Using Human Metabolic Enzyme Profiling as an Innovative Technology in the Drug Development Process

By

Hiroaki Sato

Ph.D., Pharmaceutical Sciences
University of Tokyo, 1990

Submitted to the Alfred P. Sloan School of Management in Partial Fulfillment of the Requirements of the Degree of

Master of Business Administration
at the
Massachusetts Institute of Technology

June 2000

Signature of Author _____
Hiroaki Sato
Sloan School of Management
May 8, 2000

Certified by _____
Stan N. Finkelstein, Thesis Supervisor
Senior lecturer, Sloan School of Management

Certified by _____
Anthony J. Sinskey, Thesis Supervisor
Professor, Department of Biology

Accepted by _____
Margaret C. Andrews
Executive Director of the MBA Program, Sloan School of Management

Using Human Metabolic Enzyme Profiling as an Innovative Technology in the Drug Development Process

By

Hiroaki Sato

Submitted to the Alfred P. Sloan School of Management on May 8, 1996
in partial fulfillment of the requirements for
the Degree of Master of Business Administration

ABSTRACT

Undesirable pharmacokinetic properties, such as poor bioavailability and drug-drug interactions, have been one of major reasons for the failure of new pharmaceuticals in clinical trials. These dropout risks can be reduced by early knowledge of human pharmacokinetics in the drug discovery process. Although new drug candidates have been first tested in animal-based systems, the prediction of human pharmacokinetics from animal data has been unsuccessful due to species differences in the enzymes involved in drug metabolism.

Recent progress in molecular biology made it possible to develop *in vitro* drug metabolism systems using human metabolic enzymes, such as purified microsomes or expressed cytochrome P450. These systems are useful in profiling the enzymes involved in the human metabolism and extrapolating the *in vitro* findings to *in vivo* situations. The *in vitro* systems may yield a rapid drug metabolism screening of numerous compounds generated through combinatorial chemistry and high-throughput pharmacological screening. The integration of these technologies into the drug development process will significantly reduce the dropout risks in clinical trials and shorten the period between the drug discovery and market introduction.

Thesis Supervisor: Stan N. Finkelstein
Title: Senior Lecturer, Sloan School of Management

Thesis Supervisor: Anthony J. Sinskey
Title: Professor, Department of Biology

TABLE OF CONTENTS

TABLE OF CONTENTS
(continued)

ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. Stan Finkelstein and Prof. Anthony Sinskey, for their suggestions throughout the writing of this thesis. Their guidance made the entire project a great learning experience for me. Dr. Sinskey wishes to acknowledge support of the MMBPP activities at MIT.

I extend my thanks to Kirin Brewery Co. Ltd., for giving me the opportunity to study at the Massachusetts Institute of Technology.

And finally, I give a special appreciation to my wife Miyuki, for her continuous and devoted support.

Hiroaki Sato
May, 2000
Boston, Massachusetts

CHAPTER ONE

Introduction

1.1 Background

(1) Dropout risks in clinical trials due to pharmacokinetics

Pharmaceutical developmental processes have been understood as pipelines –the flow of compounds in various development stages. It is a generally accepted idea in the pharmaceutical industry that these pipelines are pretty leaky affairs. For every approved drug that comes out of a traditional pipeline, about 10,000 molecules have got lost somewhere within the process (Carr, 1998).

The development of novel therapeutic agents has become a high-risk challenge to pharmaceutical companies. A recent survey shows that on the average $400 million will have been spent between the drug discovery and the final approval by Food and Drug Administration (Lin, 1998). The major portion of the cost is allocated to generate data that prove the clinical efficacy and safety of candidate compounds. To avoid costly withdrawal in clinical trials, pharmacological and toxicological properties of the drug candidate molecules have been evaluated with animal models and *in vitro* systems before the clinical trials are initiated. However, these preclinical efforts have not necessarily secured drug candidates from unexpected termination in the clinical development process.

A survey of 319 drug candidates that were evaluated in clinical trials revealed that 77 out of 198 failed candidates were terminated due to pharmacokinetic problems (Prentis et al., 1988). The failure rate due to poor pharmacokinetics (39.4 % = 77 / 198 x 100) exceeded the rate due to clinical inefficacy (29.3 %). This study suggests that early understanding of human pharmacokinetic properties, which enable desired pharmacological effects by drug delivery to the target site, may have significant impact on reducing the dropout risk of drug candidates in clinical stages of evaluation.

Undesirable pharmacokinetic properties in the clinical trial can be classified into four categories: Absorption, Distribution, Metabolism and Excretion (ADME) (Table 1-1). It should be emphasized that metabolism is involved in all the undesirable pharmacokinetic properties.

Table 1-1. Undesirable pharmacokinetic properties and ADME factors that may affect the pharmacokinetics of the drug

Undesirable phamacokinetic properties	ADME factors
1. Poor bioavailability	- Poor absorption - Extensive first-pass metabolism
2. Inter-individual variability	- Polymorphism in metabolism
3. Drug-drug interaction	- Inhibition of distribution and excretion - Metabolic inhibition or induction
4. Too long or too short half-life	- All ADME factors

Scientists in the pharmaceutical industry are facing significant challenges to minimize these dropout risks in clinical trial by predicting clinical pharmacokinetics in the pre-clinical stage. To achieve this goal, two methodologies have been employed and pharmacokinetic principles that can support these two methodologies have been developed (Lin and Lu, 1997; Lin, 1998; Ito et al., 1998). They are:
(i) "Scale-up" pharmacokinetic data from *in vivo* animal models to humans
(ii) "Extrapolate" pharmacokinetic data from *in vitro* systems to *in vivo* models

In reality, however, the application of the first methodology to actual drug development projects has been limited, mainly because of large species-specific differences in the enzymes involved in drug metabolism (Gonzalez, 1992). The inter-species differences have resulted in the failure of candidate drugs in various clinical stages, such as the antiviral terpenoid carbenoxolone, the antiasthmatic compound FPL 52757, and the cardiotonic drug amirinone (Easton et al., 1990).

(2) Limitations in predicting human pharmacokinetics by animal models

The primary objective of pre-clinical pharmacokinetics has been to clarify ADME properties of drug candidates in animal models in order to predict human pharmacokinetics both qualitatively and quantitatively. If pharmacokinetic properties obtained in animal models are similar to those in humans, results from pharmacological and toxicological studies in animals may be more directly related to the clinical efficacy and safety in humans. However, the intrinsic differences among species, especially in drug metabolism, make it difficult to find the "right" animal models that simulate human pharmacokinetics. There has been a popular belief that the monkey is the most comparable model to human, and monkeys are often used by the pharmaceutical industry as a representative mammalian species for drug testing. However, Stevens et al. (1993) disputed this traditionally accepted idea. They compared hepatic drug metabolism activities between the rhesus monkey and humans using purified liver subcellular fractions. This study revealed that at least seven metabolic activities in rhesus monkeys are significantly different from those in humans.

Although the cytochrome P450s, as the major drug metabolizing enzymes in mammals have been thought to evolve from a single ancestral gene, biochemical evidence provides numerous examples of differences in the genetic structure of cytochrome P450s among animal species. Although at least 14 cytochrome P450 gene families have been identified in mammals and these genes are possessing highly conserved regions of amino acid sequences, there are significant variations in the primary sequences among species (Nelson et al., 1996). Even a small change in the amino acid sequence can cause large differences in substrate specificity. This fact may lead to the significant inter-species differences in terms of metabolism rate and metabolite profile. In addition to these qualitative differences, it is reported that the enzyme level of cytochrome P450 isoforms also differs across species (Guengerich, 1995). Due to these quantitative differences, traditional animal scale-up approach could not predict the human metabolism of antipyrine (Boxenbaum, 1980). Differences exist not only in catalytic activities of drug metabolizing enzymes from species, but also in their interactions (Kobayashi et al., 1989, Diaz et al., 1990). These studies demonstrate that drugs that have no activity to induce animal cytochrome P450 enzymes may induce these enzymes in humans, and vice versa.

These examples indicate that the predictions of pharmacokinetic properties from animals to humans are difficult, largely due to intrinsic differences in drug metabolism among animal species. When significant differences exist between animals and humans, additional experiments may be required to evaluate the benefits and risks of the new drug candidate. For example, if a major metabolite in humans is not observed in the species used for toxicological evaluation, additional studies may be needed to evaluate the toxicological profile of that metabolite. Therefore, the prediction of human pharmacokinetics by animal models remains risky and costly unless an integrated model to explain the species differences is developed.

(3) Emerging opportunities of *in vitro* human metabolism systems

While the progress in molecular biology has improved the understanding of drug-metabolizing enzymes, the increased availability of commercial resources for human *in vitro* metabolism systems have made this approach more feasible to predict human pharmacokinetics at the preclinical stage. Due to the simplicity of *in vitro* systems for the study of drug metabolism, the application of *in vitro* systems has expanded largely in 1990s. Many studies have proved that the *in vitro* systems are very useful to investigate the factors influencing drug metabolism; such as profiling of metabolites, identification of involved enzymes, and the understanding of reaction mechanisms (Houston and Carlile, 1997).

On the other hand, the use of *in vitro* systems has another opportunity to be fully exploited: quantitative prediction of *in vivo* drug metabolism by *in vitro-in vivo* extrapolation. There have been arguments regarding the quantitative

predictability of *in vivo* metabolic clearance and bioavailability from *in vitro* systems. Some scientists (Sugiyama et al., 1989; Pang and Chiba, 1994) have positive views about this methodology, whereas others (Gillette, 1984) believe that it is still difficult to predict *in vivo* metabolism quantitatively from *in vitro* metabolism data.

From an industrial perspective, Lin (1998) suggested that quantitative *in vitro* metabolic data can be extrapolated reasonably to *in vivo* situations if the following conditions are satisfied.

(i) *In vitro* experimental conditions are carefully designed to simulate the *in vivo* situations

(ii) Appropriate pharmacokinetic principles are applied to the analysis of experimental results.

Some pharmaceutical companies are adopting more empirical approaches where test animals, such as rats, are used to confirm the quantitative relationship between *in vitro-in vivo* metabolism in the earliest development stage.

(4) Needs for high-throughput screening of drug metabolism

In recent years, the advent of combinatorial chemistry and high-throughput pharmacological screening necessitates the high-throughput evaluation of ADME properties, including drug metabolism. Combinatorial chemistry revolutionized the drug discovery process by creating huge, previously unattainable numbers of molecules from quite a small number of starting modules (Carr, 1998). Screening library compounds for positive targeted activities is highly mechanized with high-throughput screens. The capacity for screening these molecules against targets of interest is rapidly growing beyond 100,000 per week (Hook, 1996). These technological advances have now given discovery scientists the ability to deliver large numbers of lead compounds for final optimization. These changes have lead to pressure on the throughput of downstream functions in the traditional drug development process. Therefore, the downstream area of pharmaceutical R&D including pharmacokinetic evaluation, which has not been shaken up by technological innovation, is becoming a bottleneck in the middle of the drug development process (Tarbit and Berman, 1998). This results in the need for higher throughput method of screening for drug metabolism.

1.2 Objectives

The primary objective of this thesis is to demonstrate how pharmaceutical companies can establish their competitive advantages through the formation of a technology strategy around an innovative technology: human metabolic enzymes. Toward this objective, this thesis will focus on proposing strategies to integrate *in vitro* human metabolism systems into the drug discovery process.

In chapter 2, the latest knowledge about cytochrome P450 (CYP450), the major xenobiotic metabolizing enzymes, are reviewed in terms of their role for drug

metabolism. Clinically important issues related to CYP450 including pharmacogenetics and drug-drug interactions, and principles that quantitatively link CYP450 metabolism with pharmacokinetics are also discussed. Based on the understanding of the CYP450 system, chapter 3 explores the new *in vitro* technologies that can be used to predict human metabolism in the early drug development stage and discusses the limitation of each technology. The possible use of transgenic mice that encode human CYP450, the model that may replace current animal models in the future, is also discussed. Chapter 4 discusses the strategic options enabled by the new technologies and proposes the integration of these options. Chapter 5 illustrates how a pharmaceutical company can improve its drug discovery and development process by integrating the new technologies into combinatorial chemistry and high-throughput screening. A case study is analyzed in chapter 6, where a new drug discovery process that incorporates the *in vitro* drug metabolism system was implemented with an organizational change. The thesis concludes in chapter 7 by predicting the future technological changes in the *in vitro* drug metabolism system and their impact on the drug discovery and development system.

CHAPTER TWO

Drug metabolism systems in human

2.1 Cytochrome P450: critical roles in the drug metabolism

In terms of the numbers of drugs metabolized and clinical significance, the cytochrome P450s (CYP450) are the most important group of enzymes among drug metabolizing enzymes. The primary role for the CYP450 system seems to be the biotransformation of endogenous compounds in the process of digestion. It suggests that drug metabolism by the CYP450 system is a relatively new role in the history of evolution. The primary purpose of drug metabolism in the body is to make drugs more water-soluble and thus more readily excreted into urine or bile (Slaughter and Edwards, 1995). CYP450-mediated oxidations can make the parent compounds more hydrophilic not only by altering the functional groups on the molecules, but also by introducing polar functionality into the molecules even if they are as unreactive as saturated or aromatic hydrocarbons. The oxidation mechanisms of CYP450 are particularly critical for the metabolism of lipophilic compounds without functional groups suitable for conjugation reactions. The CYP450 enzymes involved in the oxidation of drugs are usually membrane-bound, with particularly high concentrations in the endoplasmic reticulum of the liver, intestine, kidney, and lung (Vainio and Hietane, 1980).

The nomenclature of cytochrome P450 is based on the functional identity that decreases in inverse proportion to an evolutionary distance from a common precursor. By using protein sequence information as the basis for assigning names, CYP450s are classified according to their structural and functional similarity. The cytochrome P450s are identified by: (i) a number that denotes the family, (ii) a letter that denotes the subfamily, and (iii) a number that identifies the specific member of the subfamily (Guengerich, 1995; Gonzalez, 1993). The CYP1, CYP2, CYP3, and CYP4 families dominate the cytochrome P450-dependent metabolism of drugs in humans. CYP3A4 is the most abundant isoform, which represents approximately 30% of the cytochrome P450 in the human liver, while CYP1A2 represents 13%, CYP2A6 4%, the CYP2C (CYP2C9 and 2C19) enzymes 20%, CYP2D6 2%, and CYP2E1 7% of the total (Shimada et al., 1994).

However, the significance of these isoforms to drug metabolism is determined by not only their abundance, but also their substrate specificity, historic exposure to environmental factors, and their genetic polymorphism. Though over 30 human CYP450 systems have been identified to date, the major ones responsible for drug metabolism are six isoforms: the CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. These enzymes play critical roles for the metabolism of the majority of drugs given to humans (Table 2-1; Cupp and Tracy, 1998).

Table 2-1. Substrates, inhibitors and inducers of human CYP450 isoforms

Isoform		Compounds (merchandise name)
CYP1A2	Substrates	Amitriptyline (Elavil), Clomipramine (Anafranil), Clozapine (Clozaril), Imipramine (Tofranil), Propranolol (Inderal), R-warfarin, Theophylline
	Inhibitors	Fluvoxamine (Luvox), "Quinolones" Ciprofloxacin (Cipro), Enoxacin (Penetrex) > norfloxacin (Noroxin) > ofloxacin (Floxin) > lomefloxacin (Maxaquin)
	Inducers	Omeprazole (Prilosec), Phenobarbital, Phenytoin (Dilantin), Rifampin (Rifadin, Rimactane)
CYP2C9	Substrates	"Nonsteroidal anti-inflammatory drugs", Phenytoin (Dilantin), S-warfarin, Torsemide (Demadex)
	Inhibitors	Fluconazole (Diflucan), Ketoconazole (Nizoral), Metronidazole (Flagyl), Itraconazole (Sporanox), Ritonavir (Norvir)
	Inducers	Rifampin (Rifadin, Rimactane)
CYP2C19	Substrates	Clomipramine (Anafranil), Diazepam (Valium), Imipramine (Tofranil), Omeprazole (Prilosec), Propranolol (Inderal)
	Inhibitors	Fluoxetine (Prozac), Sertraline (Zoloft), Omeprazole, Ritonavir (Norvir)
CYP2D6	Substrates	"Antidepressants": Amitriptyline (Elavil), Clomipramine (Anafranil), Desipramine (Nopramin), Doxepin (Adapin, Sinequan), Fluoxetine (Prozac), Imipramine (Tofranil), Nortriptyline (Pamelor), Paroxetine (Paxil), Venlafaxine (Effexor) "Antipsychotics": Haloperidol (Haldol), Perphenazine (Etrafon, Trilafon), Risperidone (Risperdal), Thioridazine (Mellaril) "Beta blockers": Metoprolol (Lopressor), Penbutolol (Levatol), Propranolol (Inderal), Timolol (Blocadren) "Narcotics": Codeine, Tramadol (Ultram)
	Inhibitors	"Antidepressants": Praxetine > fluoxetine > sertraline (Zoloft) > fluvoxamine (Luvox), Nafazodone (Serzone), Venlafaxine > clomipramine (Anafranil) > amitriptyline Cimetidine (Tagamet), Fluphenazine (Prolixin) "Antipsychotics": Haloperidol, Perphenazine, Thioridazine
CYP2E1	Substrates	Acetaminophen (Tylenol), Ethanol
	Inhibitors	Disulfiram (Antabuse)
	Inducers	Ethanol, Isoniazid (Laniazid)

CYP3A4	Substrates	Amitriptyline (Elavil), "Benzodiazepines": Alprazolam (Xanax), Triazolam (Halcion), Midazolam (Versed), Calcium blockers, Carbamazepine (Tegretol), Cisapride (Propulsid), Dexamethasone (Decadron), Erythromycin, Ethinyl estradiol (Estraderm, Estrace), Glyburide (Glynase, Micronase), Imipramine (Tofranil), Ketoconazole (Nizoral), Lovastatin (Mevacor), Nefazodone (Serzone), Terfenadine (Seldane), Astemizole (Hismanal), Verapamil (Calan, Isoptin), Sertaline (Zoloft), Testosterone, Theophylline, Venlafaxine (Effexor), "Protease inhibitors": Ritonavir (Norvir), Saquinavir (Invirase), Indinavir (Crixivan), Nelfinavir (Viracept)
	Inhibitors	"Antidepressants": Nefazodone > fluvoxamine (Luvox) > fluoxetine (Prozac) > Sertraline, Paroxetine (Paxil), Venlafaxine, "Azole antifungals": Ketoconazole (Nizoral) > itraconazole (Sporanox) > fluconazole (Diflucan), Cimetidine (Tagamet), Clarithromycin, Diltiazem, Erythromycin, Protease inhibitors
	Inducers	Carbamazipine, Dexamethasone, Phenobarbital, Phenytoin (Dilantin), Rifampin (Rifadin, Rimactane)

Understanding of the involvement of CYP450 systems in preclinical development has significant impact on two clinical issues:

(i) Prediction and explanation of an individual's response to a particular therapeutic regimen: If elimination of a drug occurs via a single metabolic pathway without any other elimination route, individual differences in metabolic rate leads to large differences in drug concentrations in the blood and tissues. In some cases, these differences exhibit a two-peak distribution among the investigated population, indicating a generic polymorphism of metabolic enzymes. When a generic polymorphism affects an important metabolic route of elimination, large dosing adjustments may be necessary to achieve the safe use of the drug.

(ii) Rational management of possible drug-drug interactions: Many elimination routes via CYP450 metabolism can be inhibited by concomitant drug treatment. As a result, abrupt changes in metabolism can occur in a single individual with a co-administered agent. Such interactions can lead to substantial increases in the blood and tissue concentrations of a drug and may cause the accumulation of a toxic substance. These types of changes can alter a new drug's safety profile in important ways, particularly a drug with a narrow therapeutic range.

To minimize these risks in clinical trials, it is essential to clarify whether the drug is eliminated primarily by excretion of unchanged drug or by one or more routes

of metabolism. If elimination is affected primarily by metabolism, the principal metabolizing route(s) should be thoroughly understood. This information will identify the implications of metabolic differences between and within individuals and the importance of certain drug-drug interactions.

2.2 Pharmacogenetics of cytochrome P450

(1) Polymorphisms in cytochrome P450 gene family

Inter-individual variability in drug response is a serious problem if the drug has a narrow therapeutic window. Variability in drug response can be divided into pharmacological and pharmacokinetic factors. Sources of pharmacokinetic variability are also divided into the acquired factors (environment, disease, and age) and the innate factors, genetic polymorphisms.

Genetic polymorphisms in the genes that regulate the expression of metabolizing enzymes can cause inter-individual variability in drug metabolism, depending on the type of catalytic reaction and substrates. The polymorphism in drug metabolism may cause two different types of undesirable consequences in specific population:

(i) Increase in the plasma and tissue drug concentration to the toxic level, if there is a deficiency in metabolizing enzymes responsible for the elimination of the drug

(ii) Inefficient pharmacological response, if the metabolic activation of the drug by a polymorphic enzyme is essential, or if higher than normal levels of a metabolizing enzyme cause too rapid elimination rate (Brosen and Gram, 1989).

Polymorphisms in drug metabolism were studied by use of ethnic populations involving phenotype determination. The recent progress in molecular biology has enabled the genetic understanding the polymorphisms, which are now identified in the several genes encoding various cytochrome P450 isoforms. Although the genetic polymorphisms are reported for about five isoforms, CYP 2D6 and CYP2C19 are of clinical importance because these isoforms metabolize a wide variety of commonly prescribed drugs.

CYP2D6

A lot of prescribed drugs are metabolized by the cytochrome P450 2D6: antidepressants, antipsychotics, betablockers, and antiarrythmics (Cholerton et al., 1992; Gonzalez and Idle, 1994). In terms of molecular biology, the CYP2D6 polymorphism is explained by at least 17 different allelic variation (Daly et al., 1996), where most mutations in CYP2D6 are either deletions, point mutations or amino acid substitutions (Evert et al., 1994; Marez et al., 1996). Approximately 7% of Caucasians who lack CYP2D6 activity are categorized as poor drug metabolizers (Nakamura et al., 1987), whereas the individuals with normal metabolic activity are known as extensive drug metabolizers. Although the risk

of adverse effects by elevated plasma and tissue concentration depends on the contribution of CYP2D6 to the overall metabolism, poor metabolizers may generally face at a higher risk of adverse effects than extensive metabolizers (Cholerton, 1992). For example, the increase in plasma concentrations in poor metabolizers has been reported for the antidepresseants and neuroleptic drugs in which CYP2D6 metabolism is involved (Dahl and Bertilsson, 1993; Jerling et al., 1996). Furthermore, the withdrawal of perhexilene and phenformin from the market is caused by the toxicity in poor metabolizers.

CYP2C19

Although fewer drugs are metabolized by CYP2C19 than CYP2D6, the polymorphism in CYP2C19 is no less clinically important than CYP2D6 for two reasons. First, substrates of CYP2C19 include frequently prescribed drugs, such as omeprazole, propranolol, and imipramine (Daly et al., 1993; Goldstein and de Morais, 1994; Brosen et al., 1995). Second, a considerable portion of certain ethnic group lacks this enzyme: for example, approximately 20% of Asians are poor metabolizer of CYP2C19 (Wilkinson et al., 1989).

(2) Impact of GeneChip technology on tailored therapeutics

In 1997, Affymetrix, Inc. launched its GeneChip® CYP450 assay kit for research applications. The GeneChip CYP450 assay is more informative, faster and easier to use than current genotyping methods, such as conventional dideoxy sequencing and fragment length analysis. The GeneChip CYP450 assay uses over 8,000 different oligonucleotide probes synthesized on a glass chip to identify cytochrome P450 gene sequences encoding the known variations in the CYP2D6 and the CYP2C19 gene. The assay system is the DNA probe array based assay (Chee et al., 1996) for the simultaneous detection of 15 known mutations of the CYP2D6 gene and 3 mutations of the CYP2C19 gene (Press release from Affymetrix, 1997). The validity of the assay system has been confirmed by a greater than 99.7% base calling concordance with dideoxy sequencing method for known variants of the CYP2D6 and CYP2C19 genes (Press release from Affymetrix on Nov 6, 1997).

The GeneChip CYP450 assay is a product that addresses the field of pharmacogenetics, which will play an increasingly important role in drug research and clinical trials. The assay system enables clinical investigators to screen patients according to their genetic profile in order to maximize efficacy and minimize toxicity of many important classes of drugs. It means that the assay system has a potential to change the current drug development and marketing process. These assays can not only assist in more effective prescribing for compounds that can give rise to serious toxicity in the patients with a deficiency in the metabolizing enzyme, but also help in recognizing problems with polymorphic drug metabolism during drug development process. The assay system has been marketed to clinical laboratories and pharmaceutical companies. These companies will develop more appropriate dosing regimens to reflect

individual patient's metabolic rates, with the understanding of the involvement of specific CYP450 isozymes in the metabolism of new drug candidates.

2.3 Metabolic drug-drug interactions and clinical significance

Concomitant administration of several drugs is common and is often the situation in hospitalized patients. Whenever two or more drugs are administered over similar or overlapping time periods, the possibility for drug interactions exists. As the number of marketed drugs is increasing, clinical drug-drug interaction studies have become an important aspect of the drug development process. In reality, however, clinical interaction studies are conducted with a limited number of drug combinations, because studies of all possible interactions are neither practicable nor economic. Principally, the selection of drug interaction studies is usually based on two main criteria: the therapeutic index and the likelihood of co-administration (Tucker, 1992). Even with these criteria, clinical studies to assess drug interactions with new drug candidates are still very costly and time consuming. Therefore, the significance of preclinical efforts to predict possible drug-drug interaction is increasing in whole drug development process.

(1) Metabolic induction

Metabolic induction is associated with an increase in the intracellular concentration of a drug-metabolizing enzyme, caused by an increase in transcription of the associated gene. The extent of gene expression is influenced by exposure to a variety of endogenous and exogenous factors. Induction of drug metabolizing enzymes contributes to inter- and intra-individual variation in drug efficacy and potential toxicity associated with drug-drug interactions. There exist two scenarios in the induction-based interactions. The common situation is that a drug shows reduced therapeutic efficacy and increased metabolic clearance when the drug's deactivation by metabolism is induced by another drug. The other situation is that a drug shows increased toxicity if the drug is activated to yield a toxic metabolite. In reality, the changes in drug efficacy and toxicity are affected by:

(i) the extent of enzyme induction,
(ii) the therapeutic ratio of substrate and metabolite and
(iii) the relative importance of the involved enzyme in multiple metabolism pathways.

The clinical significance of the metabolic induction is dependent on the involved isoforms of cytochrome P450 enzymes. Several human CYP450s, such as CYP1A1, CYP2E1, and CYP3A4, are induced by dietary factors, smoking, drinking, and other drugs (Guengerich, 1995). The most clinically important metabolic induction is the elevated first-pass metabolism of CYP3A substrates following prolonged antibiotic or anticonvulsant treatment. In contrast, CYP2D6 appears to undergo no metabolic induction. Well-known inter-individual variation in CYP2D6 activity is the result of genetic polymorphism (Gonzalez et

al., 1988). However, it is evident that the major part of the human drug-metabolizing CYP450s are either polymorphic or inducible, explaining the important variation in CYP450-catalyzed drug metabolism (Table 2-2).

Table 2-2. Genetic polymorphism and inducibility among the important human cytochrome P450s (Ronis and Ingelman-Sundberg, 1999)

Isozyme	Polymorphism	Inducibility
CYP1A2	No	Yes
CYP2A6	Yes	Yes
CYP2C9	Yes	Yes
CYP2C19	Yes	Yes
CYP2D6	Yes	No
CYP2E1	Yes	Yes
CYP3A4	No	Yes

Over recent years, enormous advances have been made in characterizing the isoforms of cytochrome P450 enzymes and in establishing which isoforms catalyze particular biotransformation reactions. These progresses have brought about reasonable definition for which isoforms are induced by particular inducing agents (Spatzenegger and Jaeger, 1995). This increased knowledge has made the prediction of induction-based interaction much more reliable (Table 2-1).

(2) Metabolic inhibition

Most metabolic inhibitors will increase the plasma concentrations of affected drugs, which potentially result in drug toxicity. The increase in the plasma level may improve efficacy as far as the concentration remains within the therapeutic range. The consequences of inhibitory interactions are, therefore, more clinically severe than those of induction which usually leads to diminished efficacy. For example, cimetidine (Tagamet), an H_2 receptor antagonist and a potent inhibitor of CYP3A4, inhibits the CYP450-catalyzed metabolism of many drugs. It is reported that cimetidine may pose particular hazards when combined with warfarin and other anticoagulants (Serlin et al., 1979), qunidine (Hardy et al., 1983), or lidocaine (Knapp et al., 1983). Although cimetidine has been widely prescribed for ulcers, a considerable market share was lost to the noninhibitory alternative ranitidine (Zantac) through advertising, on the basis of the prospect of drug-drug interactions.

Due to the progress of molecular biology, detailed knowledge is becoming available concerning the specificity of inhibitors (Table 2-1). CYP1A is inhibited by ciprofloxacin, enoxacin, norfloxacin, and several related drugs (Fuhr et al., 1992); CYP2B by chloramphenicol (Ciaccio et al., 1987) and secobarbital (Lunetta et al., 1989); CYP2C by cimetidine (Chang et al., 1992) and cannabidiol (Narimatsu et al., 1993); CYP2D6 by many compounds, including quinidine, fluoxetine, clomipramine, and sertraline (Crewe et al., 1992); and CYP3A4 by

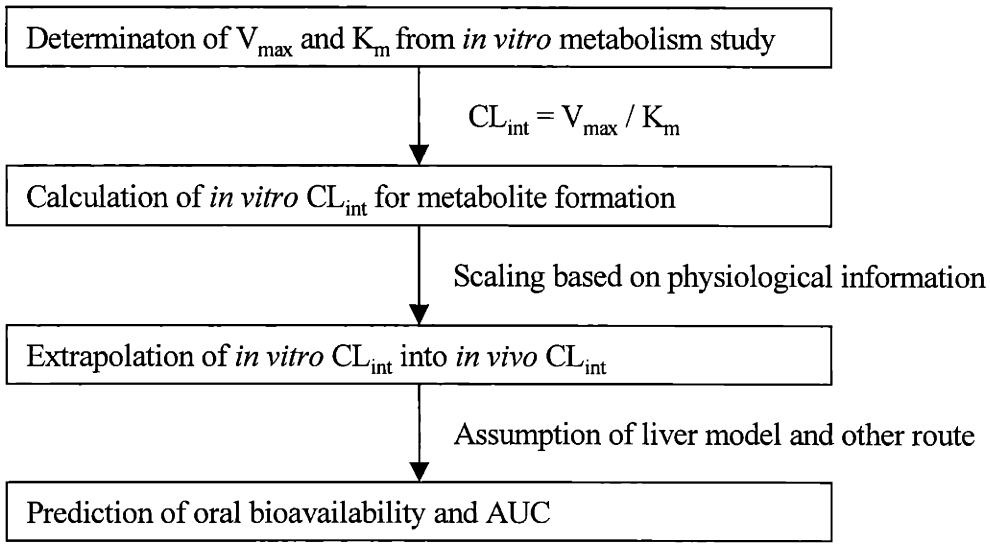
nifedipine (Pichard et al., 1990), cimetidine (Chang et al., 1992), verapamil, erythromycin, ethinyl estradiol (Pichard et al., 1990), and many other drugs. This expanding knowledge of the isozyme specificities of inhibitors makes possible the prediction of many drug interactions.

Several types of inhibition have been reported for the CYP450s. The most common mechanism is competitive inhibition. Other types of inhibition are product inhibition, tight-binding inhibition, mechanism-based inactivation, and covalent binding. For example, quinidine and most other CYP2D6 inhibitors seem to act in competitive manner (Koymans et al., 1992). Azoles are one example of ligands that are bound to the heme iron (Murray and Wilkinson, 1984). Discrimination among the types is essential when pharmacokinetic principles are applied for quantitative consideration.

2.4 Quantitative relationship between drug metabolism and pharmacokinetics

Most of *in vitro* drug metabolism studies have been interpreted in a qualitative manner, concerned with profiling metabolites, identifying the enzymes(s) involved, and investigating the reaction mechanisms. However, recent progresses in *in vitro* methodologies together with advances in analytical chemistry provide drug metabolism scientists with good opportunities to be more quantitative in the studies of drug metabolism. Figure 2-1 presents a four-stage procedure that extrapolates *in vitro* metabolism data to *in vivo* pharmacokinetic evaluation (Houston and Carlile, 1997). This approach assumes that enzyme systems *in vitro* operate in a manner comparable to the *in vivo* situation.

Figure 2-1. Four-stage procedure for the prediction of *in vivo* pharmacokinetics from *in vitro* drug metabolism systems



Intrinsic clearance (CL_{int}) is the principle parameter that can bridge the *in vitro* drug metabolism and *in vivo* pharmacokinetics (Houston and Carlile, 1997). The

term has units of flow rate (mL/min) and act as a proportional constant to describe the relationship between the rate of metabolism of a substrate and its concentration at the enzyme site (C_E) as eq. 1:

$$\text{Rate of metabolism} = CL_{int} * C_E \quad (1)$$

Where C_E is the unbound concentration of drug within the liver and assumed to equate with the concentration available to the enzymatic reaction. CL_{int} is also considered in the Michaelis-Menten relationship using V_{max} and K_m as Eq. 2:

$$\text{Rate of metabolism} = [V_{max} / (K_m + C_E)] * C_E \quad (2)$$

Where V_{max} is the maximal rate of metabolism and K_m is the Michaelis constant for the substrate-enzyme interaction. Under linear conditions, when the free substrate concentration (C_E) is 10% or less of the K_m, then eq. 2 reduces to eq. 3:

$$\text{Rate of metabolism} = (V_{max} / K_m) * C_E \quad (3)$$

By comparison of eq. 1 and eq. 3,

$$CL_{int} = V_{max} / K_m = \text{Rate of metabolism} / C_E \quad (4)$$

This equation illustrates that the Michaelis-Menten parameters (V_{max} and K_m) obtained from *in vitro* system can be used to predict *in vitro* CL_{int}. Depending on the underlying mechanism of the inhibitor, the V_{max} value of a drug can be decreased (non-competitive inhibition) or the K_m value can be increased (competitive inhibition). However, regardless of the mechanism, enzyme inhibition always results in a decrease in the intrinsic clearance ($CL_{int} = V_{max} / K_m$). Therefore, the concept of intrinsic clearance is the cornerstone for the extrapolation of *in vitro* data to the *in vivo* situation as illustrated in further discussion (Chapter 4.3).

In vivo consideration of CL_{int} is more complex because CL_{int} is not the sole determinant of hepatic clearance (CL_H), the term reflects the liver's ability to remove drug from the circulating blood. CL_H is also influenced by hepatic blood flow and the extent of binding of the drug to blood components, such as serum albumin (Wilkinson and Shand, 1975). Moreover, the pharmacokinetic parameter is expressed in terms of circulating blood concentrations and not drug concentrations at the active site of the enzyme. The quantitative relationships between CL_H, hepatic blood flow, unbound fraction of drug in the blood, and CL_{int} cannot be adequately defined without knowledge of the relationship between circulating drug concentrations and C_E. Since this cannot be determined practically, a liver metabolism model is necessary in order to relate these two concentrations and is an implicit part of defining CL_{int} *in vivo* (Wilkinson, 1987). If the liver is the main organ to metabolize the drug, the total clearance (CL_{tot}) is approximately equal to the hepatic clearance (CL_H) that can be expressed as eq. 5

$$CL_H = Q_h * E_h = Q_h * (f_b * CL_{int}) / (Q_h + f_b * CL_{int}) \quad (5)$$

Where Q_h is the hepatic blood flow, E_h is the hepatic extraction ratio, f_b is the unbound fraction of drug in blood, and CL_{int}, the intrinsic clearance, is a measure of the drug metabolizing activity ($= V_{max} / K_m$) in the liver *in vivo*. Based on this relationship, the hepatic first-pass effect reflects the hepatic bioavailability (F_h) can be expressed as:

$$F_h = 1 - E_h = Q_h / (Q_h + f_b * CL_{int}) \quad (6)$$

And the area under the blood concentration curve after oral dosing (AUC_{oral}) can be described as:

$$AUC_{oral} = F_h * dose / CL_H = dose / (f_b * CL_{int}) \quad (7)$$

As shown in eq. 7, a decrease in the CL_{int} ($= V_{max}/K_m$) caused by metabolic inhibition will yield an almost proportional increase in the AUC after oral dosing.

CHAPTER THREE

New technologies

3.1 *In vitro* human metabolism systems

In vitro models used for metabolism studies can be broadly separated into two groups. The first group consists of intact cells, including freshly isolated and cultured hepatocytes that do not require any cofactors or coenzymes added. The second group of *in vitro* systems includes subcellular fractions (e.g., microsomes) and hetrologous expression systems after reconstituted with appropriate cofactors and coenzymes. Many of the available methods are complementary and the impact of drug metabolism study is greatly increased when different approaches are combined with the understanding of the advantages and disadvantages of each system. The appropriate uses of three representative *in vitro* metabolism systems are summarized in Table 3-1.

Table 3-1. Comparison of *in vitro* drug metabolism systems

System	Appropriate Use	Comment
Hepatocytes	- Metabolite Profiling	- Liver slices are also used
	- Kinetic analysis (K_m, V_{max}, and CL_{int})	- More reliable for *in vivo* prediction
	- Drug-drug interaction	- Useful for metabolic induction and inhibition
Microsomes	- Kinetic analysis (K_m, V_{max}, and CL_{int})	- Less reliable for *in vivo* prediction
	- Identification of enzymes involved	- Regression analysis and selective inhibition are required
	- Drug-drug interaction	- Useful only for metabolic inhibition
Expression System	- Confirmation of metabolism	- For the identification of isoforms involved
	- Drug-drug interaction	- Useful only for metabolic inhibition
	- Bioreactors	- Metabolite production

(1) Freshly isolated and primary cultured hepatocytes

Freshly isolated or primary cultured hepatocytes offer a sophisticated environment to study drug metabolism. Studies are conducted within a physiological condition that contains normal concentrations of enzymes and cofactors, as well as the cellular components necessary to regulate enzyme induction. By analogy with hepatocytes found *in vivo*, drugs must cross a biological membrane, interact with cellular organelles and receptors, and compete with endogenous substrates for biotransformation. In comparison with many other *in vitro* techniques, hepatocytes are clearly more akin to the *in vivo* situation.

Hepatocytes have a multitude of applications in the field of drug metabolism from an industrial perspective, where they are used as a model system to study drugs in the discovery and development process. Hepatocytes have been isolated from a large number of laboratory animal species, as well as human liver tissue (Oldham et al., 1990). Hepatocytes have been used to study several aspects of drug metabolism, such as metabolic profiling; identification of metabolism pathways, and comparisons of metabolism among species to determine which animal species best represents the human metabolism. In suspension or culture, hepatocytes are also used to estimate *in vivo* pharmacokinetics.

Freshly isolated hepatocytes contain normal level of drug-metabolizing enzymes, making them suitable for drug metabolism studies. These cells when placed in suspension with appropriate nutrients can survive for extended periods, up to 3-4 h, after which significant cell death begins to occur (Sinz, 1999). Therefore, suspension experiments are considered short-term experiments, whereas cultured hepatocytes are considered longer-term experiments. Experiments best suited for suspension type studies include those that require appropriate levels of drug-metabolizing enzymes to form metabolites for identification purposes. These studies would generally include cross-species comparisons of rate and metabolite formation, *in vivo* estimations of drug pharmacokinetics, prediction of potential drug-drug interactions, or the formation of toxic metabolites. Although some of these studies can be performed in culture, it is more prudent to study these phenomena when the cells contain a normal complement and quantity of drug-metabolizing enzymes. Isolated hepatocytes in suspension are uniquely simple drug metabolize system requiring no additional cofactors, except for an adequate buffer system for suspension.

The application of cultured hepatocytes to the drug metabolism study is different from the experiments that employ freshly isolated hepatocytes. These differences in application stem from the changes that occur when the cells are placed in culture. Once hepatocytes are placed in culture, the concentrations of various drug-metabolizing enzymes tend to decrease, making hepatocytes in culture a less attractive model system for some studies. On the other hand, some of the architectural components of liver structure are resorted, such as attachment to a basement membrane, cell-to-cell contacts, and regeneration of membrane receptors that may have been removed during cell isolation. A major advantage

of primary cultured hepatocytes is the ability to study drug metabolism that takes longer than 2-3 h to manifest themselves, such as the effect of drugs on the expression of drug-metabolizing enzymes (Donato et al., 1995).

Although the freshly isolated and primary cultured hepatocyte as a model of drug metabolism is a close approximation to the *in vivo* functioning liver, the hepatocyte model has several limitations. These issues include (i) poor availability of human liver sample suitable for the preparation of fresh hepatocytes, and (ii) the problems inherent in primary hepatocyte cultures (Guillouzo et al., 1993). Even with these limitations, the hepatocyte serves as an excellent model system for drug metabolism. Clearly, hepatocytes are becoming more universal in their application to drug metabolism in such areas as evaluation of potential drug-drug interactions and as a model system to study CYP450 enzyme induction. A standardization of hepatocyte characterization and culture conditions would permit a more universal cross-referencing preservation of human hepatocytes.

(2) Subcellular system: microsomes

The liver is considered the major drug-metabolizing organ, and hepatic cells contain a multitude of drug-metabolizing enzymes that are usually localized to specific organelles within the cell. The major enzymes involved in drug metabolism, cytochrome P450s, are predominantly localized within the endoplasmic reticulum. Consequently, isolated liver microsomes, highly concentrated enzyme sources containing the endoplasmic reticulum, become the most popular subcellular fraction for the use of *in vitro* drug metabolism studies.

Preparation of microsomes requires disruption of the cell by homogenization, followed by differential centrifugation and resuspension of the pellet in a suitable storage buffer. Advantages of microsomes for drug metabolism include their ease of the preparation, flexibility of incubation conditions (cofactors, buffer, pH and temperature), and simple long-term storage protocols. Microsomes can be stored for a number of years at -80^{o}C and still retain their enzymatic activity reflective of fresh tissue (Pearce et al., 1996). Disadvantages of microsomes include loss of some enzymes during preparation, and limitation of sequential metabolism that requires multiple subcellular components to be complete. In this respect, freshly isolated or cultured hepatocytes and liver slices may be considered advantageous.

In the drug development process, microsomal studies are currently applied for metabolite identification, *in vitro-in vivo* extrapolation, and drug-drug interaction studies. Microsomes are also used to generate metabolites of compounds that can be rapidly identified using highly sensitive analytical techniques. After brief incubations of test drugs with microsomal suspension, gas chromatography-mass spectrometry (GC/MS) analysis has been used to identify metabolites (Woolf and Adams, 1987). The metabolites formed by microsomes can also be rapidly identified when this *in vitro* model is used with liquid chromatography-

electrospray ionization-mass spectrometry (LC/ESI-MS), a powerful technique for structural identification. A recent example of this technique is the use of LC-ESI/MS to analyze metabolism of taxol after incubation with human microsomes (Poon et al., 1996). The increasing sensitivity and automation of mass spectrometry and related technology will continue to have a considerable effect on the early drug development process.

Microsomes can provide the ideal means to estimate inter-individual variability in humans owing to the relative ease of use and availability of various human microsomes (Hoener, 1994). Human hepatic microsomes have been used successfully to estimate intrinsic clearance (CL_{int}; Chapter 2.4) (Hoener, 1994). Several studies have examined the relationship of the *in vitro* data derived from various models with the observed *in vivo* pharmacokinetic parameters (Houston, 1994; Iwatsubo et al., 1996). When compared to liver slices and hepatocytes, microsomes are found to be the inconsistent predictor of CL_{int}, possibly due to end-product inhibition (Houston and Carlile, 1997). This system, however, can produce acceptable prediction for many compounds and there is a need to define the caveat of the use of microsomes, considering where the limitations occur.

Human microsomes with the required cofactors are sophisticated representation of only part of the whole cell. They do not take into account the regulatory effects of cell-cell communication or the heterogeneous cellular nature. These considerations aside, it is clear that microsomes will continue to be the dominant *in vitro* metabolism system and play many useful roles in the pharmaceutical industry as far as the availability of fresh human tissue suitable for hepatocyte preparation is limited.

(3) Expression system via recombinant DNA technology

Major metabolic enzyme systems, such as cytochrome P450, comprise different enzymes called as isoforms. Each drug metabolism system often interacts differently with any number of drugs, and the expression of these different isoforms is under the control of different genetic factors, which contribute to the inter-individual variability. To investigate the function of each isoform, drug metabolism scientists have needed simplified *in vitro* metabolism models. With the development of heterologous expression systems (Remmel and Burchell, 1993), many investigators have turned to various "transgenic cell" lines as additional sources of human drug metabolism systems.

Heterologous expression of CYP450s has been achieved in mammalian cells, yeasts, insect cells, and bacteria. To introduce and express the genetic information of drug-metabolizing enzymes in a cell, tissue mRNA should be isolated and used as a source to synthesize the cDNA encoding the enzyme under investigation. Several methods are available for this approach (Friedberg et al., 1999). In the early studies, a few milligrams of tissue are required to obtain sufficient quantities for the generation of cDNA libraries. However, using reverse

transcription-polymerase chain reaction (RT-PCR), it is now possible to isolate a particular cDNA from a single cell (Yang et al., 1989). The cDNA, recombined with an expression vector containing a selection marker, is introduced into suitable recipient cells. Unlike the parental cells, cells that have taken up the vector should survive under selective pressure, and those that express the protein encoded by the cDNA of interest are identified by immunological and enzymatic assays.

A significant advantage of heterologous expression systems is that they can yield enough starting material so that the drug-metabolizing enzymes of choice can be purified to electrophoretic homogeneity. Although the purification of CYP450 enzymes from native human liver microsomes has been difficult, especially when trying to purify low-abundance CYP450 isoforms (Guengerich et al., 1986), many kinds of heterologously expressed CYP450 proteins, including CYP3A4, CY2C9 and CYP2D6, can be purified and reconstituted (Imaoka et al., 1996). Researchers can now perform *in vitro* metabolism studies with commercially available preparations of heterologously expressed human liver CYP450s. It should be emphasized, however, that the kinetic parameters (V_{max} and K_m) obtained from the expressed systems have to be combined with the information about the cellular concentration of the expressed enzymes to draw meaningful conclusions. Even if the catalytic activity of CYP3A4 toward a specific substrate is tenfold lower, but also if its concentration is tenfold higher than that of CYP2D6, both isoforms will contribute equally to the metabolism of this compound.

CYP450 expressed models are useful for confirming isozymes involved in specific metabolism of a drug. Furthermore, they provide powerful means to predict polymorphic drug oxidations and drug-drug interactions. Moreover, recombinant DNA technology allows the generation of sufficient material for structural studies on the drug-metabolizing enzymes. For example, heterologous expression system as a means of obtaining antigens for antibody production is an invaluable tool for the exact quantification of drug metabolizing enzymes (Friedberg et al., 1991).

3.2 *In vivo* human metabolism system: transgenic mouse

Although mammalian, yeast, insect, and bacterial systems represent rapid and simple methods for assessing the role of cytochrome P450s in drug metabolism, they are nonetheless *in vitro* models and may not necessarily reflect the *in vivo* situation. Heterologous *in vitro* expression models mimic only certain parts of the processes that determine the *in vivo* drug metabolism. One crucial difference is that *in vivo* drug disposition is determined by a range of parameters, including absorption, distribution and excretion. Therefore, *in vivo* models give us the opportunity to study the biological effects of gene expression under more physiologically relevant conditions. The development of *in vivo* models can be achieved by the use of transgenic animals either for heterologous protein

expression or for gene inactivation by homologous recombination (Fernandez-Salguero and Fonzalez, 1996). For the latter approach, the mouse is still the only species for which the necessary technology is available.

Several techniques for gene transfer into animals have been developed (Boyd and Samid, 1993). In one approach, the gene of interest is introduced by microinjection into the germline of mice. Male pronuclei of fertilized mouse oocytes are injected with several copies of the gene of interest and transferred into the oviducts of pseudopregnant foster mice. Up to 90% of the injected mice survive microinjection, approximately 20% of those develop to term, and 25% of those carry the injected DNA and, therefore, are transgenic (final yield = 4.5%). In another approach, the transgene is introduced into somatic cells *in vitro* by retroviral transfer. Cells with the heterologous gene are then implanted into the adult organism where the protein encoded by the foreign gene are produced (Valerio, 1992). The studies on transgenic animals demonstrate that these technologies are very powerful for elucidating the physiological role of drug-metabolizing enzymes and their regulatory system.

The inactivation of selected genes, knockout, is also a meaningful approach. However, the inactivation of a gene already in the germline may be problematic, for in some cases no viable offspring is obtained. Moreover, the phenotype obtained by the inactivation of a certain enzyme or regulatory protein may be diverse, depending on the generated transgenic line. Although an elegant technique has been recently devised to solve these obstacles (Rossant and Nagy, 1995), the knock-out models should be validated to confirm their applicability for *in vivo* drug-metabolizing systems in the drug development process (Tarbit et al., 1993; Rodrigues, 1994).

A number of laboratories are already expressing human drug-metabolizing enzymes in rodents (Simula, 1993). These humanized animal models for drug metabolism can facilitate the prediction of human pharmacokinetic properties at the drug discovery stage in future because they allow the evaluation of *in vivo* metabolic pathways catalyzed by the human enzymes.

CHAPTER FOUR

Strategic options enabled by the new technologies

4.1 Profiling of metabolites

In the drug development process, early information on metabolite profiles of a new drug in human is critical in selecting the appropriate animal species used for the toxicological evaluation. In the preclinical toxicity study, regulatory agencies require that the systemic exposure of a drug candidate and its metabolites in the animal species should exceed the level expected in humans in order to ensure a clinical safety margin. Therefore, it is critical to select the animal species that have metabolite profiles similar to those of humans. However, the *in vivo* human drug metabolism study is not carried out until the later stages of the drug development process, which is too late for animal selection. Recently, the increased availability of human tissues and advances in bioanalytical technologies have provided the opportunities for the *in vitro* human metabolism studies at the early stage of the drug development process before the toxicity studies are initiated (Wrighton et al., 1993).

In qualitative aspects, the *in vitro* metabolite profile of a drug can accurately reflect the *in vivo* metabolite pattern by the use of human liver slices and hepatocytes. From the physiological points of view, precision-cut liver slices are quite useful because this system retains the enzymes and cofactors of both Phase I and Phase II reactions and better simulates the *in vivo* situation (Dogterom, 1993). Although liver slices are valuable in identifying metabolite profiles, their use in obtaining kinetic parameters is limited. Worboys et al. (1996) showed that the values of CL_{int} (= V_{max} / K_m) of a series of drugs in the liver slices are significantly underestimated compared to those in hepatocytes, by a factor ranging from 2 to 20. These results suggest that distribution equilibrium between all of the cells within the liver slice and the incubation medium cannot be achieved in the incubation period, because of the slice thickness (up to 260 μm). Freshly isolated and cultured hepatocytes are free of the distribution problem and may be more useful for identifying the metabolic pathway of drugs. For example, *in vitro* metabolic profile of ketotifen, an antiasthmatic drug, by cultured human hepatocytes was consistent with the *in vivo* metabolic pathways (Le Bigot et al., 1987).

Another consideration in *in vitro* metabolite profiling is the choice of drug concentrations, because the major metabolic pathway may be shifted depending on the drug concentration used. The clinical studies indicated that N-demethylation is the major metabolic pathway of diazepam in humans. However, *in vitro* studies in human liver microsomes indicated that 3-hydroxylation was the major metabolic pathway when a high concentration (100 μM) of diazepam was

incubated (Inaba et al., 1988). When a lower substrate concentration relevant to *in vivo* situation was used (Yasumori et al., 1993), the major metabolic pathway of diazepam was confirmed to be N-demethylation in human liver microsomes. These studies indicate that the appropriate choice of drug concentration is critical to *in vitro* profiling of metabolites.

4.2 Identification of CYP450 isozymes involved in drug metabolism

Metabolism of drugs is usually complex, involving several pathways and various enzyme systems. In many cases, a single metabolic reaction may involve multiple isozymes. For example, the S-oxidation of 10-(N,N-dimethylaminoalkyl) phenothiazines in human liver microsomes is catalyzed by several cytochrome P450s, including CYP2A6, CYP2C8 and CYP2D6 (Cashman et al., 1993). For another example, amitriptyline is N-demethylated to nortriptyline in human by numerous CYP450 isoforms, including CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Venkatakrishnan et al., 1998). Thus the complexity of metabolism results from the multiplicity of enzyme systems.

Definitive identification of the CYP450 isoforms involved in drug metabolism is essential in predicting potential drug-drug interactions and inter-individual variability due to polymorphism. Best combination of the *in vitro* approaches has been studied in order to identify which cytochrome CYP450 isoform is responsible for metabolizing a drug of interest (Guengerich and Shimada, 1993). To determine the role of a CYP450 isoform in a specific metabolism pathway, several criteria should be confirmed in the drug metabolism studies (Correia, 1995):

(i) Determination of the metabolism pathway by the purified enzymes
(ii) Inhibition of the metabolic activity by isoform-specific inhibitors and/or antibodies
(iii) Correlation of the metabolic activity in various liver samples with the activities of marker substrates specific to the individual CYP450 isoform
(iv) Confirmation of the activity in heterologous expression system

In enzyme identification, selection of the substrate concentration is highly important because several cytochrome P450 isoforms with distinct K_m values may contribute to the metabolism of a single drug. For example, a CYP2C antibody shows no inhibition of diazepam N-demethylation at a substrate concentration of 200 μM but inhibits over 80% activity at a lower substrate concentration of 20μM (Kato and Yamazoe, 1994). This illustrates the importance of using clinically relevant substrate concentrations for *in vitro* studies in identifying drug-metabolizing enzymes. It is also essential to select the appropriate concentration of isoform-specific inhibitors (Newton et al., 1995).

4.3 Prediction of dropout risks in clinical trials

(1) Drug-drug interaction

In the past, drug-interaction studies were conducted at a late clinical stage during Phase II and III. With the increasing availability of human drug metabolizing enzymes, *in vitro* systems are used as screening tools to predict *in vivo* drug interaction at a much earlier stage before the drug is selected for the preclinical development. Oxidative metabolism represents a major route of elimination for many drugs, and inhibition of cytochrome P450s is one of the main reasons for drug interactions. A significant drug-drug interaction occurs only when drugs compete for the same enzyme system and when the metabolic reaction is a major elimination pathway. Therefore, before the drug-drug interaction study, it is essential to identify the isoforms involved in the metabolism of particular drugs and to evaluate the relative contribution of the isoforms to the whole metabolic pathways. An understanding of the mechanism involved in enzyme inhibition (e.g., competitive or mechanistic inhibition) is also critical in order to provide a rational basis for designing experimental conditions and interpreting drug-drug interaction data.

One of the important criteria in *in vitro* drug-drug interaction studies is the use of clinically relevant concentrations of the inhibitor and the substrate. The use of supra-therapeutic drug concentrations may produce a drug-drug interaction *in vitro*, but not *in vivo*, because the major metabolic pathway is shifted depending on the drug concentration used. Another important criterion is the protein concentration of hepatocytes, microsomes or expressed enzymes. The inhibitory activity may be underestimated in the presence of a high concentration of protein because of the depletion of the inhibitor by non-specific binding to the proteins.

Although increasing evidence has demonstrated that *in vitro* interaction studies can accurately reflect the *in vivo* situation (Wrighton and Ring, 1994; Riesenman, 1995), it must be emphasized that the correct extrapolation of the *in vitro* interaction data to the *in vivo* situation requires a good understanding of pharmacokineitc principles. For competitive inhibition, the rate of metabolism in the absence (V_0) and presence (V_i) of the inhibitor can be expressed as:

$$V_0 = V_{max} * S / (K_m + S) \quad (8)$$

$$V_i = V_{max} * S / [K_m * (1 + I / K_i) + S] \quad (9)$$

Where V_{max} is the maximum velocity, K_m is the Michaelis constant of the substrate, K_i is the inhibition constant of the inhibitor, and S and I are the substrate and inhibitor concentrations, respectively. By rearrangement of eq. 8 and eq. 9, the percent of inhibition can be described as:

$$(V_0 - V_i) / V_0 (\%) = (I / K_i) / (1 + I / K_i + S / K_m) * 100 \quad (10)$$

As shown in eq. 10, the percent of inhibition is dependent on both the ratio of [I / K_i] and [S / K_m]. Thus, an understanding of the relationship between the substrate and the inhibitor concentrations is critical to the design and interpretation of *in vitro* inhibition studies.

The goal of an *in vitro* interaction study is to predict the quantitative effect of drug inhibition *in vivo*. For the competitive inhibition, the percent of inhibition of intrinsic clearance ($CL_{int} = V_{max} / K_m$) can be described as eq. 11 (see Chapter 2.4):

$$(CL_{int,0} - CL_{int,i}) / CL_{int,0}\,(\%) = (I / K_i) / (1 + I / K_i + S / K_m) * 100 \qquad (11)$$

where $CL_{int,0}$ and $CL_{int,i}$ are the intrinsic clearance in the absence and presence of the inhibitor, respectively. After drug administration *in vivo*, both [S] and [I] continue to change as a function of time, unless they are maintained under steady-state conditions. Therefore, appropriate pharmacokinetic models to simulate the change of [S] and [I] are needed in order to obtain an accurate *in vitro-in vivo* extrapolation. The physiologically based pharmacokinetic approach can provide an accurate prediction of drug-drug interactions. For example, Lin et al., (1984) successfully applied a physiologically based pharmacokinetic model to predict product inhibition. However, this approach is very costly and time-consuming when all of the parameters needed are obtained. A literature survey (Lin, 1998) reveals that *in vitro* interaction studies are generally carried out in a qualitative sense, by comparing the relative affinities of the substrate (K_m) and the inhibitor (K_i) with their concentration ranges in clinical studies. One of the most common approaches is the use of *in vitro* K_i values together with *in vivo* values of the peak plasma concentration (C_{max}) of the inhibitor to forecast the possibility of *in vivo* drug-drug interactions.

(2) Bioavailability

One of the main objectives of the *in vitro* metabolism is the quantitative prediction of the *in vivo* pharmacokinetic parameters, such as bioavailability (F). Knowing that the *in vitro* extrapolation is an approximation, the *in vitro* metabolic data can be extrapolated quantitatively well to the *in vivo* situation by applying the appropriate pharmacokinetic principles with a good understanding of the interdependent factors involved (Houston and Carlile, 1997). There are many examples of good quantitative *in vitro-in vivo* extrapolation. Indinavir, a potent HIV protease inhibitor, exhibited marked species differences in metabolic clearance (CL_{int}). This drug is metabolized mainly by the CYP3A4 isoform to form oxidative metabolites in all species examined. The *in vitro* metabolic clearance obtained from incubations with rat, dog, and monkey liver microsomes was in good agreement with the corresponding *in vivo* metabolic clearance of indinavir (Lin et al., 1996). Iwatsubo et al. (1997 and 1998) successfully predicted the *in vivo* metabolic clearance and bioavailability of YM796, a central nervous system drug for the treatment of Alzheimer's disease, by using a

recombinant human CYP3A4 system together with the knowledge of the content of this isoform in human liver microsomes.

Although hepatic drug metabolism can make a major contribution to bioavailability, the combination of hepatic and intestinal drug metabolism often has a large influence on first-pass drug metabolism after oral administration (Hall et al., 1999). A literature review revealed that in some cases, *in vitro* metabolic data failed to predict *in vivo* clearance (Lin and Lu, 1997). Sources of inaccuracy in predicting the *in vivo* metabolic clearance include presence of extrahepatic metabolism. CYP3A4, the most abundant cytochrome P450, presents both in the liver (Guengerich et al., 1986; Shimada et al., 1994) and in the epithelial cells that line the lumen of the small intestine (Watkins et al., 1987; Kolars et al., 1992). Recently, it has become clear that some substrates for CYP3A4 also undergo substantial metabolism in small intestine during absorption process (Wu et al., 1995; Paine et al., 1996; Lown et al., 1997).

The bioavailability (F) of an orally administered drug is dependent on a number of physical factors. These include disintegration and dissolution properties of the drug formulation, solubility of the drug molecule in the gastrointestinal tract, permeability through intestinal membrane, and metabolism by intra- and extra-hepatic enzymes. By incorporating all these factors, the bioavailability (F) of a drug can be expressed as eq. 12 (see eq. 6 in Chapter 2.4):

$$F = F_{ab} * F_i * F_h = F_{ab} * (1-E_i) * (1-E_h) \qquad (12)$$

Where F_{ab} represents the net fraction of a dose absorbed across the membrane of the intestinal cell, and F_i and F_h represent the fraction escaping first-pass intestinal (I) or hepatic (H) metabolism, respectively. From a historical perspective, only the first and third terms of eq. 12 (F_{ab} and F_h) were considered to have a significant impact on the bioavailability of most drugs. However, the discovery of a major drug-metabolizing enzyme CYP3A4 in the gastrointestinal tract has lead to an evaluation of its role as another determinant of oral drug bioavailability. In recent years, it becomes possible to investigate the absorption and extra-hepatic drug metabolism coordinately, using cell lines derived from small intestine, such as Caco-2 (Crespi et al., 1996).

4.4 Bioreactors for metabolite production

The recent progress in combinatorial chemistry and its effect on the drug discovery process have increased the number of structurally diverse and complex drug candidates (Kubinyi, 1995), such as taxol (Rahman, 1994). However, the modern-day drug development also makes it difficult to synthesize the metabolites of which structures are identified. Pharmaceutical companies are looking to use bioreactors as alternative means of the bulk synthesis of such metabolites. The drug-metabolizing enzymes that carry out regio-selective or

enantio-selective biotransformation can be harnessed to generate large amounts of the desired products.

Basically, organisms that express drug-metabolizing enzymes have biotechnological applications as bioreactors for the production of valuable metabolites. Bacterial systems appear to be the most useful for this purpose, because they can be grown to relatively high cell densities, express high levels of recombinant protein, and need less cost for scale-up production. However, the application of the bacterial systems requires an establishment of separation technology, by which the metabolites are isolated from large volumes of culture medium.

An approach to resolve this difficulty is to immobilize drug-metabolizing enzymes to solid supports under mild conditions (Stempfer et al., 1996). The immobilization procedure requires:

(i) covalent attachment to solid supports,
(ii) absorption on solid supports, or
(iii) encapsulation (Kathcalski-Katzir, 1993).

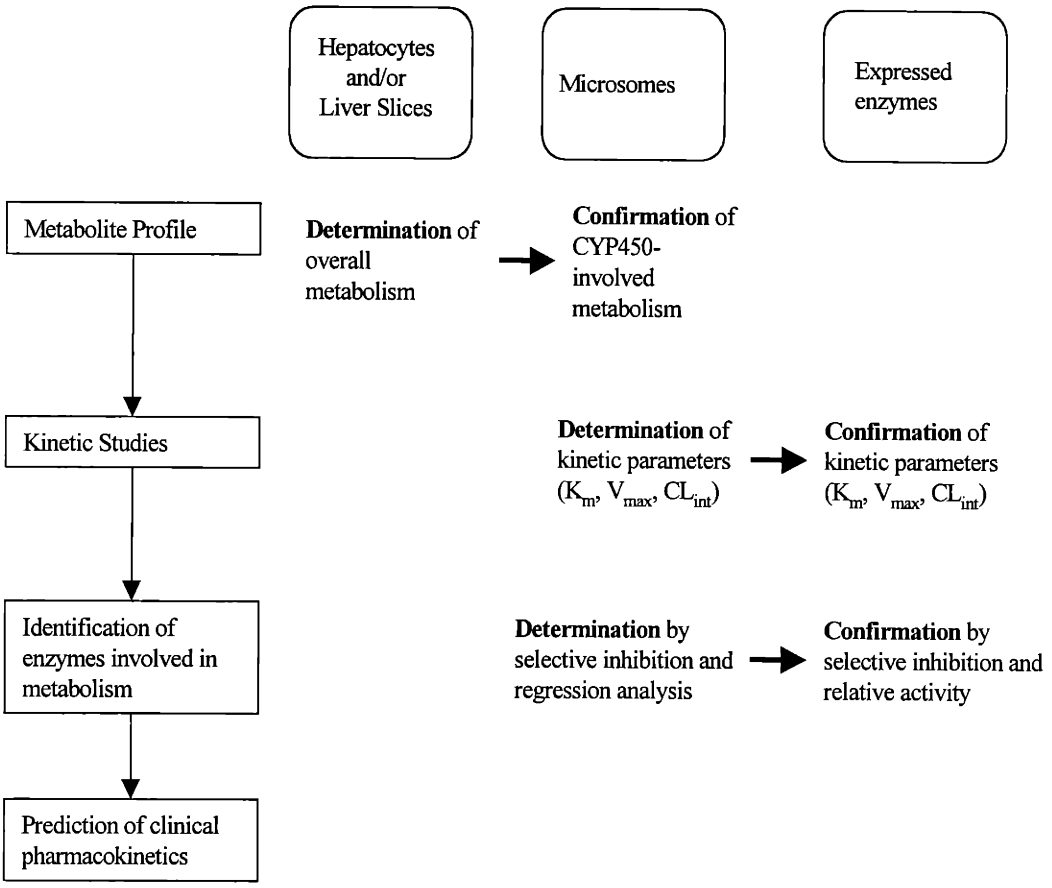
The technological advantages of the immobilized-bioreactors are easy separation of products from incubation mixtures, stabilization of the enzyme against degradation, and the opportunity to recover and reuse the enzymes (Dulik and Fenselau, 1988). It is envisioned that computer-controlled systems will enable on-line monitoring of drug loading and product recovery process in order to circumvent the end-product or substrate inhibition. The establishment of immobilized bioreactors technology will require inexpensive and large amounts of sources of drug-metabolizing enzymes. Therefore, the technological goal of the immobilized bioreactors will be met with currently available heterologous expression systems.

4.5 Integration of strategic options

Many pharmaceutical companies are establishing rigorous drug metabolism programs using expression systems, hepatic microsomes, hepatocytes, or liver slices from appropriate species, including humans. This type of approach rapidly assesses the metabolic labiality of a series of compounds and facilitates creation of a structure-metabolism relationship around the obtained information (see Chapter 5.1). Given that pharmaceutical companies are turning their attention to combinatorial methods and high-throughput screening of drugs, the importance of the *in vitro* methods for investigating the drug metabolism will continue to increase. Moreover, the combined approach of various drug metabolism systems will play an important role in the discovery and development of novel drug entities, if the different systems are used in an integrated fashion. The framework of an integrated approach proposed by Rodrigues and Wong (1997) involves the use of the cDNA-expressed enzymes in conjunction with human hepatocytes,

liver slices, and hepatic microsomes (Figure 4-1). Such an integrated approach enables one to exploit the strengths of each drug metabolism system.

Figure 4-1. Integrated approach for studying the *in vitro* drug metabolism in the drug development process.



In this approach, it is imperative that an overall metabolic profile is initially obtained with intact cell models, especially when information concerning the *in vivo* drug metabolism is not available. In turn, the data that are obtained with either cDNA-expressed enzymes or microsomes can be viewed in perspective (Rodrigues et al., 1995 and 1996). In this system, cDNA expressed enzymes should be used to confirm the results obtained with native human liver tissue. In addition, it is possible to use various cell lines (e.g., Caco-2), so that new drug absorption and metabolism can be coordinately investigated (Crespi et al., 1996).

This integrated *in vitro* approach enables one to assess the involvement of specific cytochrome CYP450 isozymes such as CYP2D6 or CYP3A4. If this information is provided early in the drug discovery process, medicinal chemists can understand the role of different functional groups on the parent molecule in altering the systemic exposure of a drug. With increasing concerns about drug-

drug interactions or inter-individual variability, early elimination of compounds that inhibit CYP3A4 or compounds that are primarily metabolized by polymorphic isozymes (CYP2D6 or CYP2C19) is of increasing importance. This knowledge can assist in predicting the existence of poor drug metabolizers with exaggerated systemic exposure and the likelihood of drug-drug interactions.

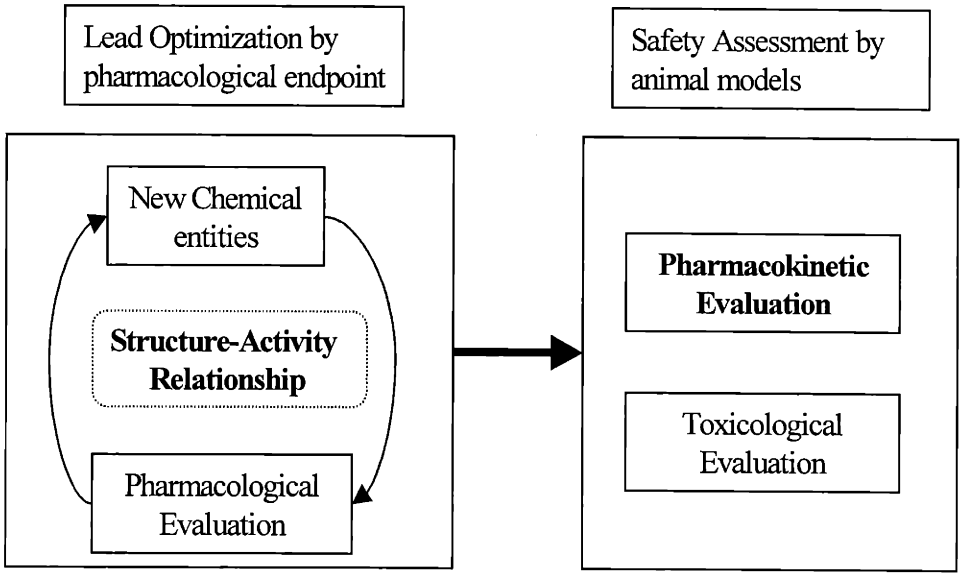
CHAPTER FIVE

Integration of *in vitro* human metabolism system into the drug discovery process

5.1 Impact of drug metabolism studies on the drug discovery process

Historically, pharmacokinetic studies have been initiated after a candidate compound was discovered and optimized based on pharmacological endpoints (Figure 5-1). However, pharmacokineticists have often experienced discrepancies between *in vitro* potency and *in vivo* pharmacological models. Although there are many pharmacokinetic reasons an early lead compound does not work *in vivo*, the first step should be the determination of an exposure-response relationship in the animal models. If the compound is present in the plasma, then target tissue levels or protein binding may be the issues to be investigated. But if the drug is not detectable in the *in vivo* animal models, then absorption or metabolism should be re-evaluated in order to examine whether these factors are limiting *in vivo* activity.

Figure 5-1. Traditional role of pharmacokinetic evaluation in the drug development process



Recently, industrial demands for greater throughput of new compounds with less dropout risks in the clinical trials have resulted in early involvement of pharmacokinetic studies in the drug discovery process. Therefore, it is now essential to design a screening process to select both pharmacologically and pharmacokinetically viable compounds in the drug discovery phase. There are

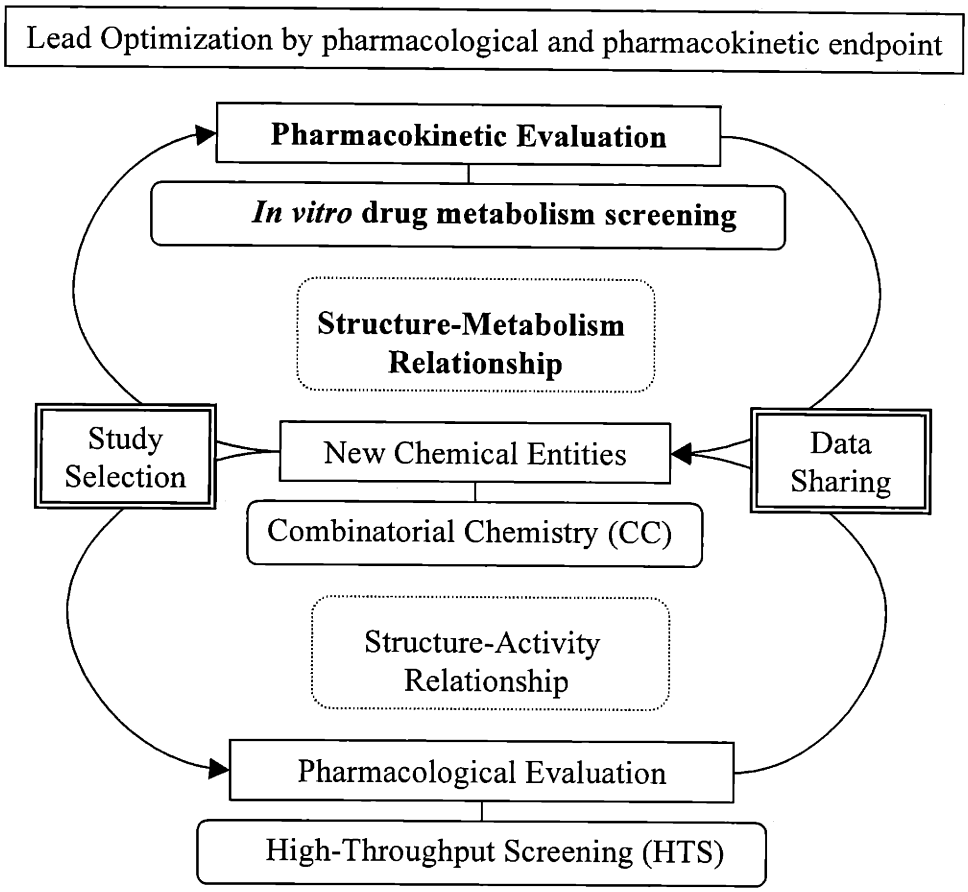
three major criteria in which drug metabolism and pharmacokinetic studies can have a significant impact on the drug discovery process (Inskeep and Day, 1999);

(i) Select compounds with pharmacokinetic parameters relevant to the target therapeutic area.
(ii) Predict human pharmacokinetics quantitatively to design phase I trial rationally.
(iii) Customize pharmacokinetic properties that can differentiate a candidate from other drugs.

However, the integration of the pharmacokinetic screening with the pharmacological screening is a critical question for the establishment of an effective drug discovery program. The vast numbers of compounds synthesized with combinatorial chemistry are increasing the needs for quick support by pharmacokinetic evaluation. One of the answers to the question is the "brute force" approach (Campbell, 1994), in which many compounds are screened until the desirable pharmacological and pharmacokinetic traits are found (Olah et al., 1997). The primary focus of this approach is fine-tuning of both the structure-activity relationship and the structure-metabolism relationship. Theoretically, this strategy involves initiating pharmacology and pharmacokinetic studies in parallel. Although this approach may provide a lot of data in a short time, it also requires extensive resources and may be practical only for big pharmaceutical companies. In reality, many companies are customizing this approach: they are carefully selecting the studies in order to sequentially obtain the information necessary for the progress in the drug discovery program and avoid studies that could be delayed until the success of the compound is more assured (Figure 5-2).

In reality, the design of new drugs is still understood by many medicinal chemists as a maximization of the desired drug activity within certain structural limits. Sometimes, however, compounds that show very high *in vitro* activity may prove later to have no *in vivo* activity, or to be highly toxic in *in vivo* models. Lack of *in vivo* activity is often attributed to undesirable pharmacokinetic properties, and the toxicity may result from the formation of reactive metabolites. Therefore, rational drug design should also take both pharmacokinetic and metabolic information into consideration, and the information should be incorporated with molecular-biochemical and pharmacological data to provide promising drug design. This lead-optimization process requires an iteration of reassessing structural changes to obtain optimal pharmacological and pharmacokinetic properties. An efficient feedback loop between the drug metabolism scientists and the medicinal chemists should be established for simultaneous consideration of both structure-activity relationship and structure-metabolism relationship (Figure 5-2).

Figure 5-2. An example of the brute force approach for pharmacology and pharmacokinetic studies in the drug discovery program



5.2 Endpoints of *in vitro* drug metabolism screening

The responsibility of drug metabolism scientists in the drug discovery process is to profile metabolic strength and weakness of new chemical entities and identify the structural attributes most favorable for the desired *in vivo* pharmacokinetics. Although most drug discovery projects have employed both *in vitro* and *in vivo* techniques in the relative proportions to meet specific needs, *in vitro* approach is far more appropriate than *in vivo* approach if a large number of compounds need examination. It is also inappropriate to combine *in vivo* animal models with combinatorial chemistry and high-throughput screening, because *in vivo* animal models are labor-intensive and their throughput is limited.

In vitro drug metabolism screening is useful in evaluating the pharmacokinetic endpoints for the lead optimization in the proposed drug discovery program (Figure 5-2). By using the intrinsic clearance (CL_{int}) as a parameter for the extrapolation of *in vitro* data to the *in vivo* situation (see chapter 2-4), one can

clearly define the pharmacokinetic endpoints to be evaluated in the *in vitro* drug metabolism screening (Table 5-1).

Table 5-1. Endpoints of *in vitro* drug metabolism screening for the lead optimization.

Pharmacokinetic properties	Endpoints of *in vitro* drug metabolism screening
Bioavailability	- Choose the compounds with the lowest CL_{int} values (see Chapter 2.4)
Drug-drug interaction	- Prioritize the compounds which are metabolized by multiple CYP450 isozymes
	- Choose the compounds with the lowest inhibition activity: $(CL_{int,0} - CL_{int,i})/ CL_{int,0}$ (%) (see Chapter 4.3)
Inter-individual variability	- Drop the compounds of which metabolism is dominated by CYP2D6 or CYP2C19
	- Choose the compounds with the smallest coefficient variation of CL_{int} calculated from several microsome preparations

There are more advantages of the *in vitro* drug metabolism screening: (i) selection of a candidate is not confounded by inter-species differences, (ii) it is easy to characterize metabolites for potential activity, and (iii) information derived from the *in vitro* models can be integrated into the ongoing synthetic efforts.

5.3 High-throughput approach of *in vitro* drug metabolism screening

Drug metabolism departments within the pharmaceutical industry are re-organizing themselves for the rapid evaluation of large numbers of compounds (Tarbit and Berman, 1998). This is partly due to the need for high-throughput approaches in drug metabolism, driven by the impact of the combinatorial chemistry and the high-throughput pharmacological screening. Of equal importance is the belief that large data sets will dramatically increase the ability to model biological processes and relate these processes to structural features of the compounds of interest. The *in vitro* drug metabolism approach can generally accommodate a large number of compounds for evaluation. Although the systems currently employed for drug metabolism screening can not be considered truly high-throughput, several of them are rapid enough to be a practical part of the screening paradigm for the fast-moving discovery programs (White, 2000). For example, a rapid microtiter plate (MTP) assay has been successfully applied for the high-throughput screening for CYP2D6 inhibitors (Favreau et al., 1999)

The linkage of *in vitro* metabolism system with another technology also has a great impact to change the drug discovery process. For example, new high-throughput physicochemical methods are being linked to appropriate *in vitro* metabolism systems. In the future, robotics and miniaturization will be further applied to the measurement of physicochemcal and drug metabolism properties (Kansy et al., 1998). Even though these approaches do not completely replace *in vivo* methods, considerable progress has recently been made towards this future perspective (Smith and van de Waterbeemd, 1999).

CHAPTER SIX

Implementation of new drug discovery process: a case study of EGT Pharmaceuticals, Inc.*

** The name of the company is disguised for confidentiality.*

6.1 Dropout in clinical trial due to pharmacokinetic problems

Since 1970s, EGT Pharmaceuticals Inc. (EGT), had focused its drug research and development (R&D) resources in developing biotechnology products for the niche therapeutic area, and successfully, by 1990, marketed novel pharmaceuticals, without experiencing any dropout of drug candidates. Since the success in developing bio-products, EGT has tried to diversify its product lines, including chemically synthesized compounds. The R&D structure was re-organized according to this corporate goal. Figure 6-1 illustrates the organizational chart of EGT since 1990, where the drug discovery process was implemented by three functional groups in the research laboratory. Drug metabolism studies, in which animal models were mainly used, were not involved in the drug discovery program, but defined as the function of the pharmacokinetic evaluation in the development laboratory (Figure 6-2).

Figure 6-1. R&D organization chart of EGT Pharmaceuticals Inc., in 1990-1999

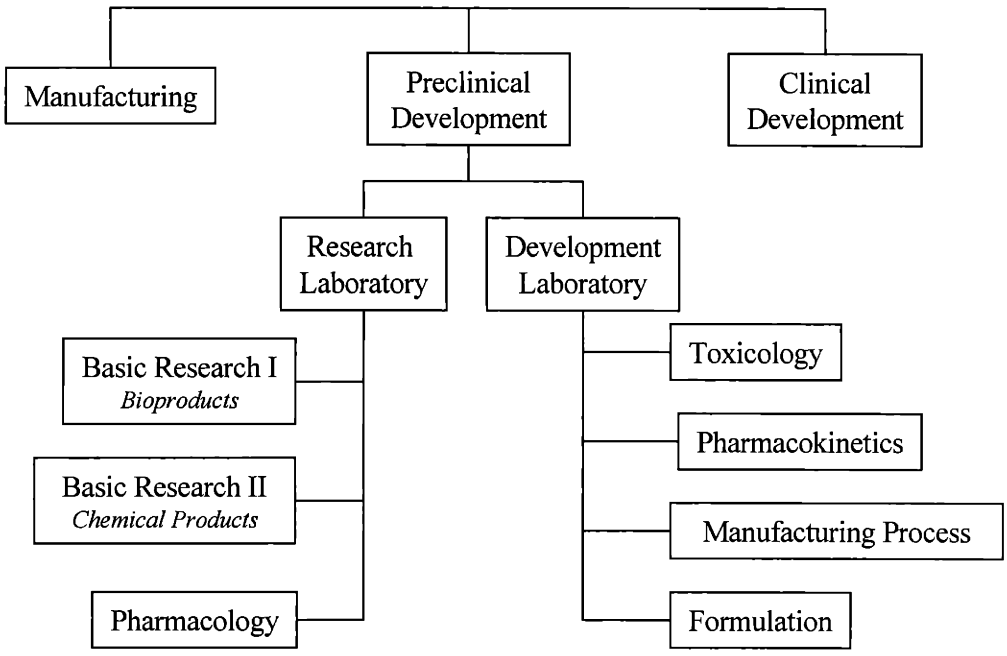
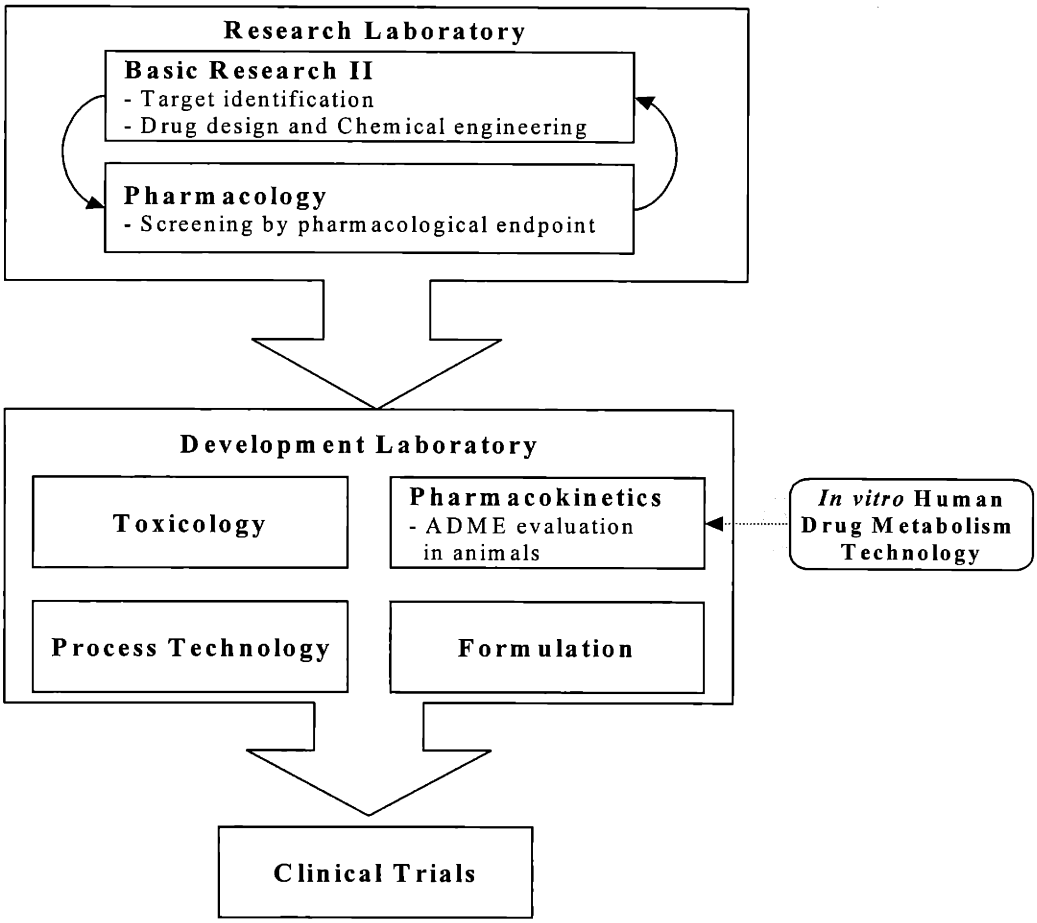


Figure 6-2. Flow chart of the drug discovery and development process for chemical products in EGT Pharmaceuticals, Inc.



In spite of these strategic attempts, the company through the 1990s suffered successive failures of the drug candidates. Five drug candidates, all chemically synthesized compounds, were forced to withdraw from their development pipelines. The last failure that the company experienced was due to poor pharmacokinetics in the phase I trial. The oral bioavailability of the candidate in healthy volunteer was less than 1%, one-tenth of the value observed in dogs in the preclinical pharmacokinetic studies. Moreover, an unmanageable inter-individual variability was observed: two of eight volunteers in the same cohort showed about ten times higher blood concentration than the other volunteers. These results strongly suggested the existence of poor drug metabolizers due to polymorphisms in the metabolism of the compound.

6.2 Project-team approach to a metabolism-driven drug discovery program

In parallel to the problematic phase I trial, new technologies of *in vitro* human drug metabolism system, such as human microsomes and cDNA expressed

systems, were introduced to the pharmacokinetic group of the development laboratory (Figure 6-2). In order to elucidate the poor pharmacokinetics observed in the clinical trial, scientists in the pharmacokinetic group were engaged in establishing *in vitro* human drug metabolism system. They revealed that the failed compound is, as speculated, metabolized extensively by CYP2D6 and inter-individual variability is due to polymorphism of the CYP450 isoform.

This study drove EGT to reconsider their drug discovery and development process. To examine the impact of the *in vitro* drug metabolism screening on the drug discovery process, a project team was formed from drug-metabolism scientists, medicinal chemists and pharmacologists. More than fifty chemical entities, which were primarily screened by pharmacological endpoints, are subsequently screened based on the metabolic labiality in the *in vitro* human metabolism system. This approach successfully identified a promising compound that had an acceptable bioavailability and little inter-individual variability.

The managers of EGT then attempted to change their drug discovery process by incorporating *in vitro* drug metabolism systems, via a technology transfer from the pharmacokinetic group in the development laboratory to the basic research group in the research laboratory. However, the managers soon realized that the transfer process was unsuccessful due to organizational constraints:

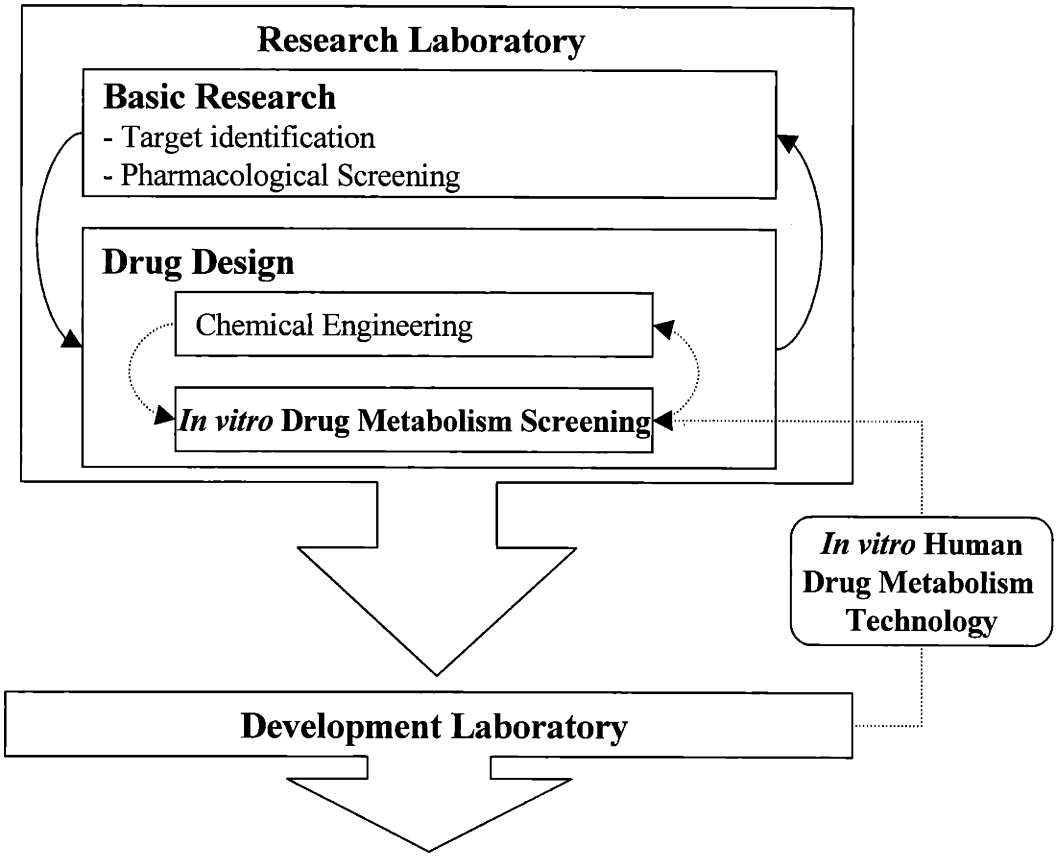
(i) The research laboratory lacks technology communicator, a scientist capable of managing the knowledge about the *in vitro* drug metabolism system in the drug discovery process.
(ii) Organizational boundaries, including physical distance between two laboratories, make people-to-people communication extremely difficult.

6.3 Implementation of new drug discovery strategy and organizational change

To settle these difficulties, an organizational change in the research laboratory was implemented in the beginning of 2000, so that the new organizational structure can accommodate the integration of the *in vitro* drug metabolism system into the drug discovery process. Figure 6-3 illustrates the flow chart of the new drug discovery process in EGT, in which a team responsible for the *in vitro* drug metabolism screening is established in the drug design group. The close communication with the chemical engineering team can lead to the establishment of a feedback loop for the lead-optimization based on the structure-metabolism relationship, as well as the structure-activity relationship between the drug design and the basic research group (Figure 6-3).

This case illustrates an example of organizational change driven by a technological innovation, where the technology can be incorporated into the new organizational structure. However, in order to keep the new drug discovery process functional, further managerial efforts should be required, such as facilitating people-to-people communication between the drug metabolism scientists and the medicinal chemists.

Figure 6-3. Flow chart for the new drug discovery process of chemical entities after the organizational change



CHAPTER SEVEN

Conclusion

The use of animal models to predict pharmacological effects in humans comes from the belief in the unity life of mammals. Scientists had believed that the central physiological functions, including the major routes of endogenous metabolism, were common in all mammals. However, in reality, pharmacokineticists have failed to find an animal species in which the drug metabolism processes are consistently the same as those in humans.

Pharmaceutical companies in recent years have placed a greater emphasis on the *in vitro* human drug metabolism studies in the drug discovery process. Early identification of pharmacokinetic problems, such as the involvement of polymorphic CYP450 isozymes may drive synthetic efforts to identify promising agents that possess an appropriate pharmacokinetic profile in humans. The establishment of predictive models using the *in vitro* human metabolism systems may provide insight into any potential weakness in the metabolism in humans. An approach adopting these models can have a greater degree of confidence before expending resources to advance a compound into the clinical trial.

The concept that the properties of the whole are the sum of the properties of the parts has had a profound impact on the manner of conducting science. Drug metabolism scientists have applied this concept by using a broad spectrum of methods, from expressed enzymes, microsomes to hepatocytes. However, the extrapolation of the *in vitro* metabolic data to the *in vivo* situation is not always straightforward. It is clear that each *in vitro* system has its advantages and disadvantages. As long as their limitations are recognized and appropriate considerations are taken into the study design, *in vitro* systems can aid in the selection of the animal species for toxicity studies as well as provide preliminary profiles of human metabolism.

With the recent breakthroughs in molecular biology, it is possible that in the very near future we can use humanized animals for studying *in vivo* human drug metabolism in the drug discovery stage. A number of genetically modified animals have been established as models for human genetic diseases, but the transgenic approach for evaluating the metabolism of drugs has not been utilized yet to any large degree. Using standard techniques, it may not be difficult to develop transgenic animals that express genes coding for both human cytochrome P450s in the liver and intestine. This type of transgenic animal would certainly provide valuable means for evaluating the intestinal and hepatic first-pass metabolism of drugs.

Based on the current technological trajectory, it is possible to predict the future impact of *in vitro* drug metabolism studies on the drug discovery and development process (Figure 7-1):

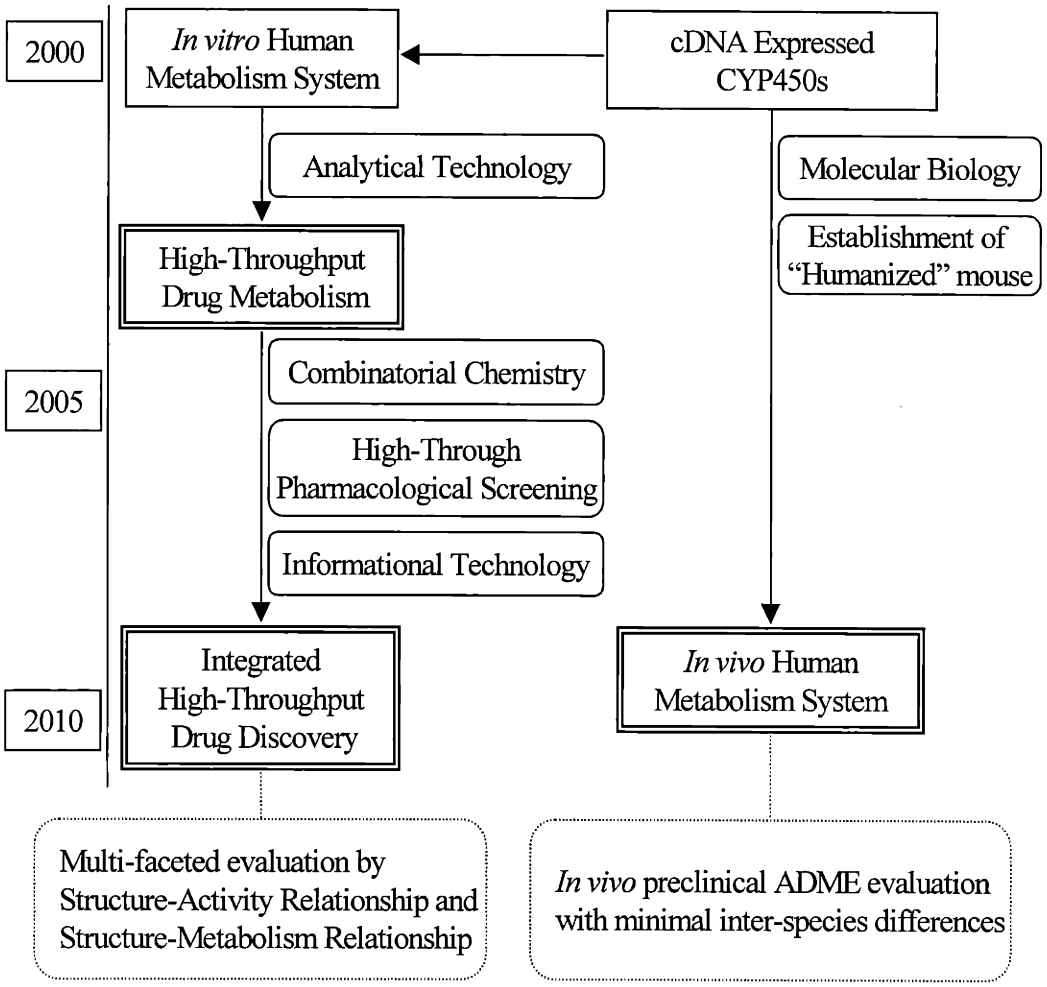
(i) High-throughput *in vitro* drug metabolism system, with enhanced data-processing capability comparable to high-throughput pharmacological screening

(ii) Integrated high-throughput screening system, in which drug candidates are rapidly evaluated by pharmaco-toxicolological and pharmacokinetic endpoints.

(iii) *In vivo* human metabolism model, which can replace animal models that are currently used in the preclinical pharmacokinetic studies

With the growth in the technology, it is not enough to just provide a pharmacologically potent agent. Depending on the therapeutic area, providing a drug with the optimal pharmacokinetic profile may give that product a competitive edge. Pharmaceutical companies are facing huge technological opportunities and threats of the human metabolic enzymes. Pharmaceutical companies that try to change their drug discovery and development process by adapting innovative technologies will obtain sustainable advantages in ever-present competition in the pharmaceutical industry.

Figure 7-1. Expected technological changes in the drug metabolism studies in next 10 years.



REFERENCES

CHAPTER ONE

Boxenbaum H
Interspecies variation in liver weight, hepatic blood flow, and antipyrine intrinsic clearance: extrapolation of data to benzodiazepines and phenytoin
J Pharmacokinet Biopharm, 1980, 8(2): 165-176

Carr G
The Pharmaceutical Industry
The Economist, 1998; Feb. 21: 3-18

Diaz D, Fabre I, Daujat M, Saint Aubert B, Bories P, Michel H, Maurel P
Omeprazole is an aryl hydrocarbon-like inducer of human hepatic cytochrome P450
Gastroenterology, 1990, 99(3): 737-747

Eason CT, Bonner FW, Parke DV
The importance of pharmacokinetic and receptor studies in drug safety evaluation.
Regul Toxicol Pharmacol, 1990, 11(3): 288-307

Gillette JR
Problems in correlating in vitro and in vivo studies of drug metabolism
Pharmacokinetics: a modern view (Benet LZ, Levy G, Ferraiolo BL ed.), Plenum press, New York, 1984, 235-252

Gonzalez FJ
Human cytochromes P450: problems and prospects.
Trends Pharmacol Sci, 1992, 13(9): 346-352

Guengerich FP
Human cytochrome P450 enzymes
Cytochrome P450: Structure, Mechanism and Biochemistry (Ortiz deMontellano PR ed.), Plenum Press, New York, 1995, 473-535

Hook D
Ultra high-throughput screening-a journey intonanoland with Gulliver and Alice
Drug Discov Today, 1996, 1:267-268

Houston JB
Utility of in vitro drug metabolism data in predicting in vivo metabolic clearance
Biochem Pharmacol, 1994, 47: 1469-1479

Houston JB, Carlile DJ
Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices

Drug Metabolism Reviews, 1997, 29(4): 891-922

Ito K, Iwatsubo T, Kanamitsu S, Nakajima Y, Sugiyama Y
Quantitative prediction of in vivo drug clearance and drug interactions from in vitro data on metabolism, together with binding and transport
Annu Rev Pharmacol Toxicol, 1998; 38: 461-499

Kobayashi S, Murray S, Watson D, Sesardic D, Davies DS, Boobis AR
The specificity of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man
Biochem Pharmacol, 1989, 38(17): 2795-2799

Lin JH
Applications and limitations of interspecies scaling and in vitro extrapolation in pharmacokinetics
Drug Metab Dispos, 1998; 26(12): 1202-1212

Lin JH, Lu AYH
Role of pharmacokinetics and metabolism in drug discovery and development
Pharmacological Reviews, 1997; 49(4): 403-449

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW
P450 superfamily, update on new sequences, gene mapping, accession numbers and nomenclature
Pharmacogenetics, 1996, 6(1): 1-42

Pang KS, Chiba M
Metabolism scaling-up from in vitro to organ and whole body
Handbook of experimental pharmacology: pharmacokinetics of drugs (Wellin PG, Balant LP ed.), Springer-Verlag, Berlin, Germany, 1994, 110: 101-187

Prentis RA, Lis Y, Walker SR
Pharmaceutical innovation by the seven UK-owned pharmaceutical companies (1964-1985)
Br J Clin Pharmacol, 1988; 25(3): 387-396

Stevens JC, Shipley LA, Cashman JR, Vandenbranden M, Wrighton SA
Comparison of human and rhesus monkey in vitro phase I and phase II hepatic drug metabolism activities
Drug Metab Dispos, 1993, 21(5): 753-760

Sugiyama Y, Sawada Y, Iga T, Hanano M
Reconstruction of in vivo metabolism from in vitro data
Xenobiotic Metabolism and Disposition (Kato R, Estabrook RW, Cayen MN ed.), Taylor&Francis, London, United Kingdom, 1989, 225-235

Tarbit MH, Berman J
High-throughput approaches for evaluating absorption, distribution, metabolism and excretion properties of lead compounds
Curr Opin Chem Biol, 1998, 2:411-416

CHAPTER TWO

Brosen K, de Morais SM, Meyer UA, Goldstein JA
A multifamily study on the relationship between CYP2C19 genotype and the S-mephenytoin oxidation phenotype
Pharmacogenetics, 1995, 5(5): 312-317

Brosen K, Gram LF
Clinical significance of the sparteine/debrisoquine oxidation polymorphism
Eur J Clin Pharmacol 1989, 36(6): 537-547

Chang T, Levine M, Bellward GD
Selective inhibition of rat hepatic microsomal cytochrome P-450. II. Effect of the in vitro administration of cimetidine
J Pharmacol Exp Ther, 1992, 260(3): 1450-1455

Chee M, Yang R, Hubbell E, Berno A, Huang XC, Stern D, Winkler J, Lockhart DJ, Morris MS, Fodor SP
Accessing genetic information with high-density DNA arrays
Science, 1996, 274(5287): 610-614

Cholerton S, Daly AK, Idle JR
The role of individual human cytochromes P450 in drug metabolism and clinical response
Trends Pharmacol Sci, 1992, 13(12): 434-439

Ciaccio PJ, Dunigan DB, Halpert JR
Selective inactivation by chloramphenicol of the major phenobarbital-inducible isozyme of dog liver cytochrome P-450
Drug Metab Dispos, 1987, 15(6): 852-856

Crewe HK, Lennard MS, Tucker GT, Woods FR, Haddock RE
The effect of selective serotonin re-uptake inhibitors on cytochrome P450 2D6 (CYP2D6) activity in human liver microsomes
Br J Clin Pharmacol, 1992, 34: 262-265

Cupp MJ, Tracy TS
Cytochrome P450: New nomenclature and clinical implications
Americal Family Physician, 1998, January 1:1-14

Dahl ML, Bertilsson L
Genetically variable metabolism of antidepressants and neuroleptic drugs in man
Pharmacogenetics 1993, 3(2): 61-70

Daly AK, Brockmoller J, Broly F, Eichelbaum M, Evans WE, Gonzalez FJ, Huang JD, Idle JR, Ingelman-Sundberg M, Ishizaki T, Jacqz-Aigrain E, Meyer UA, Nebert DW, Steen VM, Wolf CR, Zanger UM
Nomenclature for human CYP2D6 alleles
Pharmacogenetics, 1996, 6(3): 193-201

Daly AK, Cholerton S, Gregory W, Idle JR
Metabolic polymorphisms
Pharmacol Ther, 1993, 57(2-3): 129-160

Evert B, Griese EU, Eichelbaum M
A missense mutation in exon 6 of the CYP2D6 gene leading to a histidine 324 to proline exchange is associated with the poor metabolizer phenotype of sparteine
Naunyn Schmiedebergs Arch Pharmacol, 1994, 350(4): 434-439

Fuhr U, Anders EM, Mahr G, Sorgel F, Staib AH
Inhibitory potency of quinolone antibacterial agents against cytochrome P450IA2 activity in vivo and in vitro
Antimicrob Agents Chemother, 1992 36(5): 942-948

Goldstein JA, de Morais SM
Biochemistry and molecular biology of the human CYP2C subfamily
Pharmacogenetics, 1994, 4(6): 285-299

Gonzalez F
Cytochrome P450 in humans
Cytochrome P450 (Schenkman JB, Greim H, ed.), Springer-Verlag, New York, 1993, 239-257

Gonzalez FJ, Idle JR
Pharmacogenetic phenotyping and genotyping. Present status and future potential
Clin Pharmacokinet, 1994, 26(1): 59-70

Gonzalez FJ, Skoda RC, Kimura S, Umeno U, Zanger UM, Nebert DW, Gelboin HV, Hardwick JP, Meyer UA
Characterization of the common genetic defect in humans deficient in debrisoquine metabolism
Nature, 1988, 331(6155): 442-446

Guengerich FP
Huaman cyochrome P450 enzymes

Cytochrome P450: Structure, Mechanisms and Biochemistry (Ortiz de Montellano PR, ed.), Plenum Press, New York, 1995, 473-575

Hardy BG, Zador IT, Golden L, Lalka D, Schentag JJ
Effect of cimetidine on the pharmacokinetics and pharmacodynamics of quinidine
Am J Cardiol, 1983, 52(1): 172-175

Houston JB, Carlile DJ
Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices
Drug Metabolism Reviews, 1997, 29(4): 891-922

Jerling M, Dahl ML, Aberg-Wistedt A, Liljenberg B, Landell NE, Bertilsson L, Sjoqvist F,
The CYP2D6 genotype predicts the oral clearance of the neuroleptic agents perphenazine and zulcopenthixol
Clin Pharmacol Ther, 1996, 59(4): 423-428

Knapp AB, Maguire W, Keren G, Karmen A, Levitt B, Miura DS, Somberg JC
The cimetidine-lidocaine interaction
Ann Intern Med, 1983, 98(2): 174-177

Koymans L, Vermeulen NP, van Acker SA, te Koppele JM, Heykants JJ, Lavrijsen K, Meuldermans W, Donne-Op den Kelder GM
A predictive model for substrates of cytochrome P450-debrisoquine (2D6)
Chem Res Toxicol, 1992, 5(2): 211-219

Lunetta JM, Sugiyama K, Correia MA
Secobarbital-mediated inactivation of rat liver cytochrome P-450b: a mechanistic reappraisal
Mol Pharmacol, 1989, 35(1): 10-17

Mrez D, Legrand M, Sabbagh N, Lo-Guidice JM, Boone P, Broly F
An additional allelic variant of the CYP2D6 gene causing impaired metabolism of sparteine
Hum Genet, 1996, 97(5): 668-670

Murray M, Wilkinson CF
Interactions of nitrogen heterocycles with cytochrome P-450 and monooxygenase activity
Chem Biol Interact, 1984, 50(3): 267-275

Nakamura K, Goto F, Ray WA, McAllister CB, Jacqz E, Wilkinson GR, Branch RA
Interethnic differences in genetic polymorphisms of debrisoquin and mephenytoin hydroxylation between Japanese and Caucasian populations
Clin Pharmacol Ther, 1987, 38(4): 402-408

Narimatsu S, Watanabe K, Matsunaga T, Yamamoto I, Imaoka S, Funae Y, Yoshimura H

Suppression of liver microsomal drug-metabolizing enzyme activities in adult female rats pretreated with cannabidiol
Biol Pharm Bull, 1993, 16(4): 428-430

Pichard L, Fabre I, Fabre G, Domergue J, Saint Aubert B, Mourad G, Maurel P
Cyclosporin A drug interactions. Screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes
Drug Metab Dispos, 1990, 18(5): 595-606

Ronis MJJ, Ingelman-Sundberg M
Induction of human drug-metabolizing enzymes: mechanisms and implications:
Handbook of drug metabolism (Woolf TF ed.), Marcel Dekker, Inc., 1999, 239-262

Serlin MJ, Sibeon RG, Mossman S, Breckenridge AM, Williams JR, Atwood JL, Willoughby JM
Cimetidine: interaction with oral anticoagulants in man
Lancet, 1979, 2(8138): 317-319

Shimada T, Yamazaki H, Miura M, Inui Y, and Guengerich FP
Interindividual variations in human liver cytochrome P450enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals; studies with liver microsomes of 30 Japanese and 30 Caucasians.
J Pharmacol Exp Ther, 1994, 270(1): 414-423

Slaughter RL, Edwards DJ
Recent advances: the cytochrome P450 enzymes
Ann Pharmacother, 1995; 29(6): 619-624

Spatzenegger M, Jaeger W
Clinical importance of hepatic cytochrome P450 in drug metabolism
Drug Metab Rev, 1995, 27(3): 397-417

Tucker GT
The rational selection of drug interaction studies: implications of recent advances in drug metabolism
Int J Clin Pharmacol Ther Toxicol, 1992, 30(11): 550-553

Vainio H, Hietane E
Role of extrahepatic metabolism
Concepts in Drug Metabolism (Jenner P, Testa B ed.), Marcel Dekker, New York, 1980, 251-284.

Wilkinson GR
Clearance approaches in pharmacology
Pharmacol Rev, 1987, 39(1): 1-47

Wilkinson GR, Guengerich FP, Branch RA,
Genetic polymorphism of S-mephenytoin hydroxylation
Pharmacol Ther, 1989, 43(1): 53-76

Wilkinson GR, Shand DG
Commentary: a physiological approach to hepatic drug clearance
Clin Pharmacol Ther, 1975, 18(4): 377-390

CHAPTER THREE

Boyd AL, Samid D
Review: molecular biology of transgenic animals
J Anim Sci, 1993, 71(Suppl 3): 1-9

Donate MT, Castell JV, Gomez-Lechon MJ
Effect of model inducers on cytochrome P450 activities of human hepatocytes in primary culture
Drug Metab Dispos, 1995, 23(5): 553-558

Fernandez-Salguero PM, Gonzalez, FJ
Targeted disruption of specific cytochromes P450 and xenobiotic receptor genes
Method Enzymol, 1996, 272: 412-430

Friedberg T, Henderson CJ, Pritchhard MP, Wolf CR
In vivo and in vitro recombinant DNA technology as a powerful tool in drug development
Handbook of drug metabolism (Woolf TF ed.), Marcel Dekker Inc., New York, 1999, 321-362

Friedberg T, Kissel W, Arand M, Oesch F
Production of site specific P450 antibodies using recombinant fusion proteins as antigens
Methods Enzymol, 1991, 206: 193-201

Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T, Waxman DJ
Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism
J Biol Chem, 1986, 261(11): 5051-5060

Guillouzo A, Morel F, Fardel O, Meunier B
Use of human hepatocyte cultures for drug metabolism
Toxicology, 1993, 82(1-3): 209-219

Hoener BA
Predicting the hepatic clearance of xenobiotics in humans from *in vitro* data
Biopharm Drug Dispos, 1994, 15(4): 295-304

Houston JB
Utility of in vitro drug metabolism data in predicting *in vivo* metabolic clearance
Biochem Pharmacol, 1994, 47: 1469-1479

Houston JB, Carlile DJ
Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices
Drug Metabol Rev, 1997, 29(4): 891-922

Imaoka S, Yamada T, Hiroi T, Hayashi K, Sakaki T, Yabusaki Y, Funae Y
Multiple forms of human P450 expressed in Saccharomyces cerevisiae: Systematic characterization and comparison with those of the rat
Biochem Pharmacol, 1996, 51(8): 1041-1050

Iwatsubo T, Hirota N, Ooie T, Suzuki H, Sugiyama Y
Prediction of *in vivo* drug disposition from in vitro data based on physiological pharmacokinetics
Biopharm Drug Dispos, 1996, 17: 273-310

Oldham HG, Standring P, Norman SJ, Blake TJ, Beattie I, Cox PJ, Chenery RJ
Metabolism of temelastine (SK&F 93944) in hepatocytes from rat, dog, cynomolgus monkey and man
Drug Metab Dispos, 1990, 18(2): 146-152

Pearce RE, McIntyre CJ, Madan A, Sanzgiri U, Draper AJ, Bullock PL, Cook DC, Burton LA, Latham J, Nevins C, Parkinson A
Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity
Arch Biochem Biophys, 1996, 331(2): 145-169

Poon GK, Wade J, Bloomer J, Clarke SE, Maltas J
Rapid screening of taxol metabolites in human microsomes by liquid chromatography/electrospray ionization-mass spectrometry
Rapid Commun Mass Spectrom, 1996, 10(10): 1165-1168

Remmel RP, Burchell B
Validation and use of cloned, expressed human drug-metabolizing enzymes in heterologous cells for analysis of drug metabolism and drug-drug interactions
Biochem Pharmacol, 1993, 46(4): 559-566

Rodrigues AD
Use of *in vitro* human metabolism studies in drug development. An industrial perspective
Biochem Pharmacol, 1994, 48(12): 2147-2156

Rossant J, Nagy A

Genome engineering: the new mouse genetics
Nature Med, 1995, 1(6): 592-594

Simula AP, Crichton MB, Black SM, Pemble S, Bligh HF, Beggs JD, Wolf CR
Heterologous expression of drug-metabolizing enzymes in cellular and whole animal models
Toxicology, 1993, 82(1-3): 3-20

Sinz MW
In vitro Metabolism: hepatocytes
Handbook of drug metabolism (Woolf TF eds.), Marcel Dekker Inc., New York, 1999, 401-424

Tarbit MH, Bayliss MK, Herriott D, Hood SR, Hutson JL, Park GR, Serabjit-Singh CJ
Applications of molecular biology and *in vitro* technology to drug metabolism studies: an industrial perspective
Biochem Soc Trans, 1993, 21(4): 1018-1023

Valerio D
Retrovirus vectors for gene therapy procedures
Transgenic Animals (Kollias G, Grosveld F ed.), Academic press, London, 1992, 211-246

Woolf TF, Adams JD
Biotransformation of ketamine, (Z)-6-hydroxyketamine, and (E)-6-hydroxyketamine by rat, rabbit, and human liver microsomal preparations
Xenobiotica, 1987, 17(7): 839-847

Yang JL, Maher VM, McCormick JJ
Amplification and direct nucleotide sequencing of cDNA from the lysate of low numbers of diploid human cells
Gene, 1989, 83(2): 347-354

CHAPTER FOUR

Cashman JR, Yang Z, Yang L, Wrighton SA
Stereo- and regioselective N- and S-oxidation of tertiary amines and sulfides in the presence of adult human liver microsomes
Drug Metab. Dispos, 1993, 21(3): 492-501

Correia MA
Cytochrome P450: Structure, Mechanism, and Biochemistry, 2nd ed. (Ortiz de Montellano PR, ed.), Plenum, New York, 1995, 607-630

Crespi CL, Penman BW, Hu M

Development of Caco-2 cells expressing high levels of cDNA-derived cytochrome P450 3A4
Pharm Res, 1996, 13: 1635-1641

Dogterom P
Development of a simple incubation system for metabolism studies with precision-cut liver slices
Drug Metab Dispos, 1993, 21(4): 699-704

Dulik DM, Fenselau C
Use of immobilized enzymes in drug metabolism studies
FASEB J, 1988, 2(7): 2235-2240

Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T, Waxman DJ
Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism.
J Biol Chem, 1986, 261(11): 5051-5060

Guengerich FP, Shimada T
Human cytochrome P450 enzymes and chemical carcinogenesis
Human Metabolism: from molecular biology to man (Jeffery EH ed.), CRC Press, Boca Raton, FL, 1993, 5-12

Hall SD, Thummel KE, Watkins PB, Lown KS, Benet LZ, Paine MF, Mayo RR, Turgeon DK, Bailey DG, Fontana RJ, Wrighton SA
Symposium article: molecular and physical mechanisms of first-pass extraction
Drug Metab Dispos, 1999, 27(2): 161-166

Houston JB, Carlie DJ
Prediction of hepatic clearance from microsome, hepatocytes and liver slices
Drug Metab Rev, 1997, 29(4): 891-922

Inaba T, Tait A, Nakano M, Mahon WA, Kalow W
Metabolism of diazepam *in vitro* by human liver. Independent variability of N-demethylation and C3-hydroxylation
Drug Metab Dispos, 1988, 16(4): 605-608

Iwatsubo T, Hisaka A, Suzuki, Sugiyama Y
Prediction of *in vivo* nonlinear first-pass hepatic metabolism of YM796 from *in vitro* metabolic data
J Pharmacol Exp Ther, 1998, 286(1): 122-127

Iwatsubo T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, Yokoi T, Kamataki T, Sugiyama Y
Prediction of *in vivo* hepatic metabolic clearance of YM796 from *in vitro* data by use of human liver microsomes and recombinant P-450 isozymes

J Pharmacol Exp Ther, 1997, 282(2): 909-919

Katchalski-Katzir E
Immobilized enzymes--learning from past successes and failures
Trends Biotechnol, 1993, 11(11): 471-478

Kato R, Yamazoe Y
Sex-dependent regulation of cytochrome P450 expression
Principles, mechanisms and biological consequences of induction (Ruckpaul K, Rein H ed.), Taylor&Francis, New York, 1990, 82-112

Kolars JC, Schmiedlin-Ren P, Schuetz JD, Fang C, Watkins PB
Identification of rifampin-inducible P450IIIA4 (CYP3A4) in human small bowel enterocytes.
J Clin Invest, 90(5): 1871-1878

Kubinyi H
Strategies and recent technologies in drug discovery
Pharmazie, 1995, 50(10): 647-662

Le Bigot JF, Begue JM, Kiechel JR, Guillouzo A
Species differences in metabolism of ketotifen in rat, rabbit and man: demonstration of similar pathways *in vivo* and in cultured hepatocytes
Life Sci. 1987, 40(9): 883-890

Lin JH
Applications and limitations of interspecies scaling and *in vitro* extrapolation in pharmacokinetics
Drug Metab Dispos, 1998, 26(12): 1202-1212

Lin JH, Chiba M, Balani SK, Chen IW, Kwei GY, Vastag KJ, Nishime JA
Species differences in the pharmacokinetics and metabolism of indinavir, a potent human immunodeficiency virus protease inhibitor
Drug Metab Dispos, 1996, 24(10): 1111-1120

Lin JH, Lu AYH
Role of pharmacokinetics and Metabolism in drug discovery and development
Pharmacol Rev, 1997, 49(4): 403-449

Lin JH, Sugiyama Y, Hanao M, Awazu S
Effect of product inhibition on elimination kinetics of ethoxybenzamide in rabbits. Analysis by physiological pharmacokinetic model.
Drug Metab Dispos, 1984, 12(2): 253-256

Lown KS, Mayo RR, Leichtman AB, Hsiao HL, Turgeon DK, Schmiedlin-Ren P, Brown MB, Guo W, Rossi SJ, Benet LZ, Watkins PB
Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporin A.
Clin Pharmacol Ther, 1997, 62(3): 248-260

Newton DJ, Wang RW, Lu AY
Cytochrome P450 inhibitors. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes
Drug Metab Dispos, 1995, 23(1): 154-158

Paine MF, Shen DD, Kunze KL, Perkins JD, Marsh CL, McVicar JP, Barr DM, Gillies BS, Thummel KE
First-pass metabolism of midazolam by the human intestine
Clin Pharmacol Ther, 1996, 60(1): 14-24

Rahman A, Korzekwa KR, Grogan J, Gonzalez FJ, Harris JW
Selective biotransformation of taxol to 6 alpha-hydroxytaxol by human cytochrome P450 2C8
Cancer Res, 1994, 54(21): 5543-5546

Riesenman C
Antidepressant drug interactions and the cytochrome P450 system: a critical appraisal
Pharmacotherapy, 1995, 15(6 pt2): 84S-99S

Rodrigues AD, Kukulka MJ, Roberts EM, Ouellet D, Rodgers TR
[O-methyl 14C]naproxen O-demethylase activity in human liver microsomes: evidence for the involvement of cytochrome P450 1A2 and P450 2C9/10
Drug Metab. Dispos, 1996, 24(1): 126-136

Rodrigues AD, Mulford DJ, Lee RD, Surber BW, Kukulka MJ, Ferrero JL, Thomas SB, Shet MS, Estabrook RW
In vitro metabolism of terfenadine by a purified recombinant fusion protein containing cytochrome P450 3A4 and NADPH-P450 reductase. Comparison to human liver microsomes and precision-cut liver tissue slices
Drug Metab Dispos, 1995, 23(7): 765-775

Rodrigues AD, Wong SL
Application of human liver microsomes in metabolism-based drug-drug interactions: *in vitro-in vivo* correlations and the Abbott Laboratories experience
Adv Pharmacol, 1997, 43: 65-101

Shimada T, Yamazaki H, Miura M, Inui Y, Guengerich FP
Interindividual variations in human liver cytochrome P450enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals; studies with liver microsomes of 30 Japanese and 30 Caucasians.

J Pharmacol Exp Ther, 1994, 270(1): 414-423

Stempfer G, Holl-Neugebauer B, Kopetzki E, Rudolph R
A fusion protein designed for noncovalent immobilization: stability, enzymatic activity, and use in an enzyme reactor
Nat Biotechnol, 1996, 14(4): 481-484

Venkatakrishnan K, Greenblatt DJ, Von Moltke LL, Schmider J, Harmatz JS, Shader RI
Five distinct human cytochromes mediate amitriptyline N-demethylation *in vitro*: dominance of CYP2C19 and 3A4
J Clin Pharmacol, 1998, 38(2): 112-121

Watkins PB, Wrighton SA, Schuetz EG, Molowa DT, Guzelian PS
Identification of glucocorticoid-inducible cytochromes P-450 in the intestinal mucosa of rats and man.
J Clin Invest, 1987, 80(4): 1029-1036

Worboys PD, Bradbury A, Houston JB
Kinetics of drug metabolism in rat liver slices: II. Comparison of clearance by liver slices and freshly isolated hepatocytes
Drug Metab Dispos, 1996, 24(6): 676-681

Wrighton SA, Ring BJ
Inhibition of human CYP3A catalyzed 1'-hydroxy midazolam formation by ketoconazole, nifedipine, erythromycin, cimetidine and nizatidine
Pharm Res, 1994, 11(6): 921-924

Wrighton SA, Vandenbranden M, Stevens JC, Shipley LA, Ring BJ, Rettie AE, Cashman JR
In vitro method for assessing human hepatic metabolsm: their use in drug develppment
Drug Metab. Rev., 1993, 25(4): 453-484

Wu CY, Benet LZ, Hebert MF, Gupta SK, Rowland M, Gomez DY, Wacher VJ
Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine.
Clin Pharmacol Ther, 1995, 58(5): 492-497

Yasumori T, Nagata K, Yang SK, Chen LS, Murayama N, Yamazoe Y, Kato R
Cytochrome P450 mediated metabolism of diazepam in human and rat: involvment of human CYP2C in N-demethylation in the substrate concentration-dependent manner
Pharmacogenetics, 1993, 3(6): 291-301

CHAPTER FIVE

Campbell DB

Are we doing too many animal biodisposition investigations before phase I studies in man? A re-evaluation of the timing and extent of ADME studies
Eur J Drug Metab Pharmacokinet, 1994, 19(3): 283-293

Favreau LV, Palamanda JR, Lin CC, Nomeir AA
Improved reliability of the rapid microtiter plate assay using recombinant enzyme in predicting CYP2D6 inhibition in human liver microsomes
Drug Metab Dispos, 1999, 27(4): 436-439

Inskeep PB, Day WW
Preclinical drug metabolism studies and strategies
Handbook of drug metabolism (Woolf TF ed.), Marcel Dekker, Inc., New York, 1999, 551-576

Kansy M, Senner F, Gubernator K
Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes
J Med Chem 1998, 41(7): 1007-1010

Olah TV, McLoughlin DA, Gilbert JD
The simultaneous determination of mixtures of drug candidates by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry as an *in vivo* drug screening procedure
Rapid Commun Mass Spectrom, 1997, 11(1): 17-23

Smith DA, van de Waterbeemd Han
Pharmacokinetics and metabolism in early drug discovery
Curr Opin Chem Biol, 1999, 3: 373-378

Tarbit MH, Berman J
High-throughput approaches for evaluating absorption, distribution, metabolism and excretion properties of lead compounds.
Curr Opin Chem Biol 1998, 2(3): 411-416

White R
High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery
Annu Rev Pharmacol Toxicol, 2000, 40: 133-