PHARMACOKINETIC STUDIES OF CIPROFLOXACIN AND THEOPHYLLINE AND OF THE INTERACTION BETWEEN THESE TWO DRUGS IN HEALTHY MALAYSIAN VOLUNTEERS

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BY

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To my wife Jameela, my sons Abdulrahman and Ammar and my daughters Fatima and Suad and my brothers and sisters.

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Ah receptor	aryl hydrocarbon receptor
ANOVA	analysis of variance
AUC_{0-t}	area under plasma drug concentration-time curve up to t hours
$AUC_{0-\infty}$	area under drug concentration-time curve from time 0 to infinity
CAR	constitutive androstane receptor
CI	confidence interval
CL_m	formation clearance of metabolite
CL_o	total body clearance
CL_R	renal clearance
C_{max}	Peak plasma concentration
CV	coefficient of variation
CYP	cytochrome P450
1,3-DMU	1,3-dimethyluric acid
FDA	Food and Drug Administration
f_m	molar urinary recovery of the respective metabolite
f_e	cumulative fraction of dose excreted in urine
FMN	flavin mononucleotide
FAD	flavin adenine dinucleotide
FMO	flavin monooxygenase
GST	glutathione-S-transferase
HPLC	high-performance liquid chromatography
k_e	elimination rate constant

Ki	inhibition constant
K_m	Michaelis-Menten constant
MI	Metabolic intermediate
mRNA	messenger ribonucleic acid
1-MU	1-methyluric acid
3-MX	3-methylxanthine
NS	not statistically significant
PAH	polycyclic aromatic hydrocarbon
P-gp	P-glycoprotein
pK_a	negative logarithm of acid ionization constant
PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
RXR	retinoid X receptor
SD	standard deviation
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SULT	sulfotransferase
$t_{1/2}$	elimination half life
T_{max}	time at which highest concentration occurs
UDP	Uridine-diphosphate
UGT	UDP-glucuronosyltransferase
Vd	Volume of distribution
V_{max}	maximum velocity of metabolism

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KAJIAN-KAJIAN FARMAKOKINETIK SIPROFLOKSASIN DAN TEOFILIN SERTA INTERAKSI ANTARA KEDUA DRUG INI DALAM SUKARELAWAN MALAYSIA YANG SIHAT

ABSTRAK

Teofilin adalah sejenis bronkodilator yang kerap kali digunakan sebagai rawatan untuk penyakit asma dan halangan pernafasan kronik. Di dalam manusia dewasa, lebih kurang 90% teofilin dimetabolismekan di hati oleh enzim sitokrom P450 (CYP), dan hanya 10% dikumuhkan melalui ginjal dalam bentuk asal. Tujuan kajian ini adalah untuk mengkaji interaksi farmakokinetik di antara regimen berganda dos oral siprofloksasin dengan teofilin dos tunggal dan menjelaskan mekanisme interaksinya di dalam sukarelawan-sukarelawan lelaki sihat. Untuk mencapai matlamat ini, kaedah HPLC yang sensitif dan selektif dengan pengesan lampau ungu telah dibangunkan dan disahkan untuk menentukan kepekatan siprofloksasin di dalam plasma dan kepekatan teofilin di dalam plasma dan urin. Koefisi variasi untuk kepersisan dan kejituan siprofloksasin kurang daripada 7.8% melebihi julat kepekatan 50 hingga 5500 ng/mL dengan had pengesanan 25 ng/mL. Keluk penentukuran untuk teofilin di dalam plasma adalah linear (R^2 = 0.9999) dalam julat kepekatan 62.5 hingga 20000 ng/mL bagi had pengesanan 31.2 ng/mL. Koefisi variasi kepersisan dan kejituan untuk intra-hari dan antara hari kurang daripada 7.1%. Purata keseluruhan pemulihan untuk teofilin dan tiga metabolitnya di dalam urin berjulat daripada 85 hingga 102%. Had pengesanannya adalah 0.625 µg/mL untuk I-MU (asid 1- metilurik), 3-MX (3-metilxantin) dan teofilin dan 1.25 µg/mL untuk 1,3-DMU (asid 1,3-dimetilurik). Koefisi variasi intra-hari dan antara hari adalah kurang daripada 8.70%. Sejumlah sembilan orang sekarelawan

lelaki yang sihat telah mengambil bahagian di dalam kajian interaksi farmakokinetik ini. Kajian ini dijalankan secara rawak, label terbuka, dos tunggal, berpuasa dan kaedah bersilang tiga. Sukarelawan-sukarelawan ini dibahagikan secara rawak kepada tiga kumpulan dengan menerima tablet siprofloksasin sahaja, teofilin sahaja dan kombinasi siprofloksasin dan teofilin dalam semasa tempoh pertama kajian. Selepas seminggu tempoh pembersihan para sukarelawan menerima rawatan silang yang lain semasa tempoh kajian kedua dan ketiga yang juga dipisahkan selama seminggu tempoh pembersihan. Di dalam kajian gabungan, rawatan awal dengan siprofloksasin 500 mg setiap 12 jam untuk enam dos diikuti dengan dos tunggal 250 mg teofilin. Purata parameter farmakokinetik dibandingkan menggunakan ujian pelajar t-berpasangan. Keputusan kajian ini menunjukkan bahawa rawatan awal dengan siprofloksasin secara signifikan meningkatkan purata $AUC_{0-\infty}$, kepekatan plasma puncak (C_{max}) dan separuh hayat($t_{1/2}$) teofilin sebanyak 33.64% ($P < 0.001$), 15.23% ($P < 0.004$) dan 16.25% ($P < 0.034$), masing-masing dibandingkan dengan rawatan teofilin sahaja. Siprofloksasin juga dikenalpasti mengurangkan klearans keseluruhan teofilin sebanyak 27% ($P < 0.001$). Tambahan pula, keputusan kajian ini menunjukkan bahawa klearans ginjal pembentukan metabolit teofilin ; 1-MU, 3-MX dan 1,3-DMU masing-masing menurun secara signifikan (54.13%, 53.18% dan 34.98%). Kajian ini juga menyatakan bahawa siprofloksasin secara signifikan meningkatkan $AUC_{0-\infty}$ dan kepekatan plasma teofilin melalui perencatan CYP1A2 yang diperantarakan oleh metabolisme pengoksidaan secara *in vivo*. Berdasarkan penemuan kajian ini, adalah amat disarankan agar paras serum harus sentiasa dipantau rapi apabila ubat ini diberikan bersama dengan siprofloksasin di dalam

amalan klinikal. Tambahan kepada kajian interaksi ini, kajian secara *in-vivo* yang lain dijalankan untuk menilai keperolehan bandingan antara Enoxin® dan Ciprobay® di dalam para sukarelawan lelaki sihat. Tiada perbezaan yang signifikan didapati berdasarkan analisis varian (ANOVA); pada 90% selang keyakinan nilai purata untuk nisbah ujian/rujukan adalah masing-masing 0.96 hingga 1.11 untuk AUC_{0-t} dan 0.97 hingga 1.10 untuk $AUC_{0-\infty}$, manakala C_{max} adalah 0.93 hingga 1.07. Adalah disimpulkan bahawa kedua-dua formulasi ini mempunyai biokesetaraan yang saling boleh disilihgantikan.

PHARMACOKINETIC STUDIES OF CIPROFLOXACIN AND THEOPHYLLINE AND OF THE INTERACTION BETWEEN THESE TWO DRUGS IN HEALTHY MALAYSIAN VOLUNTEERS

ABSTRACT

Theophylline is a bronchodilator frequently used in the treatment of asthma and chronic obstructive pulmonary disease. In human adults, approximately 90% of theophylline is metabolised in the liver by the cytochrome P450 (CYP) enzymes, and only 10% is excreted via the kidneys in unchanged form. The aims of this work were to investigate the pharmacokinetic interaction between a multiple-dose regimen of oral ciprofloxacin and single dose of theophylline and to elucidate the mechanism of such interaction in healthy male volunteers. To achieve these aims, sensitive and selective HPLC methods with ultraviolet detection have been developed and validated for the determination of ciprofloxacin in plasma and theophylline in plasma and urine. The coefficient of variation for ciprofloxacin precision and accuracy was less than 7.8% over the concentration range of 50 to 5500 ng/mL and with limit of detection of 25 ng/mL. The calibration curve for theophylline in plasma was linear (R^2 = 0.9999) in the concentration range of 62.5 to 20000 ng/mL with a detection limit of 31.2 ng/mL. The coefficient of variation for intra-day and inter-day precision and accuracy was found to be less 7.1%. The overall mean recoveries for theophylline and its three metabolites in urine ranged from 85 to 102%. The detection limit was 0.625 µg/mL for I-MU (1-methyluric acid), 3-MX (3-methylxanthine) and theophylline and 1.25 µg/mL for 1,3-DMU (1,3-dimethyluric acid). The intra-day and inter-day coefficient of variation was less than 8.70%. A total of nine healthy male volunteers participated in the pharmacokinetic

interaction study. This study was a randomised, open label, single-dose, fasting and three way crossover design. The volunteers were randomly divided into three groups and received ciprofloxacin alone, theophylline alone and combination of ciprofloxacin and theophylline tablets in the first period of the study. After a one-week washout period the volunteers received the other crossover treatments during the second and third period which were separated also by a one-week washout period. In the combination study, pretreatment with ciprofloxacin 500 mg every 12 hr for six doses was followed by a single dose of 250 mg theophylline. Mean pharmacokinetic parameters were compared by the use of a student paired t-test. The results of the study indicated that pretreatment with ciprofloxacin significantly increased the average $AUC_{0-\infty}$, the peak plasma concentration (C_{max}) and the $t_{1/2}$ of theophylline by 33.64% ($P < 0.001$), 15.23% ($P < 0.004$) and 16.25% ($P < 0.034$), respectively compared to the treatment by theophylline alone. Ciprofloxacin also markedly reduced the total clearance of theophylline by 27% ($P < 0.001$). Furthermore, our results showed that the renal clearances of theophylline metabolites formed; 1-MU, 3-MX and 1,3-DMU were significantly decreased (54.13%, 53.18% and 34.98%, respectively). This study revealed that ciprofloxacin significantly raised the $AUC_{0-\infty}$ and the plasma concentration of theophylline by inhibiting its CYP1A2 mediated oxidative metabolism *in vivo*. Based on the findings of this study, it is strongly recommended that the serum level of theophylline should be closely monitored whenever this drug is used concomitantly with ciprofloxacin in clinical practice. In addition to the interaction study another *in-vivo* study was performed to evaluate the comparative bioavailability between Enoxin® and Ciprobay® in 24 healthy male volunteers. No significant difference was found

based on analysis of variance (ANOVA); the 90% confidence intervals of the mean values for the test/reference ratios were 0.96 to 1.11 for AUC_{0-t} and 0.97 to 1.10 for $AUC_{0-\infty}$ respectively, while that of C_{max} was 0.93 to 1.07. It was concluded that both formulations are bioequivalent and thus interchangeable.

CHAPTER 1

INTRODUCTION

1.1 PRINCIPLES OF DRUG METABOLISM

Humans are exposed daily to a wide types of foreign compounds called xenobiotics ie. substances absorbed through the lungs or skin or ingested either inadvertently as those present in food and drink or deliberately as drugs for therapeutic purposes (Katzung, 2001a). These xenobiotics, including drugs, are eliminated from the body by several means. Most of pharmacologically active compounds are lipophilic which enables them to pass through biological membranes. These lipophilic compounds need to be biotransformed into more water soluble metabolites in order to be excreted into urine or bile (Meyer, 1996). This biotransformation is catalyzed in humans by a number of drug metabolizing enzymes. Metabolites formed are often less active than the parent drug or even inactive, but the metabolites may also show enhanced pharmacological activity. In addition, the metabolism of drugs can produce harmful and toxic products which may be responsible for various forms of toxicity such as hepatotoxicity (Nelson *et al*., 1996).

Liver is the major organ for drug biotransformation (Meyer, 1996), but drug-metabolizing enzymes are also present at other sites, such as the intestinal wall, kidney, lung and skin (Krishna and Klotz, 1994). One major enzyme system involved in drug biotransformation is the cytochrome P450 (CYP) monooxygenase system. Other important enzymes include esterases, epoxide hydrolase and conjugating enzymes such as glucuronosyltransferases and sulfontransferases.

Drug metabolism is normally divided into two phases; functionalisation (phase I) and conjugation (phase II) reactions, respectively (Gibson and Skett, 2001). Phase I reactions refer to a set of reactions that result in relatively small chemical changes that make compounds more hydrophilic and also provide a functional group that is used to complete the phase II reactions. The phase I reactions are mostly oxidative, incorporating a functional molecule such as a hydroxyl (-OH) group into the drug molecule (Meyer, 1996). Generally, they render the compound less lipophilic, but additionally serve to expose reactive sites, or to add functionally reactive sites to which polar groups may be conjugated. Oxidative metabolism represents a major route of elimination for many drugs (Lin and Lu, 1998). Other types of phase I reactions are reduction such as nitro reduction (chloramphenicol) and hydrolysis. Hepatic and extrahepatic CYP enzymes play a major role in phase I human drug metabolism. Functionalization reactions of phase I are reactions, which generate functional group as in hydroxylation or unmask functional group as in ester hydrolysis.

There are also non-CYP450 oxidations, for example, monoamine oxidase reactions, different mechanism with the same result as P450 deamination; flavin monooxygenase reactions (FMO), but P450 reductases also use flavin as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Yan and Caldwell, 2001). Phase II reactions usually conjugate the drug with endogenous substrates, such as a glucuronide, glutathione or a sulfate group. Compounds that remain in the circulation after phase I metabolism often undergo phase II metabolism. Phase II reactions are characterized by conjugation with small endogenous substance, often taking advantage of

functional group added in phase I. The phase II conjugation reactions involve glucuronidation, sulphation, acetylation and conjugation with glutathione. The conjugated metabolites are highly water soluble and are readily excreted into urine and bile, which are the main routes of elimination for most drugs (Meyer, 1996). Phase II enzymes that catalyse conjugation reactions include broad specificity transferases such as UDP-glucuronosyltransferases (UGT), glutathione-S-transferases (GST) and sulfotransferases (SULT). Many drugs undergo both phase I and phase II metabolism sequentially, but some can be excreted after either phase I or phase II reactions or even while still nonmetabolized (Krishna and Klotz, 1994).

1.2 CYP ENZYMES

The CYP enzymes are a superfamily of heme-containing mono-oxygenases (Wrighton and Stevens, 1992; Meyer, 1996). CYP 450 enzymes are so called because the pigment (P) has a 450 nm spectral peak when reduced and bound to carbon monoxide (Omura and Sato, 1962). CYP enzymes are found not only in human beings but also in bacteria, fungi, plants and animals (Nelson, 1999). Originally, CYP was thought to be a single enzyme, and by the mid-1960s, it was associated with drug and steroid metabolism. After advances in messenger ribonucleic acid (mRNA) purification in the early 1980s, cloning studies have revealed dozens of different CYP enzymes (Nebert and Russell, 2002), which comprise a superfamily of heme-thiolate proteins, and are found in every class of organism. This superfamily is believed to have originated from an ancestral gene that existed over three billion years ago. They are membrane-bound, localised to the smooth endoplasmic reticulum of the cells

(Meyer, 1996), and are responsible for most of the oxidative metabolism of both xenobiotics and endogenous substrates (Dresser *et al*., 2000; Lin and Lu, 1998).

The nomenclature of all CYP enzymes is based on their amino acid sequence (Nelson, 1996). The CYP enzymes within the same family are designated by an Arabic numeral (e.g., CYP1) and share more than 40% identity in the amino acid sequence. The families are further divided into subfamilies, with enzymes within a subfamily designated by a capital letter (e.g., CYP1A) and sharing more than 55% identity in the amino acid sequence. Finally, an Arabic numeral after the letter denotes each individual isoenzyme (e.g., CYP1A2) (Nebert and Russel, 2002).

Functionally, the CYP enzymes are divided into two major classes, those with a specific role for example, in steroid biosynthesis, bile and arachidonic acid metabolism (CYP4, CYP5 and higher) and those essentially involved in the metabolism of xenobiotics such as drugs, anti-oxidants and chemicals (CYP1, CYP2, CYP3) (Nelson, 1999). Many of these enzymes are involved in metabolism of different substrates such as endogenous compounds, chemicals and natural plant products (Nelson *et al*., 1996). Among the 18 CYP gene families in humans recognised by Nelson in 1999, only the first three families (CYP1, CYP2, and CYP3) seem to be important in drug metabolism (Wrighton *et al*., 1996). The estimated proportions of the major drug metabolising CYP enzymes in human liver are as follows; CYP3A4 29%, CYP2C8 19%, CYP2E1 19%, CYP1A2 15%, CYP2C9 12%, CYP2D6 3%, CYP2C19 2%, and CYP2B6 < 1% (Pelkonen *et al*., 1998, Proctor *et al*., 2004). However, there are notable

interindividual differences in the hepatic contents of the different CYP enzymes. Furthermore, the hepatic abundance of the CYP enzymes does not correlate with the importance of the individual enzyme in drug metabolism. For example, CYP2D6 metabolises more than 75 different drugs (Nebert and Russell 2002), but CYP2E1 metabolises relatively few drugs. Together these enzymes account for approximately 70% of the total CYP hepatic content (Shimada *et al*., 1994). They are distinguished by their different substrate specificities and turnover capacities. Examples of substrates, inhibitors and inducers of these individual isoenzymes are presented in Table 1.1.

The expression and catalytic activity of CYP enzymes may vary greatly between individuals, due to genetic, non-genetic endogenous (e.g. diseases) and environmental (drugs, diet) factors (Pelkonen *et al*., 1998). Genetic polymorphism in drug metabolism greatly affects the activity of some CYP enzymes such as CYP2C19 and CYP2D6 (Nakamura *et al*., 1985; Wrighton *et al*., 1996). Some CYP enzymes are inducible by environmental factors such as drugs (Wrighton *et al*., 1996; Pelkonen *et al*., 1998). Furthermore, catalytic activity of CYP enzymes can be inhibited or inactivated.

Induction and inhibition of CYP enzymes increase the intraindividual and interindividual variability of drug metabolism. The activity of certain CYP enzymes, for example CYP2D6, CYP2C9, CYP2C19 is mainly monogenically determined, with a fraction of the population having reduced or no enzyme activity due to any of several mutations in the gene coding for that enzyme (Tanaka, 1999). This phenomenon is termed genetic polymorphism and is

defined as the presence of different alleles at a certain gene locus with a frequency in the population over 1%, resulting in at least two genotypes in the population.

Table 1.1 Examples of substrates, inhibitors and inducers of the main human hepatic drug-metabolising CYP enzymes. Modified from Bertz and Granneman (1997) and Pelkonen *et al*. (1998).

Isozyme	Substrates	Inhibitors	Inducers
CYP1A2	Caffeine, Clozapine, Imipramine Olanzapine, Ropivacaine Theophylline, Tizanidine	Cimetidine, Ciprofloxacin Fluvoxamine, Furafylline Rofecoxib	Carbamazepine Charcoal grilled meat Cigarette smoke Omeprazole
CYP2C8	Carbamazepine Ibuprofen, Paclitaxel Rosiglitazone, Tolbutamide	Gemfibrozil Trimethprim Montelukast	Phenobarbital Rifampicin
CYP2C9	Glibenclamide Glipizide, Ibuprofen Losartan, Phenytoin S-Warfarin, Tolbutamide	Amiodarone Fluconazole, Miconazole Sulfaphenazole	Phenobarbital Rifampicin
CYP2C19	Citalopram, Diazepam Proguanil Proton pump inhibitors S-Mephenytoin	Clarithromycin Fluconazole Fluvoxamine, Omeprazole	Phenobarbital Rifampicin
CYP2D6	Amitriptyline, Fluvoxamine Fluoxetine, Haloperidol Metoprolol, Ondansetron Tramadol	Flecainide, Fluoxetine Quinidine, Paroxetine	Unknown
CYP3A4	Alprazolam, Amiodarone Cyclosporin, Lovastatin Midazolam, Nifedipine Tacrolimus, Triazolam	Clarithromycin Erythromycin Grapefruit juice Itraconazole	Carbamazepine Phenobarbital Phenytoin St John's wort

1.2.1 CYP1A SUBFAMILY

CYP1A1 and CYP1A2 are the two members of the CYP1A subfamily. CYP1A1 is expressed mainly in extrahepatic tissue such as lungs, small intestine and placenta, and is of little importance in drug metabolism (Miners and McKinnon, 2000). There is genetic polymorphism in the inducibility of CYP1A1 by polycyclic aromatic hydrocarbons, with a high inducibility phenotype being more common in patients with lung cancer (Kouri *et al*., 1982; Nebert *et al*., 1991). CYP1A2 is abundantly expressed in the human liver, and it can also be expressed in some extrahepatic tissues, such as the gastrointestinal tract (Ding and Kaminsky, 2003). CYP1A2 enzyme metabolizes many commonly used drugs and this is the only isoenzyme affected by tobacco. In humans, the interindividual variability of the expression of CYP1A2 is large. For example, about 40-60 fold differences between different ethnic groups have been reported (Shimada *et al*., 1994).

CYP1A2 metabolises, for example, caffeine, tacrine, clozapine, ropivacaine, theophylline, lidocaine and melatonin (Butler *et al*., 1989; Madden et al., 1993; Bertilsson *et al*., 1994; Oda *et al*., 1995; Tjia *et al*., 1996; Wang *et al*., 2000; Härtter *et al*., 2001). The major route of caffeine metabolism in man (*N*-3-demethylation of caffeine to paraxanthine) is mediated by CYP1A2 (Lelo *et al*., 1986; Butler *et al*., 1989). Therefore, caffeine is often used as a probe substrate for CYP1A2. For example, the caffeine/paraxanthine ratio is a suitable index of CYP1A2 activity *in vivo* (Fuhr and Rost, 1994). CYP1A2 is inhibited by, for example, fluvoxamine and ciprofloxacin (Nemerof *et al*., 1996; Fuhr *et al*., 1992), and induced by cigarette smoking and other compounds containing

polycyclic aromatic hydrocarbons (PAH) and cruciferous vegetables. CYP1A enzymes transform some procarcinogens, such as PAH compounds and aflatoxin B1, to carcinogens (Miners and McKinnon, 2000).

CYP1A2 activity shows interindividual variability which seems to be explained by both genetic and environmental factors (Rasmussen *et al*., 2002, Aklillu *et al*., 2003). Even though there is genetic polymorphism in the *CYP1A2* gene (www.imm.ki.se/CYPalleles/cyp1a2.htm), no nucleotide differences which can clearly explain the phenotypic variability in *CYP1A2* gene expression or inducibility have been identified. A single nucleotide polymorphism (SNP), 164C>A, the allele *CYP1A2*1F*, has been associated with higher inducibility of CYP1A2 activity (Chida *et al*., 1999, Sachse *et al*., 1999, Bondolfi *et al*., 2005), but this has not been confirmed in all studies (Schulze *et al*., 2001, Nordmark *et al*., 2002, Shimoda *et al*., 2002, Kootstra-Ros *et al*., 2005).

1.2.2 CYP2B6

The expression of CYP2B6 in human liver, and its role in hepatic drug clearance, has earlier been considered to be rather insignificant (Mimura *et al*., 1993; Shimada *et al*., 1994). However, recent studies have suggested higher abundance of hepatic CYP2B6 with significant interindividual variability (Gervot *et al*., 1999; Hesse *et al*., 2000). Substrates of CYP2B6 are, for example, nevirapine, bupropion, propofol and ketamine (Erickson *et al*., 1999; Hesse *et al*., 2000; Court *et al*., 2001; Hijazi and Boulieu, 2002). The activity of CYP2B6 can be inhibited by fluvoxamine, sertraline, paroxetine, hormone replacement therapy, clopidogrel, and ticlopidine (Hesse *et al*., 2000, Palovaara *et al*., 2003;

Turpeinen *et al*., 2005) and induced by, for example, phenobarbital and cyclophosphamide (Gervot *et al*., 1999). The *CYP2B6* gene is highly polymorphic and the *CYP2B6*6* mutation may have clinical relevance as it increases the 4-hydroxylation of cyclophosphamide (Xie *et al*., 2003).

1.2.3 CYP2C

The human CYP2C subfamily isoforms are primarily hepatic, but they exist in the small intestine as well, and include CYP2C8, CYP2C9, CYP2C18, and CYP2C19. CYP2C18 does not seem to be clinically important. Paclitaxel is the prototypic substrate for CYP2C8, and trimethoprim and montelukast are its selective inhibitors (Rettie *et al*., 2000, Wen *et al*., 2002, Walsky *et al*., 2005). Substrates of CYP2C9 are, for example, S-warfarin and ibuprofen (Bertz and Granneman, 1997) and sulfaphenazole is a prototypic inhibitor (Rettie *et al*., 2000). For CYP2C19, hydroxylation of S-mephenytoin is a prototypic reaction, and omeprazole can serve as a selective inhibitor (Ko *et al*., 1997; Pelkonen *et al*., 1998). Other substrates, inhibitors and inducers are given in Table 1.1. CYP2C9 and CYP2C19 exhibit genetic polymorphism, with clinical consequences (Goldstein, 2001).

1.2.4 CYP2D6

Even though the amount of CYP2D6 is rather low compared to, for example, CYP3A4 and CYP1A2 in human liver, it metabolises approximately 30% of all drugs (Fromm *et al*., 1999). Codeine, fluoxetine, fluvoxamine and tramadol are some substrates of CYP2D6. CYP2D6 is inhibited by several agents, such as its own substrates fluoxetine and paroxetine (Belpaire *et al*.,

1998). Quinidine is a selective inhibitor of CYP2D6 (Newton *et al*., 1995). CYP2D6 seems not to be inducible, in contrast to all other CYP enzymes involved in human drug metabolism (Zanger and Eichelbaum, 2000). CYP2D6 is polymorphically expressed; approximately 7% of the Caucasians lack functional CYP2D6 enzyme, being poor metabolisers (PMs) (Mahgoub *et al*., 1977; Alvan *et al*., 1990). In contrast, individuals with a duplicated or multiplicated *CYP2D6* gene have very high CYP2D6 activity and are called ultrarapid metabolisers (UMs) (Johansson *et al*., 1993).

1.2.5 CYP2E1

This enzyme participates in the metabolism of only a few drugs, often forming toxic metabolites. Ethanol is both a substrate and inducer of CYP2E1 (Klotz and Ammon, 1998). In addition, CYP2E1 metabolises paracetamol (acetaminophen), halothane, chlorzoxazone and mediates activation of many carcinogens (Klotz and Ammon, 1998; Raucy and Carpenter, 2000).

1.2.6 CYP3A SUBFAMILY

CYP3A4, CYP3A5 and CYP3A7 belong to the CYP3A subfamily and seem to have similar substrate specificities (Wrighton and Thummel, 2000). CYP3A7 is poorly expressed in the adult human liver, but in foetal human liver it accounts for 50% of all expressed CYP enzymes (Wrighton *et al*., 1988; Koch *et al*., 2002).

CYP3A4 is one of the most important drug metabolising CYP enzymes in humans. In addition to the abundant expression of CYP3A4 in the liver

(approximately 30%), it is the dominant CYP enzyme in the small intestinal mucosa (Shimada *et al*., 1994; Kivistö *et al*., 1996; Zhang *et al*., 1999). It has been suggested that approximately 50% of all clinically used drugs that are metabolised are substrates of CYP3A4 (Table 1.1). Hepatic and intestinal CYP3A4 are induced by several drugs, for example rifampicin, carbamazepine, phenytoin, and St John`s wort (*Hypericum Perforatum*) (Backman *et al*., 1996a; Backman *et al*., 1996b; Pelkonen *et al*., 1998; Dürr *et al*., 2000).

CYP3A5 is, in contrast to CYP3A4, polymorphically expressed (Kuehl *et al*., 2001). CYP3A5 can be found in significant levels in approximately 10-40% of Caucasians, 40-50% of Chinese and more than 50% of African Americans (Wrighton *et al*., 1990; Tateishi *et al*., 1999; Chou *et al*., 2001; Kuehl *et al*., 2001).

1.3 INHIBITION OF CYP ENZYMES

Inhibition of CYP enzymes by xenobiotics, at certain concentrations, is often selective for a limited number of isoforms. For example, itraconazole is a potent and relatively selective inhibitor of CYP3A4 (Back and Tjia, 1991). The fluoroquinolone antibiotic ciprofloxacin inhibits mainly CYP1A2 (Bertz and Granneman, 1997) and paroxetine inhibits mainly CYP2D6 (Jeppesen *et al*., 1996). K_i is an inhibition constant related to the affinity of a compound to an enzyme, and is important in predicting *in vivo* drug-drug interactions resulting from metabolic inhibition. K_i can be estimated *in vitro* either graphically by plotting methods or by nonlinear regression analysis. The mechanisms of CYP inhibition can be divided into reversible inhibition, quasi-irreversible and

irreversible inhibition, of which reversible inhibition is probably the most common (Lin and Lu, 1998).

Reversible inhibition can be further divided, based on enzyme kinetics, into competitive, noncompetitive, and uncompetitive. In competitive inhibition, the inhibitor competes with the substrate for the active site of the free enzyme, for example the CYP3A4 inhibitor itraconazole (Von Moltke *et al*., 1996). In noncompetitive inhibition, the inhibitor binds to a different binding site of the enzyme and has no effect on the binding of the substrate for example the inhibition of the CYP3A-catalysed conversion of dextrometorphan to 3-methoxymorphinan by omeprazole, (Ko *et al*., 1997). In uncompetitive inhibition, the inhibitor binds only to an enzyme substrate complex, not to the free enzyme for example the CYP2E1 inhibitor cotinine (Van Vleet *et al*., 2001).

In quasi-irreversible inhibition, an inhibitor is first metabolically activated by the CYP enzyme, and then this inhibitory metabolite forms a stable metabolic intermediate (MI) complex with the prosthetic heme of CYP, rendering the enzyme functionally inactive. MI complexation can be reversed *in vitro* but not in vivo, hence, the name quasi-irreversible (Lin and Lu, 1998). Erythromycin is an example of a quasi-irreversible inhibitor of CYP3A (Thummel and Wilkinson, 1998).

Additionally, in irreversible inhibition, a drug is oxidized by the CYP to a reactive intermediate, which covalently binds to the enzyme causing irreversible inactivation. This kind of inhibition is also called mechanism-based or suicide

inhibition (Lin and Lu, 1998; Thummel and Wilkinson, 1998). Mechanism-based inhibitors of CYP enzymes *in vitro* are, for example, furafylline (CYP1A2), gestodene (CYP3A4) and ethinylestradiol (CYP3A4) (Guengerich, 1990; Kunze and Trager, 1993; Lin and Lu, 1998).

In quasi-irreversible and irreversible inhibition (MI complexation and mechanism-based inhibition), a time-dependent proportion of the CYP enzymes is complexed or destroyed, while the remaining enzymes show normal activity. This leads to a time-dependent decrease in V_{max} (maximum velocity of metabolism) but no change in K_m (Michaelis-Menten constant). Time-dependent loss of enzyme activity is one of the most important criteria in distinguishing between reversible and irreversible inhibition (Lin and Lu, 1998).

1.4 INDUCTION OF CYP ENZYMES

Induction is defined as an increase in amount and activity of a drug-metabolizing enzyme, as a consequence of long-term (hours and days) chemical exposure (Pelkonen *et al.*, 1998). From a biological point of view, induction is an adaptive response that protects the cells from toxic xenobiotics by enhancing the detoxification activity. However, induction may also enhance toxicity by increased formation of reactive metabolites (Lin and Lu, 1998). Many inducers are also inhibitors of the enzymes they induce, and the inductive effects of a single drug may be mediated by more than one mechanism (Fuhr, 2000).

Some, but not all, CYP enzymes are inducible. The regulation of gene transcription generally depends on inducers interacting with certain receptors. The expression of CYP1 genes is induced by the cytosolic aryl hydrocarbon (Ah) receptor, which dimerizes with the Ah receptor nuclear translocator, in response to many polycyclic aromatic hydrocarbons such as those found in industrial incineration products, cigarette smoke, and charcoal grilled food (Nebert and Russell, 2002; Rushmore and Kong, 2002). After a sequence of events, the appropriate mRNA is transcribed and translated into its corresponding protein (Lin and Lu, 1998).

The large interindividual variation in CYP1A inducibility is probably linked to genetic polymorphism in Ah receptor; studies suggest an increased risk for malignancies in individuals who have the high affinity Ah receptor phenotype (Miners and McKinnon, 2000). Sometimes the binding of inducing agents to the Ah receptor also leads to induction of phase II enzymes such as uridine diphosphate glucosyltransferases and glutathione-S-transferases (Lin and Lu, 1998).

Induction of CYP3A4 is mediated by nuclear receptors: pregnane X receptor (PXR), constitutive androstane receptor (CAR), and peroxisome proliferator-activated receptor (PPAR) (Waxman, 1999; Fuhr, 200; Gibson and Skett, 2001). In order to activate the *CYP3A4* gene, PXR and CAR first have to bind to an inducer, and thereafter form heterodimers with the retinoid X receptor (RXR) which binds to specific gene regulation elements (Gibson and Skett, 2001).

The rifampicin/glucocorticoid-type induction appears to be mediated by PXR, which is activated by CYP3A4 inducers such as rifampicin and phenobarbital (Fuhr, 2000; Goodwin *et al.*, 2002). All CYP3A4 substrates are subject to this type of induction (Fuhr, 2000). Rifampicin is a potent inducer of CYP3A4 and found to reduce the AUC of oral midazolam, triazolam, and buspirone by > 90% (Backman *et al.*, 1996a; Villikka *et al.*, 1997; Backman *et al.*, 1998; Lamberg *et al.*, 1998).

1.5 CYP INHIBITION AND INDUCTION IN DRUG-DRUG INTERACTIONS

Drug interactions are always major concern in medicine and for the pharmaceutical industry. They can cause considerable differences in drug response and lead to adverse effects of drugs. Drug interactions can be pharmacokinetic or pharmacodynamic in nature, but pharmacokinetic interactions are more common. Pharmacodynamic interactions cause changes in the effects of drugs for example alcohol enhances the sedative effect of benzodiazepines (Seppälä *et al*., 1982). Pharmacokinetic interactions can take place at the level of drug absorption, distribution, metabolism and excretion.

Environmental factors such as drugs, tobacco smoking, charcoal grilled meat, and grapefruit juice can inhibit or induce CYP enzyme. A change in drug metabolism is an important cause of pharmacokinetic drug-drug interactions (Dresser *et al.*, 2000). The clinical significance of a drug-drug interaction depends on the magnitude of change in the active parent drug and/or active metabolite concentrations at the effect site, and on the therapeutic index of the drug. For example, anticoagulants, antidepressants, and cardiovascular drugs

have a narrow therapeutic index, making patients on these drugs more prone to drug interactions (Lin and Lu, 1998). Theophylline which was used as a substrate in this pharmacokinetic interaction study has a low therapeutic index.

The administration route of the CYP substrate can influence the clinical effect of enzyme inhibition and induction, because orally administered drugs are exposed to the intestinal CYPs during absorption. For example, grapefruit juice greatly inhibits the CYP3A4 in the small intestine and thereby impairs the intestinal first-pass metabolism of orally administered CYP3A4 substrate drugs such as lovastatin (Kantola *et al.*, 1998). On the other hand, itraconazole inhibits CYP3A4 both in the gut and the liver, impairing both the first-pass and systemic elimination of CYP3A4 substrates such as midazolam (Olkkola *et al.*, 1996). Rifampicin seems to induce more the intestinal than the hepatic CYPs (Hebert *et al.*, 1992).

1.6 DRUG TRANSPORTERS

Transport processes are increasingly acknowledged as important determinants of absorption, distribution and excretion of drugs through cell membranes. It is often necessary to consider both metabolisms (by CYP enzymes) and transport mechanisms (by drug transporters) together when predicting *in vivo* pharmacokinetics (Kusuhara and Sugiyama, 2002).

The main efflux transporter is P-glycoprotein (P-gp). P-gp is involved in the excretion of substances into the gut, bile, and urine, and is considered to play an important role in the blood brain, blood testes, and blood placenta

barriers (Lo and Burckart, 1999). P-gp has overlapping substrate specificity with CYP3A4 (for example diltiazem, nifedipine, HIV protease inhibitors) (Lin and Yamasaki, 2003). Recently, P-glycoprotein induction by rifampicin was shown to be mediated by the orphan nuclear receptor PXR (Geick *et al*., 2001), which is involved in CYP3A4 induction.

However, important exceptions exist: as an example, digoxin is eliminated almost entirely by excretion, but is a prototypical P-glycoprotein substrate susceptible to clinically relevant drug interactions with P-gp inhibitors such as itraconazole, verapamil, cyclosporine and quinidine (Partanen *et al*., 1996; Fromm *et al*., 1999; Silverman, 2000). Another example, midazolam is a substrate of CYP3A4 but not of P-gp (Kim *et al*., 1999).

It seems that intact P-gp activity is of special importance in preventing central nervous system (CNS) -adverse effects of drugs (Lin and Yamazaki, 2003). Generally, P-gp inhibition leads to a much greater increase in drug concentrations in the brain than in plasma (Lin and Yamazaki, 2003). Healthy volunteers tolerated the P-gp substrate loperamide well when it was ingested with placebo, but when administered with the P-gp inhibitor quinidine, it caused respiratory depression (Sadeque *et al.*, 2000).

1.7 BIOEQUIVALENCE AS A PROOF OF THERAPEUTIC EQUIVALENCE

Generic drugs have been introduced on the pharmaceutical market mainly to reduce drug expenses by the consumers and health care authorities. This concern about lowering health care costs has resulted in a tremendous increase in the use of generic drug products. The term ‘generic product’ is used in different ways. It can mean a product marketed under the drug’s non-proprietary approved name, or it can mean a product marketed under a different brand (proprietary) name. It is sometimes used to mean any product from a company other than the innovator (research-based) manufacturer. A common use of the term (and that used by the World Health Organization (WHO)), is for a pharmaceutical product that is intended to be interchangeable with the innovator product in an individual patient, usually manufactured without a licence from the innovator company marketed after expiry of patent or other exclusivity rights (Birkett, 2003).

1.7.1 RELATIVE BIOAVAILABILITY

Bioavailability is a pharmacokinetic term that describes the rate and extent to which the active drug ingredient is absorbed from a drug product and becomes available at the site of drug action. Since pharmacologic response is generally related to the concentration of drug at the receptor site, the availability of a drug from a dosage form is a critical element of a drug product's clinical efficacy. However, drug concentrations usually cannot be readily measured directly at the site of action. Therefore, most bioavailability studies involve the determination of drug concentration in the blood or urine. This is based on the premise that the drug at the site of action is in equilibrium with drug in the blood.

It is therefore possible to obtain an indirect measure of drug response by monitoring drug levels in the blood or urine. Thus, bioavailability is concerned with how quickly and how much of a drug appears in the blood after a specific dose is administered. The bioavailability of a drug product often determines the therapeutic efficacy of that product since it affects the onset, intensity and duration of therapeutic response of the drug.

Relative or comparative bioavailability refers to the availability of a drug product as compared to another dosage form or product of the same drug given in the same dose. These measurements determine the effects of formulation differences on drug absorption. The relative bioavailability of product A compared to product B, both products containing the same dose of the same drug, is obtained by comparing their respective area under the curves (AUCs).

$$\text{Relative Bioavailabilty} = \frac{AUCA}{AUCB} \qquad (1.1)$$

where drug product B is the reference standard. When the bioavailability of a generic product is considered, it is usually the relative bioavailability that is referred to.

Numerous biopharmaceutical studies have clearly indicated that the formulation and manufacture factors as well as the physicochemical properties of the drug can markedly affect the rate and extent of absorption of a drug. This led to the realization that different products of the same drug moiety containing the same molar dose may not necessarily be bioequivalent and achieve similar

drug concentrations in the plasma. Therefore, they may not be therapeutically equivalent.

1.7.2 FACTORS AFFECTING BIOAVAILABILITY

The various factors that can influence the bioavailability of a drug can be broadly classified as dosage form-related or patient-related. Some of the factors related to the physicochemical properties of the drug are particle size, crystalline structure, degree of hydration of crystal, salt or ester form of the formulation, amount of disintegrant and lubricant and nature of diluents.

Particle size of a drug may markedly affect its dissolution rate and hence the absorption. Particle size can have a significant effect on AUC. This had been demonstrated by Neuvonen *et al*. (1977) after administration of equal doses containing micronized formulation and conventional formulation, serum levels of phenytoin were measured. Based on the AUC, almost twice as much phenytoin was absorbed after the micronized preparation.

The bioavailability of oral dosage forms decreases in the following order: solutions > suspensions > capsules > tablets > coated tablets (Hirst, 1976). Although this ranking is not universal, it does provide a useful guideline (Ashford, 2000a). On the other hand, the particular salt of a drug has been shown to affect the rate and extent of absorption. The oral hypoglycaemic tobutamide sodium is absorbed faster than that of the free acid (Ashford, 2000a).

Another important factor to be considered is the crystal form of the drug. A classic example of the influence of polymorphism (more than one crystal form) on drug bioavailability is provided by chloramphenicol palmitate. The three polymorphs of chloramphenicol in suspensions resulted in different plasma levels (Aguiar, 1967). The chloramphenicol concentration in the body depended on the percentage of β-polymorph in the suspension. Generally, the crystal form that has the lowest free energy is the most stable polymorph (Shargel and Yu, 2003). Differences in bioavailability may also occur between the anhydrous and hydrated forms of a drug. The anhydrous form of ampicillin was absorbed more than the slower dissolving trihydrate form (Ashford, 2000a).

In addition to some of the dosage form-related factors identified above, bioavailability may also be affected by a variety of physiological and clinical factors related to the patient. Considerable inter-subject differences in the bio-availability of some drugs have been observed. These can often be attributed to individual variations in such factors as gastric emptying rate, intestinal transit time, intestinal absorptive capacity along the gastrointestinal tract, variations in pH of gastrointestinal fluids, disease states, food, fluid volume and other drugs (Chereson, 1999).

Since the proximal small intestine is the optimum site for drug absorption, a change in the stomach emptying rate is likely to alter the rate, and possibly the extent of drug absorption. Any factor that slows the gastric emptying rate may thus prolong the onset time for drug action and reduce the therapeutic efficacy of drugs that are primarily absorbed from the small intestine. In

addition, a delay in gastric emptying could result in extensive decomposition and reduced bioavailability of drugs that are unstable in the acidic media of the stomach such as penicillins and erythromycin (Chereson, 1999).

There are numerous factors that affect gastric emptying rate (Blanchard and Sawchuk, 1979). Factors such as a patient's emotional state, certain drugs, type of food ingested and even a patient's posture can alter the time course and extent of drug absorption (Nimmo *et al*., 1973; Blanchard and Sawchuk, 1979; Bennett *et al*., 1984; Ashrof, 2000b). The intestinal transit time is relatively constant and unaffected by food (Mundy *et al*., 1989; Ashrof, 2000b). On the other hand, the gastric emptying of pharmaceuticals is highly variable and is influenced by food (Davis *et al*., 1984; Ashford, 2000b).

The escalating cost in health care has created a demand for cheaper drug products. As a consequence, many generic formulations of established products appeared in the market. Hence, bioequivalence assessment of generic drugs with innovator formulations is required in order to exclude any clinically significant difference in the rate and extent to which the active moiety becomes available at the site of drug action. Consequently, this will protect the consumer from any difference in activity of the generic as compared to the innovator.

Two products are considered therapeutically equivalent if they contain the same active substance or therapeutic moiety, and show the same efficacy and safety clinically (EMEA, 2001). Establishing bioequivalence between two products in adequately designed bioequivalence studies is considered sufficient

proof of therapeutic equivalence and no further clinical evidence of therapeutic equivalence is needed. From the regulatory point of view this means that, two drug products are considered interchangeable if they contain the same active drug substance and have been shown to be bioequivalent (CDER, 2000; CDER, 2001; EMEA, 2001).

1.7.3 METHODOLOGY USED FOR THE EVALUATION OF BIOEQUIVALENCE

According to current regulatory guidelines, bioequivalence studies should be designed in such a way that the effect of the formulation can be distinguished from other effects (EMEA, 2001). Basically, a bioequivalence study compares the systemic exposure of two drug products, commonly referred to as test (the new product) and reference (the existing product for which efficacy and safety have been shown). There are several test procedures that are considered adequate for the evaluation of bioequivalence. These include pharmacokinetic, pharmacodynamic, clinical, and *in vitro* studies. Pharmacokinetic bioequivalence studies are usually preferable (Chen *et al*., 2001).

For establishing pharmacokinetic bioequivalence between test and reference, the similarity of certain pharmacokinetic parameters for the products is evaluated as a measure of systemic exposure. The pharmacokinetic parameters to be determined for the test and the reference include AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, C_{max} / AUC, k_e and t ½. Adequate parameters are chosen on clinical and pharmacokinetic grounds. Usually the primary parameters for the

evaluation of bioequivalence are C_{max} and AUC. The study design has to reflect the pharmacodynamic and pharmacokinetic properties of the drug compound(s), i.e., one needs to consider how these properties relate for example to sampling times, wash-out periods, and active metabolites. In addition, several details of bioequivalence study designs are provided in regulatory guidelines (Malaysian Guidelines for the Conduct of Bioavailability and Bioequivalence Studies, 2000; CDER, 2000; EMEA, 2001).

Bioequivalence studies are generally conducted in healthy male and female volunteers who are at least 18 years old, although they may also be carried out in target indication patients. Rather recently a requirement has been issued for conducting bioequivalence studies in a population that is representative of the population of patients who will use the drug (CDER, 2000). Thus, bioequivalence studies will increasingly be conducted also in the elderly.

Three different methods may be used for the evaluation of pharmacokinetic bioequivalence. These methods comprise the evaluation of average, individual, and population bioequivalence. The average bioequivalence method remains the primary method for assessing bioequivalence in new regulatory guidelines for conducting and analysing bioequivalence studies (CDER, 2000; CDER, 2001; EMEA, 2001).

1.7.4 AVERAGE BIOEQUIVALENCE

Non-replicate study-designs: For the evaluation of average bioequivalence, a two period cross over study-design is usually considered