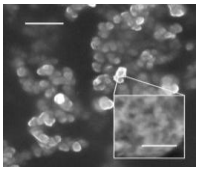
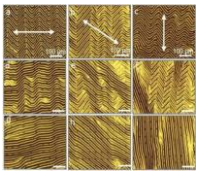
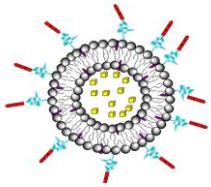


Figure 2.4: Representative nano-based pharmaceutical carriers employed for the enhancement of the oral bioavailability of poorly water-soluble drugs (Kaparissides et al., 2006).

Elan Pharma (Pty) Ltd. (Dublin, Ireland), is one of the first companies to nanosize drugs, and currently boasting of four products on the market. Its first nanotechnology based formulation of Wyeth's Rapamune (sirolimus which is an immunosuppressant to prevent organ transplant rejection, was developed and approved by US Food and Drug Administration in 2000 and has become the fastest selling drug in the transplant market. Some of the key nanotechnology-based approaches for the enhancement of drug solubility and oral bioavailability according to Saffie-Siebert and co-workers (2005) are highlighted in Table 2.3. There is a high expectation and possibility that nanotechnology will find useful application and provide a lasting solution to many challenges in pharmaceuticals and medicine as the natural biological (cellular) interface operates at the nanoscale level (i.e. 1-100nm). However, as promising as it appears to be, together with its multifaceted applications including developments in drug delivery, advancement in nanotechnology has raised safety concerns (Sun et al., 1999; Filho et al., 2007; Ahamed et al., 2008;). Known properties of some familiar materials can change when they enter the nanometer range (Filho et al., 20070). The fact that exposure of animals to nanoparticles can lead to neurological damage as well as respiratory and circulatory problems, has driven governmental research into the safety of these new technologies (Saffie-Siebert et al., 2005; Oberdoster et al., 2005).

Other issues that need attention for a wider acceptance of nanoparticle-mediated delivery of biologicals include low incorporation efficiency of hydrophilic drugs, precise control of drug release and avoidance of particle aggregation (Morishita and Peppas, 2006). With a responsible research and development strategy, including early consideration of public safety concerns, significant therapeutic advances are to be expected from this growing field within the next few years.

Table 2.3: Diverse nanotechnology-based approaches employed by various pharmaceutical companies.

Company	Nanotechnology-based formulation approach	Example	Description and Reference
Elan Pharma International (Dublin, Ireland)	Nanocrystal drug particles (<1,000nm) production by wet-milling and stabilised against agglomeration through surface adsorption of stabilizers, applicable to new molecular entities like aprepitant, also for reformulation of existing drugs e.g. sirolimus		Nanocrystal drug particle (Physorg.com, 2005)
Eurand Pharmaceuticals (Vandalia, Ohio USA)	Nanocrystal/amorphous drug production by physical breakdown of the crystal lattice and stabilised with biocompatible carriers (swellable microparticles or cyclodextrins)		Cyclodextrin nanoparticle (Bean, 2006)
SkyePharma Plc, (Piccadily, London, UK)	Use of high shear, cavitation or impaction forming micro-nanomicro particulate/droplet water insoluble drug core stabilized by phospholipids, applicable for insoluble drug delivery		A polymer stabilizing nano reactor with the encapsulated drug core (Kaparissides et al., 2006)
BioSante Pharmaceuticals (Lincolnshire, Illinois, USA)	Formation of nanoparticles that are Calcium Phosphate-based for improved oral bioavailability of hormones/proteins such as insulin; also as vaccine adjuvants		Calcium phosphate nanoparticles (Loher et al., 2005)
American Biosciences (Blauvelt, NY, USA)	Nanoparticle albumin-Bound technology: Injectable suspension of biocompatible protein with drug improves solubility/removes need for toxic solvents; e.g. paclitaxel-albumin nanoparticles		Paclitaxel albumin nanoparticles (Lvov et al., 2008)
Baxter Pharmaceuticals (Deerfield, Illinois, USA)	Nanoedge technology: drug particle size reduction to nano range by platforms including direct homogenisation, microprecipitation, lipid emulsions and other dispersed-phase technology		Nano lipid emulsion (Miller et al., 2006)
pSivida Ltd. (Watertown, MA, USA)	Structuring of drug particles within the nano-width pores of biocompatible BioSilicon nanoparticles, membranes or fibres; which gives controlled release and improves the solubility and bioavailability of hydrophobic drugs		Silicon nanoparticles (Physorg.com, 2005)
iMEDD Inc (Burlingame, CA, USA)	Use of silicon membrane with nano-width pores (10-100nm) used as part of an implantable system for drug delivery and biofiltration		Stretchable silicon nanomembrane (Physorg.com, 2008)
PharmaSol GmbH (Berlin Germany)	Nanostructured lipid carriers: nanostructured lipid particle dispersions with solid contents produced by high-pressure homogenisation; lipid-drug conjugate nanoparticles provide high-loading capacity for hydrophilic drugs for oral delivery		Drug encapsulated in lipid nanoparticles [(Kaparissides et al., 2006)

2.6. The Pro-drug Approach for Overcoming Poor Bioavailability and Chemical Instability

Classical pro-drug design often represents a non specific chemical approach to mask undesirable drug properties such as limited bioavailability, lack of site specificity and chemical instability (Patel, 2008; Xu et al., 2009). Although there is no strict universal definition for a pro-drug itself, and the definition may vary from author to author, pro-drugs can be generally defined as pharmacologically inert chemical derivatives that can be converted *in vivo* to the active drug molecule, enzymatically or non-enzymatically, to exert a therapeutic effect (Han and Amidon, 2000). Pro-drug design can be very effective in solving many of the stability, solubility, permeability and targeting problems that plague drug delivery and bioavailability.

Pro-drugs are made from several pro-moieties of various functional groups, including acyl, alkyl, alkanoic, and alkanoate groups. The principle behind this is that the chemical conversion into the active compound takes place once the stage for the unwanted property has been surpassed (Meyers and Borch, 2000; Napier et al., 2000; Rautio et al., 2008). The chemical stability of pro-drugs may be different under enzymatic conditions (Meyers and Borch, 2000). Pro-drugs of simple alkyl esters, cyclic carbonate esters and acyclic double esters often present their own advantages and disadvantages. Simple alkyl esters are not usually substrates of human blood esterases. They tend to rely on hepatic hydrolysis, and have been used successfully for many ACE inhibitors like enalapril which is converted to the active enalaprilat *in vivo* (Pang et al., 1984). Cyclic carbonate esters and acyclic double esters can be activated by human blood borne esterases and have been used successfully for antibiotics, antivirals, and angiotensin II antagonists (Rautio et al., 2008). Generally, esters are the most common type of pro-drugs and are converted back to the active parent via the ubiquitous esterases present in blood, tissues and organs. Levodopa, a drug used in the management of Parkinsonism, is a pro-drug that is converted to dopamine by DOPA-decarboxylase in the body. Dopamine itself is easily metabolized by aminases and hardly crosses the blood-brain barrier (Hall et al., 2007). Gabapentin is an anticonvulsant used for the treatment of epilepsy and post-herpetic neuralgia, but suffers from suboptimal pharmacokinetic properties including saturable absorption, high inter-patient variability, lack of dose proportionality and short half life (Hu, 2004a). To improve on these shortcomings, XP-13512 was developed by XenoPort, Inc, (Santa Clara, CA, USA) as an oral pro-drug. XP-13512 is a substrate of MCT-1, a monocarboxylate transporter (MCT) which is highly expressed in all segment of the colon as well as upper GIT, and is a sodium-dependent transport system responsible for

transfer and distribution of multiple vitamins from the various absorptive tissues (Cundy et al., 2004). Oral bioavailability increased from 25% for gabapentin to 85% for XP-13512 in monkeys, and no saturation was observed for increasing pro-drug doses (Cundy et al., 2004; Hu, 2004b). XP-13512 is cleaved to gabapentin by non specific esterases in the intestine, liver, and blood with low pro-drug exposure (<2%) in human clinical trials, while the tablet formulation of the pro-drug allows twice daily dosing. XP-13512 is currently in phase II clinical trials for post-herpetic neuralgia and restless legs syndrome (Wittmaack-Ekbom's syndrome) (Cundy et al., 2004; Hu, 2004b).

A pro-drug strategy was also found to overcome P-glycoprotein (P-gp)-mediated efflux by utilizing nutrient transporters expressed on the outer leaflet of cellular membranes (Hu, 2004a). Quinidine which is well known substrate of P-gp, was conjugated to valine in the form of an ester. Valine-quinidine does not interact with P-gp even at high concentrations. As reported by Hu (2004b), pro-drug design can effectively enhance oral bioavailability. A few examples of the successful application of pro-drug design are discussed in the following sub-sections.

2.6.1. Ximelagran as a pro-drug of melagran

Melagatran was identified in the 1990s as a potent direct inhibitor of thrombin and platelet aggregation. Its oral bioavailability was only about 5% which was further reduced when dosed with food due to the presence of two strongly basic groups and one carboxylic acid group. It is present as a zwitterion at intestinal pH. Ximelagatran is a pro-drug of melagatran designed by AstraZeneca Pharma (London, UK) to increase its permeability while maintaining its beneficial pharmacological properties. The carboxylic acid of melagatran was converted to an ester and the imidine was hydroxylated to reduce its basicity, leading to a compound that is not charged at intestinal pH, 170-fold more lipophilic and 80-fold more permeable than the parent. Ximelagran is virtually inactive towards thrombin and is rapidly converted to the active melagatran *in vivo* across a wide range of patient populations, leading to a much improved bioavailability of about 20% for the active melagran.

2.6.2. Fosamprenavir as a pro-drug of amprenavir

The HIV protease inhibitor amprenavir was approved by FDA in 1999 for use in adults and children with HIV infection, but its limited water solubility requires the use of softgel formulation for delivery and multiple pills for a single dose. Fosamprenavir calcium is a pro-drug of amprenavir developed by GlaxoSmithKline (Uxbridge, Middlesex, UK) with

improved aqueous solubility. After screening 60 pro-drugs through *in vitro* and *in vivo* assays, the phosphate pro-drug, fosamprenavir calcium (GW433908), was selected for its high water solubility, solution and solid-state stability, as well as its rapid conversion to the parent drug on the apical side of epithelium. The pro-drug is designed as a solid dosage form in such a way that the amount of pills required to be taken is significantly reduced, two tablets replacing eight amprenavir softgels. Fosamprenavir calcium was approved in October 2003 for use in combination with other antiretroviral agents for the treatment of HIV infection in adults (Hester et al., 2006).

2.6.3. Development of ampiroxicam to overcome gastric irritation associated with piroxicam

Piroxicam is an NSAID that can cause serious gastrointestinal perforation, ulceration, and bleeding. Ampiroxicam which is a pro-drug for Piroxicam was developed by Pfizer International (NY, USA) to reduce gastric irritation associated with piroxicam. Over 225 piroxicam derivatives were synthesized and screened for COX inhibition, bioavailability in the rat, solid state stability, and absorption in dogs. In a preclinical ulcerogenic study in rat, UD50 for ampiroxicam was 38.6mg/kg while for piroxicam UD50 was 17.9mg/kg. Also in clinical trials, ampiroxicam also showed comparable therapeutic efficacy.

2.6.4. Development of tenofovir disoproxil fumarate as a pro-drug to improve the permeability of tenofovir across biological membranes

Tenofovir is an acyclic nucleoside phosphonate that undergoes phosphorylation to form tenofovir diphosphonate, a potent inhibitor of viral reverse transcriptase. However, tenofovir is a dianion at physiological pH with a low partition coefficient, leading to low and erratic oral bioavailability. Tenofovir disoproxil fumarate has been developed as a pro-drug to improve the permeability of tenofovir across biological membranes. Tenofovir disoproxil, the bis-isopropoxyl carbonate, was the most stable of several evaluated phosphonate pro-drugs and was quickly converted back to the parent tenofovir in the presence of tissue homogenates. Oral bioavailability of tenofovir in beagle dogs is about 30%. Tenofovir disoproxil fumarate is chemically stable in solid state, is non-hygroscopic up to 93% relative humidity at room temperature and shows relatively, rapid dissolution. More important is that it has an oral bioavailability of about 43% in humans which therefore can allow only a once-daily dose (Cundy et al., 2004). Thus, the pro-drug design approach can be applied to enhance intestinal permeation of pharmaceuticals, to inhibit degradation by gastrointestinal and hepatic enzymes and to reduce the incidence of unwanted effects.

2.7. Concluding Remarks

Despite sophisticated new drug delivery systems, the development of satisfactory oral formulations remains a challenge since adequate drug delivery for most formulations has not been attained. This is in part a consequence of low bioavailability presented by numerous orally administered drugs. Notably, this is particularly so for peptides, proteins and nucleic acid molecules, due to their sensitivity to chemical and enzymatic hydrolysis as well as a poor cellular uptake. This low bioavailability implies a large variation in absorption/plasma levels as well as high manufacturing costs which are both unacceptable for drug development. It should be acknowledged that various novel drug delivery designs have tremendously improved drug absorption. Micronization and nanopharmaceutical application have to a large extent broken the barriers of poor GIT penetration while various novel formulation techniques have aided drug dissolution and absorption across the GIT wall. Significantly however, increased rate of absorption has not always translated into higher extent of absorption. This is due to exposure of absorbed drugs through the gut wall and the liver to CYP-induced pre-systemic metabolism. Thus, more strategies should be put forward if the improvement in oral bioavailability is to be attained. Among the possible strategies is the design and application of CYP inhibitors, the actions of which will be transcient to allow the normal course of drug elimination. In this case, competitive inhibition of the metabolic enzymes will be a preferable mechanism. This allows the utilization of the inhibitors while the drug escapes the first-pass. This is an exciting approach towards the enhancement of uptake and transport of orally administered molecules, leading to the requirement of relatively lower doses and decreasing incidences of adverse effects.

CHAPTER THREE

IN VITRO INVESTIGATION OF THE EFFECTS OF GRAPEFRUIT FLAVONOIDS ON THE METABOLISM OF FELODIPINE AND PHENACETIN EMPLOYING HUMAN LIVER MICROSOMES

3.1. Introduction

Multiple drug administration is often necessary in clinical practice for desirable therapeutic synergies and especially in the presence of co-morbidity. Major concerns however arise with the administration of drugs that share similar pharmacokinetic pathways. The resulting drug-drug interaction could produce unwanted side-effects, toxicological manifestations or therapeutic failure. The most significant mechanism of such interaction is the induction or inhibition of metabolic enzymes usually responsible for the systemic clearance of the drugs. This is the basis for most drug-drug contra-indications in pharmacotherapy (Lin and Lu, 1998; Thummel, 1998; Lin, 2003).

Recently, food-drug interaction has also become an important consideration in oral drug delivery (Sorensen, 2002; Dahan and Altman, 2004). The desirability of fed-state or fasted conditions for oral drug administration is often determined by considerations for food-drug interaction (Williams et al., 1993; Schmidt and Dalhoff, 2002). Elemental components of proteinaceous and fatty food substances are known to chelate a few drugs. A typical example is the formation of insoluble complexes between polyvalent metals and some antibiotics including tetracyclines and penicillins which significantly reduces the oral absorption and bioavailability of the latter (Gu, 2005). Bailey and co-workers (1998), reported an unexpected observation of increased plasma felodipine levels in a drug interaction study between the calcium channel antagonist, felodipine and ethanol where subjects were given grapefruit juice to obscure the taste of ethanol. A marked increase in oral bioavailability has been reported in various other CYP3A4 substrates including nisoldipine, nifedipine, midazolam, triazolam and cyclosporine following concurrent grapefruit consumption (Soons, et al., 1991; Hollander et al., 1995; Josefsson et al., 1996; Kupferschmidt et al., 1997; Andersen et al., 2002). The inability of other common fruit juices to exert the same effect has led to conclusions that certain components unique to grapefruit juice might be responsible for this observation. Grapefruit juice did not change the pharmacokinetic profile of felodipine which suggests the inhibition of pre-systemic

metabolism as its mechanism of action (Bailey et al., 1993). Grapefruit juice contains a range of flavonoids and furanocoumarins including naringin, naringenin, quercetin, kaempferol and bergapten. Further studies have suggested these phytochemical constituents could be responsible for the inhibitory effects on intestinal CYP3A4 enzymes (Minicalo, et al., 1992; Ho and Saville, 2001; Choi and Burm, 2006; Quintieri et al., 2008). Consequently, the consumption of grapefruit juice has been listed in most CYP3A4 substrates' monographs as being contra-indicated. This is for the concerns of increased bioavailability with possible toxicological consequences.

Research in drug metabolism has attracted more attention in recent times not only because it forms the basis for understanding pharmacotoxicology but for its growing influence and application in drug delivery and pharmacotherapy (Davila et al., 1998; Cross and Bayliss, 2000; Ekins et al., 2000; Ehrhardt and Forbes, 2005). Inhibition of first-pass drug metabolism is desirable in order to improve oral drug bioavailability and clinical outcomes (Agoram et al., 2001; Breedveld et al., 2006) while the induction of enzymatic metabolic activity may be applicable in clinical toxicity (Uwe, 2000). The human CYP enzyme system present in the liver and intestine is responsible for the metabolism of a wide range of xenobiotics (drugs, carcinogens, pesticides) and endobiotics (prostaglandins, bile acids, steroids) (Zhang and Benet, 2001; Handschin et al., 2002; Guengerich, 2003; Gonzalez, 2005; Fang and Xiao-yin, 2005; Nebert and Dalton, 2006). It is the ability of CYP to metabolize numerous structurally unrelated compounds (Chapter two, Section 2.2) that is responsible for the poor oral bioavailability of many drugs as they are subjected to pre-systemic CYP-mediated metabolic activity (Dresser et al., 2000; Quintieri, et al., 2008). The large number of documented drug-drug and drug-food interactions has also been attributed to intestinal and liver microsomal CYP activity (Wang et al., 2000; Ohyama et al., 2000; Obach et al., 2006).

3.2. *In Vitro* Drug Metabolism Techniques

One of the major steps involved in the development of new drug candidates is the verification of their potential for oral bioavailability and interaction with other drugs (Veber et al., 2002). Various challenges are experienced in extrapolating the results of animal studies to humans, and as a result, various *in vitro* methods have been developed employing human tissue-derived systems. *In vitro* investigations of drug metabolism provides information regarding the metabolic stability of the test drug, its potential for possible interactions with other compounds that have affinity for the same drug-

metabolizing enzymes, and indication of its oral bioavailability and toxicological tendencies (White, 2000; Masimirembwa et al., 2001; Veber et al., 2002).

One of the best characterized and validated models for drug metabolism studies is the use of microsomes fractionated from the human liver subcellular organelles by differential ultracentrifugation (Wrighton et al., 1993; Ekins et al., 2000). A human liver microsomal fraction contains a full complement of CYP which makes it a suitable tool for studying CYP-catalyzed metabolite formation and inhibitory interactions. In addition to reliability and reproducibility of *in vitro* data generated from their use, human liver microsomes are relatively easy to prepare, commercially available and stable on prolonged storage. This has made the application of liver microsomes the most widely used in metabolic and toxicological studies (Taavitsainen, 2001).

Other *in vitro* methods for drug metabolism studies include the utilization of human primary hepatocytes. Human hepatocytes are difficult to preserve and their preparation requires a large number of liver samples (Gomez-Lechon, 2003). These factors have greatly restricted the use of primary hepatocytes for *in vitro* studies. The use of permanent cell lines expressing one or more drug-metabolizing enzymes has also been described but with limited utilisation due to the lack of the complement of metabolizing enzymes present *in vivo* (Pelkonen and Raunio, 2005). As valuable alternatives to human liver microsomes for drug metabolic studies, cDNA-expressed enzymes produced in the endoplasmic reticulum of an eukaryote host-cell including bacteria, yeast, baculovirus systems and mammalian cell lines have also been described. A few researchers however have expressed reservations on the utilization of cDNA-expressed enzymes and the *in vivo* correlative value of the data generated because the enzymes lack the whole complement of hepatic metabolic enzymes and are studied in isolation (Taavitsainen, 2001; Plant, 2004).

In most studies therefore, the search for a suitable CYP enzyme inhibitor for oral bioavailability enhancement has involved the utilisation of HLM, the results of which are closer to *in vivo* studies. Such *in vitro* investigations are optimized by either measuring the rate of disappearance of substrates in incubation mixtures or the rate of formation of a specific metabolite.

3.3. Felodipine and Phenacetin as Probe Substrates for CYP-Mediated Metabolism

A list of recommended probe substrates and inhibitors for studies of drug metabolism by various CYP isozymes is presented in Table 3.1. Felodipine, a typical probe substrate is a dihydropyridine calcium channel blocker which has been effective in the management of cardiovascular diseases including hypertension, angina pectoris, arrhythmias and congestive heart failure. It is highly lipophilic and exhibits excellent tissue penetration but with a mean systemic oral bioavailability of 15% independent of the oral formulation used, due to CYP-induced pre-systemic metabolism (Dresser et al., 2000). Its primary route of inactivation is the oxidation of the dihydropyridine ring to the inactive dehydropyridine metabolite, a reaction mediated by CYP3A4 (Bailey et al., 1995). The pre-systemic abundance of CYP3A4 in the intestines and the liver is therefore responsible for the poor oral bioavailability of all the dihydropyridines.

Table 3.1: A list of recommended *in vitro* probe substrates and inhibitors for CYP isozymes (Source: Taavitsainen 2001 and Bjornsson et al., 2003).

CYP	Substrate	Specific reaction	Inhibitor
1A2	Ethoxyresorufin	*O*-deethylation	furafylline
	Phenacetin	*O*-deethyaltion	
2A6	Coumarin	7-hydroxylation	8-methoxypsoralen
2B6	*S*-mephenytoin	*N*-demethylation	Orphenadrine
			Sertraline
2C8	Paclitaxel	6α-hydroxylation	Quercetin
2C9	Tolbutamide	Methyl hydroxylation	Sulfaphenazole
		Hydroxylation	
	Diclofenac	7-hydroxylation	
	S-warfarin		
2C19	*S*-mephenytoin	4'-hydroxylation	Ticlopidine
	omeprazole	oxidation	
2D6	Dextromethorphan	*O*-demethylation	Quinidine
	Debrisoquine	4-hydroxylation	
	Bufuralol	1-hydroxylation	
2E1	Aniline	4-hydroxylation	Pyridine
	Chlorzoxazone	6-hydroxylation	Clomethiazole
3A4	Midazolam	1'- and 4 hydroxylation	Ketoconazole
			Verapamil
	Testosterone	6ß-hydroxylation	
	Felodipine	Dehydrogenation	
	Nifedipine	Dehydrogenation	

Phenacetin is an analgesic that was popular in the 70s and 80s (Sanerkin, 1971; Clissold, 1986). It is a pro-drug known to be converted *in vivo* to acetaminophen, the active metabolite. Following oral absorption, phenacetin is completely absorbed but undergoes favorable pre-systemic metabolism in the liver. Its first metabolic step is CYP1A2-catalyzed o-deethylation. This makes phenacetin a suitable probe substrate for CYP1A2 metabolic activity. Further metabolism of phenacetin usually includes phase two reactions of metabolite conjugation with glucuronide, sulfate or glutathione to form soluble complexes that are ultimately excreted in the urine.

The aim of the present study was therefore to optimize and validate *in vitro* techniques for drug metabolism employing CYP1A2- and CYP3A4-expressed HLM. In this study the rate of felodipine consumption was explored as the determinant of CYP3A4 metabolism while the rate and extent of paracetamol formation was used to measure CYP1A2-catalyzed *o*-deethylation of phenacetin. The present study also investigated the effect of quercetin, naringin and naringenin on CYP3A4-dependent metabolism of felodipine and formation of paracetamol, a specific and measurable product of CYP1A2-catalyzed *o*-deethylation of phenacetin. The effect of these flavonoids on felodipine metabolism was compared to that of verapamil, a known competitive inhibitor of CYP3A4.

3.4. Materials and Methods

The strategies employed in this study are outlined in Figure 3.1. Detailed description of the steps outlined follows in the subsequent sections.

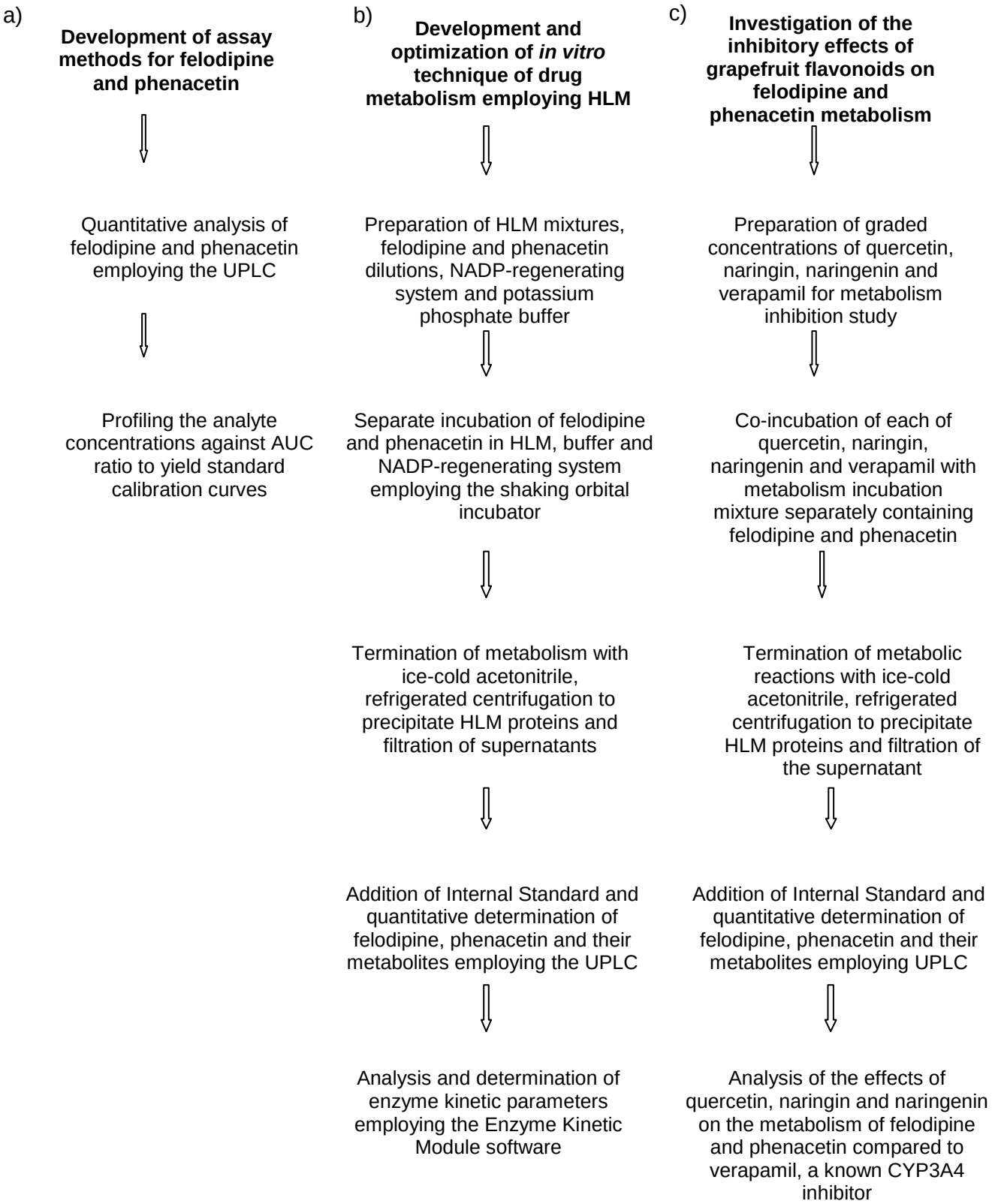


Figure 3.1: A schematic representation of the techniques employed in the current study.

3.4.1. Materials

Pooled mixed gender human liver microsomes (HLM) expressing CYP3A4, CYP2C9, CYP4A11, CYP4F2, CYP2E1 and CYP2A6 were purchased from BD Biosciences (Pty) Ltd. (Woburn, MA, USA) and stored at -70ºC until used. Supply information indicated that microsomes were prepared from donor human livers (16 male, 14 female; 26 Caucasians, 2 African-American and 2 Hispanics; age range 24-78 years; non-smokers, non-drinkers with non-liver related cause of death with no significant medical history). Felodipine ($C_{18}H_{19}Cl_2NO$; M_W=384.26g/mol), verapamil hydrochloride ($C_{27}H_{38}N_2O_4.HCl$; M_W=491.08g/mol) loperamide ($C_{29}H_{34}Cl_2N_2O_2$; M_W=477.04g/mol), phenacetin ($C_{10}H_{13}NO_2$; M_W=179.22) and sodium citrate ($C_6H_5Na_3O_7.2H_2O$; M_W=294.1g/mol; molecular biology grade) were purchased from Merck Chemicals (Pty) Ltd. (Darmstadt, Germany). Quercetin ($C_{15}H_{10}O_7$; M_W=302.236g/mol), naringin ($C_{27}H_{32}O_{14}$; M_W=580.54g/mol), naringenin ($C_{15}H_{12}O_5$; M_W=272.25g/mol), D-glucose 6-phosphate monosodium (G6P), glucose 6-phosphate dehydrogenase (G6PDH) and nicotinamide adenine dinucleotide phosphate (NADPH, reduced form) were purchased from Sigma-Aldrich (Pty) Ltd. (St Louis, MO, USA). Molecular biology grade potassium phosphate monobasic (KH_2PO_4, M_W=136.1g/mol) and dibasic (K_2HPO_4, M_W=228.2g/mol) were purchased from EMD Chemicals (Pty) Ltd. (Gibbstown, NJ, USA). All other chemicals and reagents were of standard guaranteed reagent grade and used as received under stringent bio-analytical protocols.

3.4.2. Assay method development for felodipine, phenacetin and paracetamol analysis

3.4.2.1. Quantitative analysis of felodipine concentration

A method of quantitative determination of felodipine was developed using the UPLC. Standard curves were yielded with isocratic baseline separation of felodipine and loperamide (internal standard) using UPLC technology (Waters® Acquity UPLC™ System, Milford, MA, USA) comprising a binary solvent and a sample manager; a BEH C_{18} column (1.7µm; 2.1x50mm) and a Photodiode Array (PDA) detector set at 200nm. Felodipine solutions were prepared in acetonitrile with concentrations ranging from 31.25-1000µmol/L. The mobile phase comprised 0.025M KH_2PO_4 buffer (pH 2.5) and acetonitrile (50:50) with a flow-rate of 0.2mL/min (7000psi, delta <20) while 1.7µL equal volume of sample and 50µmol/L loperamide was injected on to the column at 25ºC. Double-deionized water obtained from a Milli-Q water purification system (Milli-Q, Millipore, Billerica, MA, USA) was used for all aqueous dilutions. Loperamide eluted at 1.48±0.02 minutes while felodipine eluted at 5.1±0.02 minutes. Complete separation of

the sample and the internal standard were confirmed by two- and three-dimensional chromatographic separation.

3.4.2.2. Quantitative analysis of phenacetin and paracetamol

Using techniques similar to the one described in Section 3.4.2.1., a method of chromatographical separation and quantitative determination of paracetamol and phenacetin was developed. A complete separation was obtained by injecting 1.7µL of sample containing equal volume of 0.05M loperamide (internal standard) through a BEH phenyl column (1.7µm; 2.1x100mm) in a gradient binary mobile phase comprising 0.025M KH_2PO_4 (pH 2.5) and acetonitrile (Table 3.2) at a flow-rate of 0.25mL/min absorbing at 200nm (7000psi, delta<20). Three-dimensional chromatography was used to confirm complete separation of phenacetin, metabolites and the internal standard.

Figure 3.2: The gradient composition of the mobile phase for the separation of paracetamol, phenacetin and loperamide by UPLC.

Time (Minutes)	Flow-rate (mL/min)	K_2PO_4 (%)	Acetonitrile (%)
Initial	0.25	90	10
3	0.25	60	40
4	0.25	20	80
5	0.25	10	90

3.4.3. Optimization of *in vitro* felodipine and phenacetin metabolism employing human liver microsomes

3.4.3.1. Preparation of co-factor and NADP-regenerating solutions

Co-factor concentrates containing 400mg each of reduced $NADP^+$ and G6P; and 0.1M of magnesium chloride pentahydrate was prepared in deionized water to make a 20mL solution and stored at -20ºC until further use. G6PDH (40U/mL) was prepared in 5mM sodium citrate solution and stored at -20ºC until further use. The NADP-Regenerating system (NRS) comprised 130µL G6PDH, 650µL co-factor stock solution and made up to 4.42mL with 1.3mL 0.5M phosphate buffer (pH 7.4) and deionized water. The preparation of the NRS was completed immediately before use. It was prepared such that on addition to the incubation mixture, it contained 2.6mM $NADP^+$, 6.7mM G6P, 6.6mM magnesium chloride and 0.8units/mL G6PDH.

3.4.3.2. Preparation of felodipine, phenacetin and microsomal dilutions for incubation

Felodipine and phenacetin solutions were prepared over a range of 0.01-100.0mM in acetonitrile. Frozen HLM (-70ºC) was thawed in water at room temperature (25Cº) and 500µL (5mg protein/mL) measured with a micropipette was diluted in 0.5M phosphate buffer (pH 7.4) to produce a final protein concentration of 0.5mg/mL. This was kept on ice until further use.

3.4.3.3. In vitro metabolism of felodipine and phenacetin in human liver microsomes

In 24-well plates, 5µL felodipine and phenacetin solutions in duplicate were separately pre-warmed with 250µL 0.5mg/ml HLM for 5 minutes in a orbital shaking incubator (Labex, Stuart SBS40®, Helsingborg, Sweden) set at 100rpm and 37°C followed by the addition of 245µL NRS to initiate enzymatic metabolic reactions and a further incubation for 10 minutes. The reaction was halted by a stop solution comprising ice-cold acetonitrile (-20°C).

3.4.3.4. Analysis of felodipine, phenacetin and metabolites post-incubation

The 24-well plates were transferred to a refrigerated centrifuge (Xiang Yi L-535R™ Centrifuge, Changsha, China) and the incubation mixtures were centrifuged at 4°C at 4000*g* for 30 minutes to precipitate the HLM proteins. The supernatants were filtered through a 0.22µm Cameo Acetate membrane filters (Millipore, Billerica, MA, USA) and analyzed by UPLC as described in Sections 3.4.2.1 and 3.4.2.2 in order to quantify the extent of felodipine and phenacetin metabolism. The procedures are summarized in Figure 3.2. The rate of felodipine metabolism was determined as the rate of disappearance of felodipine from the CYP3A4-dependent reaction mixture while the rate of paracetamol formation was used as the determinant of CYP1A2-catalyzed *o*-deethylation of phenacetin. The rate of metabolism and the enzyme kinetic parameters for felodipine and phenacetin were determined employing the SigmaPlot Enzyme Kinetic Software (SigmaPlot: Enzyme Kinetic Module, Systat Software Inc., Chicago, IL, USA).

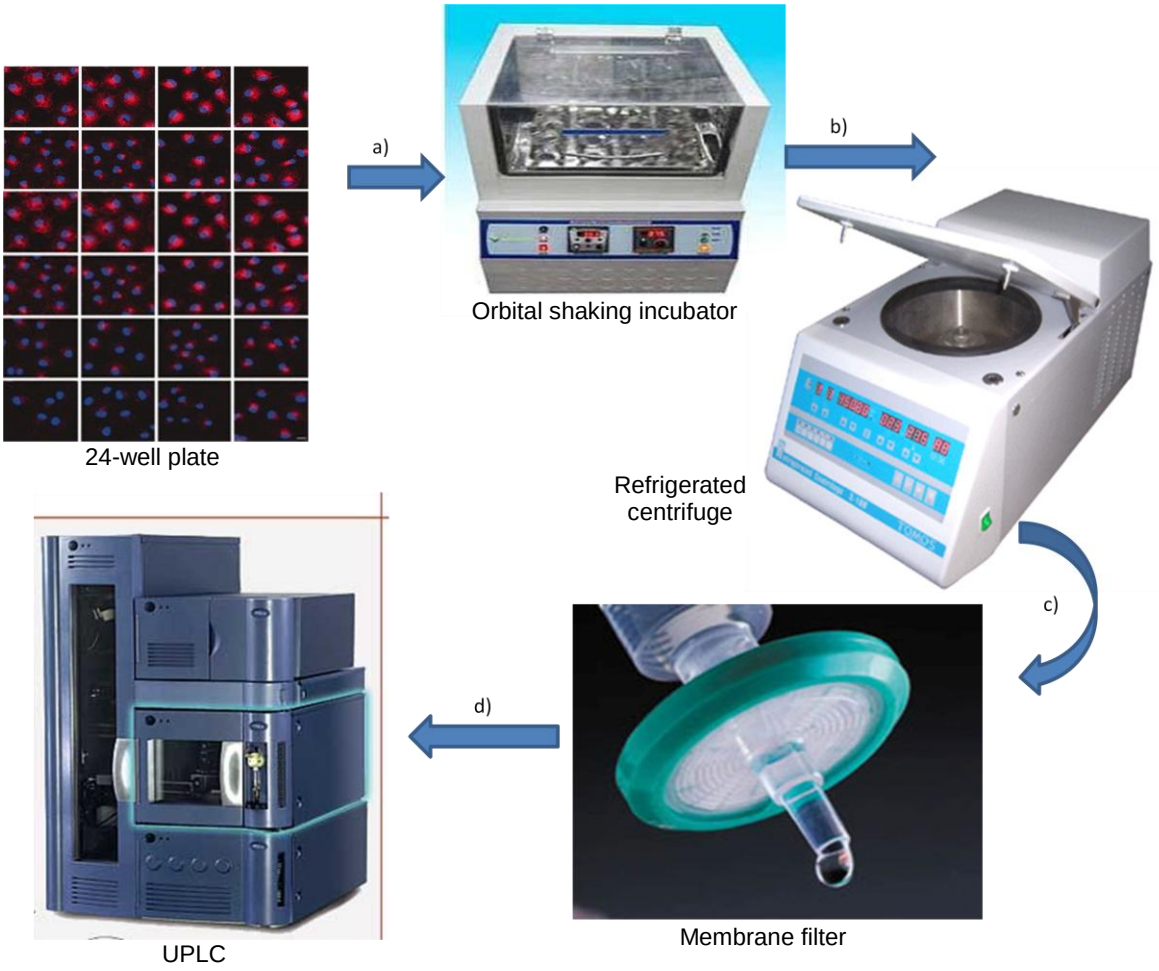


Figure 3.2: A schematic depicting the *in vitro* felodipine metabolism techniques employed that involved: a) incubation with HLM in 24-well plates placed in an orbital shaking incubator; b) refrigerated (4°C) centrifugation of the incubation mixture to precipitate HLM proteins; c) filtration of the supernatant through 0.22µm Cameo Acetate membrane filter; and d) chromatographic analysis of the filtered supernatant for substrate and metabolite.

3.4.4. Investigation of the effects of grapefruit flavonoids and verapamil on the metabolism of felodipine and phenacetin by HLM

Felodipine and phenacetin at their determined Michaelis-Menten constant (K_m) (Section 3.4.3.4) were incubated in HLM as described in Section 3.4.3 except for the addition of verapamil, and the grapefruit flavonoids. Verapamil, naringin, naringenin and quercetin solutions were prepared in solutions at graded concentrations ranging from 30-1000µM. Using a multi-channel micropipette, 5µL of each test solution was added to a 250µL diluted HLM solution (0.5mg/ml) using 24-well plates and pre-warmed for 5 minutes in a shaking incubator (100rpm; 37ºC). With the addition of the NRS, microsomal activity was initiated and the well plate incubated for 10 minutes. This was followed by the addition of 5µL of felodipine and phenacetin into each corresponding well and a further incubation for

10 minutes. The incubation time of 10 minutes was selected such that a significant fraction of felodipine and phenacetin was metabolized. Supernatants were analysed with UPLC method described in Section 3.4.2. To quantify the rate and extent of felodipine and phenacetin metabolism, control samples incubated with 0.05mg/mL microsomal proteins without co-factors and NRS were subjected to a similar procedure. Positive control incubations without test compounds allowing for maximum metabolism were also employed.

All the test compounds investigated were poorly water-soluble except for verapamil. Acetonitrile is the preferred solvent for poorly water-soluble compounds in *in vitro* determinations involving subcellular fractions (Celsis, 2009) thus, the flavonoids were dissolved in acetonitrile. At high concentration, the effect of acetonitrile on enzymatic activity could be significant (Celsis, 2009). Therefore the final concentration of acetonitrile in the incubation mixture was <1%.

3.5. Results and Discussion

3.5.1. Assay method development

3.5.1.1. Chromatographic separation and quantitative determination of felodipine

Felodipine and loperamide (internal standard) were eluted after 1.464±0.02 and 5.037±0.02 minutes, respectively (Figure 3.3). At the retention time and wavelength employed (200nm), no interfering peaks were noted as confirmed by three-dimensional chromatographical analysis (Figure 3.4). The assay method yielded a linear calibration curve through the origin with the relationship y=0.0158x where 'x' variable represented felodipine concentration and 'y' the chromatographic felodipine/internal standard Area Under the Curve (AUC) ratio (Figure 3.5). Assay method validation analysis revealed satisfactory intra and inter-day precision and accuracy (R^2=0.99).

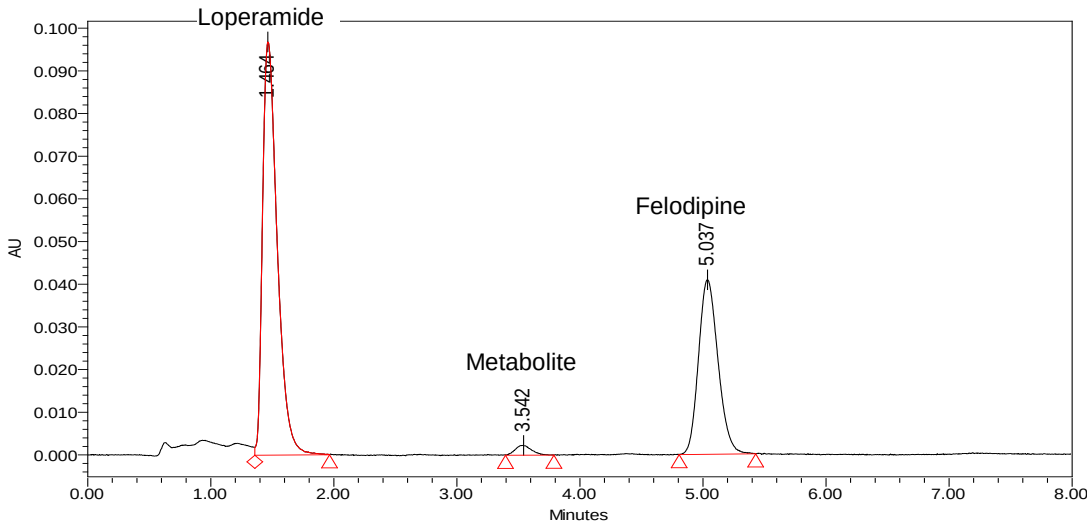


Figure 3.3: UPLC chromatograms showing the retention time of felodipine (5.037 minutes), the appearance of its metabolite (3.542 minutes) and the internal standard (1.434 minutes).

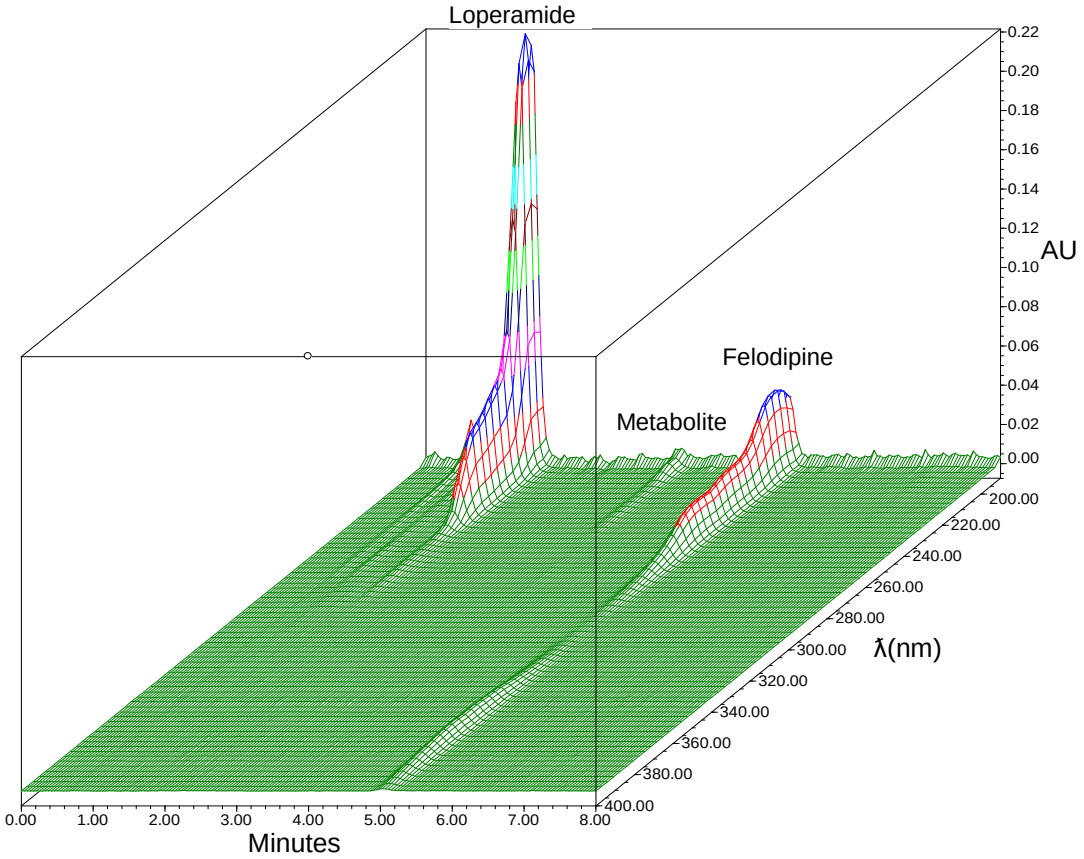


Figure 3.4: A three-dimensional chromatogram showing a complete separation of felodipine and loperamide

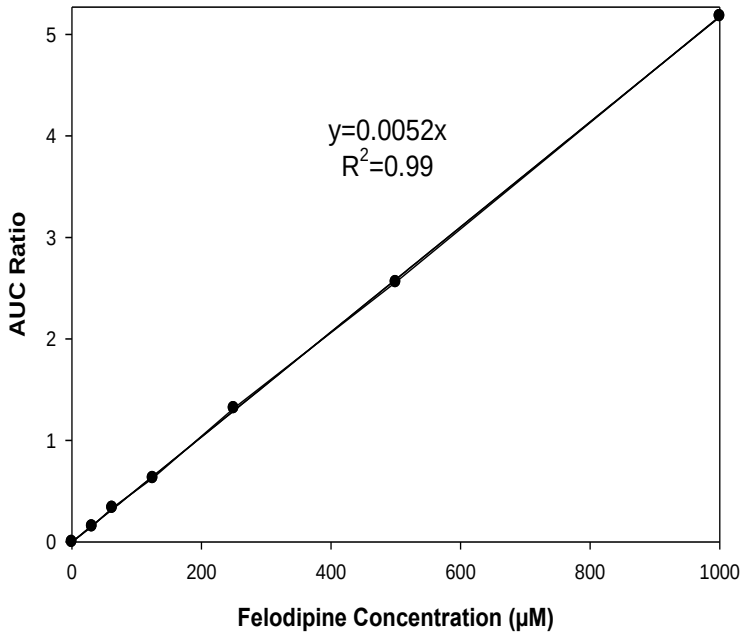


Figure 3.5: A standard calibration curve for the quantitative analysis of felodipine concentration by UPLC (N=3; S.D.<0.002 in all cases).

3.5.1.2. Chromatographic separation and quantitative determination of phenacetin and paracetamol

Paracetamol, phenacetin and loperamide (internal standard) were eluted after 1.672±0.02, 2.400±0.02 and 3.595±0.02 minutes respectively (Figure 3.6). Complete separation of sample components with no interfering peaks was confirmed through a three-dimensional chromatographic analysis (Figure 3.7). The method was validated yielding a linear standard curve through the origin with the relationships y=0.0031x (R^2=0.99) and y=0.002x (R^2=0.99) for phenacetin and paracetamol, respectively. The 'x' and 'y' variables represented the phenacetin/paracetamol concentrations and the ratio of the AUCs respectively (Figure 3.8).

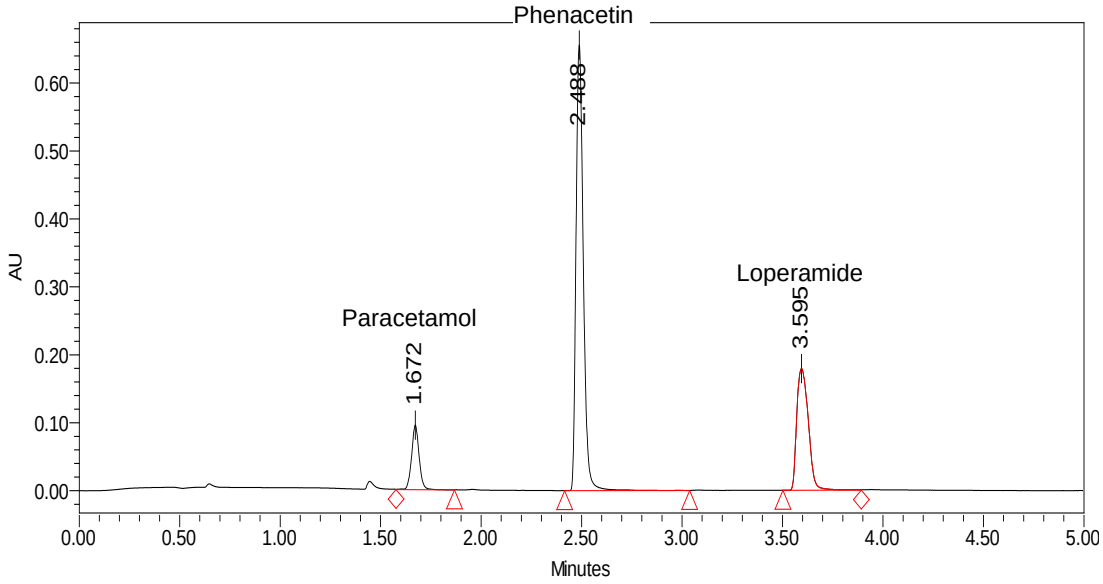


Figure 3.6: UPLC chromatograms showing the retention time of phenacetin (2.488 minutes), the appearance of paracetamol, its metabolite (1.672 minutes) and the internal standard (3.595 minutes).

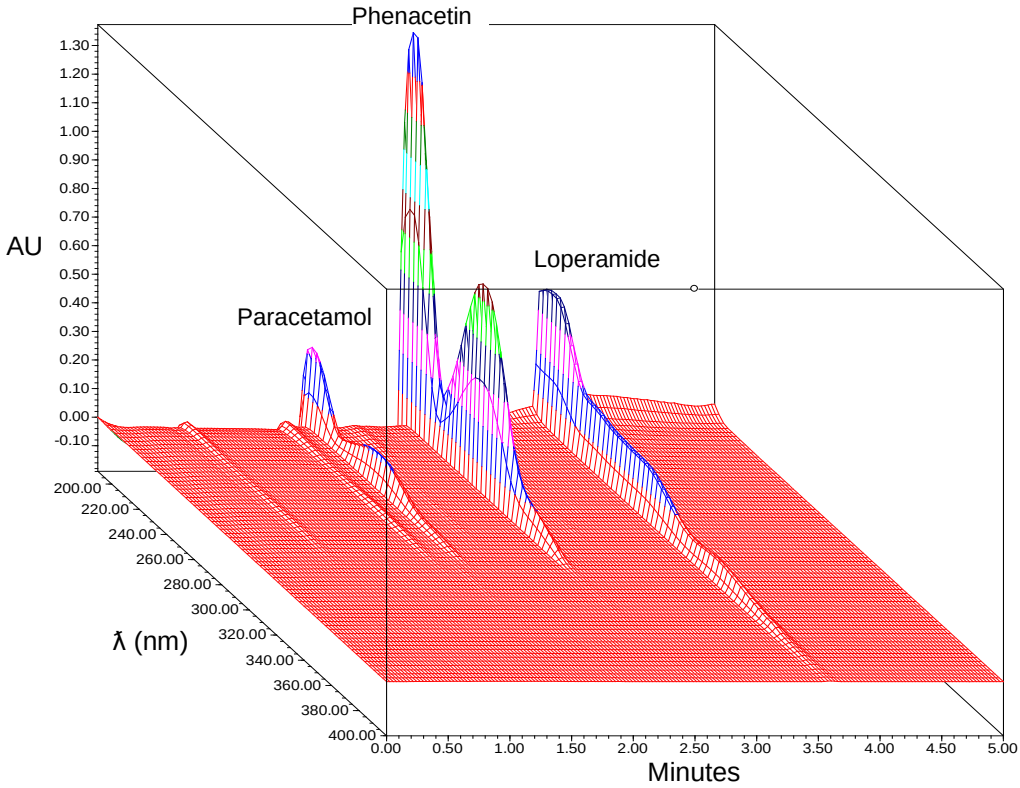


Figure 3.7: A three-dimensional chromatogram showing a complete separation of phenacetin, paracetamol and loperamide.

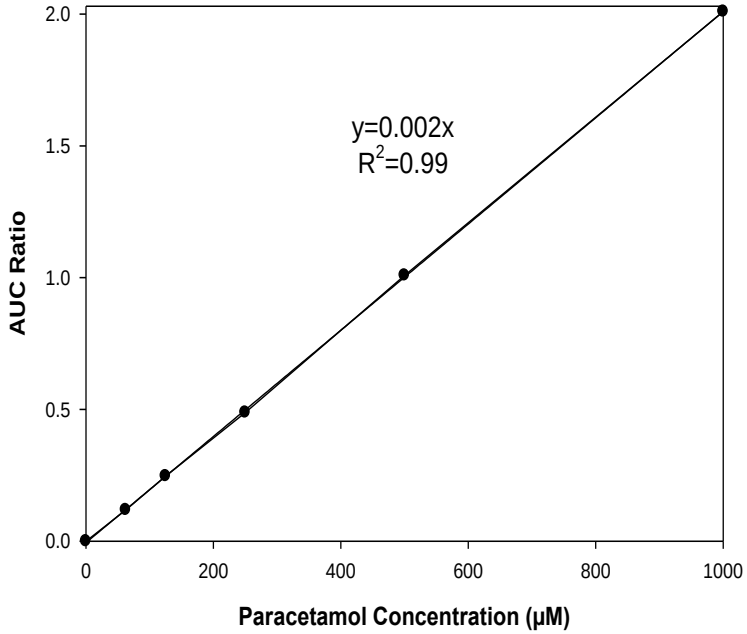


Figure 3.8: A standard calibration curve for the quantitative analysis of paracetamol concentration by UPLC (N=3; S.D.<0.002 in all cases)

3.5.2. Determination of enzyme kinetic parameters for *in vitro* felodipine and phenacetin metabolism

Decrease in felodipine concentration in the incubation mixture as determined by UPLC was used as evidence of enzymatic activity while the appearance and increase in concentration of paracetamol was used as evidence of CYP1A2-mediated *o*-deethylation of phenacetin. Similar results were obtained when the rate of phenacetin metabolism and rate of paracetamol formation were used as evidence of CYP enzymatic activity. By comparing the felodipine concentration in the metabolized mixture with the control, the difference represented the quantity of substrate metabolized.

In most enzymatic reactions, the Michaelis-Menten principle holds. According to this postulation, the initial rate of enzymatic reactions is directly proportional to the substrate concentration. It describes how the initial reaction rate, V_0 depends on the position of the substrate-binding equilibrium and the rate of metabolite formation (Kou et al., 2005). There is a substrate concentration where reaction rate is maximal (V_{max}) and beyond which the rate of metabolite formation is independent of further increase in substrate

concentration [S]. The substrate concentration corresponding to half the V_{max} is referred to as the Michaelis-Menten constant (K_m) (Hsu et al., 2001). The Michaelis-Menten equation as expressed in Equation 3.1 is the basis for most single-substrate enzyme kinetics.

$$V_0 = \frac{V_{max}\ [S]}{K_m + [S]} \qquad \textit{Equation 3.1}$$

Where V_0=the initial rate of metabolism, V_{max}=the maximum rate of metabolism, [*S*]=the substrate concentration and K_m=the Michaelis-Menten Constant

The rate of product formation depends on both the enzyme and substrate concentrations reaching its peak at equilibrium where the saturation of the enzyme binding site yields the highest rate of product formation. A typical Michaelis-Menten curve is therefore non-linear and various attempts have been made to modify it in a way that enzyme kinetic parameters can be estimated at a glance. One of the most common linearized modifications to the Michaelis-Menten curve is the Hanes-Woolf Plot. It is based on the rearrangement of Michaelis-Menten equation as depicted in Equation 3.2.

$$\frac{[S]}{V} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}} \qquad \textit{Equation 3.2}$$

As presented in Equation 3.2, a plot of the ratio of the initial substrate concentration to the reaction velocity (*[S]/V*) against [*S*] will yield a linear graph with $1/V_{max}$ as the slope, a *y*-intercept of K_m/V_{max} and an *x*-intercept of $-K_m$. Hanes-Woolf Plot has been used for rapid and easy determination of major kinetic parameters such as the K_m and the V_{max}.

The metabolic behavior of felodipine and phenacetin were characterized by Enzyme Kinetic Software capable of applying various enzyme kinetic principles including Michaelis-Menten and Hanes-Woolf in order to determine the enzyme kinetic parameters. A typical Michaelis-Menten curve yielding a V_{max} of 634.1nmol/min/mg HLM and *Km* of 7.4µM (Figure 3.9a) was generated on profiling the initial rate of felodipine metabolism against concentration. A similar curve characterized the rate of paracetamol formation from phenacetin as presented in Figure 3.9b yielding a V_{max} of 12.7nmol/minute/mg and a K_m of 12.2µM.

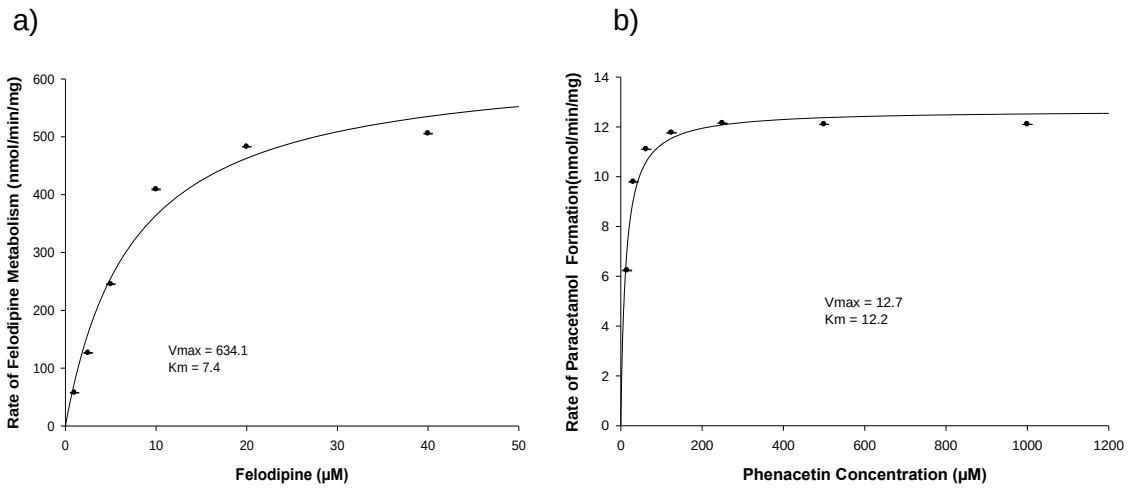


Figure 3.9: Michaelis-Menten Plot depicting the enzyme kinetic parameters of a) CYP3A4-catalyzed felodipine metabolism and b) CYP1A2-catalyzed phenacetin o-deethylation generated by Enzyme Kinetic Software (SigmaPlot: Enzyme Kinetic Module) (N=3; S.D.<0.005 in all cases).

Further analysis employing the Enzyme Kinetic Software was performed applying the Hanes-Woolf Plot. The plots generated confirmed the kinetic parameters obtained as depicted in Figure 3.10. Hanes-Woolf Plot has been used extensively for accurate and faster determination of enzyme kinetic parameters (Grace et al., 1994; Vatamaniuk et al., 2000; Lu et al., 2004). Although literature values vary widely due to inter-laboratory differences and environmental conditions, kinetic parameters obtained through this method were reproducible, thereby validating the method for further analytical enzymatic determinations.

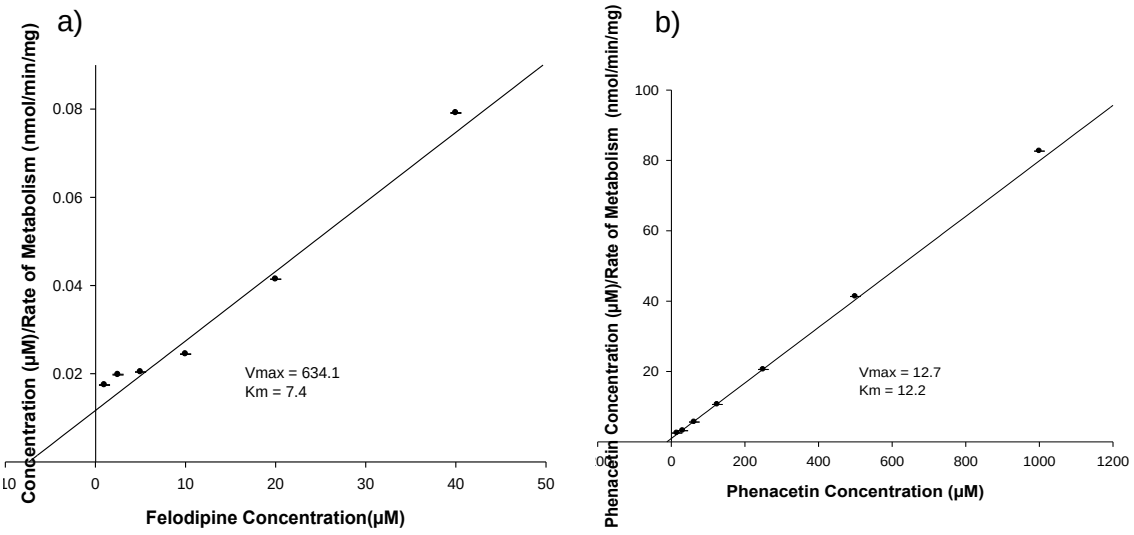
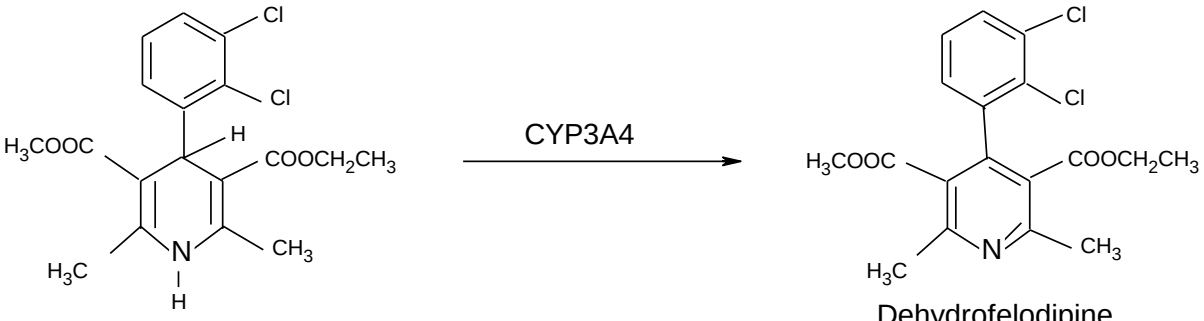


Figure 3.10: Hanes-Woolf Plots depicting the enzyme kinetic parameters of a) CYP3A4-catalyzed felodipine metabolism and b) CYP1A2-catalyzed phenacetin o-deethylation, generated by Enzyme Kinetic Software (SigmaPlot: Enzyme Kinetic Module) (N=3; S.D.<0.005 in all cases).

There is a direct correlation between *in vitro* and *in vivo* metabolism of xenobiotics by human liver and intestinal microsomal cytochromes (Moltke et al., 1999; Martinez et al., 1999; Schmider et al., 1999). As a result of this, new drug candidates are usually subjected to *in vitro* microsomal reactions to determine their susceptibility to pre-systemic metabolism, mode of elimination from the body and interaction with other drugs. Thus results obtained from standardized and validated *in vitro* techniques are useful indicators of *in vivo* behavior.

Standardization of laboratory techniques is often necessary in order to obtain reliable and reproducible results, and to determine best substrate concentration for optimal metabolic activity. HLM are sub-cellular fractions of the liver containing CYP enzymes, flavin monooxygenases and UDP glucuronyl transferases. HLM therefore, requires stringent and fastidious conditions for optimal *in vitro* activity. Incubation in buffer solutions (pH 7.4) at 37ºC, with co-factor solutions and NADP generating system has been demonstrated to enhance *in vitro* metabolic activity of HLM comparative to *in vivo* behavior (Yan and Caldwell, 2005).

The Michaelis-Menten plot and its linearized forms have remained the most reliable mechanism of evaluation of enzyme activity. An optimal metabolic concentration for felodipine was predetermined in accordance with Michaelis-Menten principle. The K_m value corresponding to the addition of 5µL of 0.75mM felodipine to a total incubation mixture of 0.5mL with 0.25mg/ml microsomal protein concentration as performed in this study agrees with earlier determinations (Miniscalo et al., 1992). Felodipine was the choice substrate in this study due to the initial observation of the influence of grapefruit juice on oral bioavailability in patients on felodipine therapy and concomitant grapefruit ingestion (Miniscalo et al., 1992). The predominant metabolic inactivation of the dihydropyridines (DHP) is the oxidation of the DHP ring to form the pharmacologically inactive pyridines (Figure 3.11). This step is mediated by CYP3A4 (Cardoza and Amin, 2002). The initial assumption therefore, was the modulation of CYP3A4 by chemical constituents of grapefruit juice.



Felodipine
(a)

Dehydrofelodipine
(b)

Figure 3.11: Oxidation of a) felodipine to the pharmacologically inactive b) dehydrofelodipine.

3.5.3. Assessment of the inhibitory effects of flavonoids on felodipine metabolism in HLM incubation mixtures

The flavonoids co-incubated with felodipine in HLM mixtures demonstrated an initial concentration-dependent inhibition of the rate and extent of felodipine metabolism. However, at higher concentrations, metabolism inhibition approached 100% (Figure 3.12). This result is in agreement with earlier determinations suggesting that flavonoids and furanocoumarins present in grapefruit juice are responsible for the observed increase in plasma felodipine concentration following a concomitant ingestion of grapefruit juice by patients on felodipine therapy (Miniscalo et al., 1992; Bailey et al., 1998). With IC_{50} values of 0.0631, 0.1032 and 0.0332µM respectively for quercetin, naringin and naringenin, all three test flavonoids compare closely to verapamil (IC_{50}=0.0310µM) in CYP3A4 inhibition. Naringenin appeared more potent than naringin. This further suggested that the aglycone portion of the naringin glycoside exerts the inhibitory activity while the presence of the sugar moiety may not be necessary for inhibitory activity. With this, naringin will have to be hydrolyzed to its aglycone, naringenin which explains why naringenin appeared more potent.

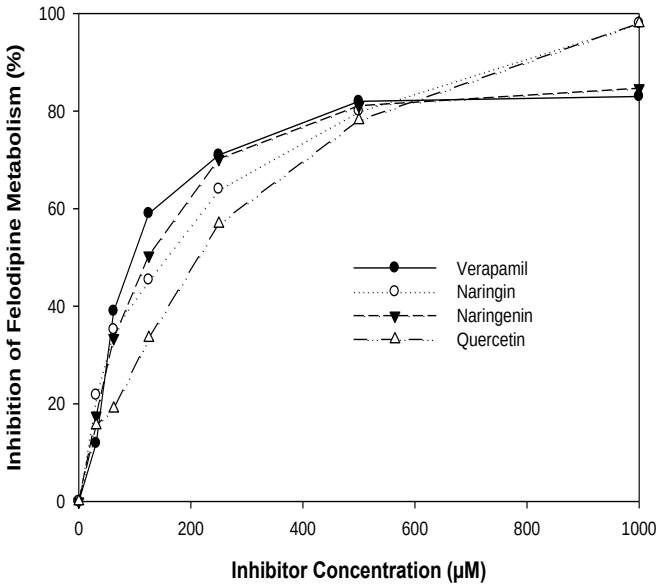


Figure 3:12: Profile of the inhibitory effects of flavonoids and verapamil on CYP3A4-dependent felodipine metabolism (N=3; S.D.<0.004 in all cases).

3.5.4. Assessment of the effects of flavonoids on the CYP1A2-catalyzed *o*-deethylation of phenacetin

All three flavonoids inhibited the rate and extent of paracetamol formation from phenacetin with IC_{50} values of 625.71, 831.43 and 8.88µM respectively for quercetin, naringin and naringenin. A steady concentration-dependent inhibition was observed with naringin and quercetin while naringenin appeared the most potent exhibiting enzyme inhibition tending towards 100% at a relatively low concentration (Figures 3.13). Compared to the effects on felodipine metabolism, the flavonoids exhibited less inhibitory activity against paracetamol production from CYP1A2-catalyzed phenacetin metabolism as demonstrated by the IC_{50} values. The influence of the flavonoids on the metabolic activity of CYP1A2 and CYP3A4 is compared in Table 3.3.

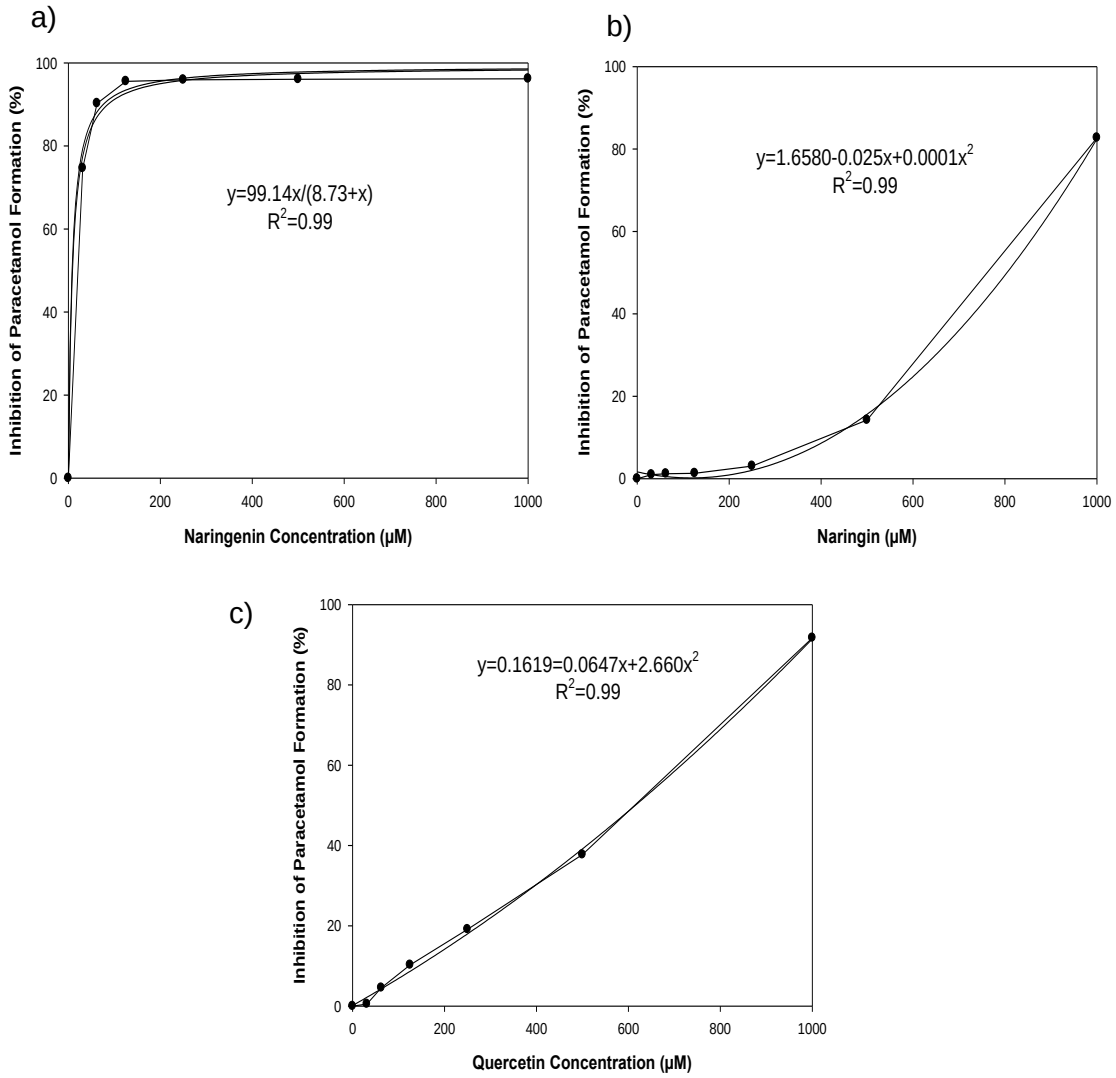


Figure 3:13: The effects of varying concentrations of a) naringenin, b) naringin and c) quercetin, on phenacetin metabolism by HLM (N=3; S.D.<0.005 in all cases).

Table 3.3: Comparison of the inhibitory properties of grapefruit flavonoids against felodipine and phenacetin metabolism by HLM.

Inhibitor	***IC*$_{50}$ (µM) (felodipine)**	***IC*$_{50}$ (µM) (phenacetin)**
Naringin	0.1032	831.43
Naringenin	0.0332	8.8800
Quercetin	0.0631	625.71

IC_{50}=the concentration of test flavonoid that exhibit 50% inhibition of metabolism

Quercetin is a phytochemical often used as a nutritional supplement. It forms part of the coloring found in a wide range of fruits. Although it has been widely used and promoted as being effective against various of diseases including cancer (Gulati et al., 2006), there is no clinical evidence that quercetin can prevent or treat cancer. Studies have shown that

quercetin has anti-inflammatory and anti-oxidant properties which explain its use in cardioprotection (Guardia et al., 2001; Kahraman et al., 2003). It has also been suggested to have ameliorating effects on obesity and other metabolic syndromes (Rivera et al., 2008). However, despite these indications of possible health benefits, quercetin has not been confirmed by any regulatory agency as a specific therapy for any pathological condition in man. It is known to competitively bind bacterial DNA gyrase and therefore contra-indicated with certain antibiotics especially fluoroquinolones (Plaper et al., 2003).

Ingested quercetin is extensively metabolized into non-active phenolic acids with >96% of the ingested dose excreted within 72 hours (Meng et al., 2004; Graf et al., 2006). The extensive first-pass metabolism of quercetin and its ability to inhibit *in vitro* CYP-catalyzed metabolism of felodipine and phenacetin as shown in this study further suggests its strong affinity for CYP enzymes. Competitive molecular binding may thus explain its mechanism of inhibitory action. Thus in addition to known interactions between grapefruit juice and CYP3A4 substrates, grapefruit juice and other quercetin-containing fruits have the potential of interacting with CYP1A2 substrates as the results of this investigation shows.

Naringin, which is a flavonoid glycoside and a conjugate of naringenin and a sugar molecule, is present in grapefruit and is responsible for its bitter taste. It is metabolized into naringenin in the body. Some of the reported pharmacologic effects of naringin include its antioxidant, blood lipid-lowering, antifungal and anticancer properties (Manthey et al., 2001). Naringenin also possesses antioxidant properties, and in addition is known to exert anti-inflammatory actions, modulate the immune system and enhance carbohydrate metabolism. It is a free radical scavenger and has been shown to reduce the hepatitis virus production by infected hepatocytes in cell culture and suppresses HMGCoA and ACAT in rats fed a high cholesterol diet (Zbarsky et al., 2005). Both naringin and naringenin demonstrated potent inhibitory effects against paracetamol production from CYP1A2-induced phenacetin metabolism. The IC_{50} values showed that naringenin is about 100-fold more potent than its glycoside naringin in its inhibitory activity against CYP1A2 (Table 3.2). This further suggests that the aglycone portion of naringin is the active inhibitor against CYP1A2.

The ability of the grapefruit flavonoids to inhibit CYP-catalyzed metabolism, apart from toxicological implications, can be applicable in drug delivery. Pre-systemic CYP inhibition can greatly improve oral drug bioavailability. Flavonoids being physiologically active compounds may not be useful in this regard but their intrinsic chemical and structural properties that enhance their binding affinity to CYP enzymes can be employed to model

pre-systemic CYP modulators that are physiologically inert and pharmaceutically acceptable.

3.6. Concluding Remarks

This chapter has been able to establish optimal *in vitro* conditions for assessing the metabolic activity of CYP-expressed HLM on felodipine and phenacetin. The flavonoids naringin, naringenin and quercetin inhibited the metabolism of felodipine and phenacetin. This confirmed that flavonoid contents of grapefruit juice are responsible for its inhibitory activity on CYP3A4. It also suggested that the effect of grapefruit juice is not limited to CYP3A4 inhibition as the study has demonstrated that the flavonoids also inhibited CYP1A2-dependent phenacetin *o*-deethylation. This further implied a potential interaction of grapefruit juice with drugs other than CYP3A4 substrates. In the search for a suitable pre-systemic metabolism inhibitor, the study of these flavonoids can be employed as leads for the design of suitable oral bioavailability enhancers. Since binding affinity to receptor sites and subsequent interaction are largely dependent on chemical structure, the parent structure of flavonoids and their structure-activity relationships with CYP can therefore be explored for further studies including modeling and design of pharmaceutical alternatives for CYP inhibition.

CHAPTER FOUR

THE GENERATION OF CYTOCHROME P450 3A4 INHIBITORS THROUGH A NOVEL APPLICATION OF COMPUTATIONAL MODELING

4.1. Introduction

In chapter three, the ability of grapefruit flavonoids to inhibit CYP3A4-induced metabolism of felodipine was demonstrated. Considering the magnitude of the influence of CYP3A4 on oral drug delivery (Chapter two, Section 2.8), pre-systemic enzymatic modulation may greatly enhance the oral bioavailability of drugs. The search for an effective pre-systemic enzyme inhibitor is therefore an important component of the earnestly sought solution to the many challenges of effective oral drug delivery. It is important that an effective pre-systemic metabolism inhibitor should be inert, biocompatible and biodegradable. Such compounds should also exert temporary and/or reversible inhibition on metabolic enzymes to allow systemic drug clearance and prevent drug accumulation. As described in Chapter three, Section 3.4, naringin, naringenin and quercetin exert various pharmacological effects and are therefore not ideal for inclusion in pharmaceutical formulations to enhance oral bioavailability.

Recent advancement in computer-based simulations has opened the possibility of exploring computational modeling in predicting natural and biological activities given specific sets of input parameters. Computational modeling simulates natural phenomena and can be employed to predict the relationship between xenobiotics and biological enzymes (Long and Walker, 2003). Understanding the complex nature of biochemical and pharmacological reactions at a cellular level which hitherto has been unexploited, has now been made possible through computational molecular and structural rationalization techniques (Ironi and Tentoni, 2003; Sibeko et al., 2009). Quantitative structure-activity relationships (QSAR) of CYP substrates, their pharmacophoric units and the distinct amino acid sequences of CYP enzymes, their molecular binding sites and overlapping substrate specificity are some of the inputs required to understand the biochemical basis of enzymatic reactions, predictability of outcomes on parameter variation and a deeper understanding of cellular reactions. Initial studies have reported the successful application of two-dimensional and three-dimensional QSAR, pharmacophoric mapping and ligand-

based computational modeling in predicting the affinity of structurally diverse compounds to CYP2C9 and other CYPs (Rao et al., 2000; Rieko, 2006).

A little is known of the crystal structure of mammalian CYPs due to their membrane-bound nature (Ekins et al., 2001). Computational modeling has therefore become a useful tool to understand the structure and the active sites of these enzymes. Understanding the substrate specificity of the CYPs is essential because of their clinically important roles in the metabolism of drugs. It has been shown that various three-dimensional QSAR (3D-QSAR) models can be generated and may be employed to classify molecules for their likely capability to be CYP substrates or inhibitors. Computational modeling and 3D-QSAR provide a visual description of how molecules may bind to CYPs as either substrates or inhibitors.

Lewis and co-workers (1996) had suggested structural requirements of CYP3A4 substrates to include a hydrogen bond acceptor atom 5.5-7.8Å from the site of metabolism and 3Å from the oxygen associated with the heme. Ekins and co-workers (1999a; 1999b and 2001) employed the Catalyst software programme (Model ChemLab, Fulton, NY, USA) to build 3D-QSAR pharmacophoric models to reveal the structural features common to competitive-type CYP3A4 inhibitors. The substrate model was described to include a hydrogen bond acceptor 5.5-7.8Å from the site of metabolism and 3Å from the oxygen molecule associated with the heme, while the inhibitor pharmacophores included three hydrophobes at distance ranging from 5.2-8.8Å from a hydrogen bond acceptor, three hydrophobes at distance of 4.2-7.1Å from a hydrogen bond acceptor and an additional 5.2Å from another hydrogen bond acceptor or one hydrophobe at distance 8.1-16.3Å from the furthest of three hydrogen bond acceptors (Ekins et al., 2001). It is believed that this molecular understanding can be explored to generate useful Modeled Pre-systemic CYP Inhibitors (MPCI) in order to improve the oral bioavailability of drug.

The aim of the present study was therefore to apply the parameters involved in flavonoid-CYP and verapamil-CYP binding and interaction as a blueprint for computational modeling to generate MPCIs and to investigate the ability of the modeled compounds to inhibit *in vitro* CYP3A4-induced felodipine metabolism. The use of verapamil and grapefruit flavonoids (established CYP3A4-inhibitors) as controls provided the platform for comparative studies of the effects of the MPCIs. Both human liver and intestinal microsomes were employed to assess the inhibitory activity of the MPCIs in order to assess their potential pharmaceutical utility.

4.2. Materials and Methods

4.2.1. Materials

Pooled mixed gender human intestinal microsomes (HIM) expressing CYP3A4, CYP2C9, CYP2J2, CYP4F12, UDT-glucuronosyl transferase and carboxylesterase prepared from matured enterocytes of both duodenum and jejunum sections of five donors (1 Male, 4 Female with non-enteric related pathology and cause of death) and pooled mixed gender human liver microsomes (HLM) expressing CYP3A4, CYP2C9, CYP4A11, CYP4F2, CYP2E1 and CYP2A6 were purchased from BD Biosciences (Pty) Ltd. (Woburn, MA, USA) and stored at -70ºC until used. Methoxy poly(ethylene glycol) (M_W=5000g/mol [MPEG 5000] and 10000g/mol [MPEG 10000]), poly(ethylene glycol) (PEG, M_W=2000g/mol [PEG 2000] and 5000g/mol [PEG 5000]), 4-arm-poly(ethylene glycol) (M_W=10000g/mol [4-arm-PEG 1000] and 20000g/mol [4-arm-PEG 20000]) and 8-arm-poly(ethylene glycol) (8-arm-PEG) were purchased from Jenkem Technology (Pty) Ltd. (Beijing, China). O-(2-aminoethyl)-O-methoxy poly(ethylene glycol) (M_W=7500g/mol) (MPEG-NH_2), poly (L-lysine), poly(methyl methacrylate), and poly(phenylalanine) (NADPH, reduced form) were purchased from Sigma-Aldrich (Pty) Ltd. (St Louis, MO, USA). Other materials employed for this phase of the study and their respective sources can be located in Chapter three, Section 3.4.1.

4.2.2. Computational modeling of Cytochrome P450-inhibitor interactions to generate pre-systemic Cytochrome P450 inhibitors

As an example, a computational comparative study of the structural and three-dimensional amino acid sequence of CYP3A4 was explored (Figure 4.1). This was simultaneously approximated with structural properties, 3D-QSAR of CYP3A4, grapefruit flavonoids and verapamil employing Hyperchem 7.5 Professional computational modeling software on a non-silicon graphics system (HyperCube Inc. Gainesville, FL, USA) (Figure 4.2). The templates derived from the known substrates (felodipine) were step-wise modified as a single variant within the structure taking into consideration the overall electronegativity/total charge density, dipole moment, bond length, bond angle, stereo-orientation and effective geometry. This provided a deeper molecular understanding of substrate specificity, binding affinity and manipulability of CYP3A4. The most stable forms of the resulting compounds were determined by estimations of the hydration energy and the energy of conformation. Polymer conjugation with known CYP3A4 inhibitors including high molecular mass flavonoids and furanocoumarins based on multi-site reactivity of the CYP variants was also explored.

Arg-Ser-Leu-Leu-Ser-Pro-Thr-Phe-Thr-Ser-Gly-Lys-Leu-Lys-Glu-Met-Val-Pro-Ile-
Ile-Ala-Gln-Tyr-Gly-Asp-Asp-Val-leu-Val-Arg-Asn-Leu-Arg-Arg-Glu-Ala-Glu-Thr-
Gly-Lys-Pro-Val-Thr-leu-Lys-Asp-Val-Phe-Gly-Ala-Tyr-Ser-Met-Asp-Val-Ile-Thr-
Ser-Thr-Ser-Phe-Gly-Val-Asn-Ile-Asp-Ser-Leu-Asn-Asn-Pro-Gln-Asp-Pro-Phe-
Val-Glu-Asn-Thr-Lys-Lys-leu-leu-Arg-PheAsp-Phe-Leu-Asp-Pro-Phe-Phe-Leu-
Ser-Ile-Ile-Phe-Pro-Phe-Leu-Ile-Pro-Ile-Leu-Glu-Val-Leu-Asn-Ile-Cys-Val-Phe-
Pro Arg-Glu-Val-Thr-Asn-Phe-Leu-Arg-Lys-Ser-Val-Lys-Arg-Met-Lys-Glu-Ser-
Arg-Leu-Glu-Asp-Thr-Gln-Lys-His-Arg-Val-Asp-Phe-Leu-Gln-Leu-Met-Ile-Asp-
Ser-Gln-Asn-Ser-Lys-Glu-Thr-Glu-Ser-His-Lys-Ala-Leu-Ser-Asp-Leu-Glu-Leu-
Val-Ala-Gln-Ser-Ile-Ile-Phe-Ile-Phe-Ala-Gly-Tyr-Glu-Thr-Thr-Ser-Ser-Val-Leu-
Ser-Phe-Ile-Met-Tyr-Glu-Leu-Ala-Thr-His-Pro-Asp-Val-Gln-Gln-Lys-Leu-Gln-Glu-
Glu-Ile-Asp-Ala-Val-Leu-Pro-Asn-Lys-Ala-pro-pro-Thr-Tyr-Asp-Thr-Val-Leu-Gln-
Met-Glu-Tyr-Leu-Asp-Met-Val-Val-Asn-Glu-Thr-Leu-Arg-Leu-Phe-Pro-Ile-Ala-
Met-Arg-Leu-Glu-Arg-Val-Cys-Lys-Lys-Asp-Val-Glu-Ile-Asn-Gly-Met-Phe-Ile-Pro-
Lys-Gly-Trp-Val-Val-Met-Ile-Pro-Ser-Tyr-Ala-Leu-His-Arg-Asp-Pro-Lys-Tyr-Thr-
Glu-Pro-Glu-Lys-Phe-Leu-Pro-Glu-Arg-Phe-Ser-Lys-Lys-Asn-Lys-Asp-Asn-Ile-
Asp-Pro-Tyr-Thr-Tyr-Thr-Pro-Phe-Gly-Ser-Gly-Pro-Arg-Asn-Cys-Ile-Gly-Met-Arg-
Phe-Ala-Leu-Met-Asn-Met-Lys-Leu-Ala-Leu-Ile-Arg-Val-Leu-Gln-Asn-Phe-Ser-Phe-
Lys-Pro-Cys-Lys-Glu-Thr-Gln-Ile-Pro-Leu-Lys-Leu-Ser-Leu-Gly-Gly-Leu-Leu-Gln-
Pro-Glu-Lys-Pro-Val-Val-Leu-Lys-Val-Glu-Ser-Arg-Asp-Gly-Thr-Val-Ser-Gly-Ala

Key

Gly-Glycine	Ile-Isoleucine	Trp-Tryptophan	Asn-Asparagine
Pro-Proline	Met-Methionine	His-Histidine	Glu-Glutamic acid
Ala-Alanine	Cst-Cysteine	Lys-Lysine	Asp-Aspartic acid
Val-Valine	Phe-Phenylalanine	Arg-Arginine	Ser-Serine
Leu-Leucine	Tyr-Tyrosine	Glutamine	Thr-Threonine

Figure 4.1: Amino acid sequence of CYP3A4 isozyme (Adapted from Nelson, 2009).

The 3D modeling of the polypeptide structure demonstrated the maneuverability of the CYP3A4 polypeptide chain for the active site (heme sub-structure, located at the cysteine-58 residue). The constantly interchanging conformation of the protein chain due to physiological conditions inherent or induced by xenobiotic factors and entities as well as the distance mapping of the active (heme) site, the different locations of the polypeptide chains and the proximity of amino acid residues by way of their conformation variability also confirmed the manipulability of the CYP3A4 active sites (Figure 4.2).

The computational simulation performed kept the distance in the range of 2-25Å and mapped at three different locations from the N-terminal, middle of the chain and the C-terminal ending of the polypeptide chain suggesting the approach of the incoming molecule to the active site which in its conformation of binding and molecular volume would reach the biochemical site of action. The proximity of other sulphur containing

residues especially from the methionine residues of the CYP3A4 enzyme may be a complimentary location to act as a scavenger (S atom) due to its high valence and ability to bind other atoms. Although cysteine-58 placement of the heme sub-structure is hypothetical, the link of the heme substructure to any other cysteine residue would not have changed the activity except for a negligible change in the resulting loop which itself does not alter the activity and approach of the chain and any xenobiotic entity attached to it for its interaction by detoxification via biochemical conversion.

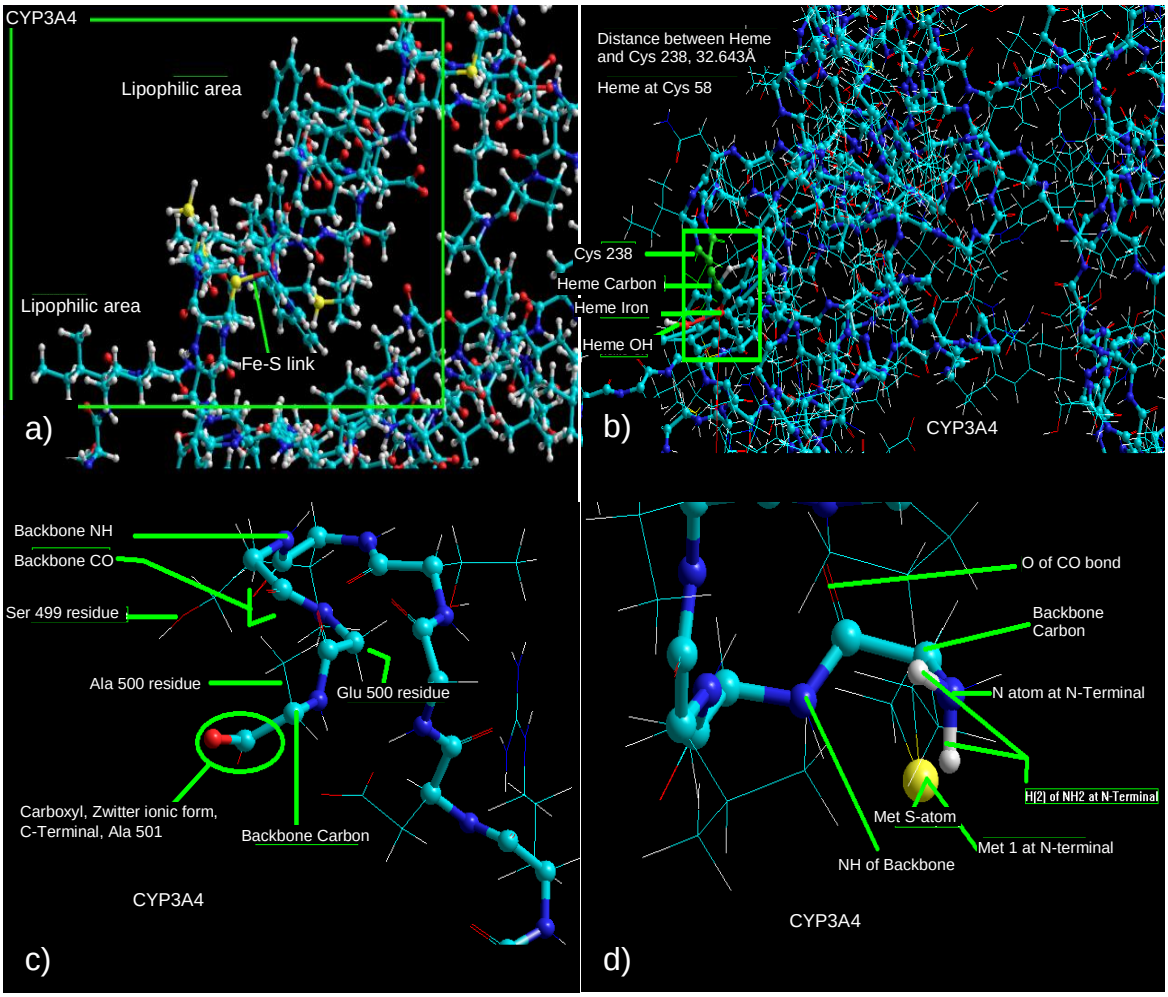


Figure 4.2: A three dimensional amino acid sequence of CYP 3A4 as shown by Hyperchem 7.5 professional software revealing the a) structural morphology, b) manipulable heme substructure, c) expanded view of the C-terminal and d) expanded view of the N-terminal.

It was therefore reasonable to propose that the versatile activity of the CYP3A4 polymorph was dependent on numerous factors. The fact that a range of molecules of different molecular mass, size, volume and charge as well as diverse classes of

compounds are substrates and inhibitors of CYP suggested a more flexible approach to the bioactivity mechanism involved in interaction with CYP3A4 and its substrates. Thus, the binding of both substrates and inhibitors to the CYP3A4 peptide chain is complimentary and conjugative to the corresponding active site of the polypeptide chains. Therefore, computational modeling of bioactivity was employed to predict and generate biodegradable, biocompatible natural and synthetic polymers and their conjugates potentially capable of interacting with the CYP3A4 active domain.

4.2.3. Investigation of the inhibitory effects of the MPCIs on felodipine metabolism employing CYP-expressed HLM and HIM

Polymers including derivatives of poly(ethylene glycol), polyacrylates, polyoxyethylene and polypeptides were prepared in a 0.01-100mg/ml concentration range and incubated with felodipine separately in HLM and HIM solutions in methods as described for the grapefruit flavonoids and verapamil in Chapter three, Section 3.5.4. Their inhibitory effects were investigated on the microsomal metabolism of felodipine. The potency of the inhibitors was measured by their IC_{50} values that were determined by profiling the percentage inhibition of felodipine metabolism against the inhibitor concentration employing the Enzyme Kinetic Software (SigmaPlot: Enzyme Kinetic Module, Systat Software Inc., Chicago, IL, USA). The total quantity of felodipine metabolized within the incubation period was determined from control incubations where metabolism was maximal. The concentration of felodipine in incubation mixtures containing the various test inhibitors was determined and the extent of metabolism was measured as a percentage of the control. The percentage inhibition observed was profiled against concentration of test inhibitors added to the incubation mixture. In each case, the inhibitor concentration responsible for 50% substrate metabolism inhibition (IC_{50}) was determined. All determinations were performed in triplicates (n=3).

4.3. Results and Discussion

4.3.1. Computational molecular and structural modeling of Cytochrome P450 3A4 inhibitors

Computational modeling was used to elucidate structural and chemical requirements for CYP3A4 binding and activity. From the constituent structural topography for biomimetism, the inter-structural cavity shape formation, which may be responsible for activity depended on the presence of structural groups and their stereo-electronic factors present in specified reactive domains of the protein fiber, which was used as a blueprint for the

formation of nearly equivalent cavities within the polymer structural groups (Figure 4.3). For a protein structure, the surrounding contribution is less significant due to the incompassive and ulta-advanced structural features of the molecule compared to polymers with less stereo-electronic and structural features at reactive domains or interactive sites.

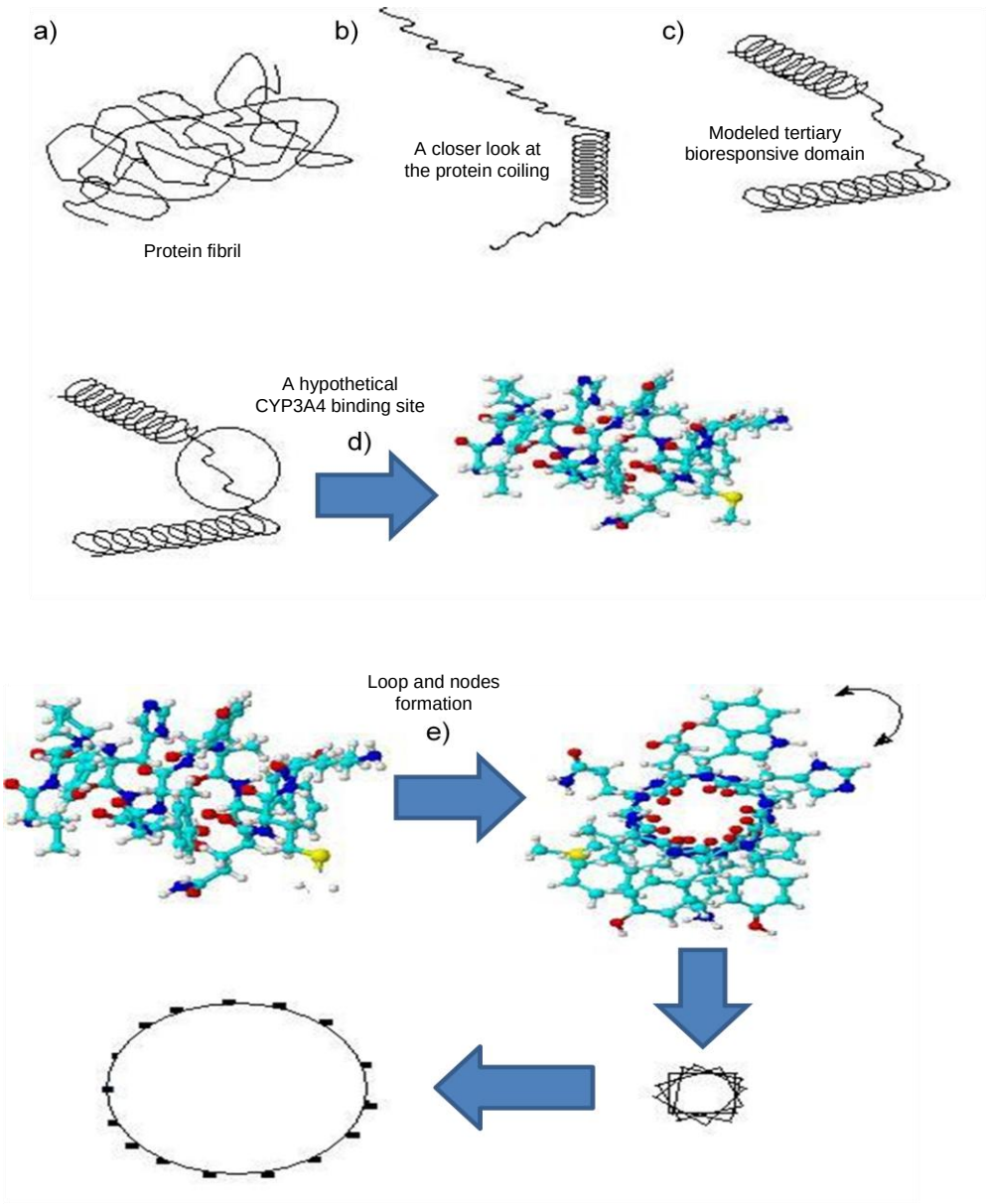


Figure 4.3: Computational models showing the molecular structure of the bio-reactive sites of CYP3A4, with a) protein fibril; b) a closer look at protein coiling; c) tertiary structural and bioresponsive domain for biomimetic activity; and d) the arrow represents hypothetical binding site on the 3-dimensional protein structure (e) with loop and nodes formation.

Further understanding of the binding of verapamil and flavonoids to active site of CYP was derived from computational modeling. Based on the computational biomolecular understanding of the inhibitory mechanism of flavonoids-CYP3A4 and verapamil-CYP3A4 complex formation and subsequent action, computational biomimetism and simulation (Figure 4.3) was employed to predict and generate high molecular weight poly(ethylene glycol) based polymers and their derivatives, homopeptides, heteropeptides, polyoxyethylene and polyacrylate derivatives and conjugations with the potential capacity to interact with CYP3A4.

4.3.2. Investigating the effects of MPCIs on *in vitro* felodipine metabolism by human liver microsomes

The MPCIs inhibited felodipine metabolism to varying degrees (Figure 4.4). The potencies of the flavonoids and the MPCIs measured as their IC_{50} values were compared (Table 4.1). However, a few of the modeled compounds did not appear to have any significant influence on the rate of felodipine metabolism by HLM. PEG 2000, PEG 5000, methoxy PEG (M_W=1000g/mol and 2000g/mol) and polyoxyethylene (M_W=750g/mol) within the test concentration range of 0.001-100mg/mL did not have a significant effect on the rate and extent of felodipine metabolism by HLM compared to the controls.

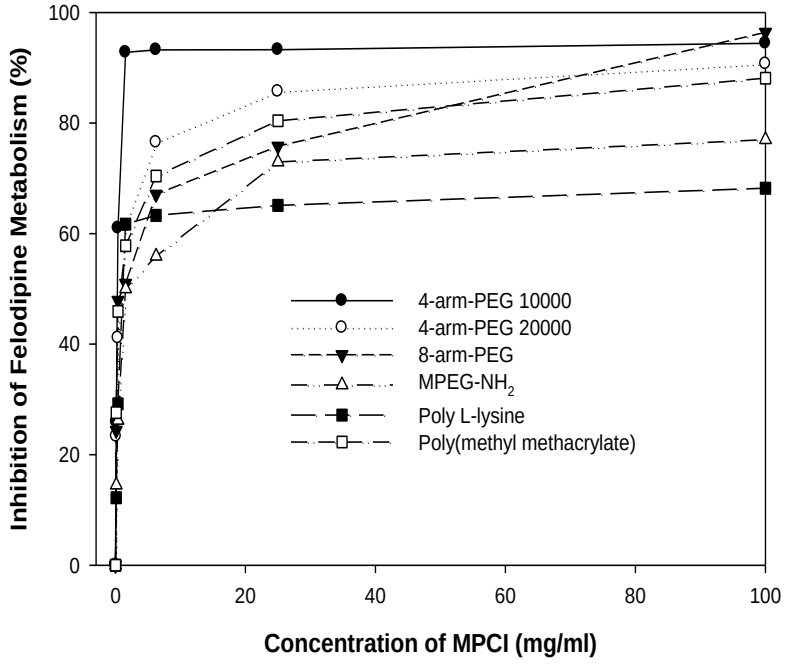


Figure 4.4: The inhibitory effects of the Modeled Pre-systemic Cytochrome P450 3A4 Inhibitors on the CYP3A4-catalyzed felodipine metabolism (N=3; S.D.<0.005 in all cases).

The concentrations of verapamil and the MPCIs in the incubation mixture were also monitored. Although *in vitro* techniques are often insufficient to prove the mechanism of enzymatic metabolism inhibition, the disappearance of the flavonoids, verapamil and the MPCIS suggested competitive receptor binding to enzymes as a possible mechanism of felodipine inhibition. This was further demonstrated by the direct relationship between the inhibitor concentration and the initial extent of inhibition observed (Figure 4.4).

Table 4.1: Comparison of the inhibitory properties of various HLM inhibitors and MPCIs.

Inhibitor	***IC*$_{50}$ (mg/ml)**	***IC*$_{50}$ (µM)**	**Highest inhibition observed (%)**	**Conc. (µM) at max inhibition**
Verapamil	0.0530	107.88	83	500
Naringin	0.1032	177.81	98	1000
Naringenin	0.0332	121.97	95	500
Quercetin	0.0631	208.65	98	750
4-Arm-PEG 10000	0.2379	23.79	95	10000
4-Arm-PEG 20000	0.6000	30	91	5000
8-Arm-PEG 10000	0.0722	7.22	96	10000
Poly (L-lysine)	0.2226	29.68	69	1000
Poly(methyl methacrylate) 10000	0.1628	16.28	88	26667
O-(2-aminoethyl)-O'-methoxy PEG 7500	0.1029	13.72	77	1000

The ability of the MPCIs modeled based on the chemical structure and binding properties of the flavonoids to inhibit the metabolism of felodipine by HLM agrees with other studies suggesting that the inhibitory effects of flavonoids are molecularly structure-dependent (Miniscalo et al., 1992). This therefore, provided the theoretical avenue for a reliable research route and mechanism in computational modeling. The ability of PEG-derivatives to inhibit CYP-induced metabolism should also draw attention to pharmaceutical grade polymers in the search for oral bioavailability enhancers. Results of this study show inhibitory potency for the multi-armed PEGs in this order: 8-arm-PEG >4-arm-PEG 20000 >4-arm-PEG 10000 (Figure 4.5). This may suggest a trend in the binding and increasing substrates affinity of multi-armed PEGs with CYP3A4.

Although enzymatic catabolism has been reported to be responsible for the high intestinal first-pass effect of dietary proteins (Stoll et al., 1998), amino acids have not been reported to inhibit HLM. The ability of poly (L-lysine) to inhibit CYP3A4-mediated metabolism of felodipine however suggests that amino acid derivatives offer potential in the search for oral bioavailability enhancers.

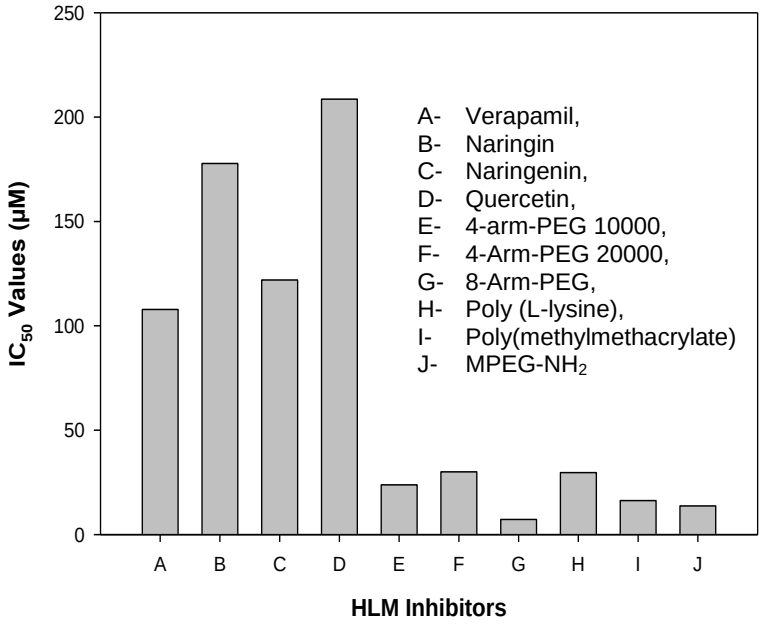


Figure 4.5: Comparison of the potencies of the investigated MPCIs.

4.3.3. Comparative inhibition of felodipine metabolism by the MPCIs in HLM and HIM

Most *in vitro* drug metabolism studies for *in vivo* correlation often employ HLM. Pre-systemic drug metabolism occurs principally in the intestinal wall and the liver due to the presence of metabolic enzymes in both organs (Table 4.2). An HLM inhibitor may not necessarily have the same effect in the intestines although cytochromic morphology may be similar. In this case, prospective oral bioavailability enhancers must necessarily be absorbed to exert hepatic activity. An enzyme inhibitor active against intestinal CYP will prevent intestinal pre-systemic metabolism and enhance oral bioavailability. It was therefore necessary to investigate intestinal activity of prospective pre-systemic metabolism inhibitors especially since they may not survive the physicochemical barriers necessary for hepatic absorption. The use of HIM estimates the potential utility of MPCIs that may be pre-systemically degradable and whose potency may be lost outside the GIT. Thus, HIM was employed to investigate the effects of HLM inhibitors on intestinal metabolism for a more reliable extrapolation.

Table 4.2: Intestinal expression and localization of CYP isozymes (Finnstrom et al., 1998).

CYP	Stomach	Duodenum	Ascending colon	Transverse colon	Descending colon
1B1	+++++	+	++++	+	+
2E1	+++	++	++	+	+++
3A4	+++	++++	++	+	++
3A5	++++	+++++	++++	+++	++

The number of + signs indicates the relative amount of CYP expressed

The MPCIs were incubated with felodipine at the pre-determined K_m value with CYP3A4 expressed HIM for *in vivo* correlational reliability. From the results obtained (Figure 4.6; Tables 4.3), the modeled HLM inhibitors demonstrated inhibitory actions against intestinal microsomal metabolism of felodipine. Thus, this further confirms the potential utility of these intestinal CYP3A4 inhibitors in oral drug delivery.

Table 4.3: Comparison of the inhibitory potencies of MPCIS in HLM and HIM.

MPCIs	***IC*$_{50}$ values in HLM (μM)**	**Inhibition in HIM (%) at the HLM *IC*$_{50}$**	***IC*$_{50}$ in HIM (μM)**
4-Arm-PEG 10000	23.79	33.90	45.18
4-Arm-PEG 20000	30	48.69	41.03
8-Arm-PEG 10000	7.22	71.03	5.78
Poly (L-lysine)	29.68	67.45	19.20
Poly(methyl methacrylate) 10000	16.28	54.51	15.92
O-(2-aminoethyl)-O´-methoxy PEG 7500	13.72	36.16	21.34

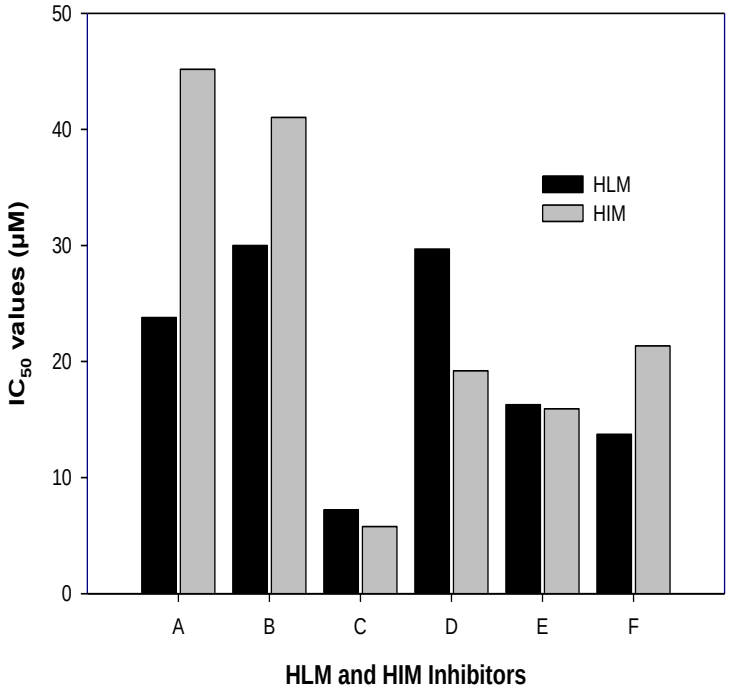


Figure 4.6: Comparison of the IC_{50} values of the MPCIs in CYP3A4-expressed HLM and HIM: A) 4-arm-PEG 1000, B) 4-arm-PEG 20000, C) 8-arm-PEG, D) poly (L-lysine), E) poly(methylmethacrylate) and F) MPEG-NH_2.

4.4. Concluding Remarks

This phase of the study has shown that the mechanism of drug-drug and drug-food interaction can be exploited through the aid of computational modeling to generate potential pharmaceutical alternatives in drug delivery and toxicological studies. From the results, 8-arm-PEG demonstrated the highest inhibitory potency against felodipine metabolism by HLM and HIM. With a satisfactory *in vitro* result, 8-arm-PEG may be a potential oral bioavailability enhancer through the inhibition of pre-systemic CYP-catalyzed drug metabolism. Further studies including *ex vivo* and *in vivo* studies are necessary to assess this potential and therefore described in Chapter five.

CHAPTER FIVE

INVESTIGATION OF THE ORAL BIOAVAILABILITY-ENHANCING PROPERTY OF 8-ARM-POLY(ETHYLENE GLYCOL) 10000

5.1. Introduction

In Chapter four, various Modeled Pre-systemic Cytochrome P450 3A4 Inhibitors (MPCIs) were generated through computational modeling and were investigated for their inhibitory activity against the *in vitro* metabolism of felodipine employing Human Liver Microsomes (HLM) and Human Intestinal Microsomes (HIM). Results showed that 8-arm-poly(ethylene glycol) 10000 (8-arm-PEG), a derivative of poly(ethylene glycol) (PEG) possessed the highest inhibitory potency.

PEG is an oligomer or polymer of ethylene oxide. It is produced by the interaction of ethylene oxide with water, ethylene glycol or ethylene glycol oligomers and has the general molecular structure as shown in Figure 5.1 where *n* represents the number of oxyethylene moieties.

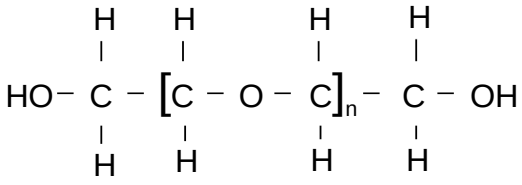


Figure 5.1: The molecular structure of poly(ethylene glycol).

PEG has properties that are pharmaceutically desirable (Harris and Chess, 2003). Commercially available PEG and most of its derivatives exist as liquid or low-melting solids depending on the molecular mass, which varies widely from 300-10,000,000g/mol. They are non-toxic, odorless, lubricating, non-volatile, non-irritating, non-antigenic and non-immunogenic; and are therefore employed in a variety of pharmaceuticals as tableting excipients, coating, ointment and suppository bases, dispensing agents, solvents, lubricants, binders and as plasticizers (Veronese and Pasut, 2005). PEG and most of its derivatives are freely soluble in water, methanol, benzene and dichloromethane but insoluble in diethylether and hexane. PEG is eliminated unchanged

from the body by either the kidney (M_W <30,000g/mol) or in the feces (M_W >20,000g/mol) (Friman et al., 1993). The United States Food and Drugs Administration (USFDA) has approved PEG for use as a vehicle or base in the food, cosmetic and pharmaceutical products including oral, injectable, topical, rectal and nasal preparations. PEG is used as a laxative base in macrogol-containing products such as Movicol® (Norgine Pharmaceutical Ltd. Middlesex, Uxbridge, UK).

Technological advances in the synthetic preparation and modification of PEG has given rise to various PEG derivatives with variable physicochemical behavior. Alterations in the molecular configuration of PEG has yielded *branched* PEGs comprising 3-10 PEG chains emanating from a central core group, *star* PEG with 10-100 PEG chains emanating from a central core group, while multiple PEG chains are normally grafted to a polymer backbone to form a *comb* PEG (Stowe et al., 2005). Pegylation, often defined as the modification of a protein, peptide or non-peptide molecule by the linking of one or more PEG chains, has evolved to include conjugation of PEG derivatives with biomolecules to enhance the pharmacokinetic and pharmacodynamic properties of protein pharmaceuticals (Hinds and Kim, 2002). It is the act of covalently coupling a PEG structure to another larger molecule, for example a therapeutic protein in which case the molecule is referred to as being pegylated (Veronese and Mero, 2008).

The active terminals of PEG are usually maintained after molecular and structural modifications yielding different PEG configurations and derivatives. These alterations however can modify the drug delivery and pharmacokinetic behavior of PEG. The most popular branched PEGs are 3-10-armed PEGs. 8-arm-PEG has been reported to improve the swellability and desirable mechanical properties such as the softness and tenacity of poly(L-lactic acid) when conjugated, enhancing the suitability of the co-polymer as an implantable soft material (Koji et al., 2006). Partially cholesterol-substituted 8-arm-PEG-*block*-poly(L-lactide) has been prepared as a novel star-shaped, biodegradable co-polymer derivative which has been reported as a potential candidate as an injectable cellular scaffold (Nagahama et al., 2008).

PEG-based hydrogels, PEG-modified liposomes and PEG-polypeptide conjugations are becoming increasingly important in drug delivery. Many proteins have been linked to one or more polymeric chains in order to protect them from rapid enzymatic degradation and to increase their half-life. Examples of such conjugates are PEG-asparaginase, PEG-superoxide dismutase and PEG-α-2a interferon (Guiotto et al., 2002).

Liposomal pegylation is also gaining increasing applicability in drug delivery. Pegylated liposomes have been demonstrated to show increased half-life, decreased plasma clearance and a shift in drug distribution favorable to disease tissues, compared to classical liposomes (Chess and Harris, 2003). The pharmacokinetic behavior of consensus interferon-α was significantly altered when pegylated with a 40,000g/mol branched PEG (Du et al., 2008). The unique nature of the Blood-Brain Barrier (BBB) prevents many therapeutic compounds from accessing the central compartment which presents a challenge in the pharmacotherapy of neurological and psychiatric disorders. There is a high physiological risk in injecting drugs directly into the brain or when the BBB is disrupted. Certain drug carriers have shown promising ability to transport drugs across the BBB. An example is polymeric nanoparticles such as n-hexadecylcyanoacrylate (PHDCA) conjugated with PEG which has been reported to demonstrate enhanced penetration into the brain compared to PHDCA alone (Chess and Harris, 2003). This conjugation is also reported to have a superior distribution into deep areas of the brain including the striatum, the hippocampus and the hypothalamus without damage to the brain structures and the BBB (Chess and Harris, 2003). Thus, pegylation is a promising drug carrier system for brain drug delivery.

The modeled compound, 8-arm-PEG is a poly-armed derivative of PEG. Its structural difference lies in the molecular arrangement of the constituent monomeric units of the parent PEG molecule (Figure 5.2). Having demonstrated potent inhibitory activity against the *in vitro* metabolism of felodipine by CYP3A4-expressed HLM and HIM as reported in Chapter four, 8-arm-PEG has the potential for further application in oral drug delivery aimed at enhancing the oral bioavailability of CYP3A4 substrates in addition to being a drug carrier. PEG and most of its derivatives, being pharmacologically inert polymers with satisfactory properties as a drug carrier, will be suitable for modifying the drug delivery kinetics and possibly enhancing the oral bioavailability of drugs susceptible to CYP3A4-catalyzed pre-systemic metabolism.

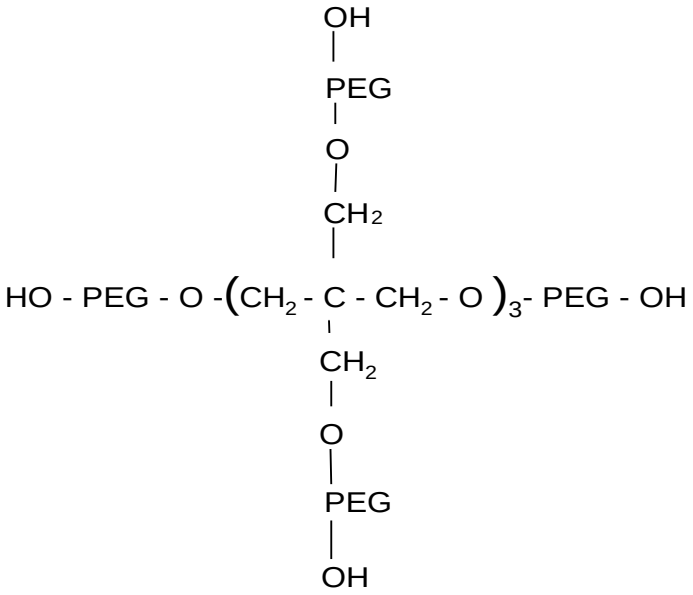


Figure 5.2: The molecular structure of 8-arm-PEG.

The aim of this phase of the study was therefore, to examine and explore the pharmaceutical formulatory capacity, drug release profile and oral bioavailability-enhancing properties of 8-arm-PEG via *ex vivo* and *in vivo* studies. Employing a direct compression technique, 8-arm-PEG was assessed for its compactibility, hardness and friability. It was included in graded concentrations with hydroxypropylmethyl cellulose (HPMC) for formulating a felodipine tablet matrix where its effects on felodipine release and oral felodipine bioavailability were investigated. The current study also aimed at developing an *ex vivo* method of felodipine metabolism employing freshly excised pig intestinal tissue. The objective was to assess the effect of 8-arm-PEG on the metabolism of felodipine by intestinal CYP3A4 after the *in vitro* study with HIM and HIM (described in Chapter four) portrayed 8-arm-PEG as a potent CYP3A4 inhibitor. In addition, the ability of 8-arm-PEG to translate its CYP3A4 inhibitory effect for enhanced oral bioavailability was investigated in the Large White pig model.

5.2. Materials and Methods

5.2.1. Materials

Chemically pure D-glucose anhydrous (M_W=180.16g/mol, molecular biology grade) was purchased from Rochelle Chemicals and Laboratory Equipment (Pty) Ltd. (Johannesburg, Gauteng, South Africa). Magnesium sulfate anhydrous ($MgSO_4$, M_W=120.415g/mol), magnesium chloride ($MgCl_2.6H_20$, M_W=203.30g/mol), sodium hydroxide pellets (NaOH,

M_W=40g/mol) and hydroxypropylmethylcellulose (HPMC) were purchased from Merck Chemicals (Pty) Ltd. (Darmstadt, Germany). Polysorbate 80 (density at 20°C =1.007g) was purchased from Saarchem (Pty) Ltd. (Krugersdorp, Gauteng, South Africa) while heparin (1000IU/mL) and normal saline were purchased from Bodene (Pty) Ltd. (PE, Eastern Cape, South Africa). Other materials employed in this part of the study and their respective sources can be located in Chapter three, Section 3.4.1 and Chapter four, Section 4.2.1.

5.2.2. Formulation of the 8-arm-PEG-incorporated tablet matrices

Employing a mortar and pestle, 8-arm-PEG was manually triturated until a free-flowing fine powder was formed. The powdered polymer was then weighed employing a calibrated digital analytical balance (Boeco BBL Model Balances, Hamburg, Germany, accuracy=±0.05mg) and triturated with 10mg felodipine and HPMC such that the final tablet matrix comprised varied quantities of 8-arm-PEG made up to final mass (600mg) with HPMC in order to maintain a constant tablet mass. The triturated mixture was directly compressed on a Caver® Press (Caver® Laboratory Equipment, Wabash, Indiana, USA) under 5 tons of compression force employing a flat-faced punch and die (13mm in diameter). Tablet matrices comprising only 8-arm-PEG only were also compressed for comparative studies with native HPMC tablets. In-process validation tests were performed on randomly selected tablets.

5.2.3. Evaluation of tablet hardness

In order to assess the suitability of 8-arm-PEG for tableting, hardness testing was conducted on the matrices employing a TA.XT*plus* Texture Analyzer (Stable Micro systems, Godalming, Surrey, England, UK) fitted with a ball probe with a spherical indenter of 3.125mm in diameter and a 5kg load cell. The hardness of the tablet matrices was determined from Force–Distance profiles obtained by converting the indentation hardness to the Brinell Hardness Number (BHN). Equation 5.1 shows the mathematical representation of BHN expressing its relationship with the Force (F) generated from indentation, the diameter (D) of the spherical probe (3.175mm) and the dept (d) of indentation.

$$\mathrm{BHN} = \frac{2\mathrm{F}}{\pi \mathrm{D}[\mathrm{D} - (\mathrm{D}^2 - \mathrm{d}^2)]^{1/2}} \qquad \textit{Equation 5.1}$$

The textural parameter settings employed are summarized in Table 5.1. All tests were performed in triplicate and the BHN was computed in each case.

Table 5.1: The textural settings employed for BHN value calculations.

Test Parameters	Settings
Test mode	Compression
Pre-test speed (mm/sec)	1
Test speed (mm/sec)	0.5
Post-test speed (mm/sec)	1
Indentation dept (mm)	0.5
Trigger type	Auto (force)
Trigger force	0.05

5.2.4. Investigation of tablet friability

Friability tests were conducted employing a Friabilator (Heusenstamm, Germany) set at a 1% upper limit of friability. This test was performed by weighing 10 tablets each of 8-arm-PEG and HPMC and subjecting the weighed samples to 4 minutes of rotation in the friabilator. The tablets were weighed after friabilation and friability was expressed as the percentage loss on the pre-test tablet mass. All determinations were performed in triplicate.

5.2.5. Assessment of the effect of compression on the structure and morphology of 8-arm-PEG

Tablet matrices of 8-arm-PEG were prepared as described in Section 5.2.2. Fourier Transform Infrared Spectrometer (FTIR) was employed to assess infrared spectra of powdered and compressed 8-arm-PEG matrices in order to ascertain the integrity of the structural backbone of 8-arm-PEG. Quadruple scanning was performed on each sample following background spectra collection at wave numbers ranging from 4000-400cm^{-1}. Spectra generated from powdered and compressed samples were superimposed for comparison to observe possible conformational changes in the polymeric structure as a result of compression.

5.2.6. Investigation of the influence of varying concentration of 8-arm-PEG on the *in vitro* felodipine release from tablet matrices

The effects of graded concentrations of 8-arm-PEG (0, 16.7, 33.3, 50, 66.7, 83.3 and 100%$^{w}/_{w}$) on felodipine release from the HPMC tablet matrix was investigated employing a

modified USP 32 rotating paddle method in a calibrated six-station dissolution apparatus (Caleva Dissolution Apparatus, model 7ST; G.B. Caleva Ltd. Dorset, UK). Tablets were immersed at time zero in media containing 1%$^{w}/_{v}$ polysorbate 80 in 900mL phosphate buffer (pH 6.8) and agitated at 50rpm after dissolution conditions were equilibrated to 37ºC. At predetermined intervals, 5mL of release medium was withdrawn and analyzed by UPLC for felodipine content. Equal volumes of drug-free medium were replaced after sampling to maintain sink conditions. The poor aqueous solubility of felodipine precludes the use of plain buffer media in determining its *in vitro* release pattern. The USP dissolution medium for felodipine is 1% polysorbate 80 in phosphate buffer (pH 6.8). Polysorbate 80 as a surfactant is expected to facilitate the dissolution of felodipine in the medium and enhance even dispersion and distribution of insoluble felodipine molecules which are made available for analysis upon sampling. There is limited knowledge of intestinal drug dissolution especially for drugs with poor aqueous solubility (Persson et al., 2005). This could be due to the non-physiological aspects of *in vitro* systems. The use of buffers at intestinal pH for example provides a simulated physicochemical environment for dissolution studies. However, the presence of enzymes, lipophic contents and other organic components in the intestinal fluid makes *in vitro* systems lack biochemical equivalents. This may lead to over-estimation or under-estimation of actual *in vivo* behavior.

5.2.7. *Ex vivo* investigation of the inhibitory property of 8-arm-PEG on the intestinal tissue metabolism of felodipine

In an approach similar to a method by de Kanter and co-workers (1999, 2002 and 2005), felodipine was co-incubated with precision-cut slices of small intestinal tissue obtained from a Large White pig for *ex vivo* metabolism. The method was optimized and repeated with the separate addition of graded concentrations of naringenin (62.5, 125, 250, 500, 1000 and 2000µM) (as the control inhibitor) and 8-arm-PEG. The effect of 8-arm-PEG on felodipine metabolism was determined and compared to that of naringenin.

5.2.7.1. Preparation of intestinal tissue slices

A healthy female Large White pig (55kg) was euthanized with 200mg/mL intravenous sodium pentobarbitone (0.48mL/kg body weight). This was followed by dissection of the GIT where intestinal slices were excised from the jejunum with a 4mm biopsy core. After coring, the mucosa was carefully separated from the muscle layer. The mucosa was sliced using a tissue slicer into equal slices (1mm thickness) and the slices were

incubated immediately in Kreb-Heinseleit Buffer (KHB) employing a humidified Galaxy Standard incubator (RS Biotech Laboratory Equipment (Pty) Ltd. Ayrshire, Scotland, UK).

5.2.7.2. Preparation of Kreb-Heinseleit Buffer

KHB, a modification of Ringer's solution used to maintain physiological activity during *in vitro* investigations involving liver and other living tissues was developed in the early 1930's by Hans Krebs and Kurt Henseleit (Nickelsen and Graβhoff, 2008). The powdered salt contents of KHB listed in Table 5.2, were weighed, triturated and added to 900mL double deionized water at 20°C under agitation. This was followed by the addition of 0.373g of $CaCl_2.2H_2O$ (calcium chloride dehydrate) and 28mL of 7.5%$^{w}/_{v}$ Na_2CO_3 (sodium bicarbonate). The solution was agitated until dissolved while the pH was adjusted with 1N hydrochloric acid to 6.8. The final buffer was made up to 1L volume with double deionized water, sterilized by filtration through a 0.22µm filter system and utilized immediately.

Table 5.2: Components of Krebs-Henseleit Buffer (Sigma-Aldrich Product Information, 2009).

Components	**g/L**
D-Glucose	2.000
Magnesium sulphate (anhydrous)	0.141
Potassium phosphate (monobasic)	0.160
Potassium chloride	0.350
Sodium chloride	6.900

5.2.7.3. Investigation of the effect of 8-arm-PEG on felodipine metabolism using the excised intestinal tissues

Freshly excised and precision-cut intestinal tissue was incubated with freshly prepared KHB employing a six-well culture plates in a humidified incubator (Galaxy Incubators, RS Biotech Laboratory Equipment (Pty) Ltd. Ayrshire, Scotland, UK) circulated with 95:5 O_2/CO_2 (Afrox (Pty), Alrode, Gauteng, South Africa) and set at 37ºC). Each well contained 2mL KHB, 4mm cored intestinal tissue and 500µM felodipine. The felodipine metabolic rate was determined between 0 and 3 hours of control incubations while graded concentrations of naringenin and 8-arm-PEG (62.5, 125, 250, 500, 1000 and 2000µM) were added. The tests were performed such that tissue slices lasted for <4 hours after the source pig was euthanized. This was necessary in order to maintain tissue survival and enhance the maximum physiologic activity of the tissue enzymes. All determinations were performed in triplicate. The rate and extent of felodipine metabolism in 8-arm-PEG co-incubated well plates were compared to controls (8-arm-PEG-free as a negative control) and naringenin co-incubated metabolism (as a positive control).

5.2.7.4. Analysis of the effects of naringenin and 8-arm-PEG on intestinal tissue metabolism of felodipine

At 3 hours, the metabolic activity of the excised intestinal tissues were halted by the addition of 1mL ice-cold acetonitrile (-20ºC) into each well. The fluid contents of the incubation system were transferred by pipeting into a separate labeled six-well plate. The excised intestinal tissues were further rinsed in 1mL acetonitrile to extract felodipine and its metabolites. The transferred fluid content was centrifuged at 4000*g* at 4ºC for 30 minutes in a refrigerated centrifuge (Xiang Yi L-535R^{TM} Centrifuge, Changsha, China) to precipitate the protein content. The supernatants were then filtered through a 0.22µm Cameo Acetate membrane filter (Millipore, Billerica, MA, USA) and subjected to UPLC analysis to identify and quantify felodipine content.

5.2.8. *In vivo* animal studies to investigate the oral bioavailability-enhancing property of 8-arm-PEG

With the initial results from *in vitro* and *ex vivo* studies demonstrating the inhibitory property of 8-arm-PEG on CYP3A4, the ability of 8-arm-PEG to inhibit *in vivo* CYP3A4 activity and translate such inhibition to an increase in oral bioavailability was investigated in the Large White pig model.

5.2.8.1. Procurement and habituation of pig specimens

Four Large White female pigs weighing 29.2±0.8kg were purchased from Animal Nutrition and Products Institute of the Agricultural Research Council (Pretoria, Gauteng, South Africa) and housed in standard animal rooms (Central Animal Service Unit, University of the Witwatersrand, Johannesburg, South Africa) where they were maintained in a 12-hour light/darkness cycle. The pigs were ascertained healthy by veterinary experts, fed with commercial pig feed and monitored for adaptation to the new environment. They were allowed a 10-day habituation period during which they were domesticated through feeding and frequent visiting (Figure 5.3). This was done to acculturate the pigs to subsequent processes of catheter flushing and plasma sampling.



Figure 5.3: Typical habituation process of the procured Large White female pigs.

5.2.8.2. Surgical catheterization for long-term repetitive blood sampling

The four pigs weighing 28.5kg, 28.8kg, 29.4kg and 30kg, respectively were anaesthetized with ketamine (11mg/kg) and midazolam (0.3mg/kg) administered intramuscularly. Analgesia was maintained by intramuscular administration of buprenorphine (0.05mg/kg) and carprofen (4mg/kg). The pigs were then intubated under anesthesia with 2% isoflurane in 100% oxygen. A 7 french gauge double lumen 35cm catheter (CS-28702) (Arrow Deutschland GmdH, Erding, Germany) was surgically inserted into the jugular vein of each pig under aseptic conditions. An incision, dorsal to the jugular groove on the left lateral side of the neck was made to expose the jugular vein. The vein was isolated and the catheter inserted 10cm into the lumen of the vein via a blunt dissection. The lumen of the catheter was fastened to the wall of the vein employing a purse suture procedure. With the use of trocar, the remaining 25cm length of the catheter was tunneled subcutaneously to an exit point cranial to the dorsal aspect of the scapular. In order to limit excessive movement and kinking, the externalized injection ports of the catheter were sutured to the skin of the pig. The catheter was cleaned by withdrawal of blood through it, followed by flushing with heparinized saline (1000 IU/Liter of 0.9% saline). The surgical catheterization procedure is depicted in Figure 5.4. The animals were observed over a period of 1 hour for recovery from anesthesia and then allowed further 8 days to recover from the surgical procedure before the commencement of dosing and sampling. During this period, the catheter was flushed with heparinized saline (1000IU/L normal saline) every 12 hours to prevent blood coagulation and to maintain aseptic conditions.

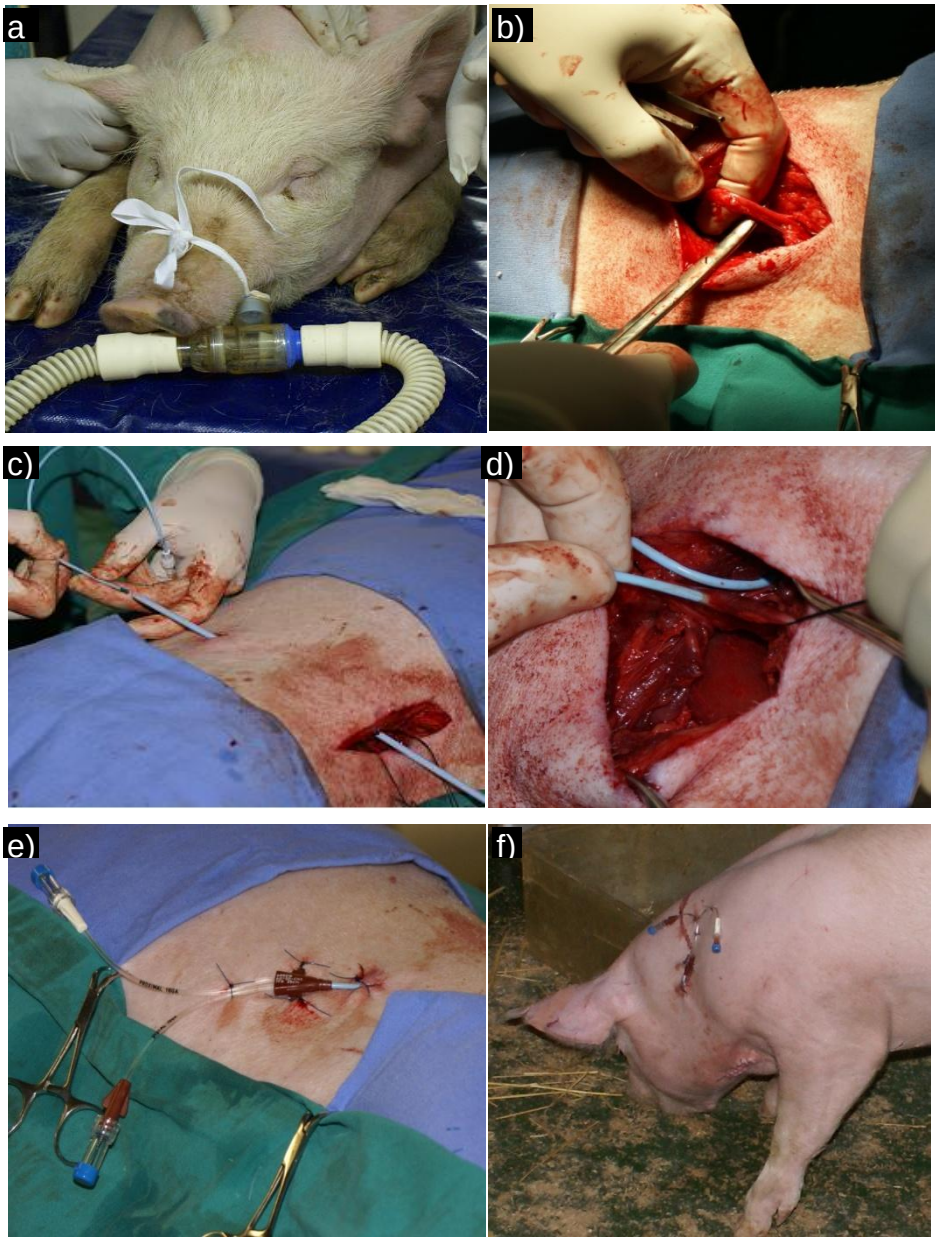


Figure 5.4: The surgical procedure for the jugular vein catheterization of experimental pigs depicting a) anesthesia and skin preparation for surgery, b)surgical incision into the jugular area, c) transverse catheterization through the neck tissue, d) isolation and catheterization of the jugular vein, e) subcutaneous tunneling of the catheter and skin suturing and f) recovery after 2 hours.

5.2.8.3. Oral dosing of the pigs with the felodipine tablet matrix containing graded concentrations of 8-arm-PEG

Dosing of the pigs was commenced after 10 post-surgery days. The jugular catheters were flushed with heparinized normal saline followed by blood sampling to obtain a pre-dosing baseline sample for control analysis. The pigs were anesthetized with intravenous ketamine (4mg/kg) and midazolam (0.3mg/kg). Anesthesia was maintained by gaseous administration of 2% isoflurane in 100% oxygen via facemask inhalation. The pigs were then held in an upright position and intubated with a bore gastric tube via the mouth

through the esophagus into the stomach. The felodipine tablet was then introduced via the tube and flushed down the stomach with 20mL of water. All intravenous felodipine dosing were performed through the jugular catheter. A minimum wash-out period of 96 hours was allowed between felodipine administrations. With an average half-life of 8.16 hours (Takanaga et al., 2000), this wash-out period allowed for the satisfactory plasma elimination of felodipine. A schematic detailing the dosing and the contents of the felodipine formulation is presented in Figure 5.5.

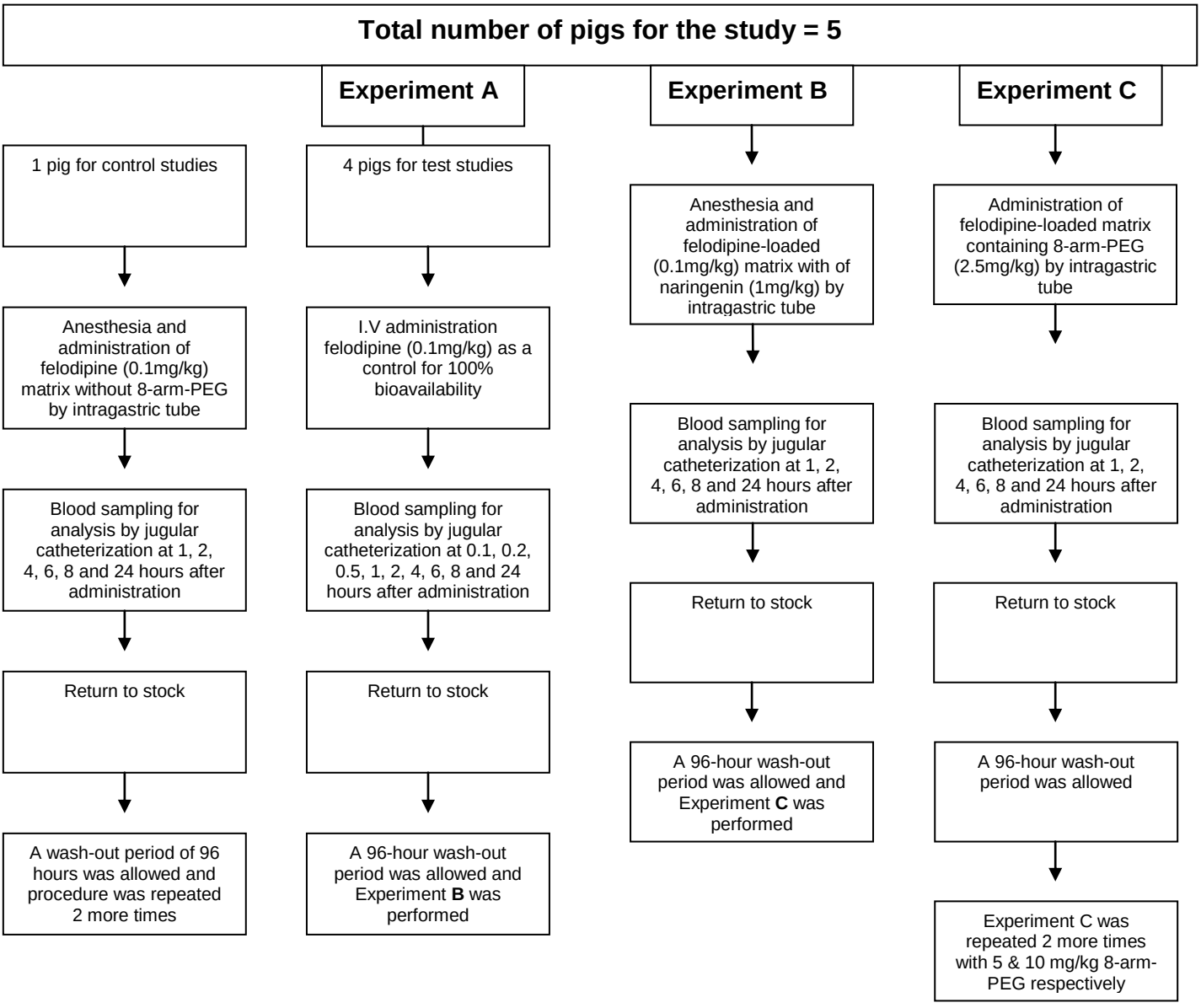


Figure 5.5: A schematic illustrating the study design for the *in vivo* experimentation in the Large White pig model.

5.2.8.4. Blood sampling for quantitative analysis of felodipine

Blood sampling from the pigs was preceded by catheter flushing with heparinized normal saline. For intravenous administration, samples were drawn after 0.1, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20 and 24 hours while sampling after oral dosing was performed at similar intervals but starting at 1 hour after dosing. All blood samples were immediately transferred into heparinized vacutainers (BD Vacutainers®, Franklin Lakes, NJ, USA) and stored at (4°C) when necessary but not for longer than 2 hours. The samples were thereafter centrifuged at 2000*g* for 20 minutes yielding a clear plasma supernatant which was pipetted with an adjustable calibrated micropipette (Boeco GmbH, Hamburg, Germany). All clear plasma samples were stored at -70°C until further analysis.

5.2.8.5. Development of an Ultra Performance Liquid Chromatographic (UPLC) method for the quantitative determination of felodipine in plasma

A method of quantitative determination of felodipine was developed employing Ultra Performance Liquid Chromatography (UPLC) (Waters® Acquity UPLC™ System, Milford, MA, USA). Isocratic baseline separation was achieved with the instrumental settings listed in Table 5.3. All solutions and solvents were filtered through a 0.22µm pore size Cameo Acetate membrane filter (Millipore Co., Billerica, MA, USA). Complete separation of felodipine and the internal standard were confirmed by three-dimensional chromatographic separation.

Table 5.3: UPLC instrumental settings for the determination of felodipine in plasma samples.

Parameters	**Values/Conditions**
Strong wash	90%$^{v}/_{v}$ acetonitrile : 10%$^{v}/_{v}$ water
Weak wash	10%$^{v}/_{v}$ water : 90%$^{v}/_{v}$ acetonitrile
Mobile phase	0.025M potassium dihydrogen phosphate, pH 2.5 and acetonitrile (40:60)
Flow-rate	0.2mL/min
Column specification	BEC C_{18} 1.7µM, 2.1x50mm
Injection volume	2µL
Run time	3 minutes
Internal Standard	10µg/mL phenacetin
Column temperature	25°C
Sample temperature	25°C
Pump pressure	6000±500psi
Delta value	<20
Wavelength of absorption	200nm

5.2.8.6. Extraction of felodipine from plasma samples

A procedure of extracting felodipine from plasma similar to that of Miglioranca and co-workers (2005) was developed for further quantitative analysis. The procedure combined liquid-liquid and solid-phase extraction techniques for optimal results. Aliquots (0.5mL) of felodipine-containing pig plasma was transferred into polypropylene tubes followed by the addition of 2mL diethyl ether:hexane mixture (80:20$^{v}/_{v}$). The samples were then vortexed for 1 minute and centrifuged at 2000*g* for 5 minutes. The supernatant was transferred to clean propylene tubes. A further addition of 2mL diethyl ether:hexane mixture was performed on the lower layer, vortexed and centrifuged for further felodipine extraction. The supernatants were combined in clean propylene tubes and loaded onto acetonitrile-conditioned Oasis® HLB cartridges enabled with the a Visiprep Vacuum Manifold (Waters Milford, MA, USA) followed by a further wash with 1mL diethyl ether:hexane mixture and collected in clean test tubes. The collected felodipine extracts were evaporated under nitrogen at 40°C. The dry residue was dissolved with 500µL acetonitrile and filtered through 0.22µm Cameo Acetate membrane filters. Equal volumes of the filtrate and the internal standard (phenacetin) were then transferred into UPLC vials, capped and placed in the autosampling compartment of the UPLC for analysis.

5.2.8.7. Calibration standards and limits of quantification for plasma felodipine

Stock solution of felodipine was prepared in acetonitrile at concentrations of 1mg/mL. The internal standard (phenacetin) solution of 10µg/mL was similarly prepared. Blank (drug-free) pig plasma samples were spiked with serially diluted felodipine solution yielding final theoretical plasma felodipine concentrations of 0.2441 0.9760, 3.90625, 15.625, 31.25, 62.5, 125, 250, 500 and 1000nmol/L. The spiked plasma samples were subjected to the extraction method described in Section 5.2.8.6. The ratio of the AUC (of the chromatogram) of felodipine/internal standard was profiled against the corresponding felodipine concentrations expressed in ng/mL. Linear regression and correlation coefficients were obtained by means of the least square method. The sensitivity of the method was assessed by employing a large number of serial dilutions and low felodipine concentrations were employed to estimate the lower limit of quantification. The limit of quantification according to Mandrioli and co-workers (2006) is the analyte concentration which produced chromatographic peaks with heights at least 3 times that of the baseline noise.

5.2.8.8. Validation of the extraction method

Standard solutions of felodipine at three different concentrations were separately added to 0.5mL blank plasma in order to obtain plasma felodipine concentrations of 100mmol/L, 1µmol/L and 0.25nmol/L. The prepared mixtures were subjected to the described extraction procedure and analyzed by UPLC. By comparing the chromatogram AUC of the felodipine obtained employing the extraction method with those obtained from standard solutions at the same theoretical concentrations, the extraction yield (%) for the samples was calculated. Intra-day reproducibility was determined by multiple injections (N=3) during a 24-hour period while the inter-day uniformity was assessed by multiple injections of samples over 3 consecutive days (N=3) for each day. The within- and between-run precisions were determined as the Relative Standard Deviation (RSD) using Equation 5.4 where *M* is the mean.

$$RSD\ (\%) = \frac{100SD}{M} \qquad \text{Equation 5.4}$$

The accuracy was assessed as the percentage Relative Error (RE) using Equation 5.5.

$$RE\ (\%) = (E - T)(\frac{100}{T}) \qquad \text{Equation 5.5}$$

where *E* is the experimentally determined felodipine concentration and *T* the theoretical concentration.

5.3. Results and Discussion

5.3.1. Assessment of the tablet formulatory properties of 8-arm-PEG

The routine tableting formulation properties investigated with 8-arm-PEG indicated that the polymer has satisfactory tableting characteristics. Its possible inclusion in a tablet matrix may not negatively impact on the tablet features. The specific outcomes of the investigations are discussed in the following sub-sections.

5.3.1.1. Formulation of the 8-arm-PEG tablet matrix by direct compression

The triturated 8-arm-PEG powder was free-flowing and when compressed yielded uniform-sized and smooth-surfaced tablets. Compared to the brittle-textured powder, the tablets were sufficiently compact and robust. The ability of 8-arm-PEG to be easily compressed without the need for pre-compression granulation indicated its suitability as

an effective excipient in direct compression tableting. The tablets formed were free of tableting flaws such as capping, chipping and sticking. This was observed despite the non-inclusion of a lubricant, binder and/or anti-adherents. Due to the superior compactibility, capability of self-lubrication and non-adherence to the punch or the die wall, 8-arm-PEG possessed desirable properties for effective tableting. This is consistent with the pharmaceutical application of PEG as a lubricant, binder and/or a diluent to improve the rheological properties of tablet formulations and granules (Larhrib and Wells, 1998). These observed properties confirmed the retention of the tablet-formulation properties of PEG in 8-arm-PEG as a derivative of PEG.

5.3.1.2. Investigation of mass uniformity of the 8-arm-PEG tablet matrix

The mass of 10 randomly selected 8-arm-PEG tablet matrices are listed in Table 5.4. Results show that the tablets had relatively uniform weights within the USP permitted range of 1%. This further confirmed the desirable tableting properties of 8-arm-PEG. The absence of capping and chipping in the compression process due to the free-flowing nature of 8-arm-PEG enhanced the mass uniformity of the tablets.

Table 5.4: Evaluation of mass uniformity of 10 randomly selected 8-arm-PEG tablet matrices.

Tablet	Weight	Weight deviation (%)
1	597.55	0.02
2	596.71	0.16
3	599.83	0.35
4	595.35	0.39
5	598.11	0.07
6	599.30	0.27
7	597.56	0.02
8	596.99	0.12
9	598.17	0.08
10	597.37	0.05

5.3.1.3. Matrix Indentation Hardness

The BHN values of equal mass (600mg) 8-arm-PEG and HMPC tablet matrices were compared. Results indicated that HPMC tablet matrices were slightly harder than 8-arm-PEG tablet matrices although both compare favorably (Table 5.5).

Table 5.5: The comparative hardness of compressed 8-arm-PEG and HPMC.

Tablets	8-arm-PEG		HPMC	
	Indentation Force (N)	BHN	Indentation Force (N)	BHN
1	2.830	1.460	2.962	1.526
2	2.827	1.459	2.961	1.528
3	2.829	1.460	2.959	1.527

5.3.1.4. Comparative friability tests on 8-arm-PEG and HPMC tablet matrices

The result of the friability tests is summarized in Table 5.6. The fact that the tests yielded results within the 1% limit with only 0.73% of the tablet mass lost after four minutes of friabilator rotation is another indication that 8-arm-PEG as an excipients can be employed to produce tablets with pharmaceutically acceptable hardness.

Table 5.6: Instrumental settings and results of friability tests on 8-arm-PEG tablet matrices.

Parameters for Friability Test	Values	
	8-arm-PEG	HPMC
Number of Tablets used	10	10
Test Duration (minutes)	4	4
Weight of tablets before test	5937.32	5980.33
Weight of tablets after test	5894.15	5951.74
The difference (weight of dust)	43.17	28.59
Friability (weight loss, %)	0.73	0.48

The results thus depicted 8-arm-PEG as a potential multi-purposed pharmaceutical excipient with physicochemical and formulation properties compatible with tablet formulation requirements.

5.3.1.5. Assessment of chemical structural changes of 8-arm-PEG due to direct compression

Possible change to the structural and morphological configuration of 8-arm-PEG was verified. Polymeric materials often undergo complex stress under compression leading to successive and simultaneous molecular and atomic rearrangement, elastic and plastic deformation, fracture, formation and/or breakage of inter-particulate bonds, cold sintering, structural deformation among others (Baker et al., 2000). The response of individual pharmaceutical materials to compression depends on the physicochemical properties of the material. Crystalline materials may exhibit different responses compared to polymers and amorphous substances. Possible deformation to the molecular and structural

arrangement of 8-arm-PEG was therefore verified employing FTIR. The spectrum of powdered samples of 8-arm-PEG served as a control for comparing with the directly compressed 8-arm-PEG. As seen in Figure 5.6, the superimposed spectra exhibited visual similarity with a 99.98% spectral similarity between the powdered and directly compressed 8-arm-PEG samples. This suggested that 8-arm-PEG was structurally stable to direct compression within the limit of pressure applied (5 metric tons).

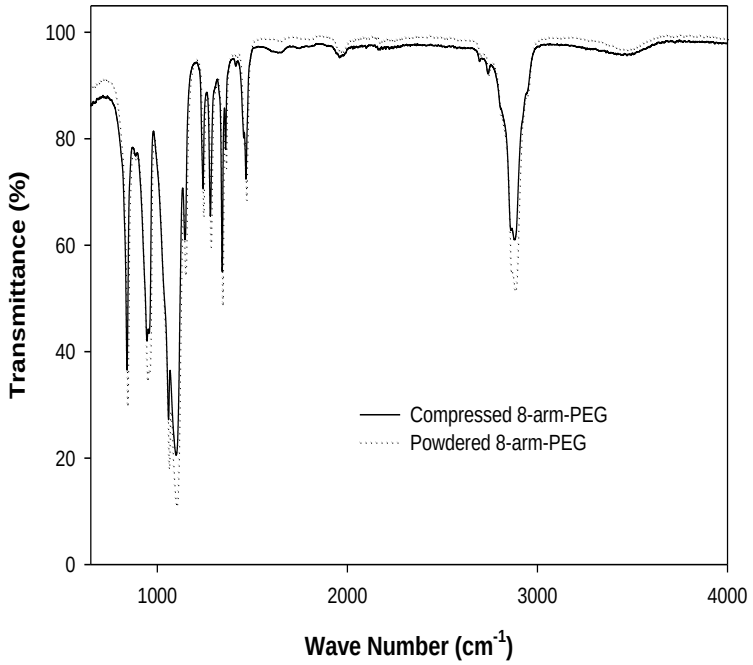


Figure 5.6: FTIR spectra of powdered and compressed 8-arm-PEG generated.

5.3.2. The influence of 8-arm-PEG on *in vitro* felodipine release

In vitro release profiles of felodipine from graded concentrations (0-100%$^{w}/_{w}$) of 8-arm-PEG in HPMC is presented in Figure 5.7. The release pattern from HPMC (0% 8-arm-PEG) was fairly consistent over 10 hours. In the 100% 8-arm-PEG matrix, there was an initial burst-release of 65% of felodipine in 1 hour. This may be attributed to the free aqueous solubility of 8-arm-PEG and deposition of surface drug on the tablet matrix. The subsequent phase of drug release was gradual over 3 hours suggesting dissolution rate-dependent release of felodipine due to its poor water-solubility. In the other tablet matrices containing graded concentrations of 8-arm-PEG, felodipine release was generally faster with increasing concentration of 8-arm-PEG.

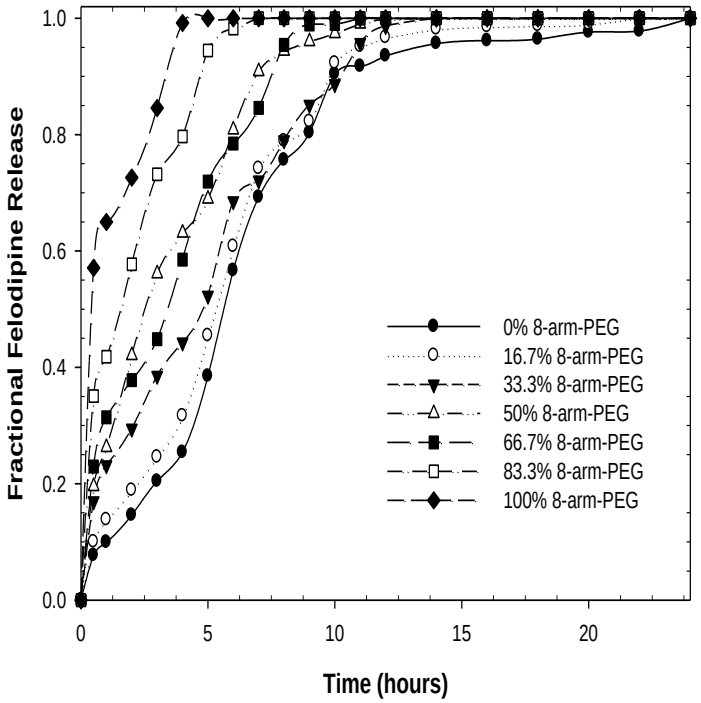


Figure 5.7: The influence of graded concentrations of 8-arm-PEG on *in vitro* felodipine release (N=3; S.D.<0.003 in all cases).

5.3.3. The influence of 8-arm-PEG on the intestinal tissue metabolism of felodipine

An *ex vivo* method for felodipine metabolism was developed employing pig intestinal tissue in order to assess the effects of 8-arm-PEG on intestinal tissue metabolism of felodipine. Results obtained from the *ex vivo* studies are shown in Figure 5.8. There was concentration-dependent inhibition of felodipine metabolism by naringenin such that at 1mM concentration, complete inhibition of felodipine metabolism was observed. With the aid of Enzyme Kinetic Module software (SigmaPlot: Enzyme Kinetic Module, Systat Software Inc., Chicago, IL, USA), the IC_{50} was determined to be 179.88µM. When compared with results from the *in vitro* study where the IC_{50} value was 0.0332µM, naringenin appeared less potent. Several factors can be responsible for this. The incubation of felodipine and naringenin in HLM mixtures in the *in vitro* study provided direct contact with the enzyme, confining binding interactions between naringenin, felodipine and the enzyme. However, in the *ex vivo* studies, other enzymes and non-enzyme protein entities were potentially capable of binding these substrates. Efflux proteins and cellular transporters such as p-glycoproteins are capable of interacting with chemical molecules (Cong et al., 2001). Although the binding affinities at different receptor sites vary, the existence of multiple binding sites in intestinal tissues would make

naringenin appear less potent at CYP3A4 sites just as higher felodipine concentrations are required for maximal metabolic activity. This can be said to account for the wide difference in the IC_{50} values of the *in vitro* and *ex vivo* felodipine metabolism inhibition by naringenin.

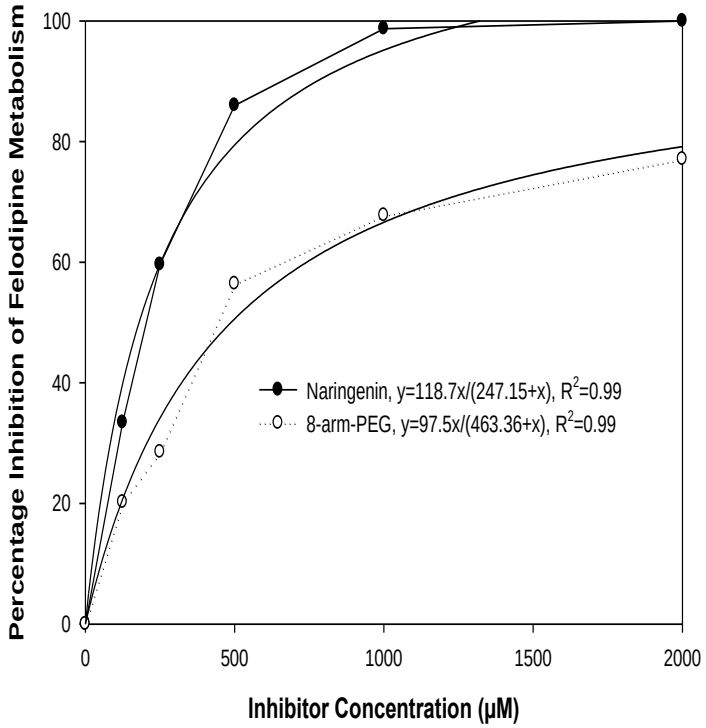


Figure 5.8: The influence of naringenin and 8-arm-PEG on intestinal tissue metabolism of felodipine (N=3; S.D.<0.01 in all cases).

Results also show that 8-arm-PEG exerted inhibitory effects on felodipine metabolism by intestinal CYP3A4. Its IC_{50} value was 487.75µM. With an IC_{50} value of 7.22µM, the *in vitro* result demonstrated >50 times potency of 8-arm-PEG against felodipine metabolism. Similar factors may be responsible for the differences in the *in vitro* and *ex vivo* potencies of 8-arm-PEG. At a concentration corresponding to the *ex vivo* IC_{50}, the pharmaceutical implication is that at a 0.5mM concentration, 8-arm-PEG has the potential of inhibiting the first-pass metabolism of felodipine by 50%. Given that the oral systemic bioavailability of felodipine is 15-20% with 80-85% metabolized by pre-systemic CYP3A4, 0.5mM 8-arm-PEG thus has the potential of reducing this loss to 40-42.5% leading to 58.5-60% oral felodipine bioavailability.

The European Centre for the Validation of Alternative Methods (ECVAM) promotes the scientific and regulatory acceptance of alternative methods which are of importance to the

biosciences and which reduce, refine or replace the use of laboratory animals (Eric et al., 2001). The need to use co-factors at high concentrations for the *in vitro* functionality of HLM implies a physiologically disproportional condition for enzyme activity. This variation in physiological and biochemical environment may pose a significant challenge in extrapolating *in vitro* metabolic results for *in vivo* correlation. Metabolic studies employing intestinal tissues presents a closer *in vivo* behavior than the use of HLM because of the presence of an operable cell membrane and physiological and biochemical environment for intestinal enzyme activity. The use of caco-2 cell layer has also been promoted as a suitable alternative to *in vitro* HLM use. This however could also be challenging. According to Sun and co-workers (2002), inter-laboratory reproducibility of caco-2 cells is difficult. These cell layers also lack metabolic enzymes such as CYP3A4 at concentrations comparable to physiological values (Sun et al., 2002).

The intestinal epithelium serves as the gatekeeper, controlling the passage of nutrients and xenobiotics out of the intestinal compartment into the system circulation (Masaoka et al., 2006). The rich expression of metabolic enzymes in intestinal tissues makes it a suitable model for drug metabolism studies (Kanter et al., 2005). The study of the intestinal enzyme development has revealed a higher level of similarity between human and pig CYP450 than other commonly used experimental animals including rat, mouse and rabbit (Shulman et al., 1998). Apart from the well-known similarity between pig and human gastrointestinal anatomy and physiology compared to other non-primate mammals, Shulman and co-workers (1998) concluded that the degree of small intestinal maturation and the similar intestinal enzyme distribution compared with that of the human infant suggest that the pig is an excellent model for studies of intestinal enzyme development, regulation and physiological functions. In a similar study, Anzenbacher and co-workers (1998) employed nifedipine oxidation and testosterone 6β-hydroxylation which are specific CYP3A4 markers to identify the presence of CYP3A4 in pigs with similar anatomical distribution as in humans.

The present study was able to develop and apply a method for the preparation of slices from the intestines that retain adequate viability and activity. It was expected that a closer *in vivo* correlation would be obtainable through *ex vivo* tissue studies. It was also envisaged that *in vivo* metabolic activity will be replicated in freshly excised and cultured metabolic enzyme-expressed tissues in simulated physiologic fluids. Although all *ex vivo* activity including metabolism inhibition by test compounds may not necessarily be similar in magnitude *in vivo*, it was reasoned that a test inhibitor that failed *ex vivo* investigation may likely lack *in vivo* effectiveness (Kanter et al., 2002; 2005). This is important for

exclusion of unnecessary utilization of laboratory animals except where confirmatory studies are prompted by positive *ex vivo* results.

5.3.4. The influence of 8-arm-PEG on the oral bioavailability of felodipine in the pig model

5.3.4.1. Recovery of felodipine from pig plasma

The developed extraction technique was applied for plasma felodipine extraction in order to remove plasma constituent interference and isolate felodipine from the plasma samples. Diethyl ether has poor aqueous solubility and is immiscible with water. Hexane with similar properties dissolves most non-polar organic compounds. Both compounds are volatile. Liquid-liquid extraction of felodipine from human and dog plasma through the application of a diethyl ether: hexane mixture has been reported to be effective for accurate analytical purposes (Kim et al., 2003; Miglioranca et al., 2005). In addition, the employment of Oasis HLB cartridges for the extraction provided an added advantage of impurity removal. The passage of the dissolved felodipine residue through 0.22µm Cameo Acetate membranes filter prepared the sample for UPLC analysis.

5.3.4.2. Quantitative UPLC analysis of plasma felodipine

A typical chromatogram depicting the retention of felodipine and phenacetin (internal standard) at 1.795±0.01 and 0.590±0.01 minutes respectively is displayed in Figure 5.9. This indicates that the extraction procedure was effective in isolating and eluting felodipine. A positive control for the extraction method containing blank plasma was also subjected to UPLC analysis yielding a chromatogram displayed in Figure 5.10. Complete separation of felodipine and the internal standard devoid of interference was confirmed through three-dimentional chromatography (Figure 5.11).

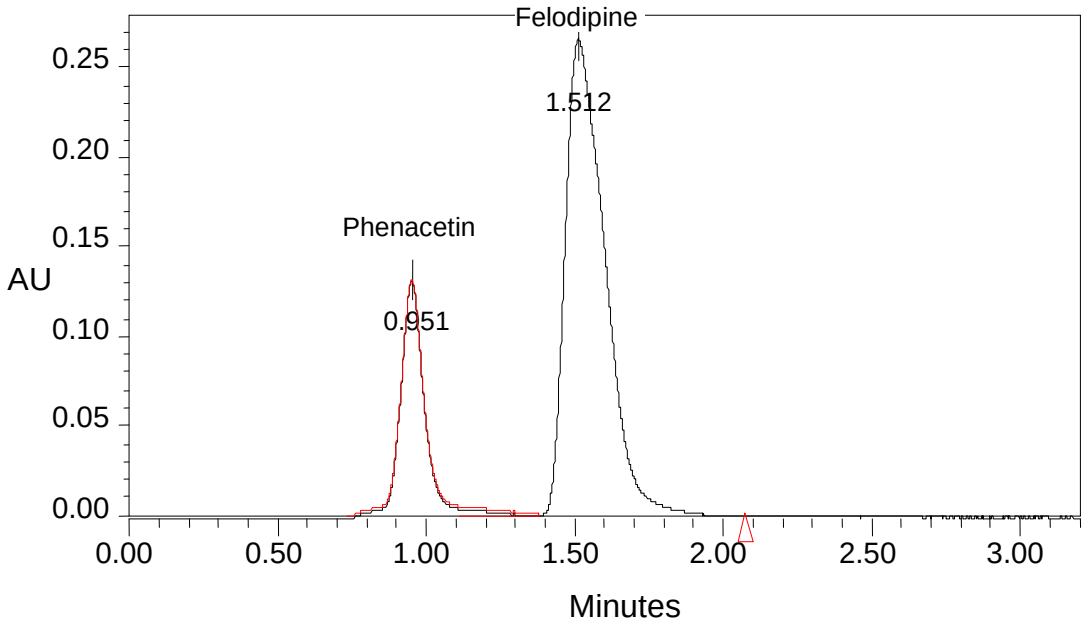


Figure 5.9: A typical UPLC chromatogram indicating the separation and retention time of felodipine (1.795) and the internal standard (0.590 minutes).

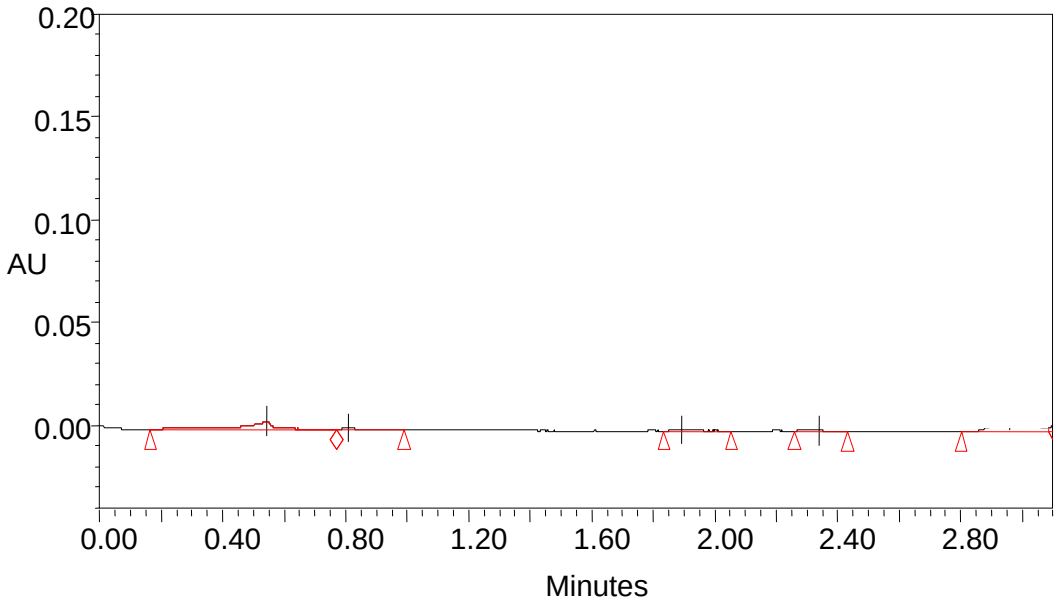


Figure 5.10: A typical UPLC chromatogram obtained from felodipine-free pig plasma.

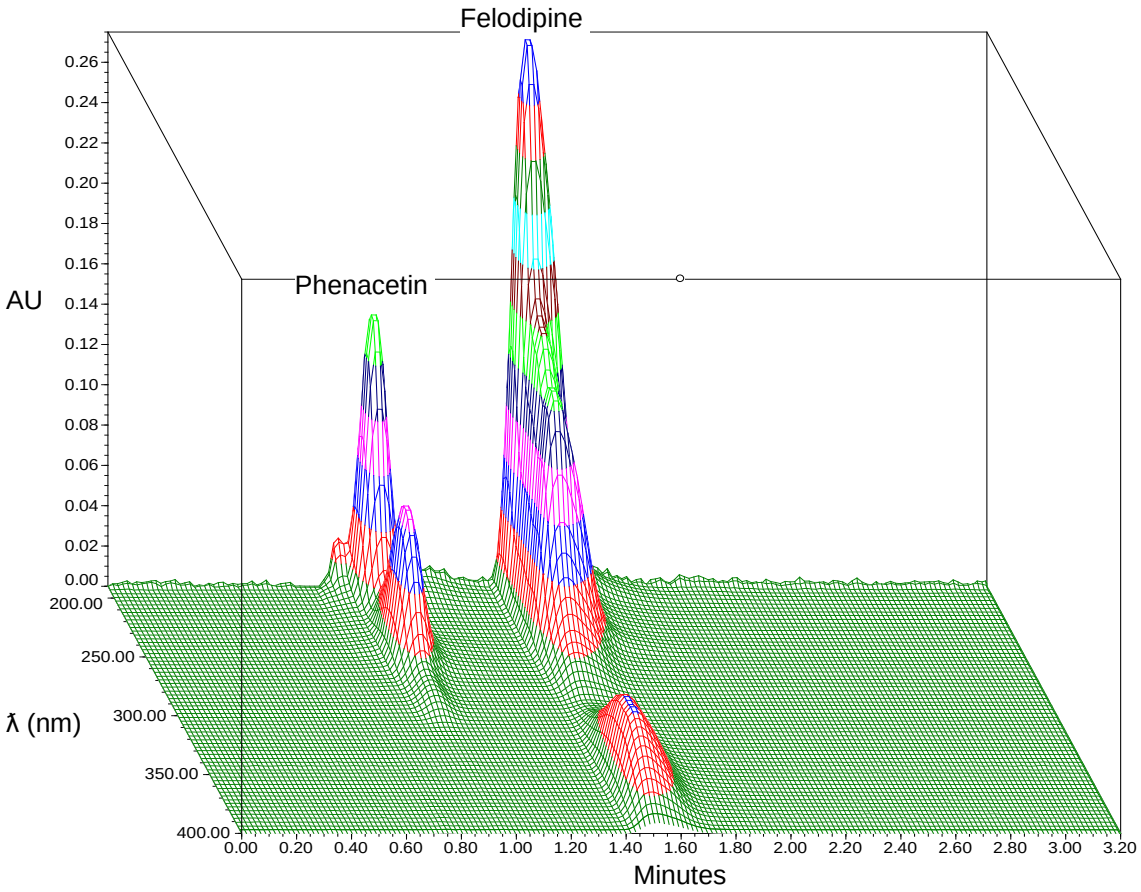


Figure 5.11: A three-dimensional chromatogram depicting a complete separation of felodipine and the internal standard and the absence of interference.

5.3.4.3. Calibration curve and the limit of quantification of plasma felodipine

Figure 5.12 shows the calibration curve for the quantitative determination of plasma felodipine. Plasma felodipine concentration range of 0.25-1000nmol/L offered satisfactory linearity (R^2=0.99) with a lower limit of quantification of 0.244nmol/L.

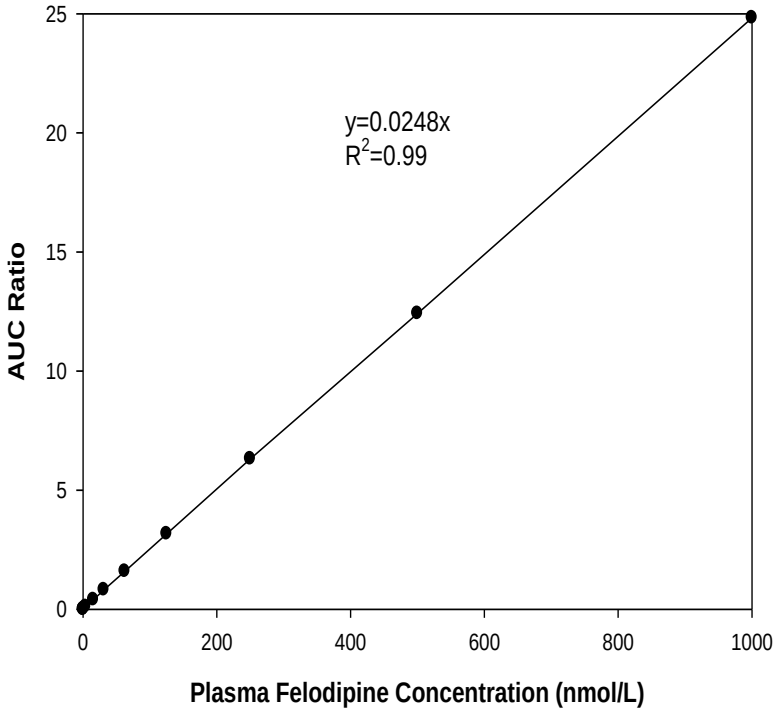


Figure 5.12: A standard calibration curve generated for quantifying felodipine concentration from plasma samples employing UPLC (N=3; S.D.<0.002 in all cases).

5.3.4.4. Method validation for plasma felodipine extraction and UPLC analysis

The recovery of felodipine from plasma samples employing three sample concentrations of 0.25nmol/L, 1µmol/L and 1mmol/L evaluated over 3 consecutive days ranged from 96.71 to 101.06%. The method validation data depicting the extraction efficiency, intra- and inter-day variation/uniformity are summarized in Table 5.7.

Table 5.7: Data for assessing the extraction efficiency of plasma felodipine.

Theoretical plasma felodipine concentration	**Intra-day**		**Inter-day**	
	Extraction yield (%)	CV (%)	Extraction yield (%)	CV (%)
0.25nmol/L	96.71±0.00008	0.017	96.67±0.00018	0.0051
1µmol/L	98.93±0.0006	0.015	98.90±0.00071	0.021
1mmol/L	101.06±0.0031	0.0053	0.053±0.0041	0.047

CV = Coefficient of Variation

5.3.4.5. The influence of graded concentrations of 8-arm-PEG on the oral bioavailability of felodipine in Large White pig model

Figures 5.13 and 5.14 depict the influence of graded concentrations (2.5mg/kg, 5mg/kg and 10mg/kg) of 8-arm-PEG on the mean plasma concentration of felodipine following oral administration in pig models. Theoretical 100% absolute bioavailability which was equivalent to a peak plasma felodipine concentration (C_{max}) of 33.137nmol/L was yielded following intravenous administration of 10mg felodipine. This served as the reference bioavailability measurement. The control inhibitor-free formulation produced a C_{max} of 6.310nmol/L which was equivalent to 19.04% absolute bioavailability. This agrees with literature values of oral felodipine bioavailability independent of the formulation to be 15-20% (Dorne et al., 2003). Table 5.8 summarizes the effects of the enzyme inhibitors on the oral bioavailability of felodipine.

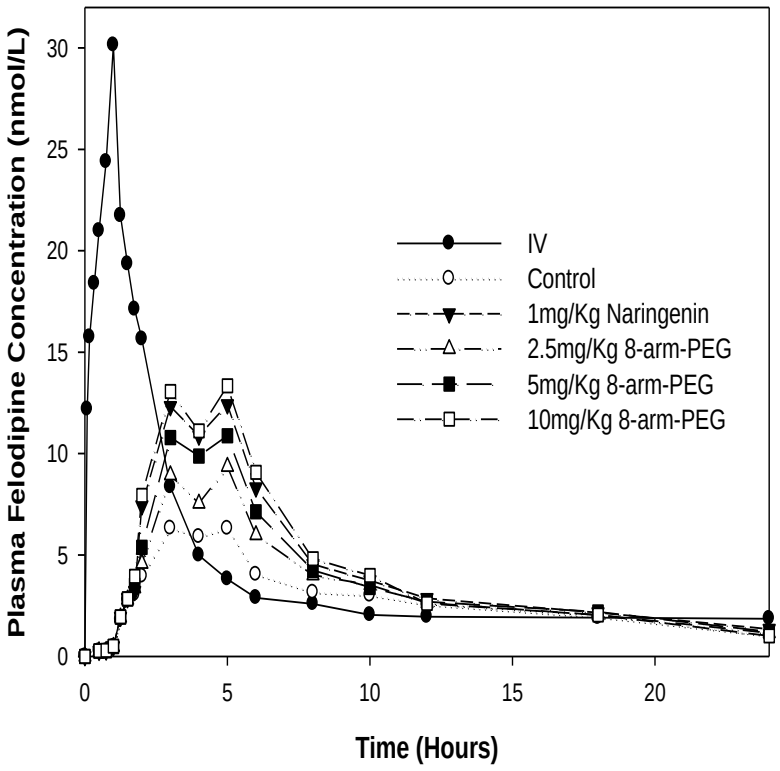


Figure 5.13: Plasma felodipine concentration- time profiles demonstrating the influence of graded concentrations of 8-arm-PEG and naringenin on oral felodipine bioavailability (N=5; S.D.<0.006 in all cases).

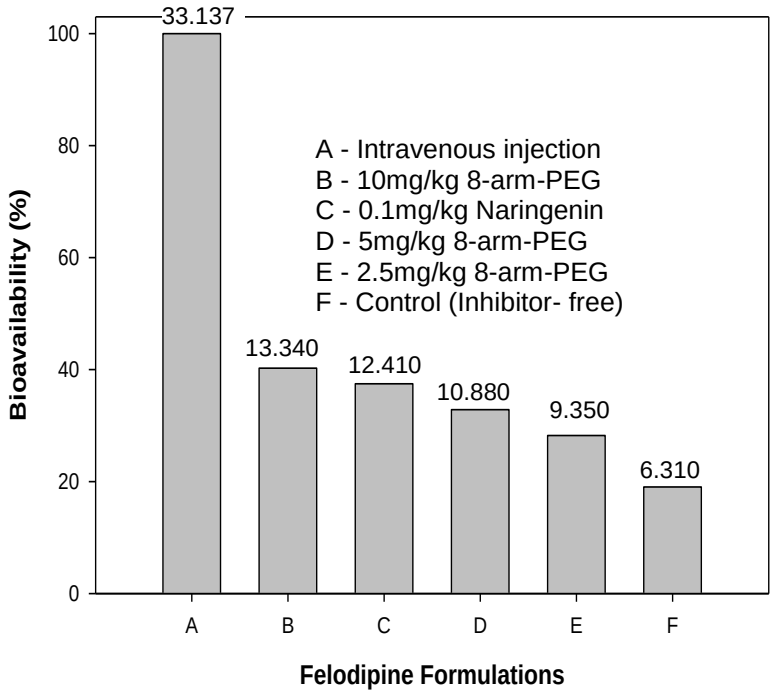


Figure 14: The effects of 8-arm-PEG on the oral bioavailability of felodipine in pigs (indicating peak plasma felodipine concentrations in nmol/L)

As displayed in Figure 5.13, only the C_{max} was significantly affected by the presence of the enzyme inhibitors while the time required for the orally administered felodipine to reach peak plasma concentration (t_{max}) and elimination profile were relatively unchanged. This suggested that the enzyme inhibition was limited to the pre-systemic sites allowing systemic felodipine clearance. This has a desirable drug delivery implication as the enzyme inhibition poses no toxicological significance at a reduced therapeutic dose. From Table 5.8, 8-arm-PEG caused a dose-dependent increase in the oral bioavailability of felodipine. Thus, with the inclusion of 10mg/kg of 8-arm-PEG in felodipine oral formulations, a 55.5% reduction in the conventional dose can achieve similar oral bioavailability. This implies that 4.5mg, in the presence of 10mg/kg 8-arm-PEG can achieve a C_{max} of 10mg felodipine in conventional formulations. The required frequency of administration of the felodipine may, however, not be affected since the elimination profile was not altered by the presence of the enzyme inhibitor.

Table 5.8: The effects of 8-arm-PEG on oral bioavailability of felodipine in pigs.

Formulation content	Increase in oral felodipine bioavailability (%)
2.5mg/kg 8-arm-PEG	48.21
5mg/kg 8-arm-PEG	72.48
10mg/kg 8-arm-PEG	111.40

Although alternative methods for routine drug absorption metabolism and other investigations are desirable to replace the use of laboratory animals, the use of experimental animals is necessary in certain cases where biochemical and physiological conditions closely resembling that in humans are desired. The use of pigs offers ease of breeding and handling advantages compared to non-human primates. This, in addition to anatomomical and morphological similarities in absorption physiology as mentioned earlier (Section 5.3.3), makes pigs the preferable models for drug absorption and metabolism studies.

In a study of the effects of PEG 400 on p-glycoprotein efflux and enterocyte-based metabolism utilizing rat jejunal tissue mounted in diffusion chambers, Johnson and co-workers (2002) reported that the presence of PEG 400 from a concentration between 1-20%$^{w}/_{v}$ exerted an inhibitory effect on p-glycoprotein efflux regardless of whether the polymer was added to the mucosal side, the serosal side or both sides of the intestinal tissue. Molecular interaction of PEG derivatives at such molecular level could only have suggested further investigations for enzymatic modulation since p-glycoprotein and intestinal CYP are closely related and synergistic in activities leading to first pass effects.

5.4. Concluding Remarks

This phase of the study investigated and demonstrated that 8-arm-PEG possesses multi-purpose tableting features including self-lubricating, disintegrant and binding properties which are desirable in tableting. The *ex vivo* tissue metabolism studies also demonstrated that 8-arm-PEG is capable of inhibiting intestinal CYP3A4 significantly inhibiting the CYP3A4-dependent metabolism of felodipine. Employing pig models, the CYP3A4-inhibiting features of 8-arm-PEG was successfully translated into improved oral felodipine bioavailability. The systemic elimination profiles of felodipine appeared unaltered by the presence of 8-arm-PEG suggesting that 8-arm-PEG exerts its inhibitory properties at pre-systemic sites of drug absorption, thus allaying toxicological fears. This study, therefore,

proposes that 8-arm-PEG possesses the potential of significantly reducing the required oral dose of felodipine in clinical practice with no influence on the required frequency of drug administration.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The various biochemical, anatomical and physiological barriers to oral drug absorption and bioavailability have received considerable attention in drug delivery studies. The various approaches aimed at enhancing oral drug delivery were presented in this study. Being responsible for the metabolism and pre-systemic metabolism of greater than 50% of all administered drugs, the CYP enzyme family was identified as the single most important factor influencing oral drug delivery for enhanced bioavailability. Various oral drug delivery systems designed to enhance trans-mucosal permeation and afford modified drug release including site-targeting were described in this study. This study also noted that while these drug delivery systems significantly enhance oral drug absorption, only minimal effects were reported on absolute bioavailability. An effective inhibition of pre-systemic CYP presents a potentially effectual means of improving the oral bioavailability of the many poorly bioavailable drugs.

The phenomena of food-drug and drug-drug interactions, often considered in clinical pharmacotherapy for toxicological purposes, have been known to occur via interferences with CYP activity. The principles involved in these interactions leading to altered plasma drug levels and modified pharmacokinetic profiles was explored with the intention of preventing pre-systemic CYP-induced drug loss.

In this research, methods for *in vitro* utilization of human liver microsomes were validated through *in vitro* metabolism of felodipine and production of acetaminophen from CYP1A2-catalyzed phenacetin o-deethylation.The study also confirmed that the component flavonoids, the major phytochemicals found in grapefruit juice are responsible for grapefruit-drug interactions. Specific flavonoids investigated including quercetin, naringenin and its glycoside, naringin did inhibit CYP-induced felodipine metabolism to varying degrees.

Computational modeling and biomimetism was employed through a multi-disciplinary biochemical approach to simulate flavonoid-CYP3A4 reactions and consequently to

generate biocompatible and biodegradable pharmaceutical grade polymers with the potential of interacting with CYP3A4 metabolic activity. A number of the generated polymers demonstrated potent inhibitory ability against felodipine metabolism through *in vitro and ex vivo* studies with 8-arm-PEG being the most potent. The successful development and validation of an *ex vivo* technique employing pig intestinal tissue for drug metabolism also confirmed the *in vitro* results prompting animal studies.

White Pig models, widely accepted to possess an acceptable level of similarity with human gastrointestinal physiology and anatomy, and closer biochemical similarities in cytochromal proteins, were employed to investigate the ability of 8-arm-PEG to translate CYP-3A4-inhibiting property to enhanced oral bioavailability of felodipine. A two-fold increase in peak plasma felodipine after 10mg/Kg 8-arm-PEG administration confirmed 8-arm-PEG as a promising oral bioavailabilty enhancer.

6.2. Recommendations

Due to the widely held fear of toxicological consequences of CYP inhibition more extensive research is required to confirm that prospective oral bioavailability enhancers are not absorbed thus localizing enzyme inhibition to pre-systemic sites alone. The roles of gastrointestinal CYP also need to be further studied in order to establish its roles in detoxification and the safety in its activity inhibition.

The association of intestinal CYP with other enzymes and efflux proteins may also arouse research curiosity. It may be necessary to investigate the effects of 8-arm-PEG on other physiologic enzymes and proteins in order to ascertain its absolute safety. Further studies may also be advisable employing primates for a closer clinical extrapolation. Importantly, studies with human subjects are recommended to ascertain the ultimate pharmaceutical applicability of 8-arm-PEG in improving the oral bioavailability of drugs.

This study presents the potential utility of 8-arm-PEG in enhancing the oral bioavailability of CYP3A4 substrates. The cost-effectiveness of its use and the pharmaco-economic considerations are worth further research. The results of this study are however novel and very promising. It is believed that the outcome of this study will be of significant value to the polymer scientist, drug delivery expert and the toxicologist.

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APPENDIX

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2009/01/05

APPLICANT: Prof V Pillay

SCHOOL: Pharmacy and Pharmacology
DEPARTMENT:
LOCATION:

PROJECT TITLE: In vivo assessment of novel biocompatible polymeric drug delivery systems in pigs

Number and Species

40 pigs

Approval was given for the use of animals for the project described above at an AESC meeting held on 27.01.2009. This approval remains valid until 27.01.2011

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

a) Clinical monitoring of the animals for the first 24 hours after drug dosing to ensure that no unwanted side-effects occur. This monitoring should be performed by the investigators as guided by the CAS veterinarian.
b) A written report should be submitted to the AESC on the clinical safety of the drug combinations observed in one animal per drug combination before proceeding to further animals in each drug combination group.
c) A letter addressed to the chairperson of AESC should be provided to indicate the exact pharmacological combinations being studied and the route of delivery of these combinations . Obviously this should be provided when the appropriate combinations are known.
d) The protocol should be revised with the inclusion of a detailed description of the procedure that will be employed for intraduodenal infusion and the person qualified to do this.
e) The protocol should be revised with a justification and rationale for the use of 2 calcium channel blockers employed together as implied by the present protocol.
f) Discussion should occur with the CAS veterinarian regarding the precise procedures required for the collection of blood and CSF samples.

Signed: _____ (Chairperson, AESC) Date: 06/02/2009

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: _____
(Registered Veterinarian)

cc: Supervisor:
Director: CAS
Works 2000/Iain0015/AESCCert.wps