# INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

**UMI**

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

# UNIVERSITY OF ALBERTA

CYTOCHROME P450 ENZYMES

AND THE METABOLISM

OF TRAZODONE AND NEFAZODONE

by

SUSAN E. ROTZINGER 

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DIVISION OF NEUROSCIENCE - PSYCHIATRY

EDMONTON, ALBERTA

FALL, 1998

# UNIVERSITY OF ALBERTA

## LIBRARY RELEASE FORM

NAME OF AUTHOR: Susan Elizabeth Rotzinger

TITLE OF THESIS: Cytochrome P450 enzymes and the metabolism of trazodone and nefazodone

DEGREE: Doctor of Philosophy

YEAR THIS DEGREE GRANTED: 1998

Permission is hereby granted to the UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

7703-77 Street
Edmonton, Alberta
T6C 2L4

DATED: July 2, 1998

# UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CYTOCHROME P450 ENZYMES AND THE METABOLISM OF TRAZODONE AND NEFAZODONE by SUSAN E. ROTZINGER in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

D Treit (Chair and Examiner)

GB Baker (Supervisor)

IL Martin (Co-Supervisor)

RT Coutts (Committee Member)

J-M Le Mellédo (Committee Member)

RM Palmour (External Examiner)

DATED: July 2, 1998

# ABSTRACT

This thesis represents the results of an investigation of the involvement of the cytochrome P450 (CYP) enzymes in the metabolism of two related antidepressants, trazodone and nefazodone. Several converging lines of *in vitro* experimentation were used to identify the enzymes involved in the metabolism of NEF to its active metabolites hydroxy-nefazodone (OH-NEF), triazoledione (TD), and m-chlorophenylpiperazine (mCPP). The metabolism of trazodone to the active metabolite mCPP was also investigated, as was the metabolism of mCPP to its major metabolite hydroxy-mCPP (OH-mCPP). The identification of the major enzymes involved in a particular metabolic pathway was accomplished *in vitro* using human liver microsomes and cells expressing human CYP enzymes. Novel analytical methods were developed for the detection of trazodone, NEF, and their metabolites using high pressure liquid chromatography (HPLC) and gas chromatography (GC). The major findings of the present thesis were that CYP3A4 is an important enzyme in the metabolism of NEF to OH-NEF and mCPP, and of OH-NEF to TD and mCPP. Furthermore, CYP3A4 was found to be important in the metabolism of trazodone to mCPP, and CYP2D6 was found to be important for the metabolism of mCPP to OH-mCPP. These findings have important implications for predicting metabolic drug-drug interactions involving trazodone and nefazodone.

# DEDICATION

This thesis is dedicated to my parents, Marcel Rotzinger and Beverley Tallon, for the support and encouragement they have provided unconditionally throughout my life, and to Bryan, for teaching me life's truly important lessons.

# ACKNOWLEDGEMENTS

First and foremost, I must acknowledge the exceptional leadership of Dr. Glen Baker, whose hard work, integrity, and dedication to excellence serves as an example of how research should be conducted. I thank him for providing me with the opportunity to conduct this research in an atmosphere fostering learning and growth, and for the guidance and encouragement necessary to make me believe that this thesis could one day become a reality.

I also wish to thank Drs. RT Coutts, IL Martin, J Fang, and A Holt for their willingness to listen to my questions, and for their patience and enthusiasm in providing the answers. I am deeply indebted to Gail Rauw, for her limitless patience and intelligence, and to Jordyce Van Muyden for her exceptional technical and organizational support. The friends that I have made during the course of my schooling have enhanced my education and my life in countless ways. I gratefully acknowledge the constant support and encouragement from my family, as well as Bryan's patience, understanding, and insight, which has helped me inestimably.

Finally, I wish to thank the Medical Research Council of Canada and the University of Alberta (Walter Johns Graduate Fellowship) for the financial support which made this research possible.

## TABLE OF CONTENTS

## LIST OF FIGURES

# ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| AUC | area under the time concentration curve |
| cAMP | cyclic adenosine monophosphate |
| CBZ | carbamazepine |
| cDNA | complementary DNA |
| CI | chemical ionization |
| $C_{max}$ | peak plasma concentration |
| p-CPEA | p-chlorophenylethylamine |
| CPZ | chlorpromazine |
| CSF | cerebrospinal fluid |
| CYP | cytochrome P450 |
| DA | dopamine |
| DNA | deoxyribonucleic acid |
| ECD | electron capture detection |
| EI | electron impact |
| EM | extensive metabolizer |
| ER | endoplasmic reticulum |
| FID | flame ionization detector |
| FLU | fluoxetine |
| FVX | fluvoxamine |

| | |
|---|---|
| GC | gas chromatography |
| GLC | gas liquid chromatography |
| GSC | gas solid chromatography |
| h | hour |
| 5-HIAA | 5-hydroxyindole-3-acetic acid |
| HP | Hewlett Packard |
| 5-HT | 5-hyrdoxytryptamine (serotonin) |
| ip | intraperitoneal |
| $K_i$ | concentration of inhibitor at which 50% of the enzyme is bound by the inhibitor |
| $K_m$ | substrate concentration at which reaction velocity equals 50% of $V_{max}$ |
| MS | mass spectrometry |
| µg | microgram |
| µl | microliter |
| µM | micromolar |
| MAO | monoamine oxidase |
| MAOI | monoamine oxidase inhibitor |
| mCPP | meta-chlorophenylpiperazine |
| Min | minutes |
| MR | metabolic ratio |
| NA | noradrenaline |
| NADPH | β-nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| NEF | nefazodone |
| NFLU | norfluoxetine |
| ng | nanogram |
| NPD | nitrogen phosphorus detection |
| ODS | octadecylsilane |
| OH-mCPP | p-hydroxy-meta-chlorophenylpiperazine |
| OH-NEF | hydroxynefazodone |
| PCR | polymerase chain reaction |
| PM | poor metabolizer |
| PRX | paroxetine |
| RDD | rational drug design |
| RIMA | reversible inhibitor of monoamine oxidase A |
| SCOT | support coated open tubular |
| SEM | standard error of the mean |
| SERT | sertraline |
| SSRI | selective serotonin reuptake inhibitor |
| TCA | tricyclic antidepressant |
| TCD | thermal conductivity detector |
| TD | triazoledione |
| TPA | oxotriazolepyridinpropionic acid |
| UV | ultraviolet |
| $V_{max}$ | maximal reaction velocity |
| WCOT | wall coated open tubular |

# Chapter 1

# Introduction

## 1.1 INTRODUCTION

The work described in this thesis centers around the identification of the cytochrome P450 (CYP) enzymes involved in the metabolism of two structurally and functionally related antidepressants, trazodone and nefazodone, and their common metabolite m-chlorophenylpiperazine (mCPP) (see Figure 1.1). Knowing which enzymes are involved in a drug's metabolism will help to identify the factors that may alter the drug's metabolism, and therefore will aid in the optimization of drug therapy and the prevention of adverse outcomes. Many factors, including age, sex, dietary components, and exposure to environmental compounds such as tobacco smoke, can potentially affect an individual's metabolism of a drug (Guengerich, 1992). In particular, metabolic drug-drug interactions can occur when drugs which inhibit or induce metabolic enzymes are taken concurrently, interfering with the therapeutic efficacy of a drug, with consequences ranging from lack of therapeutic effect to serious and life-threatening conditions (Coutts, 1994). The issue of drug interactions is particularly relevant in psychiatry, where combination drug therapy is standard practice in the treatment of some illnesses, and is often an unavoidable consequence of chronic drug therapy (Rosenbaum, 1995).

The cytochromes P450 are a family of oxidative enzymes involved in steroidogenesis and the metabolism of xenobiotics. Interindividual differences in drug metabolism can result from genetically determined differences in endogenous enzyme activities, or from enzyme induction or

inhibition by other compounds. If the particular isoforms that are primarily involved in a drug's metabolism can be identified, then potential interactions can be predicted and avoided. It is also important to take into account the formation and degradation of metabolites, which may contribute to the therapeutic effects or side effects of the parent compound, as well as having their own effects on the CYP enzymes.

*In vitro* studies of drug metabolism using human-derived materials have provided a way to study human drug metabolism and predict interactions before they occur (Guengerich, 1996). Although many factors will influence pharmacokinetics and biotransformation *in vivo*, data obtained from *in vitro* studies provide a useful starting point from which to predict interactions. Postmarketing databases and isolated case reports have traditionally been the source of information that certain drug combinations are potentially dangerous (Rosenbaum, 1995). Clearly, this is neither a safe nor efficient way to deal with the issue. Furthermore, it is difficult to know with any certainty whether a drug-drug interaction actually occurred when examining data from retrospective case reports in which a variety of environmental and individual factors could have been involved (Rosenbaum, 1995). Therefore, *in vitro* drug metabolism studies are a necessary first step towards an understanding of the pharmacokinetics of a drug *in vivo.*

The work presented in this thesis aims to identify the CYP isoforms involved in the metabolism of two novel antidepressants, trazodone and nefazodone, and their respective active metabolites. This was accomplished

using a combination of several *in vitro* methods employing human liver microsomes and microsomes prepared from a cell line genetically engineered to express the human cDNA for single specific CYP enzymes.

Trazodone is a novel antidepressant that is often prescribed to offset antidepressant-induced insomnia, and therefore has a high potential for drug-drug interactions. Indeed, many drug interactions with trazodone have been noted clinically (e.g. Aranow et al., 1989; Metz & Shader, 1990; Nierenberg et al., 1992; Reeves & Bullen, 1995; George & Godleski, 1996; Maes et al., 1997). However, the specific metabolic pathways and enzymes responsible for these interactions are not known, since detailed *in vitro* analyses have not been conducted previously. Trazodone has several identified metabolites (Figure 1.2), but of particular interest is m-chlorophenylpiperazine (mCPP), a psychopharmacologically active 5-HT agonist with prominent behavioral and physiological effects (Conn & Sanders-Bush, 1987). This metabolite could have clinically significant consequences if it reaches higher than normal plasma concentrations, since it has been shown to have anxiogenic and psychotomimetic effects in humans (Kahn et al., 1988; Krystal et al., 1993; Germine et al., 1994; Cowen et al., 1995).

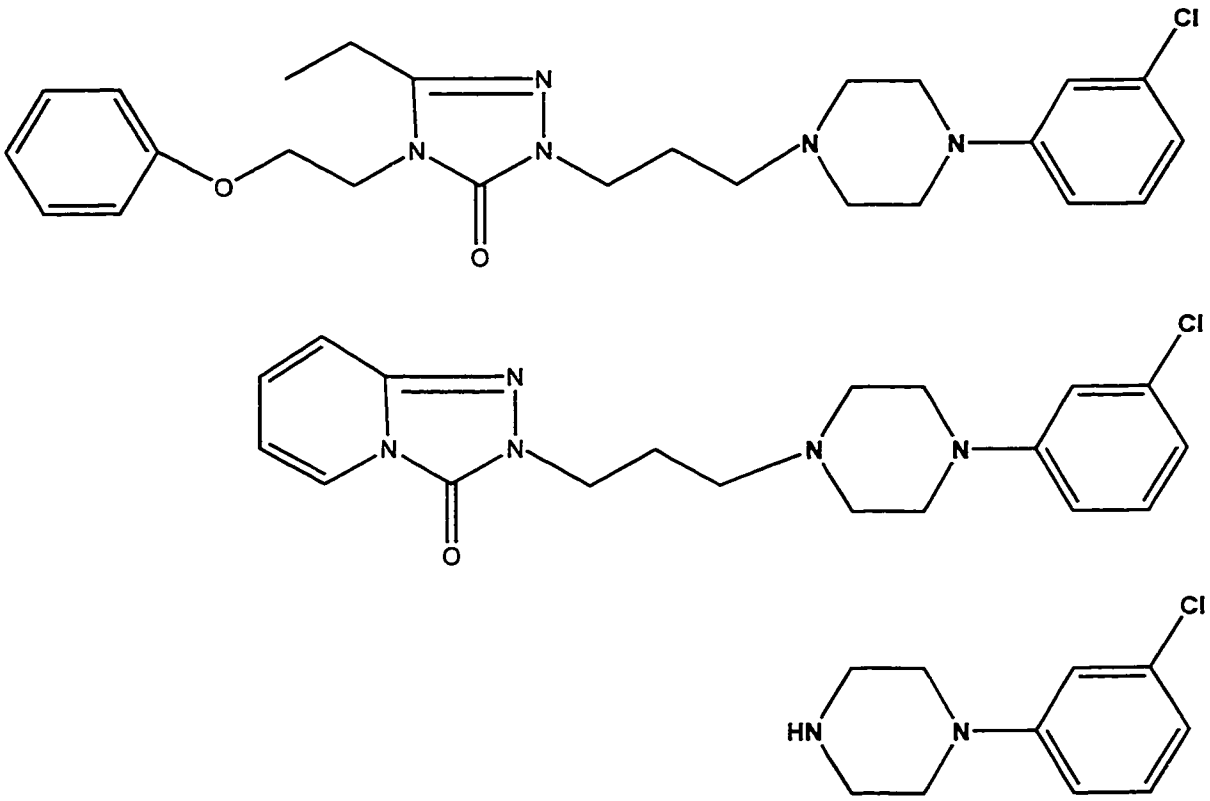



FIGURE 1.1 Chemical Structures of nefazodone (top), trazodone (middle), and m-chlorophenylpiperazine (mCPP) (bottom).

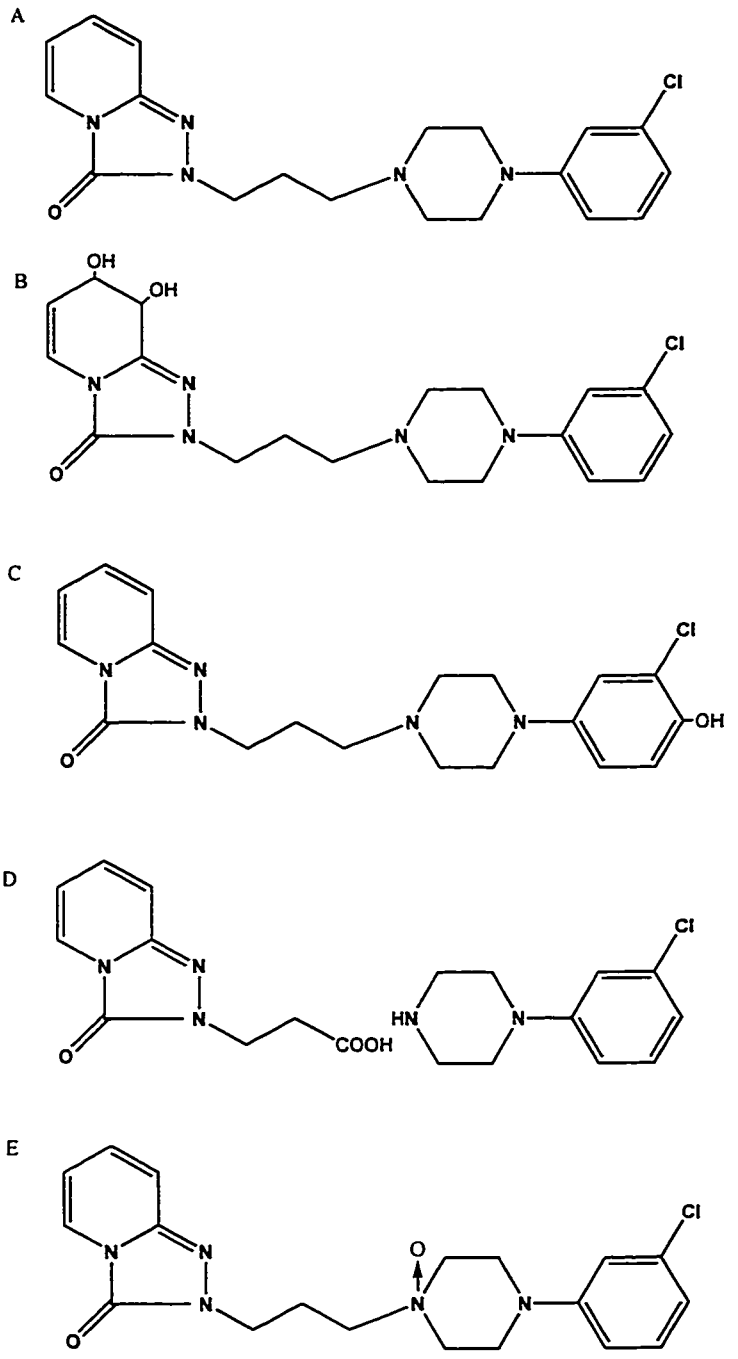



FIGURE 1.2 Structures of trazodone (A), and some of its identified metabolites: dihydrodiol metabolite (B), p-hydroxytrazodone (C), oxotriazolepyridinpropionic acid (TPA), mCPP (D), and trazodone N-oxide (E).

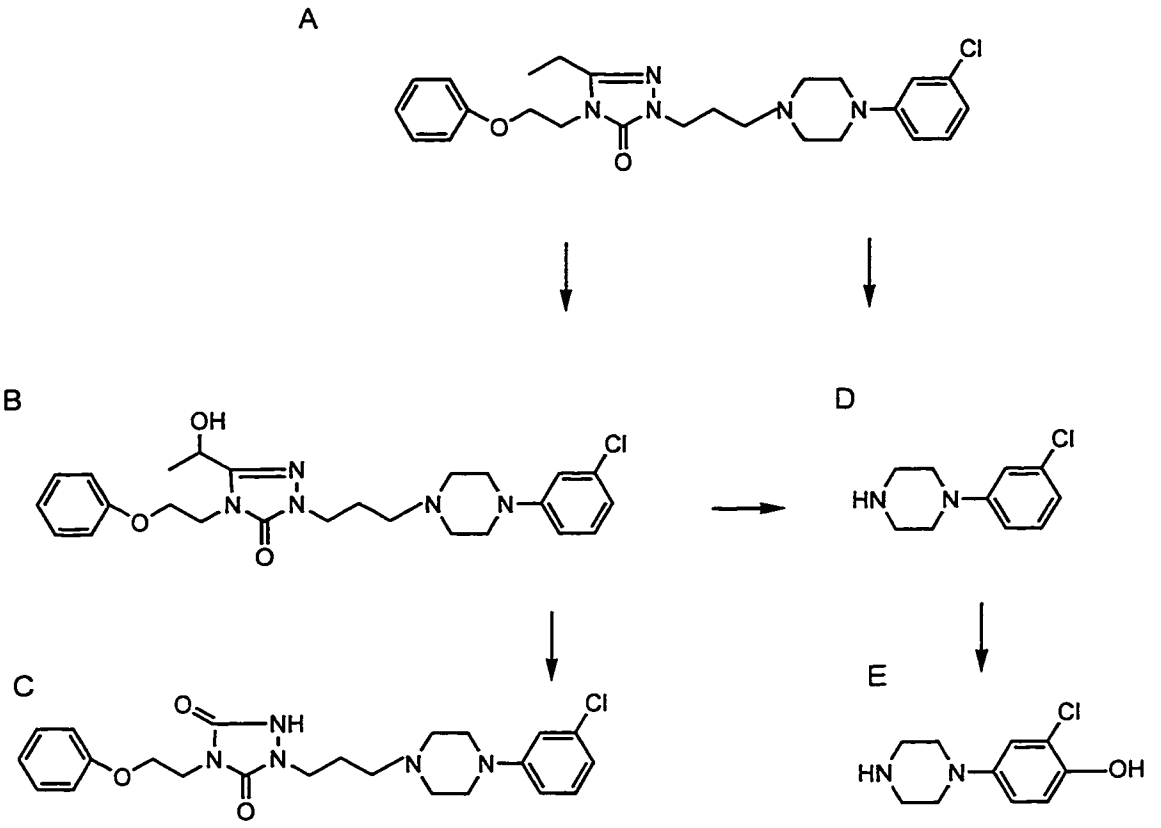



FIGURE 1.3 Structure of nefazodone (A) and its metabolites hydroxy-nefazodone (B), triazoledione (C), mCPP (D), and p-OH-mCPP (E).

Nefazodone is structurally and functionally similar to trazodone, and shares the common metabolite mCPP (Ellingrod & Perry, 1995) [Figure 1.3]. In addition to mCPP, NEF also has two other active metabolites, hydroxy-nefazodone (OH-NEF), and a triazoledione (TD) metabolite (see Figure 1.3). Although preliminary reports suggest that NEF inhibits CYP3A4 *in vitro* (von Moltke et al., 1996a; 1996b), its metabolism by the CYP enzymes has not been studied in detail.

In the present work, four different *in vitro* methods were used to identify the enzymes involved in the metabolism of trazodone, nefazodone, and their metabolites. First, each drug was incubated separately with microsomes from 16 different human livers, each of which had been previously characterized for its activity of seven different CYP isoforms (CYP1A2, CYP2A6, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11). The rate of metabolite formation from the substrate drugs was then correlated with the endogenous activity of each isoform across the 16 livers. A significant correlation between a specific enzyme activity and metabolite formation is an indication that the same enzyme is responsible for both reactions (Guengerich, 1996).

The correlational data was further validated using microsomes prepared from a cell line engineered to express the cDNA for a single human CYP. The drugs under investigation were incubated individually with microsomes expressing the cDNA for CYP1A1, CYP1A2, CYP2C8,

$CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. The incubation media were then assayed for metabolite production. This type of experiment provides a qualitative indication of the involvement of a particular isoform in a specific metabolic pathway (Guengerich, 1996).

A third line of evidence came from studies using chemical inhibitors for specific CYP isoforms. The drug substrates were incubated with human liver microsomes and varying concentrations of inhibitors specific for a single isoform. If metabolite formation decreased in the presence of increasing concentrations of a specific inhibitor, then the inhibited isoform would be implicated in that particular metabolic pathway.

Finally, potential drug interactions were investigated *in vitro* by co-incubating the drug substrates with potentially co-administered drugs at physiologically relevant concentrations.

In order to use the *in vitro* methods, sensitive and specific assays for the detection of the drugs and their metabolites were also required. Therefore, assays were developed for the detection of trazodone, nefazodone, and their respective metabolites in the incubation medium using gas chromatography with nitrogen-phosphorus detection (GC-NPD) for mCPP detection, and high performance liquid chromatography with ultraviolet detection (HPLC-UV) for nefazodone and its metabolites. A GC assay with electron capture detection (GC-ECD) was also developed for the detection and quantification of fluvoxamine (FVX), a selective serotonin reuptake

inhibitor (SSRI), was also developed in initial studies on the effects of this drug on metabolism of nefazodone and trazodone.

Thus, the main objective of the present work was to identify the specific human CYP enzymes involved in the metabolism of trazodone, nefazodone and their active metabolites. The rationale behind such an investigation was to facilitate the prediction and prevention of adverse drug interactions, and to increase knowledge regarding the metabolism of these drugs. The methods used to achieve this objective were the *in vitro* incubations of the drug substrates with human liver microsomes or microsomes prepared from cells expressing human cDNA for specific CYP isoforms. Studies with specific enzyme inhibitors helped to further implicate specific isoforms in drug metabolism, and to predict the clinical relevance of potential drug interactions. Assays were developed for the *in vitro* detection of these drugs and their metabolites, and were extended to tissues for possible future *in vivo* animal studies. This work adds to the growing literature investigating *in vitro* drug metabolism, and characterizes previously unknown metabolic pathways for trazodone and nefazodone. These studies will aid in the prevention of adverse drug interactions and in the optimization of drug therapy.

## 1.2 LITERATURE REVIEW

### 1.2.1 Drug Metabolism

#### 1.2.1.1 General Principles

Drug metabolism is the process by which a drug is chemically modified by the body, with one of the main purposes being to convert it to more water-soluble and easily excreted compounds (Meyer, 1996). Since most xenobiotics are lipophilic, metabolic pathways have evolved to convert them to more hydrophilic substances which can be excreted in the urine (Friedberg & Wolf, 1996). This process is generally carried out in the liver in two phases. In Phase 1, a new chemical group is inserted into the molecule, most often by oxidation, reduction, or hydrolysis (Friedberg & Wolf, 1996; Meyer, 1996). This provides a reactive site where, in Phase 2, an endogenous molecule such as glucuronic acid, sulphate, glycine, acetate or a methyl group is added to the drug molecule to facilitate renal excretion (Meyer, 1996).

Oxidation is a major route of xenobiotic metabolism, and there are several enzyme systems involved in oxidative reactions, one of which is the CYP system. Examples of some other oxidative enzymes are monoamine oxidases, diamine oxidase, xanthine oxidase, alcohol dehydrogenase, and aldehyde oxidase (Meyer, 1996). The majority of xenobiotic oxidations, are however, carried out by the CYP enzymes, which are important in pharmacokinetic drug interactions, and which are the subject of this thesis.

### 1.2.1.2 The Cytochrome P450 enzymes

#### *1.2.1.2.1 Classification and nomenclature*

The CYP enzymes play an essential role in the metabolism of many diverse endogenous, environmental, and synthetic chemicals, and have been identified in at least 85 eukaryote and 20 prokaryote species, to date (Nelson et al., 1996).

Most of the CYPs have been cloned and sequenced, allowing classification on the basis of their amino acid sequence homology. Individual genes and gene products are named using the root symbol "CYP", representing **cy**tochrome **P**450, followed by an Arabic number denoting the family, a letter identifying the subfamily, and an Arabic number representing the individual gene within the subfamily (Nelson et al., 1996). Families contain at least 40% sequence homology, and subfamilies at least 55% (Coon et al., 1992; Nelson et al., 1996).

It is thought that the xenobiotic-metabolizing P450s probably evolved from the steroidogenic P450s to help animals degrade dietary chemicals and plant toxins (Gonzalez, 1992). Currently, 14 CYP gene families have been identified in all mammals. The human CYPs can be divided into two main groups, those that are involved in the synthesis of steroids and bile acids, and those that primarily metabolize xenobiotics (Gonzalez, 1992). Families 1, 2, 3, and 4 are primarily involved in xenobiotic metabolism, whereas families 5, 7, 8, 24, 27, and 51 are involved in the biosynthesis and metabolism of eicosanoids, vitamins and bile acids, and families 11, 17, 19,

and 21 in the biosynthesis of steroid hormones from cholesterol (Nelson et al., 1996).

#### *1.2.1.2.2 Structure, Function and Mechanism of Action*

The CYP enzymes are hemoproteins made up of 2 parts, a heme moiety containing a porphyrin ring with iron, and a protein component (Smith et al., 1996). The heme moiety is identical between enzymes, and forms the catalytic center. The protein component differentiates the isoforms, with different amino acid sequences altering the topography of the active site (Smith et al., 1996). The enzyme active site binds the drug substrate and confers specificity on the enzyme for catalyzing specific types of reactions. One of the advantages of the CYP system in metabolizing xenobiotics is the broad range of substrates that it can metabolize (Korzekwa & Jones, 1993). This is due in part to the large number of CYP enzymes, each of which has its own range of preferred substrates. As well, several different forms of CYP may be involved in the metabolism of a single drug, each acting at a different site on the drug molecule.

Most of the CYP enzymes are located in the liver, although they are also located extra-hepatically in the small intestine, kidneys, lungs, brain and nasal tissue (Guengerich, 1995). Xenobiotic-metabolizing CYP enzymes in the liver are found in the endoplasmic reticulum (ER), whereas the steroid-synthesizing enzymes are located in the mitochondria (Friedberg & Wolf, 1996). In the ER, the CYP enzymes mainly catalyze oxidations, which

require three components: the CYP enzyme, which acts as the substrate and oxygen binding site; the NADPH-cytochrome P450 reductase, which acts as the electron carrier and transfers an electron from NADPH to the CYP enzyme; and a phospholipid component which facilitates electron transfer. This system also requires NADPH and molecular oxygen (Coon et al., 1992).

Together, these components form a multicomponent electron transfer system which can oxidize numerous compounds. The general mechanism involves the initial binding of the substrate to the ferric form of the CYP enzyme (Hollenberg, 1992; Friedberg & Wolf, 1996). An electron is then transferred from NADPH via NADPH-P450 reductase to the iron of the ferric P450 to give the ferrous P450 substrate complex, which then binds molecular oxygen. A second electron is then added, and the oxygen-oxygen bond is cleaved with one atom of oxygen being released and forming water. The other atom of oxygen remains associated with the heme iron as an activated oxygen, which is then inserted into the substrate, releasing ferric P450 for another catalytic cycle (Hollenberg, 1992). The overall reaction is:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Through this general reaction mechanism, the CYPs are able to catalyze a wide variety of different reactions. Some examples of these are aromatic and aliphatic hydroxylations, epoxidations, N-, O-, and S-

dealkylations, oxidative deaminations, and N-oxidations. Thus, very different chemical end-points can be achieved all starting with a single hydroxylation step producing a reactive intermediate. In this way the CYPs can act on a wide range of substrates to produce many different metabolites (Hollenberg, 1992).

#### *1.2.1.2.3 Factors Affecting Drug Metabolism*

The same dose of a drug can result in large interindividual variations in drug levels and response which presents a challenge for physicians and pharmacists in the optimization of treatment. Many environmental and genetic factors can affect drug levels, putting effective and safe pharmacotherapy in jeopardy. Knowledge of what these factors are, and how they affect drug metabolism, can increase therapeutic efficacy and safety.

##### 1.2.1.2.3.1 Inhibition

The CYP enzymes can have their activity reduced through the process of inhibition, which can be competitive or non-competitive, and reversible or irreversible. The structure of the CYP enzyme allows for two different binding modes, a productive binding to the hydrophobic substrate binding site (substrate binding), as well as an inhibitory binding to the haem iron (ligand binding) (Testa, 1990). The simplest case of enzyme inhibition is by the interaction of two substrates competing for the same enzyme active site (Testa, 1990). Thus, two substrates can compete for the substrate binding

site, resulting in a mutual inhibition of metabolism, or a ligand can bind to the ligand binding site, inhibiting the enzyme, without itself being a substrate. This is an important point with respect to the study of drug interactions in that it illustrates that an inhibitor of an enzyme is not necessarily a substrate for that enzyme. An example is quinidine, which is a potent inhibitor of CYP2D6, but is a substrate of CYP3A4 (Muralidharan et al., 1991). In addition, a drug may be both a substrate and an inhibitor of an enzyme. For example, the antidepressant fluoxetine (FLU) is a substrate and an inhibitor of CYP2D6 (Preskorn, 1996). Therefore, FLU will also inhibit its own metabolism, leading to nonlinear pharmacokinetics. The degree to which a particular drug inhibits an enzyme will depend upon the affinity of the drug for the enzyme as well as its concentration at the enzyme (Riesenman, 1995).

In summary, substrates will act as competitive inhibitors of the enzymes that metabolize them, but may also inhibit the activity of other enzymes without themselves being substrates (noncompetitive inhibition) (Testa, 1990).

1.2.1.2.3.2 Induction

Enzyme activity can also be induced. Often an inducer is also a substrate for the CYP enzyme that it induces, thereby regulating its own metabolism (Okey, 1990; Denison & Whitlock, 1995). However, since most of the inducible CYPs have a wide number of possible substrates, the metabolism of many other compounds may be stimulated as well (Okey,

1990). In addition, some inducers will induce several different forms of CYP, as well as phase 2 conjugating enzymes (Okey, 1990; Denison & Whitlock, 1995).

Not all CYP isoforms are inducible. For example, there is no evidence that CYP2D6 is inducible (Okey, 1990). However, many enzymes are induced by a wide range of commonly encountered drugs, foods, and environmental compounds. For example, some commonly encountered inducers of CYP1A2 are caffeine (Berthou et al., 1995), barbecued meats and cruciferous vegetables (Wrighton et al., 1996), tobacco smoke, and oral contraceptive steroids (Bock et al., 1994).

Induction is usually the result of an increase in the rate of gene transcription for a specific CYP isoform (Okey, 1990). There are several methods by which xenobiotics can induce CYP gene transcription, depending on the nature of the inducer (Denison & Whitlock, 1995). The exact mechanisms of enzyme induction are complex and poorly understood (Denison & Whitlock, 1995).

1.2.1.2.3.3 Genetics

Interindividual variations in drug plasma levels can also occur due to genetically determined differences in enzyme activities (Eichelbaum, 1984; Coutts, 1994). Inborn genetic errors can lead to alterations in the functional activity of an enzyme ranging from a complete lack of enzyme, to enzyme overexpression (Eichelbaum, 1984; Lennard et al., 1990; Kroemer &

Eichelbaum, 1995). These genotypic alterations can result in phenotypic differences in drug metabolism. When a trait under monogenic control occurs in the population in at least two genotypes (and their corresponding phenotypes) with a frequency of at least one percent, it is known as a polymorphism (Kroemer & Eichelbaum, 1995). Polymorphisms have been characterized for N-acetyltransferase, S-methyltransferase, and enzymes involved in O-methylation (Gibaldi, 1992a). More recently, polymorphic oxidations involving the CYP enzymes have also been characterized for CYP2D6 and CYP2C19 (Kivisto & Kroemer, 1997).

Individuals can be phenotyped for their metabolic status by assessing their ability to metabolize a probe drug (Coutts, 1994). The general procedure is to administer a probe drug, collect a urine sample, and determine the ratio of parent drug to a specific metabolite, the formation of which is known to be catalyzed by the CYP isoform of interest. This ratio is known as the metabolic ratio (MR), and poor metabolizers (PMs) will have much lower metabolite levels, and therefore higher MRs than extensive metabolizers (EMs). Phenotyping is relatively easy to perform, and can be used to achieve appropriate drug levels and avoid severe toxic reactions. However, it is important that the patient has received a sufficient drug washout period so that no competing drugs or metabolites will interfere with the phenotyping process. This is particularly important for a drug such as FLU which strongly inhibits CYP2D6, as does its major metabolite norfluoxetine (NFLU). FLU administration can essentially switch an individual

to the PM phenotype during drug therapy, an event known as phenocopying (Preskorn, 1996). NFLU has a half life of 7-14 days, which could have an effect on phenotyping during the washout period. Possible ethical issues in administering probe drugs, and in withdrawing current medications must be taken into account when considering phenotyping.

An individual's metabolic status can also be determined by genotyping, which does not require administration of a probe drug, and is unaffected by environmental influences. DNA from peripheral lymphocytes can be isolated, and a restriction fragment length polymorphism can be followed by multiple polymerase chain reaction (PCR) assays to assign the individual's genotype (Kroemer & Eichelbaum, 1995).

The most intensively studied and well-known polymorphism is that of CYP2D6. It was originally known as the debrisoquin or sparteine oxidation polymorphism for its role in the metabolism of these prototype drugs (Brosen, 1990). There is considerable ethnic variation in the prevalence of this polymorphism, with 5-10% of the Caucasian population being deficient in this enzyme (Gonzalez, 1990), but less than 1% of Orientals (Lennard, 1990). This polymorphism is caused by a mutation on the CYP2D6 gene, which is located on chromosome 22, and which may result in the absence of CYP2D6 in the livers of PMs (Brosen, 1990).

The other major polymorphism is of CYP2C19, and is detected clinically by the use of mephenytoin or omeprazole as the probe drugs (Flockhart, 1995). It displays ethnic variations, with approximately 20% of

Orientals, but only 2-6% of Caucasians deficient (Goldstein & de Morais, 1994; Flockhart, 1995). There are two known mutant alleles of CYP2C19 which can be determined by a PCR-based method (Flockhart, 1995). Known substrates for CYP2C19 include diazepam, propranolol, imipramine, clomipramine and amitriptyline, and it is inhibited by FLU, FVX , omeprazole and fluconazole (Flockhart, 1995; Bertz & Granneman, 1997).

Therefore, genetic factors can affect the metabolism of a variety of drugs to a significant degree, and must be considered when prescribing drug therapy, or investigating unexpected drug effects.

#### 1.2.1.3 Consequences of altered enzyme levels

As can be seen from the preceding discussion, a multitude of factors can contribute to interindividual variations in drug levels, and must be taken into consideration in the course of any drug therapy. Of course, the clinical consequences of altered drug metabolism will also depend upon the pharmacological effects of the drug in question, its therapeutic window, and the importance of the affected enzymes in the drug's overall metabolism (Coutts, 1994). However, there are several possible situations to consider.

When there is more enzyme present than expected, as in the case of induced enzymes, substrates may be metabolized more quickly, resulting in lower than expected plasma drug levels. This can lead to reduced therapeutic effect, and possible accumulation of toxic metabolites. On the other hand, active metabolites which contribute to a drug's effectiveness may

also be metabolized more quickly, decreasing the effectiveness of a drug. Finally, if an inducing agent is suddenly removed, any concurrently administered medications may have their rate of metabolism reduced, with possible toxic consequences.

If enzyme activity is for some reason lower than would normally be expected, as in the case of enzyme inhibition or genetically poor metabolizers, drug metabolism will proceed at a slower than expected rate. A substrate or metabolite may build up to toxic plasma levels, resulting in an increase in adverse side effects, and possible drug discontinuation, when a dosage adjustment is all that is required. Another possible adverse outcome of decreased enzyme activity is seen in the case of drugs that require metabolic activation to intermediates for pharmacological effectiveness. For example, codeine must be converted to morphine via CYP2D6 for its analgesic effect, and may lack therapeutic effectiveness if CYP2D6 activity is less than expected (Desmeules et al., 1991). Similarly, terfenadine is marketed as an antihistamine, but must be metabolized by CYP3A4 to its active metabolite fexofenadine, which possesses the antihistaminic properties (Stachulski & Lennard, 1997). Serious cardiotoxic interactions can result if terfenadine is co-administered with inhibitors of CYP3A4, which prevent its metabolism.

Therefore, the importance of knowing which specific CYP enzymes are involved in a drug's metabolism is readily apparent. Potentially interacting factors can be identified and controlled ahead of time, so that therapeutic drug levels can be achieved and maintained.

#### 1.2.1.4 Important CYPs in human drug metabolism

The major CYP isoforms involved in drug metabolism are CYP1A2, CYP2D6, CYP3A, CYP2C and CYP2E1 (Bertz & Granneman, 1997). Many reviews of the major characteristics, substrates, inducers, and inhibitors of the specific CYP enzymes have been written (e.g. Bertz & Granneman, 1997; Guengerich, 1995; Meyer, 1996; Wrighton et al., 1996). The following is not meant to be such a review, but rather a brief overview of the major characteristics of the two CYP enzymes of importance to the work presented in this thesis, namely CYP3A4 and CYP2D6.

##### *1.2.1.4.1 CYP3A4*

The CYP3A subfamily is the most abundant of the CYPs, and makes up about 60% of all human liver CYPs (Guengerich, 1990). CYP3A3 and CYP3A4 are so closely related that they cannot be distinguished by immunochemcial methods or by measures of catalytic activity, and there is question as to whether CYP3A3 actually exists, or is just a cloning artifact (Wilkinson, 1996). CYP3A4 is known to be involved in the metabolism of 50% of all drugs which have had their metabolism characterized (Guengerich,

1996). In addition to its abundance in the liver, CYP3A4 is also located extra-hepatically in the intestine, and therefore can begin to metabolize drugs before they reach the hepatic enzymes (Riesenman, 1995; Wilkinson, 1996). This extra-hepatic localization contributes to the marked inter-individual variability in the metabolizing ability of CYP3A4 (Wilkinson, 1996).

CYP3A4 has a very broad substrate specificity due in part to a large and flexible binding site, and is therefore involved in a wide variety of metabolic reactions (Guengerich, 1995). It is involved in the metabolism of endogenous substrates such as testosterone and other steroid hormones (Wrighton & Stevens 1992). It is also involved in the metabolism of xenobiotics from virtually every drug class, including antifungals, antibiotics, antihistamines, anticonvulsants, antidepressants, antipsychotics, and antiarrhythmics (Nemeroff et al., 1996; Wilkinson, 1996). Although CYP3A4 is known to be involved in a wide variety of metabolic reactions, some very general features of its preferred substrates are that they are lipophilic, neutral, or basic molecules with the site of oxidation often being basic nitrogen (N-dealkylations) or allylic positions (Smith et al., 1996). CYP3A4 is particularly known for catalyzing metabolic N-dealkylations (Coutts et al., 1994).

The 3A family is highly susceptible to induction and inhibition by many compounds, which again may contribute to its marked inter-individual variation in activity, and increased likelihood for drug-drug interactions (Watkins et al., 1985; Wilkinson, 1996). Common inducers are macrolide

antibiotics, rifampicin, glucocorticoids, barbiturates, and carbamazepine (Pichard et al., 1990; Shen, 1995; Wilkinson, 1996). CYP3A4 is strongly inhibited by certain macrolide antibiotics, imidazole antifungals, calcium-channel antagonists, and corticosteroids (Wrighton & Stevens, 1992; Shen, 1995; Wilkinson, 1996), and is weakly inhibited by naringenin, found in grapefruit juice (Guengerich & Kim, 1990). Therefore, there is a wide variety of substrates and inducers of CYP3A4, leading to many opportunities for drug-drug interactions, and illustrating the importance of knowing if a new drug is a substrate for CYP3A4.

#### *1.2.1.4.2 CYP2D6*

CYP2D6 is an extremely well-studied CYP enzyme, in part because it displays polymorphism, and in part because it is involved in the metabolism of such a wide array of xenobiotics, with substrates in virtually every drug class, including common over-the-counter medications such as dextromethorphan (e.g. Ereshefsky et al., 1995; Guengerich, 1995; Bertz & Granneman, 1997). In psychiatry, CYP2D6 is involved in the metabolism of antipsychotics such as haloperidol, perphenazine and thioridazine (Guengerich, 1995), as well as most tricyclic antidepressants (TCAs) and SSRIs (Ereshefsky et al., 1995). CYP2D6 can also be inhibited by many drugs (Otton et al., 1984), often with enough potency to temporarily change the individual to the PM phenotype (Guengerich, 1995). Preferred substrates of CYP2D6 are usually lipophilic compounds that have a basic N which

becomes protonated at physiological pH, and is approximately 0.5 to 0.7 nm from the site of oxidation (Coutts, 1994; Smith et al., 1996). Substrates also possess a planar, often aromatic ring system, which helps orientate the substrate at the CYP2D6 active site (Coutts, 1994). These structural requirements influence the types of reactions that CYP2D6 can catalyze. CYP2D6 has been implicated in a wide array of hydroxylation reactions, especially aromatic ring hydroxylations of the TCAs (Coutts et al., 1994). However, CYP2D6 is also involved in many N-dealkylation reactions (Coutts et al., 1994), which emphasizes that the reactions catalyzed by a specific enzyme cannot easily be predicted based on structure or type of reaction catalyzed, and require *in vitro* experimentation for confirmation.

#### 1.2.1.5 Drug metabolism in psychiatry

The issues of drug metabolism are particularly relevant to psychiatry for several reasons. First, polypharmacotherapy is frequently encountered, which introduces the risk for drug interactions. Second, differences in metabolism can lead to very different clinical outcomes, due to the different pharmacological properties and the presence of active metabolites of many psychiatric drugs. Third, optimization of drug and dose is critical in psychiatry, where clinical efficacy may take weeks to show up, and the consequences of lack of therapeutic effect can be devastating.

Combination drug therapy is increasingly common in psychiatry (Corruble & Puech, 1993; Rosenbaum, 1995; Carson, 1996; Laird, 1996).

Indeed, there are many situations in which polypharmacotherapy is indicated (Corruble & Puech, 1993; Dufresne, 1996). Since there is a high prevalence of co-morbidity of psychiatric illnesses, it is common for patients to be prescribed more than one medication concurrently to treat multiple conditions (Rosenbaum, 1995). Multiple drugs may also be required to treat residual symptoms not alleviated by the primary drug, especially in cases of complex disorders such as schizophrenia or obsessive-compulsive disorder (Carson, 1996). In addition, drugs may be co-prescribed to alleviate unwanted side effects of the primary drug (Laird, 1996). An example of this is the prescribing of antidepressants with sedatives, to treat antidepressant-induced insomnia (Corruble & Puech, 1993). Augmentation strategies are also employed to enhance the therapeutic efficacy of a given medication, such as lithium augmentation of antidepressants, the combination of TCAs and SSRIs, and more recently, the combination of SSRIs with drugs selective for 5-$HT_{1A}$ receptors (Artigas et al., 1996; Blier et al, 1997). Due to the chronic nature of most psychiatric illnesses, it is also likely that drug treatment for an intercurrent medical condition will occur at some point during drug therapy, necessitating polypharmacotherapy, since estimates of the comorbidity of physical illness in patients with psychiatric illness range from 40 to 60% (Honig et al., 1992). Finally, drug interactions may also be encountered during drug switch-overs from one therapy to another, particularly with drugs with long half-lives and active metabolites (Rosenbaum, 1995).

Drug metabolism is also relevant to psychiatry in that differences in metabolism can lead to important differences in therapeutic outcome. For example, the benzodiazepines are chosen for different therapeutic uses depending upon their pharmacokinetic parameters such as biological half-life or route of metabolism (i.e. oxidation vs. conjugation) (Teboul & Chouinard, 1991). The TCAs are mainly metabolized by CYP-mediated hydroxylation or demethylation (Potter & Manji, 1990). These metabolites can accumulate at levels equal to, or greater than, those of the parent compounds, and may contribute to the therapeutic efficacy or side effect profile of the compound (Caccia & Garattini, 1992). For example, the secondary amine TCAs have more noradrenaline reuptake inhibiting potency than do their tertiary amine parents, which are more potent 5-HT reuptake inhibitors (Potter & Manji, 1990). The tertiary amines are also much more potent blockers of histaminic, muscarinic, and $\alpha$-adrenergic receptors (Potter & Manji, 1990). Blockade of these receptors can lead to intolerable side effects, and treatment discontinuation. This is particularly true in elderly populations, in which these side effects can have very serious complications (Skerritt et al., 1997). Thus, it can be appreciated that any factor which alters the metabolism of these drugs can substantially alter the mechanism of action, side effect profile, and the therapeutic outcome.

Finally, knowledge of the factors affecting the metabolism of a given drug are important in the optimization of drug dose. Optimization of dose is probably one of the most important steps to improving the risk-benefit ratio of currently available antidepressant agents (Corruble & Puech, 1993). Wide inter-individual differences in drug metabolism can lead to higher than expected plasma drug levels and toxic side effects on the one hand, and lower than expected subtherapeutic doses on the other (Coutts, 1994). These variations may lead to a lengthy drug substitution procedure, when all that is required is a dosage adjustment (Coutts, 1994). Antidepressant medications take at least 1 to 2 weeks to cause mood-elevating effects, but a trial of a given medication can only be said to be adequate if the patient has received an adequate dosage for at least 6 weeks (Richelson, 1994). Therefore, a knowledge of the factors which will affect a drug's metabolism will help in prescribing the correct dosage, and will help to expedite the treatment process.

#### 1.2.1.6 Pharmacokinetic and pharmacodynamic drug interactions

The prevalence of polypharmacotherapy in psychiatry leads to increased risks of drug interactions, which can be either pharmacodynamic or pharmacokinetic. Pharmacokinetic interactions generally involve alterations in the absorption, distribution, metabolism, or excretion of the parent drugs and their metabolites (Carson, 1996). A pharmacokinetic interaction will

result in a change in the concentration of a drug at its site of action, which will present clinically the same result as would changing the dose of the drug (Preskorn, 1996). This change can be either a lowering of one drug's concentration, resulting in a loss of efficacy, or the elevation of a concentration to toxic levels (Laird, 1996). Such an interaction may be difficult to detect clinically in that it may be interpreted as either a lack of response or as a super-sensitive response, and the drug may be abandoned unnecessarily instead of simply adjusting the dose.

The other type of interaction is a pharmacodynamic interaction, which presents itself as a more qualitative change. In this type of interaction, the mechanism of action of one drug increases or decreases the effects produced by the mechanism of action of another drug (Preskorn, 1996). For example, the serotonin syndrome is a serious interaction that can occur when an SSRI and a monoamine oxidase inhibitor (MAOI) are taken together, and results from the combined indirect serotonin agonism caused by the inhibition of serotonin degradation by the MAOI, and the reduced serotonin uptake produced by the SSRI (Preskorn, 1996). Together, these drugs cause a dysregulation of the serotonin system, which results in symptoms which include hyperthermia, diaphoresis, gastrointestinal distress, myoclonus, and mental confusion (Preskorn, 1996).

Thus, pharmacodynamic interactions occur when the mechanisms of action of co-administered drugs combine to have an unexpected result, and are more of a qualitative change. Pharmacokinetic interactions result in a

quantitative change in drug response, as would be expected from either increasing or decreasing the drug dose. Pharmacokinetic interactions occur at the level of drug metabolism, and thus are of interest with respect to the CYP enzymes, and this thesis.

Historically, pharmacokinetic interactions have been difficult to predict, and could only be avoided after they had already been noted to occur clinically (Carson, 1996). Now, however, advances in molecular biology and pharmacogenetics have allowed for a better understanding of the mechanisms of drug interactions at the level of the CYP enzymes, and a more systematic way to study, classify and predict drug interactions. The CYP enzymes involved in a drug's metabolism can be identified through *in vitro* assays, and thus interactions *in vivo* can be predicted.

#### 1.2.1.7 Methods for Studying Drug Interactions

It was originally hoped that an individual's ability to metabolize drugs could be characterized by the administration of a probe drug which would then allow predictions of how other drugs would be handled by that individual (Eichelbaum, 1984). However, since multiple forms of CYP exist, and may be involved to varying extents in the metabolism of any particular drug, such simplistic models were not useful. Rather, predicting an individual's metabolic response to a particular drug involves a complex interplay of factors. Foremost, the isoforms involved in a drug's metabolism must be

determined. Only then can specific individual factors which will affect drug metabolism such as genetic and environmental influences be identified.

#### 1.2.1.7.1 *In vitro*

Important information about drug metabolism can now be garnered from *in vitro* experiments, and used to predict metabolic drug interactions *in vivo* (Guengerich, 1996). This information can be used early in the drug development process to make inferences about first pass metabolism, individual differences in clearance, and likelihood of drug interactions (Bertz & Granneman, 1997). Since large differences exist between species in CYP expression and specificities, animal models are useful only in certain situations (Guengerich, 1996). Due to the difficulty, expense, and ethical issues involved in working with human subjects, methods were developed to study human metabolism *in vitro*. Although *in vitro* work will never completely model all the aspects of human drug metabolism, important insights can be gained from such models.

Several *in vitro* methods have been developed and validated, and are routinely used to study drug metabolism (Tucker, 1992; Birkett et al., 1993; Remmel & Burchell, 1993; Wrighton et al., 1993; Guengerich, 1996). These models can be roughly divided into two groups; complex and simple metabolism systems. Complex metabolism systems try to model more aspects of human drug metabolism, and use human hepatocytes, liver slices, and hepatoma cell cultures (Skett et al., 1990; Tucker 1992; Friedberg &

Wolf, 1996). One of the major problems with complex models is the difficulty and expense of obtaining and preserving suitable human liver samples in the quantities necessary for metabolism experiments (Skett et al., 1995).

The other group of models, simple drug metabolism systems, aims to study defined steps in the drug metabolism process, and employs heterologous expression systems, enzymes purified from tissue, and human liver microsomes (Friedberg & Wolf, 1996; Guengerich, 1996). These simple models are useful in characterizing specific metabolic pathways in order to predict metabolic drug-drug interactions and individual differences in metabolism (Skett et al., 1995; Friedberg & Wolf, 1996). Simple models are relatively inexpensive and easy to use, while providing valid and reliable models of human drug metabolism, and therefore are much more commonly found in the literature.

Originally, purified enzymes were prepared and used as simple models (Friedberg & Wolf, 1996). However, there were difficulties with this process and the reliability of the data (Brosen, 1990; Birkett et al., 1993; Friedberg & Wolf, 1996). For example, the purification process itself is difficult, and involves several successive chromatographic steps to separate the specific enzymes, which may result in non-homogenous purification and batch-wise variations (Freidberg & Wolf, 1996). Furthermore, purification is sometimes impossible due to the close similarities in amino acid sequences of related isoforms (Birkett et al., 1993). Advances in molecular biology made it possible to isolate mRNA from human sources, generate cDNA libraries, and

introduce the cDNA into recipient cells on an expression vector (Friedberg & Wolf, 1996). Microsome products prepared from metabolically competent derivatives of such cell lines are now available commercially, and provide stable, reliable systems in which to study drug metabolism. Using singly-expressed enzymes in recombinant protein expression systems, questions regarding the capability of a particular enzyme to catalyze a given reaction can be answered (Guengerich, 1996).

Human liver microsomes are also widely used to study drug metabolism *in vitro*. Microsomes are readily available commercially, and can be obtained already characterized for their catalytic activities for a number of different CYP enzymes.

Using simple models of drug metabolism, several types of experiments are used to identify and characterize the enzymes involved in a particular metabolic pathway. The most common of these are correlations with marker reactions, activity in a recombinant P450 vector system, and attenuation by selective chemical inhibitors or antibodies (Guengerich, 1996), which will each be described in more detail below.

The identity of the specific high affinity enzyme involved in a particular catalytic pathway can be established by correlating product formation with activity levels of specific isoforms in a panel of human livers (Brosen, 1990). In such experiments, the substrate is incubated individually with liver microsomes from a panel of human livers which have been characterized for their activity at a number of different CYP isoforms. Product formation is

correlated with these activity levels, and significant correlations strongly suggest that the same enzyme is involved in both biotransformations (Wrighton et al., 1993; Ring et al., 1996).

The correlational experiments can be corroborated using the cDNA-expressed CYPs in heterologous systems (Wrighton et al., 1993; Ring et al., 1996). If a drug is incubated with microsomes prepared from cells expressing only a single isoform, and metabolite formation is seen, then this isoform may play a role in the metabolism of the drug *in vivo*. These recombinant enzyme systems can be used to address questions of the catalytic specificity of an enzyme, and whether it is capable of catalyzing a particular reaction (Guengerich, 1996). Heterologous expression systems also allow the analysis of the role of a specific enzyme without the interference of other drug metabolizing enzymes (Friedberg & Wolf, 1996).

Specific inhibitors of individual enzymes are also used to study the contributions of various isoforms to overall drug metabolism (Birkett et al., 1993; Wrighton et al., 1993; Guengerich, 1996). The specificity and potency of a number of chemical inhibitors has been established to provide guidelines for accurate testing (Newton et al., 1995; Bourrie et al., 1996). Inhibitory antibodies can also be used, although there are questions regarding specificity (Brosen, 1990) and inhibitory capacity (Schmider et al., 1996a). The extent of inhibition by a selective inhibitor gives an indication of the contribution of that isoform to that metabolic pathway (Birkett et al, 1993).

Assessing the competitive inhibition of isoform-selective substrates in the presence of the drug under investigation is also useful to get an idea of the relative potency with which the new drug binds to various isoforms. However, these studies do not differentiate between substrates and inhibitors, provide no information as to which particular metabolic pathway is involved, and are generally only used before assays for the new drug and its metabolites are developed (Birkett et al, 1993).

Although *in vitro* systems cannot account for all of the factors which determine drug metabolism *in vivo*, important information regarding the specific enzymes involved in a drug's metabolism can be achieved using *in vitro* models, This information can be used to predict the major genetic and environmental factors which may influence a drug's metabolism *in vivo*, and can help to explain unexpected therapeutic outcomes.

1.2.1.7.2 *In vivo*

*In vivo* approaches to studying metabolic reactions involve the same principles as *in vitro* approaches, but tend to be more difficult, time consuming, and potentially problematic than are *in vitro* assays. One approach is to compare the rate of a particular metabolic reaction with the rate of a marker reaction (Guengerich, 1995). However, the validation of marker reactions *in vivo* is difficult, due to all of the potential influences on metabolism that are present *in vivo*.

Another method is to use chemical or antibody inhibitors, or enzyme inducers *in vivo* to observe their effects on a given metabolic pathway (Coutts et al., 1994). There are several inhibitors and inducers that can be used safely *in vivo* (Guengerich, 1995). Ethical issues must be considered, as well as the specificity of the inhibitors and inducers, and potential confounding variables such as individual differences in metabolism due to genetic and environmental factors.

Finally, individuals who have been phenotyped for their metabolic status can be studied (Coutts et al., 1994). Allele-specific PCR assays may be used to identify mutant alleles in PMs, and metabolism of novel compounds can be studied in these individuals to assess the role of a specific enzyme in a given metabolic pathway (Kroemer & Eichelbaum, 1995).

*1.2.1.7.3 Clinical relevance*

*In vitro* work affords the opportunity to study drug metabolism in a well-controlled environment at a minimum of time and expense. In addition, manipulations that might not be ethical in human subjects are possible. However, there are still important differences between the *in vitro* and *in vivo* situations, which have brought into question the clinical relevance of *in vitro* findings with respect to drug-drug interactions *in vivo*.

The main criticism of *in vitro-in vivo* comparisons is that the plasma concentration of drug is often lower than the $K_i$ values obtained *in vitro*, and therefore enzyme inhibition would occur to a clinically insignificant extent

(Ring et al., 1995). However, these criticisms are not supported by experimental findings. This is because *in vivo* interactions occur in the liver, where the drug concentration can be much higher than the plasma levels due to the partitioning of lipophilic drugs between the plasma and the liver tissue (von Moltke et al., 1995; Greenblatt et al., 1996; Preskorn, 1996). Therefore, any model predicting *in vivo* interactions based on *in vitro* data must take into account not only the $K_i$, but also the partitioning of the inhibitor between liver and plasma (von Moltke et al., 1995). There is currently no clearly established or validated model for *in vitro-in vivo* scaling; however, there are many models in the literature with which accurate predictions of pharmacokinetic interactions have been made (von Moltke et al., 1995; Preskorn, 1996; von Moltke et al., 1996a; Bertz & Granneman, 1997; Iwatsubo et al., 1997).

### 1.2.2 Antidepressant Drug Development

#### 1.2.2.1 History

The first antidepressant drugs were discovered by chance when iproniazid was being used as an antitubercular agent in the early 1950s, and it was discovered that it elevated mood in some patients (Pinder & Wieringa, 1993; Charney et al., 1995; Potter et al. 1995). Since the antidepressant mechanism of action was unknown, and since iproniazid was a monoamine oxidase inhibitor (MAOI), other compounds that inhibited MAO were synthesized for use as antidepressants. Although effective as

antidepressants in some patients, MAOIs did have some undesirable side effects due to their non-specific actions. In addition to anti-cholinergic, anti-histaminic and anti-adrenergic effects, their nonspecific inhibition of MAO resulted in potentially life-threatening interactions with tyramine-containing foods. Tyramine is normally metabolized by MAO, but in the presence of MAOIs its metabolism is impaired. The excess tyramine is taken up into noradrenergic neurons where it displaces noradrenaline (NA) from storage vesicles, causing an increase in NA release and possible hypertensive crisis. People on MAOI therapy were therefore placed on tyramine-restricted diets, which had the potential for serious safety and compliance issues. Reversible inhibitors of MAO-A (RIMAs), were developed to try to improve upon the original MAOIs (Pinder & Wieringa, 1993). However, the safety and tolerability of these drugs has still not been fully determined (Berlin & Lecrubier, 1996).

The other class of early antidepressants was the tricyclic antidepressants (TCAs). Iminodibenzyl, the core of the tricyclic molecule, was synthesized in 1889, but was not in clinical use until 1948, when derivatives of iminodibenzyl were synthesized to be screened as possible antihistamines, sedatives, analgesics, and antiparkinson drugs (Potter et al., 1995). Imipramine, the first TCA, came out of this effort (Charney et al., 1995, Potter et al., 1995). In 1952, reports of the effectiveness of chlorpromazine (CPZ) on psychosis were described. Since imipramine is structurally related to CPZ, with the replacement of the sulfur with an ethylene

linkage, imipramine was tried in 1958 to quiet psychotic patients, but was found to be relatively ineffective. However, it did seem to produce an improvement in a subset of patients who were identified as depressed (Potter et al., 1995). The primary biochemical effect of imipramine was subsequently determined to be the ability to inhibit the reuptake of NA and 5-HT (Charney et al., 1995).

The TCAs and MAOIs were effective drugs in a subset of patients, but were still not the ideal in that they took at least 2 weeks for clinical efficacy, were only effective in 60-75% of patients, and carried with them a wide range of side effects due to their actions at histaminic, muscarinic and α-adrenergic receptors. This resulted in bothersome side effects such as dry mouth, blurred vision, drowsiness and sedation, as well as more serious effects such as cardiovascular irregularities and seizures (Corruble & Puech, 1993). It was discovered that the antidepressant effects of the MAOIs and TCAs were not due to action at histaminic, muscarinic or α-adrenergic receptors, but rather seemed to depend on their ability to enhance monoaminergic transmission (Charney et al., 1995). Therefore, the search began for drugs that would selectively enhance the function of one of the monoamine systems, without direct effects on other neurotransmitter receptors.

#### 1.2.2.2 Rational drug design (RDD) and the 5-HT system

Rational drug design (RDD) is the process of designing drugs that have specific actions on specific neural sites based on a knowledge of the underlying mechanisms of disease, while avoiding actions at sites not associated with the disease process (Preskorn, 1996). The goals of RDD for antidepressants are to try to enhance treatment efficacy, that is, to get a clinical response in a greater number of patients, to speed up the onset of clinical response, and to reduce side effects (Pinder & Wieringa, 1993). With the ability to design drugs for very specific receptor subtypes came the next generation of antidepressant drugs.

The 5-HT system has been a major focus in theories of depression and in its treatment, as evidence from several lines of investigation have implicated this system in depression. Low levels of brain 5-HT and cerebrospinal fluid (CSF) 5-hydroxyindole-3-acetic acid (5-HIAA) have been reported in subgroups of depressed patients, as have alterations in pre- and post-synaptic 5-HT receptors, and alterations in peripheral markers of central 5-HT function such as platelet 5-HT uptake, platelet $^{3}$H-imipramine binding, platelet 5-$HT_2$ receptor density, and whole blood 5-HT content (Risch & Nemeroff, 1992). Furthermore, dietary depletion of tryptophan, the amino acid precursor to 5-HT, can cause a lowering of mood in certain susceptible individuals such as females, and males with a family history of depression (Benkelfat et al., 1994; Young et al., 1996), and a relapse into depression in recovered depressed patients (Young, 1993). Finally, most treatments for

depression involve augmentation of 5-HT function (Blier & de Montigny, 1994; 1997). In addition, the 5-HT system is involved in the regulation of many psychological and physiological functions that may be dysregulated in depression, such as mood, anxiety, arousal, eating, sleeping, aggression, cognitions, and impulse control (Dubovsky, 1994; Dubovsky & Thomas, 1995).

Since the 5-HT system had been so strongly implicated in depression, it became a target for rational drug design. A very successful group of rationally designed drugs was the selective serotonin reuptake inhibitors (SSRIs) (Preskorn, 1996). The SSRIs have the common mechanism of action of selectively inhibiting the reuptake of 5-HT in the synaptic cleft. This uptake inhibition is thought to result in excess 5-HT in the synaptic cleft, which initially causes a decrease in the firing rate of 5-HT neurons due to inhibitory autoreceptor activation. However, it is theorized that prolonged treatment results in autoreceptor desensitization, and a subsequent return to normal firing rates accompanied by an increase in 5-HT transmission (Blier & de Montigny, 1994). The drugs in this class, namely fluoxetine (FLU), paroxetine (PRX), sertraline (SERT), citalopram (CIT), and fluvoxamine (FVX), are all structurally unique and have unique pharmacokinetic profiles. They have reduced side effects as compared to the TCAs and MAOIs, since they do not interact significantly with histaminic, muscarinic, or adrenergic receptors (Preskorn, 1997). The SSRIs have been shown to be at least as effective as the TCAs and MAOIs in the treatment of depression (Pinder &

Wieringa, 1993). However, they are not without their own side effects, most commonly gastrointestinal distress, anxiety, and sexual dysfunction (Asberg & Martensson, 1993). These side effects can interfere with the quality of life of the depressed patient, and can result in treatment discontinuation. In addition, the SSRIs are still only effective in 60-70% of depressed patients, and take approximately 2 weeks for therapeutic efficacy. Therefore, there is still room for improvement in the rational design of antidepressant drugs.

Since depression is such a complex disorder, with many different possible etiologies and manifestations, it is to be expected that certain drugs will work in some patients and not in others. It has been stated that for major depressive episodes, no matter which antidepressant is used, approximately one-third of patients will recover, one-third will show partial improvement, and the remaining third will fail to respond (Corruble & Puech, 1993). Therefore, it is necessary to search for other drugs which act through different mechanisms of action, to perhaps target different subgroups of depression.

The diverse actions 5-HT are mediated by a variety of receptor subtypes at many different neuronal and brain locations, using several different intracellular signaling mechanisms (Dubovsky, 1994; Dubovsky & Thomas, 1995). At present, there are at least seven identified families of 5-HT receptors, many of which have been further divided into subfamilies (Roth, 1994). With respect to depression and antidepressant drugs, the most important 5-HT receptor families are 5-$HT_1$ and 5-$HT_2$ (Berendsen, 1995). Within the 5-$HT_1$ family, 5-$HT_{1A}$ receptors are thought to play an important

role in the etiology and treatment of depression and anxiety disorders (Asberg & Martensson, 1993; Benkelfat, 1993; Schreiber & De Vry, 1993). They are located both pre- and post-synaptically, and mediate the inhibitory actions of 5-HT through two independent G-protein linked effector systems, one of which leads to the activation of basal cyclic adenosine-monophosphate (cAMP) and inhibition of stimulated cAMP, and the other leads to the opening of a potassium channel and cellular hyperpolarization (Lesch et al., 1992; Dubovsky, 1994). The $5\text{-HT}_2$ family of receptors is made up of the $5\text{-HT}_{2A}$, $5\text{-HT}_{2B}$, and $5\text{-HT}_{2C}$ receptors. The $5\text{-HT}_{2A}$ receptor is the classical 5-HT receptor, and is often referred to as simply the $5\text{-HT}_2$ receptor. The $5\text{-HT}_{2C}$ receptor was formerly classified as a member of the $5\text{-HT}_1$ family, but was reclassified as a $5\text{-HT}_2$ receptor since it has 69% sequence homology with other $5\text{-HT}_2$ receptors, binds many of the same ligands, and shares the same second messenger systems as the $5\text{-HT}_2$ receptors (Dubovsky & Thomas, 1995). Activation of the $5\text{-HT}_2$ receptors can result in increased cellular excitability through a G-protein mediated closing of a potassium channel, or in a decreased cellular excitability through phosphotidylinositol-mediated protein kinase C activation and subsequent potassium channel opening (Leysen & Schotte, 1992; Dubovsky, 1994). Thus, the effects of 5-HT stimulation are complex, and depend on the state of the receptor at the time of activation, and on which signaling mechanism predominates at the time of activation (Dubovsky, 1994). In addition, the

influences of other 5-HT receptors and neurotransmitters will affect the net result of 5-HT receptor activation.

### 1.2.2.3 5-$HT_2$ receptors as targets for rational drug design

It has been proposed that depression is the result of a disturbed balance in the regulation of 5-HT receptors, particularly 5-$HT_{1A}$, 5-$HT_{2A}$, and 5-$HT_{2C}$ receptors, and that the function of serotonergic antidepressant treatment is to restore this balance (Berendsen, 1995). Functional interactions between 5-HT receptors exist, with 5-$HT_{1A}$ and 5-$HT_2$ exerting mutually inhibitory effects (Darmani et al., 1990; Leysen & Schotte, 1992; Barrett & Vanover, 1993; Schreiber & De Vry, 1993). It is hypothesized that these receptors may be either super- or sub-sensitive, and that the type of depression which emerges is a result of the particular imbalance. Therefore, for certain subtypes of depression, a 5-$HT_2$ receptor antagonist may be more useful. Indeed, antidepressants with 5-$HT_2$ antagonistic effects have been shown to be particularly efficacious in depression associated with anxiety, and psychotic depression (Dubovsky, 1994). In support of this theory is evidence that 5-$HT_{1A}$ activation results in an anxiolytic or sedative effect, whereas the activation of 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors is anxiogenic and has mild psychotogenic properties (Deakin, 1988; Benkelfat, 1993). In addition, many effective antipsychotic medications are also 5-$HT_2$ antagonists (Dubovsky, 1994; Roth, 1994).

The 5-$HT_2$ receptor is also a target for antidepressant drug therapy because many antidepressants, given chronically, lead to 5-$HT_2$ receptor down-regulation, which may mediate antidepressant efficacy (Deakin, 1988; Baker & Greenshaw, 1989; Berendsen, 1995). Indeed, many antidepressant and antipsychotic agents have 5-$HT_2$ antagonistic effects (Glennon, 1990). An endogenous dysregulation of 5-$HT_2$ receptors in depression has been suggested by findings that platelet 5-$HT_2$ receptor densities are higher in depressed patients than in controls (Sheline et al., 1995), and that 5-$HT_2$ binding is increased in frontal cortex of depressed suicides (Arora & Meltzer, 1989). The search for novel targets of antidepressant action led to the development of trazodone, a 5-$HT_2$ antagonist. The success of trazodone was utilized in the development of nefazodone, which was rationally designed to preserve trazodone's antidepressant efficacy without its sedating side effects.

### 1.2.3 Trazodone

#### 1.2.3.1 Introduction

Trazodone is pharmacologically and structurally unique among antidepressants in that it is a phenylpiperazine-derivative (Figure 1.2) which inhibits 5-HT reuptake and potently blocks 5-$HT_2$ receptors (Fuller et al., 1984; Marek et al., 1992), although it has a ten-fold higher affinity for 5-$HT_{2A}$ than for 5-$HT_{2c}$ receptors (Jenck et al., 1994). Given chronically, it leads to 5-$HT_2$ receptor down-regulation (Marek et al., 1992) and receptor subsensitivity

(Georgotas et al., 1982). It is superior to placebo and is as effective as amitriptyline, imipramine, fluoxetine, and mianserin in the treatment of major depression (Marek et al., 1992; Workman & Short, 1993; Haria et al., 1994). It is particularly effective in the treatment of anxiety associated with depression (Marek et al., 1992). Trazodone has a favorable side effect profile compared to the TCAs and SSRIs, lacks anticholinergic and cardiotoxic effects, and is relatively safe in overdose (Feighner & Boyer, 1988; Haria et al., 1994). The most common side effects are dizziness, somnolence, and nausea (Feighner & Boyer, 1988; Cunningham et al., 1994). Trazodone also has hypnotic effects, which provide a beneficial effect on sleep (Georgotas et al., 1982; Gershon, 1990). In fact, trazodone is often prescribed for antidepressant-associated insomnia (Jacobsen, 1990; Nierenberg et al., 1994).

#### 1.2.3.2 Pharmacokinetics

Trazodone is highly lipophilic and is well absorbed 1-2 hours post-administration (Nilsen & Dale, 1992; Haria et al., 1994; Golden et al., 1995), with bioavailability ranging from 63-91% (Haria et al., 1994). The elimination half-life is biphasic, with the first component approximately 3-6 hours and the second approximately 5-9 hours (Nilsen & Dale, 1992; Golden et al., 1995). Trazodone has linear kinetics (Nilsen et al., 1993; Haria et al., 1994) and is extensively metabolized, with less than 1% excreted unchanged in urine (Caccia et al., 1981; Nilsen & Dale, 1992; Haria et al., 1994). It is 89-95%

bound to plasma proteins (Golden et al., 1995). Large individual variations in the metabolism of trazodone are seen (Ishida et al., 1995).

#### 1.2.3.3 Metabolism

Trazodone undergoes extensive hepatic metabolism, mainly through hepatic hydroxylation, oxidation, and N-oxidation (Baiocchi et al., 1974; Haria et al., 1994; Golden et al., 1995). Trazodone is hydroxylated to a dihydrodiol metabolite [Figure 1.2] (Baiocchi et al., 1974). It is also hydroxylated in the para position on the m-chlorophenyl ring in rats (Yamato et al., 1974b) and humans (Baiocchi et al.,1974) [Figure 1.2]. N-Dealkylation at the piperazinyl nitrogen results in the formation of oxotriazolepyridinpropionic acid (TPA) and mCPP [Figure 1.2] (Melzacka et al., 1979; Yamato et al., 1974a). The piperazinyl nitrogen also undergoes N-oxidation (Baiocchi et al., 1974) [Figure 1.2]. Following an oral dose of trazodone, 20% is excreted in the urine as TPA and its conjugates, 9% as the dihydrodiol metabolite (Haria et al., 1994), and less than 1% is present as free or unconjugated mCPP (Caccia et al., 1982).

#### 1.2.3.4 Drug-Drug Interactions

The specific CYP enzymes that are involved in the metabolism of trazodone are presently unknown, but drug interaction studies and case reports have helped to suggest which ones may be involved. Several interactions with FLU have been reported. Plasma concentrations of

trazodone were increased by 31% in one case report, although FLU levels were not measured (Aranow et al., 1989). Another investigation found both trazodone and mCPP levels were elevated following the addition of 20 mg/day FLU (Maes et al., 1997). Excessive sedation following trazodone and FLU coadministration has also been reported (Metz & Shader, 1990; Nierenberg et al., 1992), which may be the result of increased trazodone plasma concentrations resulting from a pharmacokinetic interaction. In addition, serotonin syndrome has been reported following trazodone and FLU coadministration (George & Godleski, 1996), as well as following trazodone and PRX coadministration (Reeves & Bullen, 1995). This interaction may be due to a pharmacodynamic interaction since trazodone, FLU, and PRX all potentiate the 5-HT system. However, the interactions may also be due to pharmacokinetic interactions resulting from competition for CYP enzymes, and the resultant higher blood levels of trazodone, FLU or PRX precipitating the 5-HT syndrome. FLU and PRX strongly inhibit CYP2D6 (Brosen, 1993; Preskorn & Magnus, 1994; Jeppesen et al., 1996), and FLU also inhibits CYP3A4 (Greenblatt et al., 1992; Preskorn & Magnus, 1994), so the interaction could be through either or both of these pathways.

Thioridazine, a CYP2D6 inhibitor, increases plasma concentrations of both trazodone and mCPP, implicating CYP2D6 in the metabolism of both (Yasui et al., 1995). Smoking is known to induce CYP1A2 (Bock et al., 1994), and smokers have significantly lower plasma concentrations of trazodone, and higher mCPP/trazodone ratios (Ishida et al., 1995),

suggesting that CYP1A2 may be involved in trazodone metabolism. Carbamazepine, an inducer and substrate of CYP3A4 (Bertilsson & Tomson, 1986; Johnson et al., 1992; Kerr et al., 1994), decreases trazodone and mCPP plasma concentrations (Otani et al., 1996), suggesting that CYP3A4 may be involved in the metabolism of trazodone and mCPP.

In summary, there is some *in vivo* evidence implicating CYP2D6, CYP1A2, and CYP3A4 in the metabolism of trazodone. However, the specific metabolic pathways involved are not known, and definitive *in vitro* studies are required. Specifically, the enzymes involved in the formation of the active metabolite mCPP are of interest, since this metabolite has been shown to have psychopharmacological effects on its own (Kahn et al., 1988; Krystal et al., 1993; Cowen et al., 1995), and may contribute to the antidepressant efficacy of trazodone (Maes et al., 1997).

### 1.2.4 m-Chlorophenylpiperazine

#### 1.2.4.1 Introduction

m-Chlorophenylpiperazine (mCPP) is a pharmacologically active metabolite of the antidepressants trazodone, nefazodone and etoperidone, and of the minor tranquilizer mepiprazole (Melzacka et al., 1979; Caccia et al., 1981; Fong et al., 1982; Mayol et al., 1994b). It is primarily a 5-$HT_{2A}$ antagonist and 5-$HT_{2C}$ agonist (Conn & Sanders-Bush, 1987; Kahn & Wetzler, 1991; Fiorella et al., 1995), although it also binds to 5-$HT_{1A}$, 5-$HT_{1D}$,

and $\alpha_2$-adrenergic receptors equipotently in human cortex (Hamik & Peroutka, 1989). In animals and humans, mCPP causes a dose-dependent elevation of adrenocorticotropic hormone (ACTH), cortisol, and prolactin (Kahn et al., 1990a; Kahn & Wetzler, 1991; Cowen et al., 1995), measures for which it is often used as a probe of 5-HT receptor function (Kahn & Wetzler, 1991). Behaviorally, mCPP decreases food intake in both animals (Samanin et al., 1979; Kahn & Wetzler, 1991) and humans (Walsh et al., 1994; Cowen et al., 1995). It also increases measures of anxiety in animals (Gibson et al., 1994) and humans (Kahn et al., 1988; Krystal et al., 1993; Cowen et al., 1995). In addition, mCPP worsens the core symptoms of many psychiatric disorders such as the positive symptoms of schizophrenia (Krystal et al., 1993) and panic symptoms in patients with panic disorder (Kahn et al., 1988; Germine et al., 1994). These behavioral effects have been noted to occur at blood levels of mCPP which are seen at therapeutic doses of trazodone (Ishida et al., 1995). Although under normal circumstances the 5-$HT_{2C}$ antagonistic activity of trazodone may counteract these effects of mCPP, conditions under which mCPP levels are increased may lead to the presentation of side effects. It has been suggested that the 5-$HT_{2C}$ agonistic effects of mCPP may be responsible for some of trazodone's side effects such as trazodone-related priapism (Jenck et al., 1994). In addition, since many antidepressants are antagonists at 5-$HT_{2C}$ receptors, it has been proposed that elevated mCPP levels could counteract the therapeutic effects

of these drugs (Jenck et al., 1994). However, mCPP has also been suggested to be involved in the therapeutic effects of trazodone, since fluoxetine-induced increases in plasma mCPP concentrations have been found to be related to clinical response (Maes et al., 1997). The hypothesized mechanism for this effect of mCPP is an augmentation of trazodone's ability to inhibit 5-HT reuptake, desensitization of 5-$HT_{2C}$ receptors, and indirect antagonism of 5-$HT_{2A}$ receptors via partial agonistic effects at 5-$HT_{1A}$ and 5-$HT_{2C}$ receptors (Maes et al., 1997). Despite uncertainty regarding its precise mechanism of action, mCPP does have significant effects on the 5-HT system, which make the pathways leading to the formation and metabolism of this metabolite of interest.

#### 1.2.4.2 Pharmacokinetics

In the rat, mCPP is rapidly hydroxylated in the phenyl ring to p-OH-mCPP and is excreted in urine as both glucuronide and sulfate conjugates with no detectable free mCPP or p-OH-mCPP seen (Yamato et al., 1974b; Melzacka et al., 1979; Mayol et al., 1994a). Following trazodone or NEF administration, mCPP reaches peak plasma concentrations within 2-4 hours, and amounts to approximately 1- 20% of parent drug plasma concentration (Caccia et al., 1982; Kaul et al, 1995; Preskorn et al., 1995; Yasui et al., 1995; Barbhaiya et al., 1995a, 1996d; Otani et al., 1996). mCPP exhibits linear pharmacokinetics, has an elimination half life of 4-14 hours, and

reaches higher concentrations in the brain than in plasma in rats (Caccia et al., 1981).

#### 1.2.4.3 Metabolism and drug interactions

mCPP clearance is decreased in poor metabolizers of dextromethorphan, who are genetically deficient in CYP2D6, which suggests that CYP2D6 may play a role in the hydroxylation of mCPP (Buch et al., 1993; Barbhaiya et al., 1996b). Co-administration of NEF and propranolol results in an increase in the area under the time concentration curve (AUC) of mCPP, likely as a result of propranolol's inhibition of CYP2D6 (Ellingrod & Perry, 1995). Similarly, inhibition of CYP2D6 is thought to be responsible for increased mCPP levels when FLU and NEF (Marino et al., 1996), or FLU and trazodone (Maes et al., 1997) are co-administered. However, the role of other CYP enzymes such as CYP3A4 cannot be ruled out, and although these observed clinical interactions indicate CYP enzymes which may be involved in the metabolism, they do not provide definitive evidence.

### 1.2.5 Nefazodone

#### 1.2.5.1 Introduction

Nefazodone (NEF) is a phenylpiperazine-derivative antidepressant drug which is similar in structure to trazodone (Ellingrod & Perry, 1995) (Figure 1.3). It was designed to incorporate the beneficial effects of trazodone with a reduced side effect profile by decreasing the affinity for the $\alpha_1$-adrenergic receptor (Taylor et al., 1986). Like trazodone, NEF's most potent action is 5-$HT_2$ antagonism (D'Amico et al., 1990; Eison et al., 1990). Trazodone and NEF are equipotent in their ability to block 5-$HT_2$ receptors, and both cause 5-$HT_2$ down-regulation in rat cerebral cortex following chronic administration (Taylor et al., 1986). NEF also possesses 5-HT uptake inhibiting capabilities (D'Amico et al., 1990; Preskorn et al., 1995), as well as norepinephrine uptake inhibition (Taylor et al., 1986). NEF also is a 5-$HT_{2C}$ antagonist. Although NEF has $\alpha_1$-adrenergic antagonistic properties, it is not as strong an antagonist as is trazodone (Eison et al., 1990), and therefore is not as sedating (Taylor et al., 1995). NEF has no significant affinity for $\alpha_2$-adrenergic, β-adrenergic, histaminergic, dopaminergic, or cholinergic receptors (Taylor et al., 1995). It is as effective as IMI, FLU, and PRX in placebo-controlled clinical trials in the treatment of all types of depression (Montgomery, 1996), and appears to have a particular therapeutic advantage in treating anxiety and agitation associated with depression (Fawcett et al., 1995; Malik, 1996; Montgomery , 1996; Nutt, 1996). NEF is well tolerated

and has very few side effects (Robinson et al., 1996; Marcus, 1996), probably due to its relative lack of affinity for receptors other than 5-HT.

#### 1.2.5.2 Pharmacokinetics

NEF is rapidly and completely absorbed following oral administration (Malik, 1996), and is subject to extensive first pass metabolism (Mayol et al., 1994b; Barbhaiya et al., 1995a, 1996c). Peak plasma levels of NEF occur in a median time of 1.2 hours, and its metabolites in 1.7 hours (Ellingrod & Perry, 1995). Because of extensive metabolism by N-dealkylation, only trace amounts of NEF and its metabolites are found in urine (Mayol et al., 1994b; Barbhaiya et al., 1995a). The plasma half life of NEF is 2-4 hours (Malik, 1996), and steady state is achieved in 3-4 days (Malik, 1996). NEF is 99% protein bound, and has an oral bioavailability of 15-23% (Golden et al., 1995). NEF exhibits non-linear pharmacokinetics, and there are very large individual differences in plasma levels of NEF and its metabolites (Ferry et al., 1994; Kaul et al., 1995; Barbhaiya et al., 1995a, 1996d). Non-linear pharmacokinetics may be due to saturation of the metabolic pathways or the inhibition of metabolism by NEF or its metabolites (Kaul et al., 1995). Plasma levels of NEF are higher in the elderly than in the young, possibly due to decreased CYP content with age (Barbhaiya et al., 1996a).

### 1.2.5.3 Metabolism

NEF has three pharmacologically active metabolites that have been quantified in clinical pharmacokinetic studies, namely hydroxynefazodone (OH-NEF), triazoledione (TD), and mCPP (Figure 1.3). OH-NEF is present in the plasma at levels approximately one third of those of NEF (Barbhaiya et al., 1995a; Preskorn et al., 1995), but is considered to have similar clinical efficacy to NEF (Ellingrod & Perry, 1995). It has the same pharmacological profile as NEF in that it is a 5-$HT_{2A}$ and 5-$HT_{2C}$ antagonist, and it inhibits 5-HT uptake and $\alpha$-adrenergic receptors (Taylor et al., 1995). The elimination half life of OH-NEF is 3-4 hours, and it has nonlinear pharmacokinetics and a time course which parallels that of NEF (Ferry et al., 1994). The similarities in the neuropharmacological profiles of NEF and OH-NEF suggests that OH-NEF probably contributes to the clinical efficacy of NEF (Preskorn et al., 1995).

Another of NEF's major metabolites is triazoledione (TD). It inhibits the 5-$HT_{2A}$ receptor more selectively, but with only 15-30% of the potency of NEF (Mayol et al., 1994b; Taylor et al., 1995). As well, TD does not block the uptake of 5-HT (Ellingrod & Perry, 1995). However, TD does reach plasma levels that are approximately 10 times those of NEF (Preskorn et al., 1995; Barbhaiya et al., 1996d), and therefore probably contributes to NEF's efficacy. In addition, the long half life of TD (18-30 h) helps to stabilize the drug levels.

The third of NEF's main metabolites is mCPP, which is a major metabolite of trazodone, but which is only a minor metabolite of NEF. mCPP is formed directly from N-dealkylation of the piperazinyl nitrogen of NEF or OH-NEF (Mayol et al., 1994b). Although mCPP is a 5-$HT_{2C}$ agonist and 5-$HT_{2A}$ antagonist (Conn & Sanders-Bush, 1987; Fiorella et al., 1995), it is not likely to have any clinically significant effects with NEF treatment since mCPP accumulates at levels only one-tenth to one one-hundredth those of NEF, and has a half life of only 4-9 h (Kaul et al, 1995; Preskorn et al., 1995; Barbhaiya et al., 1995a, 1996d). NEF and OH-NEF are antagonists at the 5-$HT_{2C}$ receptor, and thus probably oppose the effects of mCPP under normal circumstances (Preskorn et al., 1995); however, if mCPP levels were increased due to altered rates of metabolism, it is possible that adverse effects could result.

#### 1.2.5.4 Drug-drug interactions

NEF has been reported to be a strong *in vitro* inhibitor of CYP3A4 (Ellingrod & Perry, 1995; Marcus, 1996; von Moltke et al., 1996a; 1996b), and a very weak *in vitro* inhibitor of CYP1A2 (von Moltke et al., 1996c) and CYP2D6 (Schmider et al., 1996b). Its potency for CYP3A4 inhibition is approximately one tenth that of ketoconazole, but is at least three times more potent than the SSRIs (von Moltke et al., 1996). This inhibiting potency is equivalent to the ability of FLU to inhibit CYP2D6, which is clinically

significant (Preskorn et al., 1995). OH-NEF and the inactive metabolite p-OH-NEF are also potent *in vitro* inhibitors of CYP3A4, as measured by the ability to inhibit the formation of 4-OH-alprazolam (von Moltke et al., 1996). In addition, ketoconazole and antibodies against rat CYP3A1 (analog of human CYP3A4) inhibit the formation of all metabolites of NEF *in vitro,* suggesting that NEF is also a substrate for CYP3A4 (von Moltke et al., 1996).

*In vivo,* NEF has been shown to have pharmacokinetic interactions with triazolam and alprazolam, increasing plasma concentrations and clearance times of both benzodiazepines without any effect on NEF or its metabolites (Greene et al., 1995a; Barbhaiya et al., 1995c; Kroboth et al., 1995). This interaction is clinically significant in that the psychomotor, sedative and memory effects of triazolam and alprazolam are greater in the presence of NEF than in its absence (Kroboth et al., 1995). This interaction appears to be a result of CYP3A4 inhibition by NEF since both alprazolam and triazolam are CYP3A4 substrates. However, plasma levels of lorazepam, a benzodiazepine which is metabolized by glucuronic acid conjugation rather than hydroxylation, were not affected by NEF coadministration (Greene et al., 1995b). Carbamazepine (CBZ), a substrate for, and inducer of CYP3A4 activity (Johnson et al., 1992; Kerr et al., 1994), shows elevated plasma levels when co-administered with NEF (Ashton et al., 1996). However, CBZ is also a substrate for CYP2C8 and CYP1A2, which may also play a role in the observed interaction (Johnson et al., 1992). NEF has also been reported to interact with cyclosporine (Helms-Smith et al.,

1996), another CYP3A4 substrate (Bertz & Granneman, 1997). These findings suggest the potential for interactions with other substrates or inhibitors of CYP3A4, and co-prescription with the CYP3A4 substrates terfenadine and astemizole has been contraindicated (Ellingrod & Perry, 1995; Robinson et al., 1996).

The clearance of NEF and OH-NEF is similar in extensive and poor metabolizers of dextromethorphan, and therefore these pathways appear to be independent of CYP2D6 (Buch et al., 1993; Barbhaiya et al., 1996b). However, mCPP clearance is slowed in PMs of dextromethorphan, which indicates the potential for interactions with CYP2D6 inhibitors and substrates *via* this pathway. Similarly, FLU co-administration does not alter pharmacokinetics of NEF or OH-NEF, but does increase levels of mCPP and TD (Marino et al., 1996). Therefore, interactions of NEF with CYP2D6 substrates or inhibitors is possible through these pathways.

Co-administration of NEF with propranolol results in a decrease in the peak plasma levels ($C_{max}$) and area under the time concentration curve (AUC) of propranolol, but an increase in the AUC of mCPP, likely as a result of propranolol's inhibition of CYP2D6 (Ellingrod & Perry, 1995). NEF increases digoxin levels (Dockens et al, 1996), and modestly increases the AUC of haloperidol (36%) (Barbhaiya et al., 1996e), but the enzymes responsible for these interactions have not been determined. Cimetidine, a nonspecific inhibitor of CYP enzymes (Bertz & Granneman, 1997), has no effect on levels of NEF (Barbhaiya et al., 1995b).

NEF has no effect on theophylline pharmacokinetics (Dockens et al., 1995), or the clearance of R-warfarin (both CYP1A2-mediated) (Salazar et al., 1995). Similarly, there is no difference in NEF pharmacokinetics between smokers and non-smokers (Kaul et al., 1995), indicating that NEF is probably not a strong inhibitor or substrate of CYP1A2. NEF causes only a slight decrease in peak plasma levels ($C_{max}$) of S-warfarin (Salazar et al., 1995), and does not interact with phenytoin (Robinson et al., 1996), both of which are CYP2C9 substrates (Bertz & Granneman, 1997).

In summary, caution should be exercised in the coadministration of NEF with other CYP3A4 substrates and inhibitors, since NEF appears to be both an inhibitor of, and substrate for, CYP3A4. Interactions with substrates or inhibitors of CYP2D6 are also possible due to interactions with mCPP metabolism. However, detailed *in vitro* analyses of the metabolism of NEF and its metabolites have not been published, and would be helpful to clarify and characterize these metabolic pathways.

### 1.2.6 Relevant Analytical Techniques

#### 1.2.6.1 Chromatography

Chromatography refers to a group of processes used to achieve the separation of a mixture of solutes by differential distribution between two phases, a mobile phase and a stationary phase (Tabor, 1989). These methods are generally classified according to the physical state of the mobile phase, which is either gas or liquid, and which carries the mixture of solutes over the stationary phase. These methods can be subdivided on the basis of solute interactions with the stationary phase, with the most common ones being adsorption and partition, and less common ones being ion exchange and gel-filtration (Tabor, 1989). Adsorption is the mechanism of solute interactions in liquid-solid (LS) and gas-solid (GS) chromatography, and refers to differences in attraction of the solute molecules to the stationary versus the mobile phase. In partition chromatography, the separation is achieved by differences in the distribution of solute molecules between two liquid phases [liquid-liquid (LL)], or a gas and a liquid phase [gas-liquid (GL)]. The goal of chromatography is to achieve a separation of a mixture of components, and the physicochemical basis of the separation is a distribution equilibrium between two immiscible phases. Polarity is a major determining factor in chromatography, and affects the interaction of a molecule with both stationary and mobile phases.

#### *1.2.6.1.1 Gas Chromatography*

##### 1.2.6.1.1.1 Principles

Gas chromatography utilizes a gas mobile phase and either a liquid or solid stationary phase to separate the components of a sample based on the differential partitioning of the sample molecules between the two phases in vapour form (Coutts et al., 1985). The components will partition between the stationary and mobile phases depending in part upon their polarity, with polar solutes being retained longer on polar stationary phases than on nonpolar phases. There are several components to the basic gas chromatographic system, which can be manipulated to affect the separation of components, and which will be discussed in detail below. In gas chromatography, an inert carrier gas carries the volatilized solute molecules through the gas chromatographic column, where the components are separated, to a detector, where solute molecules produce a signal which is amplified, recorded, and displayed graphically as a number of peaks corresponding to the individual sample components. The retention time is the time between the injection of the sample into the chromatographic system, and the apex of the peak corresponding to a specific component on the graphic output. In addition to the polarity of the sample, mobile phase and stationary phase, many other factors will affect the retention of a compound, such as temperature, carrier-gas flow rate, and column length and diameter. Retention times are characteristic of a given component under a controlled set of conditions, and can be used as a qualitative means of identification.

The size of the peak, in terms of area or height, is proportional to the size of the signal arriving at the detector, and is a means of quantification. For structural determination a mass spectrometer may be coupled to the system (see section 1.2.6.2).

1.2.6.1.1.2 Instrumentation

A gas chromatograph consists of the following basic parts: a carrier gas supply with flow control; a heated injection port; a chromatographic column located in an oven with temperature control; a detector; an integrator; and a data recorder.

The carrier gas is an inert gas such as nitrogen, helium, or argon. The type of gas used depends on the type of detector used, and may influence the efficiency, resolution, and retention time of the analysis. Nitrogen can be used for flame ionization, electron capture, or thermal conductivity detectors. Helium may be used with flame ionization, electron capture, or thermal conductivity detectors, and nitrogen admixed with argon-methane is used with the electron capture detector. Precise control of the flow rate of the carrier gas is required to ensure reproducible retention times.

The sample to be introduced may be a solid, liquid, or gas. Solids may be dissolved in a suitable solvent and injected in liquid form. Liquids are injected into the carrier gas stream with a microliter syringe through a self-sealing septum. Gases may be introduced by gas-tight syringes or by gas-sampling valves. The injection port is kept at a high temperature to vaporize

the sample components so that they may be swept onto the column by the carrier gas. The sample components are adsorbed onto the stationary phase at the head of the column, and are gradually desorbed by fresh carrier gas (Tabor, 1989). Efficiency of separation is affected by the sample volume, which should be kept as small as possible (1-10 μl) while still providing sufficient sample for adequate detection.

Since the column is where the separation of the sample mixture takes place, there are many different types of columns available to achieve different goals of separation. Columns are generally evaluated on their *efficiency* (ability to produce narrow peaks) and their *resolution* (ability to separate two adjacent peaks). Longer columns can achieve better resolution and efficiency, but require high carrier-gas pressures which can lead to problems at the injector with gas leaks (Poklis, 1989). An increase in column diameter increases sample capacity, but decreases separation efficiency. There is also a wide variety of column materials available such as stainless steel, glass, fused silica, borosilicate, nickel, copper, aluminum, and nylon (Coutts et al., 1985). The choice of material depends mostly upon the substances to be analyzed. Two general types of columns have been used; packed columns and capillary columns. Packed columns are 1-2 m long, have internal diameters (ID) of 2-4 mm, and are packed with solid support coated with stationary phase. Capillary columns are very long tubes (10-150 m) of very small internal diameter (0.02-0.05 cm), and are made of glass or fused

silica (Baker et al., 1982). A liquid phase can be deposited directly on the inner glass of the column, known as wall-coated open tubular (WCOT) columns, or onto a thin layer of solid support material, known as support-coated open tubular (SCOT) columns (Poklis, 1989). The present experiments employed WCOT columns, which permit analysis of larger sample volumes, and thus detection of lower analyte concentrations.

The stationary phases in gas chromatography can be either solid [gas-solid chromatography (GSC)] or liquid [gas liquid chromatography (GLC)]. In GSC the column is packed with an adsorptive solid material on which sample components are partitioned by adsorption on the surface of the solid. The most common solid phases are made from diatomaceous earth, with each one possessing individual properties which make it appropriate for a particular application (Poklis, 1989). In GLC, the stationary phase is a thin film of liquid held on an inert support (or the walls of the tubing in WCOT). The support materials used in GLC are often the same ones used as stationary phases in GSC. The support must be inert, and therefore acid washing is often required to remove active sites caused by mineral impurities, and silanization of active silanol and siloxane groups may also be required (Poklis, 1989). A wide range of liquid phases are available with differing solution properties and different affinities for various analytes, which provides great versatility.

The oven contains the column, and must be able to maintain constant and reproducible temperatures. The chromatography can be conducted

under isothermal conditions, in which the oven temperature remains constant throughout the run. However, this can result in poor separations and long running times. Greater control and efficiency can be achieved by using temperature programming. At initial lower temperatures, low boiling point components and components with low affinity for the stationary phase are separated and elute first. The temperature can then be set to increase to elute higher boiling point and higher stationary-phase affinity components.

The column effluent containing the individual separated components enters the detector and generates a signal proportional to the amount of sample present. Several types of detectors, such as thermal conductivity (TCD), flame ionization (FID), electron capture (ECD), and nitrogen-phosphorus (NPD), are available for detecting different properties of the sample. The mass spectrometer (MS) can also be used as a detector (see section 1.2.6.2). The two types of detectors used in the present investigations were ECD and NPD, and will be discussed in more detail below.

The ECD is the most sensitive detector, but can only detect compounds with the ability to capture electrons, such as halogenated compounds. The detector has a radioactive source such as tritium ($^{3}H$) or $^{63}Ni$, which ionizes the carrier gas as it enters the detector, and an electrode, which collects the ions and electrons to produce a small standing current. As components with electron-capturing properties enter the detector, they capture electrons from the standing current, diminishing the signal. This

change is amplified by the integrator, and recorded as a peak on the chart paper.

As its name suggests, the NPD is used to detect compounds which contain nitrogen or phosphorus. It consists of an electrically heated chamber in which the eluant from the GC column is mixed with hydrogen and air in the presence of a small alkali salt pellet (often cesium or rubidium salt). When the mixture is ignited, a low-temperature plasma is formed which emits a current that is amplified. If the GC eluant contains a nitrogen- or phosphorus-containing compound, the current produced by the plasma is greatly enhanced (Coutts et al., 1985). The NPD is selective and highly sensitive for nitrogen and phosphorus-containing compounds.

1.2.6.1.1.3 Derivatizations

Derivatization is a process of chemically altering the properties of a compound to make it more suitable for analysis by gas chromatography. It usually involves the replacement of the active H in polar groups (-NH, -OH, -SH) via acylation, alkylation, silylation or condensation (Knapp, 1979). Reasons for derivatization include the need to form a more volatile derivative so that the required vaporization can occur, to decrease the polarity of the compound to minimize "tailing" and the adsorption of the compound on the column, to increase the stability of thermally reactive structures, to increase the extraction efficiency from aqueous solutions, or to introduce a functional

group that will be sensitive to the detector being employed (Baker et al., 1982; Poklis, 1989).

#### *1.2.6.1.2 High Performance Liquid Chromatography (HPLC)*

##### 1.2.6.1.2.1 Principles

HPLC is a type of liquid chromatography in which a mixture of compounds is separated into its components by flowing a liquid mobile phase under pressure through a column, and over a stationary phase consisting of small particles which may be coated with another liquid. Separation is achieved *via* the differential equilibration of the analytes between the mobile and stationary phases (Tabor, 1989).

##### 1.2.6.1.2.2 Instrumentation

The basic HPLC system consists of a solvent reservoir which contains the mobile phase, a pump which is used to drive the mobile phase from the reservoir through the injector, the column, and the detector where the separated components create a signal which is amplified and recorded as a peak on a chromatogram.

Different types of solvent delivery systems are available, such as pneumatic amplifiers, syringe, diaphragm, and the small displacement reciprocating pump, which is the most common. This type of pump uses two or more pump heads operating out of phase to ensure a constant delivery of solvent, and was the type of pump used in the current experiments.

There are two types of injector systems, the syringe and fixed-loop. The fixed-loop system is more common, and was employed in the current experiments. In this system, the sample is loaded into an external loop of stainless steel tubing. The valve is then rotated so that the sample in the external loop is flushed onto the column by the mobile phase. Returning the valve to the original position allows loading of the next sample (Bowers, 1989).

The column is where the separation takes place, and is typically made of stainless steel with an internal diameter of 2.1 - 4.6 mm, and a length of 50 -1500 cm (Bowers, 1989). A guard column may be located between the injector and the analytical column, and is packed with the same material as the analytical column to trap any particulate matter and to protect the analytical column. A precolumn may also be used, and is packed with silica and located between the pump and the injection valve to saturate the mobile phase with silicate to prevent dissolution of the packing material of the analytical column. There are relatively few stationary phases in HPLC, as compared to GC, and the power and versatility of HPLC comes from the ability to alter the solvent strength, pH, and composition of the mobile phase. The most common type of HPLC is the bonded-reversed phase HPLC which utilizes a non-polar, bonded stationary phase such as octadecylsilane (ODS or C-18) and a polar mobile phase such as acetonitrile. In this type of system, more polar molecules will elute first, and less polar molecules will be retained on the column and elute later.

As in GC, there are several types of detectors available for HPLC analysis, including ultraviolet-visible absorbance detector (UV-Vis), fluorescence detectors, electrochemical detectors, and MS as a detector. A UV-Vis detector, which was used in the current work, consists of a tungsten-halide or mercury-arc lamp which emits light, and a photometer, which monitors wavelengths at either a fixed, variable, or array of wavelengths. Molecules with functional groups that absorb UV-Vis radiation, such as those with aromatic rings, can be detected.

#### 1.2.6.2 Mass Spectrometry

Mass spectrometry (MS) is a technique used in chemical structure elucidation that provides highly specific and reproducible spectra of ionic fragments formed by bombarding a volatilized compound with high energy electrons. The production of the mass spectrum involves first ionizing the sample, then filtering the fragments according to mass, and finally detecting the fragments and producing a plot of the relative abundance of the ions versus their mass to charge (m/z) ratios (Poklis, 1989).

Three different modes of ionization are used in MS, namely electron impact (EI), chemical ionization (CI), and negative chemical ionization (NCI). In EI, the mode used in the present thesis, the sample molecules are bombarded with a beam of high energy electrons, which fragments them into positive, negative, and neutral fragments. The charged fragments are then separated according to mass by accelerating them out of the ion source and

toward an ion collector. Electrical signals are produced by the ions striking the collector, and these are amplified and recorded to produce the mass spectrum.

### 1.2.7 Thesis Objectives and Rationale

Understanding the role of the CYP enzymes in drug metabolism is a critical issue in medicine, and particularly in psychiatry where pharmacotherapy has the additional challenges of a multitude of complex disorders with poorly understood etiologies. Coupled with this is the challenge of dealing with side effects that range from bothersome to life-threatening in a patient population that may already be in an unstable emotional state. A slight change in dose can mean the difference between successful drug therapy and relief from psychiatric symptoms, or therapeutic failure and possible exacerbation of symptoms. Therefore, with an understanding of the enzymes involved in a drug's metabolism, one can predict potential sources of metabolic variation, prescribe the appropriate dose, and prevent adverse interactions (Glue & Banfield, 1996).

The main objective of the present work was to identify the CYP enzymes involved in the metabolism of trazodone to its active metabolite, mCPP, as well as the enzymes involved in the metabolism of mCPP to its main metabolite, OH-mCPP, and the enzymes involved in the metabolism of NEF and OH-NEF to their main metabolites. The drug-drug interactions issue is a particularly salient one for trazodone and nefazodone, since they are

both often intentionally combined with other drugs to optimize treatment therapy, and since they both possess an active metabolite with pronounced physiological and psychological effects. Furthermore, achieving and maintaining therapeutic drug concentrations is critical, and therefore, it is important to know which enzymes metabolize a drug, so that factors which may alter enzyme activity, and thus alter metabolism, may be predicted.

In meeting the objectives of the present thesis, assays were developed for the rapid and sensitive detection of trazodone, nefazodone, and their respective metabolites in *in vitro* metabolic systems, so that the major CYP enzymes involved in the drugs' metabolism could be identified using several convergent lines of evidence. The *in vitro* methods employed will add to and further validate the growing body of work demonstrating that human drug metabolism can be studied *in vitro*, and that this work is clinically relevant. It is hoped that this information can be used in the prediction and prevention of metabolic drug interactions with trazodone, nefazodone, and mCPP, and will be used to optimize their use clinically. In the course of investigating the effects of fluvoxamine (FVX) on the metabolism of trazodone, it became apparent that the GC procedure developed for mCPP was also readily applicable to FVX. This assay was thus modified for FVX and is incorporated in this thesis.

## 1.3 MATERIALS

### 1.3.1 Chemicals

| Chemical | Supplier |
|---|---|
| acetonitrile | British Drug Houses (BDH) (Toronto, ON) |
| ammonium acetate | Fisher Scientific (Edmonton, AB) |
| ammonium hydroxide | Fisher Scientific (Nepean, ON) |
| p-chlorophenylethylamine·HCl | Sigma (St. Louis, MO), HCl salt prepared by Dr. RT Coutts, University of Alberta |
| m-chlorophenylpiperazine | Sigma |
| p-OH-m-chlorophenylpiperazine | Bristol-Myers Squibb (Wallingford, CT) |
| ethyl acetate | BDH |
| fluoxetine HCl | Lilly Research Laboratories Indianapolis, IN, USA |
| fluvoxamine maleate | Solvay Duphar (Hanover, Germany) |
| glacial acetic acid | BDH |
| glucose 6-phosphate | Sigma |
| glucose 6-phosphate dehydrogenase | Sigma |
| magnesium chloride | Sigma |
| nefazodone | Bristol-Meyers Squibb |
| OH-nefazodone | Bristol-Meyers Squibb |

| | |
|---|---|
| β-nicotinamide adenine dinucleotide phosphate | Sigma |
| norfluoxetine | Lilly Research Laboratories |
| paroxetine HCl | Dr. M Bourin, University of Nantes |
| pentafluorobenzoyl chloride | Aldrich(Milwaukee, WI) |
| potassium bicarbonate | Fisher Scientific |
| potassium carbonate | Fisher Scientific |
| potassium phosphate dibasic | J.T.Baker |
| potassium phosphate monobasic | J.T.Baker (Phillipsburg, N.J.) |
| toluene, glass-distilled | BDH |
| trazodone HCl | Research Biochemicals International, Natick, MA) |
| triazoledione | Bristol-Meyers Squibb |

### 1.3.2 Microsomal Products

#### 1.3.2.1 Human liver microsomes

Human liver microsomes were obtained from the International Institute for the Advancement of Medicine (Exton, PA), and had been characterized by this company for protein content and specific enzyme activities. The protein content was determined by the manufacturer using the Pierce protein assay, and the following enzyme activities were also determined: CYP1A2 activity by the rate of phenacetin O-deethylation (pmol/mg/min), CYP2A6 activity by the

rate of coumarin 7-hydroxylation (nmol/mg/min), CYP2C19 activity by the rate of mephenytoin 4-hydroxylation (pmol/mg/min), CYP2D6 activity by the rate of dextromethorphan O-demethylation (pmol/mg/min), CYP2E1 activity by the rate of chlorzoxazone 6-hydroxylation (pmol/mg/min), CYP3A4 activity by the rate of fractional production of [$^{14}$C] 6-hydroxy-testosterone (nmol/mg/min), and 4A11 activity by the rate of fractional production of [$^{14}$C] omega-hydroxy-lauric acid (nmol/mg/min). The microsomes were stored at -80° C, and were thawed in a hot water bath as needed.

1.3.2.2 Microsomes from cDNA-expressed human cell lines

Microsomes were prepared from metabolically competent cell lines expressing cDNA for human CYP1A2, CYP2C8, CYP2C9$_{arg}$, CYP2C9$_{cys}$, CYP2C19, CYP2D6 or CYP3A4 were purchased from the Gentest Company (Woburn, MA). The human cDNAs were isolated from human tissue cDNA libraries, and were introduced into the AHH/TK+/- cell line on extrachromosomal plasmid vectors conferring resistance to either l-histidinol or hygromycin B. The microsomes were characterized for protein and CYP content by the manufacturer. Upon arrival, the microsomes were thawed, aliquoted, and stored at -80° C until used.

### 1.3.3 Instrumentation and Apparatus

#### 1.3.3.1 Gas Chromatography

##### *1.3.3.1.1 Nitrogen-Phosphorus Detection*

Analysis of mCPP metabolism from trazodone was conducted on a Hewlett-Packard (HP) model 5890 gas chromatograph equipped with an NPD and linked to an HP 3392A integrator. A 15 m fused silica capillary column coated with a 0.25 μm film thickness of 5% phenylmethylpolysiloxane (internal diameter of 0.25 mm) was used. The carrier gas was helium at a flow rate of 3.5 ml/min, and the make-up gas was helium at a flow rate of 30 ml/min. Hydrogen and air were used at flow rates of 4 ml/min and 80 ml/min, respectively. The oven temperature was set at 105° C for an initial time of 0.5 minutes, and was then set to increase at a rate of 12° C/min to a final temperature of 295° C. The injection port temperature was set at 270° C, and the detector temperature was 325° C. All injections were in the splitless mode with a purge off time of 0.5 minutes.

##### *1.3.3.1.2 Electron-Capture Detection*

Analysis of FVX in brain tissue was conducted using a Hewlett Packard (HP) Model 5890 gas chromatograph equipped with a $^{63}$Ni ECD and linked to a HP 3392A integrator/printer. A fused silica capillary column (25 m x 0.32 mm) coated with 1.05 μm film thickness of 5% phenylmethylsilicone was employed. The carrier gas was pure helium at a flow rate of 3 ml/min.

The make-up gas was argon-methane (95:5) at a flow rate of 35 ml/min. The oven temperature was set at an initial temperature of 105°C for 0.5 min. The temperature was then increased by 10°/min to 255°, followed by a 1°/min increase to 270°C. Injection port and detector temperatures were 270°C and 325°C, respectively. All injections were carried out using the splitless mode of injection with a purge off time of 0.5 min.

#### 1.3.3.2 High Performance Liquid Chromatography

A high performance liquid chromatographic (HPLC) system with a Waters model 510 pump linked to a Waters Intelligent Sample Processor model 710B was used. Detection was performed using a Waters Lambda Max Model 481 variable-wavelength UV detector linked to a Hewlett Packard model 3392A integrator.

The HPLC mobile phase consisted of acetonitrile and ammonium acetate buffer (100: 98: 2 ml (v/v) acetonitrile: distilled $H_2O$: 1M ammonium acetate) adjusted to pH 5.4 with acetic acid. The flow-rate was 1.5 ml/min. A Phenomenex Hypersil CN (250 x 4.6 mm I.D., 5 μm particle size) column was used and was coupled to a guard column which was packed with the same material as the analytical column. Detection was performed using UV absorbance at 254 nm.

#### 1.3.3.3 Mass Spectrometry

Combined GC-MS was used to confirm the chemical structures of the derivatives of mCPP and FVX formed in the GC assays described in this thesis. The GC-MS system utilized an HP 5840 A GC inlet coupled to an HP 5895 A MS with dual electron impact/ chemical impact sources and an HP 7920 data system. The system also included an HP2648 A graphics terminal, and HP 9876 A printer, PH7920 disc drive (software) and HP 21 MX series E computer (hardware). Operating conditions were as follows: ion source temperature, 200°C; interface temperature, 275°C; column pressure, 34.5 kPa; accelerating voltage, 2200 eV; ionization voltage, 70 eV; scan speed, 100 amu/sec; and dwell time, 200 msec. The columns and oven conditions were as described in sections 1.3.3.1.1 and 1.3.3.1.2).

#### 1.3.3.4 Shaker-mixer

An Ika Vibrex VXR vortex mixer (Janke & Kunkel, Staufen, Germany) was used.

#### 1.3.3.5 Hot water bath

All microsomal incubations were carried out in a Fisher Isotemp Hot Water Bath.

#### 1.3.3.6 Centrifuges

Centrifugation of microsomal incubations was carried out in a Beckman Microfuge (Palo Alto, CA, USA). A Sorvall GLC-2B general laboratory centrifuge (DuPont, Wilmington, DE, USA) was used for low speed-small volume centrifugations required for sample extraction and derivatization. Higher speed and/or large volume centrifugations of tissues were carried out in a Damon-1EC B-20 (Needham Heights, MA, USA) refrigerated high-speed centrifuge or a Beckman model J-21B refrigerated preparative centrifuge (Palo Alto, CA, USA).

#### 1.3.3.7 Savant evaporator

A Savant Speed Vac SC 110 (Fisher Scientific) was used to evaporate samples.

#### 1.3.3.8 Tissue homogenizer

A TRI-R S63C (TRI-R Instruments, Rockville Center, NY, USA) variable speed laboratory motor with a Teflon pestle was used with a glass grinding tube for homogenizing tissue samples.

#### 1.3.3.9 Glassware Cleaning

All glassware was rinsed with tap water and washed in a dishwasher (Miele Electronic 6715) with Sparkleen™ (Fisher Scientific Co.). Test tubes were sonicated (ultra-sonic cleaner, Mettler Electronics) in a 2-5% solution of Decon 75 concentrate (BDH Chemicals) before washing in the dishwasher. All glassware was then air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, USA).

## 1.4 GENERAL EXPERIMENTAL METHODS

### 1.4.1 Potassium phosphate buffer (pH 7.4)

A 0.1 M stock solution of potassium phosphate buffer was prepared by adding 0.1 M potassium phosphate monobasic to 0.1 M potassium phosphate dibasic until a pH value of 7.4 was attained.

### 1.4.2 NADPH-generating system

An NADPH-generating system was prepared fresh daily and, according to manufacturer's specifications (Gentest Co., Woburn, MA), consisted of 1 mg/ml β-nicotinamide adenine dinucleotide phosphate, 1 mg/ml D-glucose-6-phosphate, 0.4 U/ml D-glucose-6-phosphate dehydrogenase, and 0.66 mg/ml $MgCl_2$ in 0.1 M potassium phosphate buffer. These are final concentrations in 100 µl of final incubation volume, achieved

by adding 25 μl of NADPH-generating system to the incubation at four times the final concentration.

### 1.4.3 Microsomal incubation conditions

The incubations for the drug metabolism studies were carried out in 1.5 ml polypropylene microcentrifuge tubes (Fisher, Ottawa, ON). In general, the incubation mixtures consisted of 25 μl of the NADPH-generating system, 10 μl of microsomal enzyme preparation (either human liver microsomes or microsomes from cDNA expressed cells, see relevant chapters for protein content for individual incubations), 50 μl of drug substrate in buffer, and enough 0.1 M potassium phosphate buffer to bring the final volume to 100 μl. Some incubations also contained inhibitors in a volume of 10 μl, in which case the volume of buffer was adjusted so that the final incubation volume remained 100 μl.

The tubes were incubated at 37°C in a hot water bath for the time periods indicated in the individual sections. Following the incubation period, the tubes were placed on ice and the reaction was terminated by the addition of either 100 μl ice-cold 25% potassium carbonate (trazodone metabolism) or 50 μl ice-cold acetonitrile (nefazodone metabolism). The tubes were left on ice for 10 min to precipitate the proteins, and were then centrifuged for 5 min in a Beckman Microfuge. The supernatant was then removed for chromatographic analysis.

### 1.4.4 Correlation with human liver microsome enzyme activities

Drug substrates were incubated with microsomes prepared from a panel of 16 different human livers which had been previously characterized for their catalytic activities for CYP1A2 (phenacetin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2C19 (mephenytoin 4-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2E1 (chlorzoxazone 6-hydroxylation), CYP3A4 (β-hydroxylation of [$^{14}$C]-testosterone), and CYP4A11 (omega-hydroxylation of [$^{14}$C]-lauric acid). The rate of metabolite formation was correlated with the activities of the specific enzymes for each of the 16 livers.

### 1.4.5 Metabolism with single cDNA-expressed human CYP enzymes

The drug substrates were incubated with 10 μl of microsomal product (approximately 10 mg microsomal protein/ml incubation volume) prepared from cells expressing human cDNA for one of the following enzymes: CYP1A2, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6, or CYP3A4.

### 1.4.6 Inhibition studies

To assess the effects of specific isoform inhibitors on metabolite formation, varying concentrations of inhibitors were pre-incubated in a volume of 10 μl with either human liver microsomes or microsomes from cells

expressing specific human enzymes, and the NADPH-generating system at 37°C for 10 min. Following pre-incubation, the drug substrate was added and incubation continued for a further 10 min.

## 1.5 REFERENCES


Aranow RB, Hudson JI, Harrison GP, Grady TA, Laage TA, Bell IR, Cole JO (1989) Elevated antidepressant levels after addition of fluoxetine. *Am J Psychiatr* 146:911-913.

Arora RC, Meltzer HY (1989) Serotonergic measures in the brains of suicide victims: 5-$HT_2$ binding sites in the frontal cortex of suicide victims and control subjects. *Am J Psychiatry* **146**:730-736.

Artigas F, Romero L, de Montigny C, Blier P (1996) Acceleration of the effect of selected antidepressant drugs in major depression by 5-$HT_{1A}$ antagonists. *Trends Neurosci* **19**:378-383.

Asberg M, Martensson B (1993) Serotonin selective antidepressant drugs: Past, present, future. *Clin Neuropharmacol* **16**:S32-S44.

Ashton AK, Wolin RE (1996) Nefazodone-induced carbamazepine toxicity [letter]. *Am J Psychiatry* **153:**733

Baiocchi BL, Frigerio A, Giannangeli M, Palazzo G (1974) Basic metabolites of trazodone in humans. *Arzneim-Forsch (Drug Res)* **24**:1699-1706.

Baker GB, Coutts RT, LeGatt DF (1982) Gas chromatographic analysis of amines in biological systems. In Baker GB, Coutts TR (eds) *Analysis of Biogenic Amines.* Elsevier Scientific Publishing Company, New York, pp 109-128.

Baker GB, Greenshaw AJ (1989) Effects of long-term administration of antidepressants and neuroleptics on receptors in the central nervous system. *Cell Mol Neurobiol* **9**:1-44

Barbhaiya RH, Buch AB, Greene DS (1996a) A study of the effect of age and gender on the pharmacokinetics of nefazodone after single and multiple doses. *J Clin Psychopharmacol* **16**:19-25.

Barbhaiya RH, Buch AB, Greene (1996b) Single and multiple dose pharmacokinetics of nefazodone in subjects classified as extensive and poor metabolizers of dextromethorphan. *Br J Clin Pharmacol* **42**:573-581.

Barbhaiya RH, Dandekar KA, Greene DS (1996c) Pharmacokinetics, absolute bioavailability, and disposition of [$^{14}$C]Nefazodone in humans. *Drug Metab Dispos* **24**:91-95.

Barbhaiya RH, Marathe PH, Greene DS, Mayol RF, Shukla UA, Gammans RR, Pittman KA, Robinson D (1995a) Safety, tolerance, and preliminary pharmacokinetics of nefazodone after administration of single and multiple oral doses to healthy adult male volunteers: A double-blind, Phase I study. *J Clin Pharmacol* **35**: 974-984.

Barbhaiya RH, Shukla UA, Chaikin P, Greene DS, Marathe PH (1996d) Nefazodone pharmacokinetics: assessment of nonlinearity, intra-subject variability and time to attain steady-state plasma concentrations after dose escalation and de-escalation. *Eur J Clin Pharmacol* **50**:101-107.

Barbhaiya RH, Shukla UA, Greene DS (1995b) Lack of interaction between nefazodone and cimetidine: a steady state pharmacokinetic study in humans. *Br J Clin Pharmacol* **40**:161-165.

Barbhaiya RH, Shukla UA, Greene DS, Breuel H-P, Midha KK (1996e) Investigation of pharmacokinetic and pharmacodynamic interactions after coadministration of nefazodone and haloperidol. *J Clin Psychopharmacol* **16**:26-33.

Barbhaiya RH, Shukla UA, Kroboth PD, Greene DS (1995c) Coadministration of nefazodone and benzodiazepines: II. A pharmacokinetic interaction study with triazolam. *J Clin Psychopharmacol* **15**:320-326.

Barrett JE, Vanover KE (1993) 5-HT receptors as targets for the development of novel anxiolytic drugs: models, mechanisms and future directions. *Psychopharmacol* **112**:1-12.

Benkelfat C (1993) Serotonergic mechanisms in psychiatric disorders: new research tools, new ideas. *Int Clin Psychopharmacol* **8**:53-56.

Benkelfat C, Ellenbogen MA, Dean P, Palmour RM, Young SN (1994) Mood-lowering effect of tryptophan depletion: enhanced susceptibility in young men at genetic risk for major affective disorders. *Arch Gen Psychiatry* **51**:687-697.

Berendsen HHG (1995) Interactions between 5-hydroxytryptamine receptor subtypes: Is a disturbed receptor balance contributing to the symptomatology of depression in humans? *Pharmacol Ther* **66**:17-37.

Berlin I, Lecrubier Y (1996) Food and drug interactions with monoamine oxidase inhibitors: How safe are the newer agents? *CNS Drugs* **5**:403-413.

Bertilsson L, Tomson T (1986) Clinical pharmacokinetics and pharmacological effects of carbamazepine and carbamazepine-10,11-epoxide. An update. *Clin Pharmacokinet* **11**:177-198.

Bertz RJ, Granneman GR (1997) Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet* **32**:210-258.

Blier P, Bergeron R, deMontigny C (1997) Selective activation of postsynaptic 5-HT1A receptors induces rapid antidepressant response. *Neuropsychopharmacol* **16**:333-338.

Blier P, de Montigny C (1994) Current advances and trends in the treatment of depression. *Trends Pharmacol Sci* **15**:220-225.

Blier P, deMontigny C (1997) Involvement of the serotonin system in the antidepressant response. *Médecine & Science* **13**:519-526.

Bock KW, Schrenk D, Forster A, Griese E-U, Morike K, Brockmeier D, Eichelbaum M (1994) The influence of environmental and genetic factors on CYP2D6, CYP1A2 and UDP-glucuronosyltransferases in man using sparteine, caffeine, and paracetamol as probes. *Pharmacogenet* **4**:209-218.

Bowers LD (1989) Liquid chromatography. In Kaplan LA, Pesce AJ (eds) *Clinical Chemistry Theory, Analysis and Correlation.* 2nd Ed., The CV Mosby Company, Toronto, pp 94-109.

Brosen K (1990) Recent developments in hepatic drug oxidation: Implications for clinical pharmacokinetics. *Clin Pharmacokinet* **18**:220-239.

Brosen K (1993) The pharmacogenetics of the selective serotonin reuptake inhibitors. *Clin Investig* **71**:1002-1009.

Buch AB, Shukla UA, Pittman K, Barbhaiya RH (1993) Pharmacokinetics of nefazodone in extensive and poor metabolizers of dextromethorphan. *Pharm Res* **10**:S314.

Caccia S, Ballabio M, Samanin R, Zanini MG, Garattini S (1981) (-)-m Chlorophenylpiperazine, a central 5-hydroxytryptamine agonist, is a metabolite of trazodone. *J Pharm Pharmacol* **33**:477-478.

Caccia S, Fong MH, Garattini S, Zanini MG (1982) Plasma concentrations of trazodone and 1-(3-chlorophenyl)piperazine in man after a single oral dose of trazodone. *J Pharm Pharmacol* **34**:605-606.

Caccia S, Garattini S (1992) Pharmacokinetic and pharmacodynamic significance of antidepressant drug metabolites. *Pharmacol Res* **26**:317-329.

Charney DS, Miller HL, Licinio J, Salomon R (1995) Treatment of depression. In Schatzberg, A.F. and Nemeroff, C.B. (Eds). *American Psychiatric Press Textbook of Psychopharmacology.* American Psychiatric Press, Inc.:Washington. pp. 575-601.

Conn JP, Sanders-Bush E (1987) Relative efficacies of piperazines at the phosphoinositide hydrolysis-linked serotonergic (5-HT-2 and 5-HT-1C) receptors. *J Pharmacol Exp Ther* **242**:552-557.

Coon MJ, Ding X, Pernecky SJ, Vaz AND (1992) Cytochrome P450: progress and predictions. *FASEB J* **6**:669-673.

Corruble E, Puech AJ (1993) How to improve the risk-benefit ratio of antidepressants. *Int Clin Psychopharmacol* **8**:237-241.

Coutts RT (1994) Polymorphism in the metabolism of drugs, including antidepressant drugs: Comments on phenotyping. *J Psychiatr Neurosci* **19**:30-44.

Coutts RT, Baker GB, Nazarali AJ (1985) Gas chromatography of amines and their metabolites in tissues and body fluids. In Boulton AA, Baker GB, Baker JM (eds) *Neuromethods 2: Amines and their metabolites.* The Humana Press Inc., Clifton, NJ, pp45-85.

Coutts RT, Su P, Baker GB (1994) Involvement of CYP2D6, CYP3A4, and other cytochrome P-450 isozymes in N-dealkylation reactions. *J Pharmacol Toxicol Meth* **31**:177-186.

Cowen PJ, Sargent PA, Williams C, Goodall EM, Orlikov AB (1995) Hypophagic, endocrine and subjective responses to m-chlorophenylpiperazine in healthy men and women. *Hum Psychopharmacol* **10**:385-391.

Cunningham LA, Borison RL, Carman JS, Chouinard G, Crowde JE, Diamond BI, Fischer DE, Hearst E (1994) A comparison of venlafaxine, trazodone, and placebo in major depression. *J Clin Psychopharmacol* **14**:99-106.

D'Amico MF, Roberts DL, Robinson DS, Schwiderski UE, Copp J (1990) Placebo-controlled dose-ranging trial designs in Phase II development of nefazodone. *Psychopharmacol Bull* **26**:147-150.

Darmani NA, Martin BR, Pandey U, Glennn RA (1990) Do functional relationships exist between 5-HT1A and 5-HT2 receptors? *Pharmacol Biochem Behav* **36**:901-906.

Davis R, Whittington R, Bryson HM (1997) Nefazodone: A review of its pharmacology and clinical efficacy in the management of major depression. *Drugs* **53**:608-636.

Deakin JFW (1988) $5HT_2$ receptors, depression and anxiety. *Pharmacol Biochem Behav* **29**:819-820.

Denison MS, Whitlock, JP (1995) Xenobiotic-inducible transcription of cytochrome P450 genes. *J Biol Chem* **270**:18175-18178.

Desmeules J, Gascon MP, Dayer P, Magistris M (1991). Impact of environmental and genetic factors on codeine analgesia. *Eur J Clin Pharmacol* **41**:23-26.

Dockens RC, Greene DS, Barbhaiya RH (1996) Assessment of pharmacokinetic and pharmacodynamic drug interactions between nefazodone and digoxin in healthy male volunteers. *J Clin Pharmacol* **36**:160-167.

Dockens RC, Rapoport D, Roberts D (1995) Lack of an effect of nefazodone on the pharmacokinetics and pharmacodynamics of theophylline during concurrent administration in patients with chronic obstructive pulmonary disease. *Br J Clin Pharmacol* **40**:598-601.

Dubovsky SL (1994) Beyond the serotonin reuptake inhibitors: Rationales for the development of new serotonergic agents. *J Clin Psychiatry* **55(suppl 2)**:34-44.

Dubovsky SL, Thomas M (1995) Serotonergic mechanisms and current and future psychiatric practice. *J Clin Psychiatry* **56(suppl 2)**:38-48.

Dufresne RL (1996) Issues in polypharmacotherapy: Focus on major depression. *Psychopharmacol Bull* **32**:547-553.

Eichelbaum M (1984) Polymorphic drug oxidation in humans. *Federation Proc* **43**:2298-2302.

Eison AS, Eison MS, Torrente JR, Wright RN, Yocca FD (1990) Nefazodone: Preclinical pharmacology of a new antidepressant. *Psychopharmacol Bull* **26**:311-315.

Ellingrod VL, Perry PJ (1995) Nefazodone: A new antidepressant. *Am J Health-Syst Pharm* **52**:2799-2812.

Ereshefsky L, Riesenman C, Lam YWF (1995) Antidepressant drug interactions and the cytochrome P450 system: The role of cytochrome P450 2D6. *Clin Pharmacokinet* **29(suppl 1)**:10-19.

Fawcett J, Marcus RN, Anton SF, O'Brien K, Schwiderski U (1995) Response of anxiety and agitation symptoms during nefazodone treatment of major depression. *J Clin Psychiatry* **56(suppl 6)**:37-42.

Feighner JP, Boyer WF (1988) Overview of USA controlled trials of trazodone in clinical depression. *Psychopharmacol* **95**:S50-S53.

Ferry N, Bernard N, Cuisinaud G, Rougier P, Trepo C, Sassard J (1994) Influence of hepatic impairment on the pharmacokinetics of nefazodone and two of its metabolites after single and multiple oral doses. *Fundam Clin Pharmacol* **8**:463-473.

Fiorella D, Rabin RA, Winter JC (1995) The role of the 5-HT2A and 5-HT2C receptors in the stimulus effects of m-chlorophenylpiperazine. *Psychopharmacol* **119**:222-230.

Flockhart DA (1995) Drug interactions and the cytochrome P450 system: The role of cytochrome P450 2C19. *Clin Pharmacokinet* **29(suppl 1)**:45-52.

Fong MH, Garattini S, Caccia S (1982) 1-m-Chlorophenylpiperazine is an active metabolite common to the psychotropic drugs trazodone, etoperidone, and mepiprazole. *J Pharm Pharmacol* **34**:674-675.

Friedberg T, Wolf CR (1996) Recombinant DNA technology as an investigative tool in drug metabolism and research. *Adv Drug Deliv Rev* **22**:187-213.

Fuller RW, Snoddy HD, Cohen ML (1984) Interactions of trazodone with serotonin neurons and receptors. *Neuropharmacol* **23**:539-544.

George TP, Godleski LS (1996) Possible serotonin syndrome with trazodone addition to fluoxetine. *Biol Psychiat* **39**:383-386.

Georgotas A, Forsell TL, Mann JJ, Kim M, Gershon S (1982) Trazodone hydrochloride: A wide spectrum antidepressant with a unique pharmacological profile. *Pharmacother* **2**:255-265.

Germine M, Goddard AW, Sholomskas DE, Woods SW, Charney DS, Heninger GR (1994) Response to meta-chlorophenylpiperazine in panic disorder patients an healthy subjects: Influence of reduction in intravenous dosage. *Psychiat Res* **54**:115-133.

Gershon S (1990) Insights in the use of trazodone in depressed patients. *J Clin Psychiat* **51(suppl 9)**:4-5.

Gibaldi M (1992a) Pharmacogenetics: Part I. *Annals Pharmacotherapy* **26**:121-126.

Gibaldi M (1992b) Pharmacogenetics: Part II. *Annals Pharmacotherapy* **26**:255-261.

Gibson EL, Barnfield AMC, Curzon G (1994) Evidence that mCPP-induced anxiety in the plus-maze is mediated by postsynaptic 5-HT(2C) receptors but not by sympathomimetic effects. *Neuropharmacol* **33**:3-4.

Glennon RA (1990) Serotonin receptors: Clinical implications. *Neurosci Biobehav Rev* **14**:35-47.

Glue P, Banfield C (1996) Psychiatry, psychopharmacology and P-450s. *Hum Psychopharmacol* **11**:97-114.

Golden RN, Bebchuk JM, Leatherman ME (1995) Trazodone and other antidepressants. In Schatzberg AF and Nemeroff CB (Eds). *American Psychiatric Press Textbook of Psychopharmacology.* American Psychiatric Press, Inc.:Washington. pp. 195-213.

Goldstein JA, de Morais SM (1994) Biochemistry and molecular biology of the human CYP 2C subfamily. *Pharmacogenet* **4**:285-299.

Gonzalez FJ (1990) Molecular genetics of the P450 superfamily. *Pharmacol Ther* **45**:1-38.

Greenblatt DJ, Preskorn SH, Cotreau MM, Horst WD, Harmatz JS (1992) Fluoxetine impairs clearance of alprazolam but not of clonazepam. *Clin Pharmacol Ther* **52**:479-486.

Greenblatt DJ, von Moltke LL, Schmider J, Harmatz JS, Shader RI (1996) Inhibition of human cytochrome P450-3A isoforms by fluoxetine and norfluoxetine: In vitro and in vivo studies. *J Clin Pharmacol* **36**:792-798.

Greene DS, Salazar DE, Dockens RC, Kroboth P, Barbhaiya RH (1995a) Coadministration of nefazodone and benzodiazepines: III. A pharmacokinetic interaction study with alprazolam. *J Clin Psychopharmacol* **15**:399-408.

Greene DS, Salazar DE, Dockens RC, Kroboth P, Barbhaiya RH (1995b) Coadministration of nefazodone and benzodiazepines: IV. A pharmacokinetic interaction study with lorazepam. *J Clin Psychopharmacol* **15**:409-416.

Guengerich FP (1990) Mechanism-based inactivation of human liver microsomal cytochrome P450 IIIA4 by gestodene. *Clin Res Toxicol* **3**:363:371.

Guengerich FP (1992) Human cytochrome P-450 enzymes. *Life Sci* **50**:1471-1478.

Guengerich FP (1995) Human cytochrome P450 enzymes. *Cytochrome P450*. Ortiz de Montellano, PR (Ed) Plenum Press: New York, pp 473-535.

Guengerich FP (1996) In vitro techniques for studying drug metabolism. *J Pharmacokinet Biopharmaceut* **24**:521-533.

Guengerich FP, Kim D-H (1990) In vitro inhibition of dihydropyridine oxidation and aflatoxin $B_1$ activation in human liver microsomes by naringenin and other flavonoids. *Carcinogenisis* **11**:2275-2279.

Haria M, Fitton A, McTavish D (1994) Trazodone: A review of its pharmacology, therapeutic use in depression and therapeutic potential in other disorders. *Drugs & Aging* **4**:331-355.

Harvey AT, Preskorn SH (1996) Cytochrome P450 enzymes: Interpretation of their interactions with selective serotonin reuptake inhibitors. Part I. *J Clin Psychopharmacol* **16**:273-285.

Helms-Smith KM, Curtis SL, Hatton RC (1996) Apparent interaction between nefazodone and cyclosporine [letter]. *Ann Intern Med* **125**:424.

Hollenberg PF (1992) Mechanisms of cytochrome P450 and peroxidase-catalyzed xenobiotic metabolism. *FASEB J* **6**:686-694.

Honig A, Pop P, de Kemp E, Philipsen H, Romme MA (1992) Physical illness in chronic psychiatric patients from a community psychiatric unit revisited. *Br J Psychiatry* **161**:80-83.

Ishida M, Otani K, Kaneko S, Ohkubo T, Osanai T, Yasui N, Mihara K, Higuchi H, Sugawara K. (1995) Effects of various factors on steady state plasma concentrations of trazodone and its active metabolite m-chlorophenylpiperazine. *Int Clin Psychopharmacol* **10**:143-146.

Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, Sugiyama Y (1997) Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacol Ther* **73**:147-171.

Jacobsen FM (1990) Low-dose trazodone as a hypnotic in patients treated with MAOIs and other psychotropics: A pilot study. *J Clin Psychiat* **51**:298-302.

Jenck F, Moreau J-L, Mutel V, Martin JR (1994) Brain 5-HT1C receptors and antidepressants. *Prog Neuro-Psychopharmacol & Biol Psychiat* **18**:563-574.

Jeppesen U, Gram LF, Vistisen K, Loft S, Poulsen HE, Brosen K (1996) Dose-dependent inhibition of CYP1A2, CYP2C19, CYP2D6 by citalopram, fluoxetine, fluvoxamine, and paroxetine. *Eur J Clin Pharmacol* **51**:73-78.

Johnson CM, Thummel KE, Kroetz DL, Korzekwa KR, Gonzalez FJ, Kerr BM, Levy RH (1992) Metabolism of CBZ by cytochrome P450 isoforms 3A4, 2C8 and 1A2. *Pharmaceutical Res* **9**:S-301.

Kahn RS, Wetzler S (1991) m-Chlorophenylpiperazine as a probe of serotonin function. *Biol Psychiat* **30**:1139-1166.

Kaul S, Shukla UA, Barbhaiya RH (1995) Nonlinear pharmacokinetics of nefazodone after escalating single and multiple oral doses. *J Clin Pharmacol* **35**:830-839.

Kerr BM, Thummel KE, Wurden CJ, Klein SM, Kroetz DL, Gonzalez FJ, Levy RH (1994) Human liver carbamazepine metabolism: Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation. *Biochem Pharmacol* **47**1969-1979.

Kivisto KT, Kroemer HK (1997) Use of probe drugs as predictors of drug metabolism in humans. *J Clin Pharmacol* **37**:S40-S48.

Knapp DR (1979) *Handbook of Derivatization Reactions.* John Wiley and Sons, Toronto, pp 3-24.

Korzekwa KR, Jones JP (1993) Predicting the cytochrome P450 mediated metabolism of xenobiotics. *Pharmacogenet* **3**:1-18.

Kroboth PD, Folan MM, Lush RM, Chaikin PC, Shukla UA, Barbhaiya RH, Salazar DE (1995) Coadministration of nefazodone and benzodiazepines: I. Pharmacodynamic assessment. *J Clin Psychopharmacol* **15**:306-319.

Kroemer HK, Eichelbaum M (1995) "It's the genes, stupid" Molecular bases and clinical consequences of genetic cytochrome P450 2D6 polymorphism. *Life Sci* **56**:2285-2298.

Kronbach T, Mathys D, Umeno M, Gonzalez FJ, Meyer UA (1989) Oxidation of midazolam and triazolam by human liver cytochrome P450IIIA4. *Mol Pharmacol* **36**:89-96.

Krystal JH, Seibyl JP, Price LH, Woods SW, Heninger GR, Aghajanian GK, Charney DS (1993) m-Chlorophenylpiperazine effects in neuroleptic-free schizophrenic patients. *Arch Gen Psychiatry* **50**:624-635.

Lennard MS (1990) Genetic polymorphism of sparteine/debrisoquin oxidation: a reappraisal. *Pharmacol Toxicol* **67**:273-283.

Lennard MS, Tucker GT, Woods HF (1990) Inborn "errors" of drug metabolism; pharmacokinetics and clinical implications. *Clin Pharmacokinet* **19**:257-263.

Lesch K-P, Aulakh CS, Wolozin BL, Murphy DL (1992) Serotonin (5-HT) receptor, 5-HT transporter and G protein-effector expression: implications for depression. *Pharmacol Toxicol* **71**:49-60.

Leysen JE, Schotte A (1992) Role and localization of serotonin 5-$HT_2$ receptors. *Prog Histocytochem* **26**:241-249.

Maes M, Westenberg H, Vandoolaeghe E, Demedts P, Wauters A, Neels H, Meltzer HY (1997) Effects of trazodone and fluoxetine in the treatment of major depression: Therapeutic pharmacokinetic and

pharmacodynamic interactions through formation of meta-chlorophenylpiperazine. *J Clin Psychopharmacol* **17**:358-364.

Malik K (1996) Nefazodone: structure, mode of action and pharmacokinetics. *J Psychopharmacol* **10 (Suppl. 1)**: 1-4.

Marcus RN (1996) Safety and tolerability profile of nefazodone. *J Psychopharmacol* **10 (suppl 1)**:11-17.

Marek GJ, McDougle CJ, Price LH, Seiden LS (1992) A comparison of trazodone and fluoxetine: implications for a serotonergic mechanism of antidepressant action. *Psychopharmacol* **109**:2-11.

Marino MR, Langenbacher KM, Uderman HD (1996) Interaction of nefazodone (N) and fluoxetine (F) [abstract]. *Clin Pharmacol Ther* **59**:180.

Mayol RF, Cole CA, Colson KE, Kerns EH (1994a) Isolation and identification of the major urinary metabolite of m-chlorophenylpiperazine in the rat. *Drug Metab Dispos* **22**:171-174.

Mayol RF, Cole CA, Luke GM, Colson KL, Kerns EH (1994b) Characterization of the metabolites of the antidepressant drug nefazodone in human urine and plasma. *Drug Metab Dispos* **22**:304-311.

Melzacka M, Boksa J, Maj J (1979) 1-(m-Chlorophenyl)piperazine: a metabolite of trazodone isolated from rat urine. *J Pharm Pharmacol* **31**:855-856.

Metz A, Shader RI (1990) Adverse interactions encountered when using trazodone to treat insomnia associated with fluoxetine. *Int Clin Psychopharmacol* **5**:191-194.

Meyer UA (1996) Overview of enzymes of drug metabolism. *J Pharmacokinet Biopharmaceut* **24**:449-459

Montgomery SA (1996) Efficacy of nefazodone in the treatment of depression. *J Psychopharmacol* **10 (suppl 1)**:5-10.

Muralidharan G, Hawes EM, McKay G, Korchinski ED, Midha KK (1991) Quinidine but not quinine inhibits in man the oxidative metabolic routes of methoxyphenamine which involve debrisoquine 4-hydroxylase. *Eur J Pharmacol* **41**:471-474.

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

Nemeroff CB, DeVane CL, Pollock BG (1996) Newer antidepressants and the cytochrome P450 system. *Am J Psychiatry* **153:**311-320.

Nierenberg AA, Cole JO, Glass L (1992) Possible trazodone potentiation of fluoxetine: A case series. *J Clin Psychiat* **53**:83-85.

Nierenberg AA, Adler LA, Peslow E, Zornberg G, Rosenthal M (1994) Trazodone for antidepressant-associated insomnia. *Am J Psychiat* **151**:1069-1072.

Nilsen OG, Dale O (1992) Single dose pharmacokinetics of trazodone in healthy subjects. *Pharmacol Toxicol* **71**:150-153.

Nilsen OG, Dale O, Huesbo B (1993) Pharmacokinetics of trazodone during multiple dosing to psychiatric patients. *Pharmacol Toxicol* **72**:286-289.

Nutt D (1996) Early action of nefazodone in anxiety associated with depression. *J Psychopharmacol* **10 (suppl 1)**:18-21.

Okey AB (1990) Enzyme induction in the cytochrome P-450 system. *Pharmac Ther* **45**:241-298.

Otani K, Ishida M, Kaneko S, Mihara K, Ohkubo T, Osanai T, Sugawaara K (1996) Effects of carbamazepine coadministration on plasma concentrations of trazodone and its active metabolite, m-chlorophenylpiperazine. *Ther Drug Monit* **18**:164-167.

Otton SV, Inaba T, Kalow W (1984) Competitive inhibition of sparteine oxidation in human liver by β-adrenoceptor antagonists and other cardiovascular drugs. *Life Sci* **34**:73-80.

Pichard L, Fabre I, Fabre G, Domergue J, Saint Aubert B, Mourad G, Mourel P (1990) 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* **18**:595-606.

Pinder RM, Wieringa JH (1993) Third-generation antidepressants. *Med Res Rev* **13**:259-312.

Poklis A (1989) Gas chromatography. In Kaplan LA, Pesce AJ (eds) *Clinical Chemistry Theory, Analysis, and Correlation.* 2nd Ed., The CV Mosby Company, Toronto, pp 110-125.

Potter WZ, Manji HK (1990) Antidepressants, metabolites, and apparent drug resistance. *Clin Neuropharmacol* **13(Suppl. 1)** S45-S53.

Potter WZ, Manji HK, Rudorfer MV (1995) Tricyclics and tetracyclics. In Schatzberg, A.F. and Nemeroff, C.B. (Eds). *American Psychiatric Press Textbook of Psychopharmacology.* American Psychiatric Press, Inc.:Washington. pp. 141-160.

Preskorn SH, Janicak PG, Davis JM, Ayd FJ (1995) Advances in the pharmacotherapy of depressive disorders. In Janicak, PG (Ed.), *Principles and Practice of Psychopharmacotherapy* 1:1-24.

Preskorn SH, Magnus RD (1994) Inhibition of hepatic P450 isoenzymes by serotonin selective reuptake inhibitors. In vitro and in vivo findings and their implications for patient care. *Psychopharmacol Bull* **30**:251-259.

Preskorn SH (1997) Clinically relevant pharmacology of selective serotonin reuptake inhibitors: An overview with emphasis on oxidative drug metabolism. *Clin Pharmacokinet* **32:**1-21.

Reeves RR, Bullen JA (1995) Serotonin syndrome produced by paroxetine and low-dose trazodone. *Psychosomatics* **36**:159-160.

Richelson E (1994) Pharmacology of antidepressants - Characteristics of the ideal drug. *Mayo Clin Proc* **69**:1069-1081.

Ring BJ, Binkley SN, Roskos L, Wrighton SA (1995) Effect of fluoxetine, norfluoxetine, sertraline and desmethylsertraline on human CYP3A catalyzed 1'-hydroxy midazolam formation in vitro. *J Pharmacol Exp Ther* **275**:1131-1135.

Risch SC, Nemeroff CB (1992) Neurochemical alterations of serotonergic neuronal systems in depression. *J Clin Psychiatr* **53**:3-7.

Robinson DS, Roberts DL, Smith JM, Stringfellow JC, Kaplita SB, Seminara JA, Marcus RN (1996) The safety profile of nefazodone. *J Clin Psychiatry* **57(suppl 2)**: 31-38.

Rosenbaum JF (1995) Managing selective serotonin reuptake inhibitor-drug interactions in clinical practice. *Clin Pharmacokinet* **29(Suppl. 1)**:53-59.

Roth BL (1994) Multiple serotonin receptors: Clinical and experimental aspects. *Ann Clin Psychiat* **6**:67-78.

Salazar DE, Dockens RC, Milbrath RL, Raymond RH, Fulmor IE, Chaikin PC, Uderman HD (1995) Pharmacokinetic and pharmacodynamic evaluation of warfarin and nefazodone coadministration in healthy subjects. *J Clin Pharmacol* **35**:730-738.

Schmider J, Greenblatt DJ, von Moltke LL, Harmatz JS, Duan SX, Karsov D, Shader RI (1996a) Characterization of six in vitro reactions mediated by human cytochrome P450: Application to the testing of cytochrome P450-directed antibodies. *Pharmacology* **52**:125-134.

Schmider J, Greenblatt DJ, von Moltke LL, Harmatz JS, Shader RI (1996b) Inhibition of cytochrome P450 by nefazodone *in vitro:* studies of dextromethorphan *O*- and *N*-demethylation. *Br J Clin Pharmacol* **41**:339-343.

Schreiber R, De Vry J (1993) 5-$HT_{1A}$ receptor ligands in animal models of anxiety, impulsivity and depression: multiple mechanisms of action? *Prog Neuro-Psychopharmacol & Biol Psychiat* **17**:87-104.

Sheline YI, Bardgett ME, Jackson JL, Newcomer JW, Csernansky JG (1995) Platelet serotonin markers and depressive symptomology. *Biol Psychiatr* **37**:442-447.

Shen WW (1995) Cytochrome P450 monooxygenases and interactions of psychotropic drugs: a five year update. *Int J Psychiatry Med* **25**:277-290.

Skerritt U, Evans R, Montgomery SA (1997) Selective serotonin reuptake inhibitors in older patients: A tolerability perspective. *Drugs & Aging* **10**:209-218.

Skett P, Tyson C, Guillouzo A, Maier P (1995) Report on the international workshop on the use of human in vitro liver preparations to study drug metabolism in drug development. *Biochem Pharmacol* **50**:280-285.

Smith DA, Jones BC, Walker DK (1996) Design of drugs involving the concepts and theories of drug metabolism and pharmacokinetics. *Medicinal Res Rev* **16**:243-266.

Spina E, Avenoso A, Campo GM, Scordo MG, Caputi AP, Perucca E (1997) Effect of ketoconazole on the pharmacokinetics of imipramine and desipramine in healthy subjects. *Br J Clin Pharmacol* **43**:315-318.

Stachulski A, Lennard M (1997) Helping medicine go down. *Chemistry in Britain* **Sept**, 25-28.

Tabor MW (1989) Chromatography: theory and practice. In Kaplan LA, Pesce AJ (eds) *Clinical Chemistry Theory, Analysis, and Correlation.* 2nd Ed., The CV Mosby Company, Toronto, pp 73-93.

Taylor DP, Carter RB, Eison AS, Mullins UL, Smith HL, Torrente JR, Wright RN, Yocca FD (1995) Pharmacology and neurochemistry of nefazodone, a novel antidepressant drug. *J Clin Psychiat* **56 (suppl 6)**:3-11.

Taylor DP, Smith DW, Hyslop DK, Riblet LA, Temple DL (1986) Receptor binding and atypical antidepressant drug discovery. In O'Brien RA (Ed) *Receptor Binding in Drug Research.* New York: Marcel Dekker, pp. 151-165.

Teboul E, Chouinard G (1991) A guide to benzodiazepine selection. Part II. Clinical aspects. *Can J Psychiatry* **36**:62-73.

van Praag (1977) Significance of biochemical parameters in the diagnosis, treatment and prevention of depressive disorders. *Biol Psychiat* **12**:101-131.

von Moltke LL, Greenblatt DJ, Duan SX, Schmider J, Kudchadker L, Fogelman SM, Harmatz JS, Shader RI (1996c) Phenacetin *O*-deethylation by human liver microsomes in vitro: inhibition by chemical probes, SSRI antidepressants, nefazodone and venlafaxine. *Psychopharmacol* **128**:398-407.

von Moltke LL, Greenblatt DJ, Harmatz JS, Duan SX, Harrel LM, Cotreau-Bibbo MM, Pritchard GA, Wright CE, Shader RI (1996a) Triazolam biotransformation by human liver microsomes *in vitro*: Effects of metabolic inhibitors and clinical confirmation of a predicted interaction with ketoconazole. *J Pharmacol Exp Ther* **276**:370-379.

von Moltke LL, Greenblatt DJ, Schmider J (1996b) Nefazodone *in vitro*: Metabolic conversions, and inhibition of P450-3A isoforms [abstract]. *Clin Pharmacol Ther* **59**:176.

von Moltke LL, Greenblatt DJ, Schmider J, Harmatz JS, Shader RI (1995) Metabolism of drugs by cytochrome P450 3A isoforms: Implications for drug interactions in psychopharmacology. *Clin Pharmacokinet* **29(Suppl 1)**:33-44.

Watkins PB, Wrighton SA, Maurel P, Schuetz EG, Mendez-Picon G, Parker GA, Guzelian PS (1985) Identification of an inducible form of cytochrome P-450 in human liver. *Proc Natl Acad Sci USA* **82**:6310-6314.

Wilkinson GR (1996) Cytochrome P4503A (CYP3A) metabolism: Prediction of in vivo activity in humans. *J Pharmacokinet Biopharmaceut* **24**:475-489.

Workman EA, Short DD (1993) Atypical antidepressants versus imipramine in the treatment of major depression: A meta-analysis. *J Clin Psychiat* **54**:5-12.

Wrighton SA, Stevens JC (1992) The human hepatic cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* **22**:1-21.

Wrighton SA, VandenBranden M, Ring BJ (1996) The human drug metabolizing cytochromes P450. *J Pharmacokinet Biopharmaceut* **24**:461-473.

Yamato C, Takahashi T, Fujita T (1974a) Studies on the metabolism of trazodone, I. Metabolic fate of [$^{14}$C]trazodone hydrochloride in rats. *Xenobiotica* **4**:313-326.

Yamato C, Takahashi T, Fujita T, Kuriyama S, Hirose N (1974b) Studies on metabolism of trazodone II. Metabolic fate after intravenous administration and effects on liver microsomal drug-metabolizing enzymes in rats. *Xenobiotica* **4**:765-777.

Yasui N, Otani K, Kaneko S, Ohkubo T, Osanai T, Ishida M, Mihara K, Kondo T, Sugawara K, Fukushima Y (1995) Inhibition of trazodone metabolism by thioridazine in humans. *Ther Drug Monit* **17**:333-335.

Young SN (1993) The use of dietary components in the study of factors controlling affect in humans: A review. *J Psychiatr Neurosci* **18**:235-244.

Young SN, Pihl RO, Benkelfat C, Palmour R, Ellenbogen M, Learquand D (1996) The effect of low brain serotonin on mood and aggression in

humans. In Fillipini GA (ed.) *Recent Advances in Tryptophan Research* Plenum Press: New York, pp. 45-50.

# Chapter 2

## Trazodone is metabolized to m-chlorophenylpiperazine (mCPP) by CYP3A4 from human sources

A version of this chapter has been accepted for publication, Rotzinger et al., Drug Metab Dispos, 1998)

## 2.1 INTRODUCTION

Adverse pharmacokinetic drug interactions can occur when drugs which are substrates or inhibitors of the same cytochrome P450 (CYP) enzymes are co-administered, potentially altering the expected rate of metabolism of both compounds. The clinical consequences can range from a lack of therapeutic efficacy to severe toxicity, and, in extreme cases, fatality. Therefore, it is important to identify the major enzymes involved in the metabolism of a drug so that such interactions can be predicted and avoided.

Trazodone is a phenylpiperazine-derivative antidepressant drug (Figure 2.1), which is thought to act through combined 5-$HT_2$ antagonism and 5-HT reuptake blockade (Haria et al., 1994). It is often co-prescribed with other antidepressants as a sleep-inducing agent because of its sedative side effects (Fabre, 1990; Jacobsen, 1990; Nierenberg, 1994), or as an augmentation strategy (Maes et al., 1997). This co-prescription introduces the potential for metabolic drug interactions.

Trazodone is extensively metabolized in the liver by hydroxylation, dealkylation, and N-oxidation (Baiocchi et al., 1974; Yamato, 1974a). The active metabolite mCPP is formed by N-dealkylation at the piperazinyl nitrogen (Yamato et al., 1974b, Melzacka et al., 1979). The metabolite mCPP is of interest because it has 5-$HT_{2C}$ agonistic and 5-$HT_{2A}$ antagonistic properties (Conn and Sanders-Bush, 1987; Fiorella et al., 1995), as well as behavioral effects which are consistent with 5-HT agonistic properties such as worsening of psychoses in humans, and anxiogenesis and anorexia in animals and humans

(Kahn and Wetzler, 1991). It has also been suggested by some that mCPP may contribute to the antidepressant efficacy of trazodone (Maes, 1997). Therefore, a drug interaction which alters the production of mCPP could have clinically significant effects.

Current information available on the metabolism of trazodone by the CYP enzymes comes mainly from drug interaction studies, which provide only suggestive evidence of the enzymes involved, and do not examine specific metabolic pathways, whether it is the parent compound or a metabolite causing the interaction, or whether the interaction is competitive or non-competitive. For example, thioridazine, a CYP2D6 inhibitor, increases plasma concentrations of both trazodone and mCPP, suggesting that both are substrates for CYP2D6, but providing no information as to which metabolic pathways are involved (Yasui et al., 1995). Plasma levels of trazodone, but not mCPP, are lower in smokers than in non-smokers, suggesting a possible role of the smoking-inducible CYP1A2 in trazodone, but not mCPP, metabolism (Ishida et al., 1995). Carbamazepine, a CYP3A4 inducer and substrate, decreases plasma concentrations of both trazodone and mCPP, but mCPP to a lesser extent (Otani et al., 1996). Clinical interactions between trazodone and fluoxetine (FLU) have been reported in the form of adverse side effects such as headaches, dizziness and excessive sedation (Metz and Shader, 1990; Nierenberg et al., 1992), as well as increased plasma levels of trazodone (Aranow et al., 1989; Maes et al, 1997) and mCPP (Maes et al., 1997). However, the causes of the interactions cannot easily be determined since both FLU and its main metabolite norfluoxetine (NFLU) are

inhibitors of both CYP2D6 and CYP3A4 (Crewe et al., 1992; Greenblatt et al., 1996).

A detailed *in vitro* investigation was thus necessary to identify the individual enzymes involved in the various interactions of trazodone. In particular, the pathway leading to the formation of mCPP from trazodone was of interest given the psychopharmacological effects of this metabolite. Several *in vitro* methods are routinely used to identify the CYP enzymes involved in the oxidation of a compound (e.g. Guengerich, 1996; Iwatsubo et al., 1997). The current experiments were designed to directly identify the major CYP enzymes involved in the metabolism of trazodone to mCPP using human liver microsomal preparations and cDNA-expressed human CYP enzymes.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Development of a GC-NPD method for the detection of mCPP in microsomal incubation medium

#### *2.2.1.1 Sample preparation*

Following the microsomal incubation period in the hot water bath, the samples were placed on ice and basified with 100 µl of a 25% potassium carbonate solution to terminate the reaction. To the basified incubation mixture, 300 µl of double distilled $H_2O$ ($ddH_2O$) and 1000 ng of the internal standard p-chlorophenylethylamine (p-CPEA, in 100 µl $ddH_2O$) were added. The incubation mixtures were then transferred to screw cap culture tubes (160 mm x 15 mm),

and mCPP was extracted and derivatized by shaking the tubes for 15 min on an Ika Vibrex VXR vortex mixer with 2 ml of a solution of toluene and pentafluorobenzoyl chloride (PFBC) in a ratio of 1000:1. The tubes were then centrifuged at 1000 g for 5 min in a benchtop centrifuge. The organic phase was pipetted to 100 mm x 13 mm screw cap culture tubes and taken to dryness in a Savant evaporator. The residue was reconstituted in 150 μl of toluene for GC analysis.

Calibration standards of 20, 10, 5, 2.5, and 1.25 μM mCPP were prepared and run in parallel with the assay; each tube contained the same amount of internal standard (1000 ng) as the extracts of the incubation mixtures.

*2.2.1.2 Instrumental analysis*

A 1 μl aliquot of the solution in toluene was injected on a Hewlett-Packard (HP) model 5890 gas chromatograph equipped with a nitrogen-phosphorus detector and linked to an HP 3392A integrator. A 15 m fused silica capillary column (internal diameter of 0.25 mm) coated with a 0.25 μm film thickness of 5% phenylmethylpolysiloxane was used. The carrier gas was helium at a flow rate of 3.5 ml/min, and the make-up gas was helium at a flow rate of 30 ml/min. Hydrogen and air were used at flow rates of 4 ml/min and 80 ml/min, respectively. The oven temperature was set at 105° C for an initial time of 0.5 min, and was then set to increase at a rate of 12° C/min to a final temperature of 295° C. The injection port temperature was set at 270° C, and the detector

temperature was 325° C. All injections were in the splitless mode with a purge off time of 0.5 min.

### 2.2.2 Incubation Conditions

The drug metabolism experiments were carried out as described in General Methods in an incubation medium consisting of 25 µl of NADPH generating system, 10 µl of microsomal enzyme preparation (1.5 mg microsomal protein/ml incubation mixture for human microsomes; 1.0 mg microsomal protein/ml incubation mixture for cells expressing human CYP enzymes), 50 µl of trazodone solution (100 µM) in 0.1 M potassium phosphate buffer, and 15 µl 0.1M potassium phosphate buffer (pH 7.4). The tubes were incubated for 10 min at 37° C in a water bath. The incubation time was chosen based on experiments showing that the formation of mCPP was linear for the first 20 min of incubation time. Following the incubation period, the tubes were placed on ice and 100 µl of ice-cold 25% potassium carbonate solution was added to terminate metabolism.

### 2.2.3 Time Course for mCPP production from trazodone

The time course for the production of mCPP from trazodone was assessed by incubating 100 µM trazodone for 5, 10, 20, 30 or 60 min with either human liver microsomes or cells expressing human CYP3A4.

### 2.2.4 Determination of the kinetic constants for mCPP formation from trazodone

The kinetic constants of $K_m$ and $V_{max}$ were estimated for the formation of mCPP from trazodone by incubating varying concentrations of trazodone (450, 300, 200, 133, 88.89, 59.26, 39.51, 26.34, 17.56, 11.71, 7.80, 5.20, 3.47, 2.31, and 0 μM) with human liver microsomes under the conditions described above. The data were analyzed by iterative nonlinear least squares regression analysis (GraphPad Prism), fitting the data to the equation:

$$v = (V_{MAX} \cdot S) / (K_M + S)$$

where v is the reaction velocity corresponding to S, the substrate concentration (trazodone), $V_{max}$ is the maximal velocity, and $K_m$ is the substrate concentration at which the reaction velocity equals 50% of $V_{max}$.

### 2.2.5 Correlation with human liver microsome enzyme activities

Trazodone (100 μM final concentration) was incubated with the NADPH-generating system and microsomes prepared from a panel of 16 human livers characterized for their catalytic activity for CYP1A2 (phenacetin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2C19 (mephenytoin 4-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2E1 (chlorzoxazone 6-hydroxylation), CYP3A4 (6-hydroxylation of [$^{14}$C]-testosterone, and CYP4A11

(omega-hydroxylation of [$^{14}$C]-lauric acid). The rate of formation of mCPP was then correlated with the activities of the specific enzymes for each of the 16 human livers (GraphPad Prism).

### 2.2.6 Incubations with single expressed enzymes

Trazodone (100 μM final concentration, added in a volume of 50 μl) was incubated in NADPH-generating system (25 μl), potassium phosphate buffer (15 μl), and 10 μl of a microsomal preparation (1 mg microsomal protein/ml incubation mixture) expressing one of the enzymes CYP1A1, CYP1A2, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6, or CYP3A4, for 30 min. These incubations were repeated in four separate experiments.

### 2.2.7 Inhibition with ketoconazole

The CYP3A4 inhibitor ketoconazole (final concentrations in 100 μl of 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.0 μM, added in 10 μl of buffer) was pre-incubated for 10 min with 25 μl of the NADPH-generating system, 5 μl of potassium phosphate buffer, and 10 μl of either human liver microsomes (1.5 mg microsomal protein/ml incubation mixture) or microsomes prepared from cells expressing human CYP3A4 (1mg microsomal protein/ml incubation mixture). Trazodone (100 μM final concentration, added in a volume of 50 μl) was then added and the incubation was continued for a further 10 min. As a control, quinidine, a specific inhibitor of CYP2D6, was also incubated as described for ketoconazole, using

concentrations of 6.0, 3.0, 1.5, 0.75, 0.375, and 0 μM. The inhibitions were repeated in three separate experiments.

### 2.2.8 Interactions with SSRIs

Microsomes prepared from cells expressing human cDNA for CYP3A4 (10 μl, 10 mg protein/ml incubation) were pre-incubated for 10 min with the NADPH-generating system (25 μl), and a solution of one of either fluvoxamine (FVX), paroxetine (PRX), FLU, or NFLU in potassium phosphate buffer (100 μM final concentration in 100 μl, added in a volume of 15 μl). Following pre-incubation, trazodone was added (100 μM final concentration in 100 μl, added in a volume of 50 μl), and the incubation was continued for a further 10 min. The data were analyzed by one-way analysis of variance (ANOVA) with drug group as the between factors variable. *Post hoc* analyses comparing the individual drug groups to the control group were carried out using Dunnett's Multiple Comparison Test.

## 2.3 RESULTS

### 2.3.1 mCPP levels in microsomal incubation mixtures

The assay procedures described for the detection of mCPP in the microsomal medium resulted in the formation of a derivative of mCPP with excellent chromatographic properties (Figure 2.2). The structure of the derivative was confirmed using combined GC-mass spectrometry in the electron-

impact mode, and the proposed mass fragmentation pathways are shown in Figure 2.3.

The chromatographic peak height ratios of derivatized mCPP to derivatized internal standard for the calibration standards were subjected to regression analysis, and consistently yielded a linear relationship with $r^2 > 0.99$. The limit of detection for the assay was < 140 pg on column, and was defined as the concentration of mCPP which was detectable at 3 times the signal-to-noise ratio. Within-assay reproducibility was assessed in five samples spiked with 250 ng of mCPP and 1000 ng of the internal standard p-CPEA, and carried through the procedure in parallel. The peak height ratios of derivatized mCPP to derivatized p-CPEA for the five samples had a coefficient of variation of 9.61%. Between assay reproducibility was assessed by comparing peak-height ratios for standards of known concentration across 5 separate assays. The coefficient of variation between assays was 9.94% (n=5).

Recovery of mCPP during extraction was assessed by comparing recovery of mCPP from five samples taken through the extraction procedure to five samples in which mCPP was added directly to the toluene:PFBC mixture. The mean recovery of mCPP from the extracted samples was 93%.

### 2.3.2 Time course

The time course of the formation of mCPP from trazodone in human liver microsomes and cells expressing CYP3A4 is shown in Figure 2.4. The production of mCPP is approximately linear until 20 min, at which time

production appears to level off. Therefore, an incubation time of 10 min was chosen for subsequent analyses.

### 2.3.3 Kinetic analyses

Incubations of various concentrations of trazodone with human liver microsomes resulted in a concentration-dependent formation of mCPP, as shown in Figure 2.5. The apparent $K_m$ was 311.3 ± 32.19 μM, and apparent $V_{max}$ 4.95 ± 0.29 nmol/min/mg protein.

### 2.3.4 Correlation with human liver microsome enzyme activities

The rate of formation of mCPP showed significant correlation ($r=0.81$, $p<0.0001$) with CYP3A4 activity (Figure 2.6). Correlations with all other CYP enzymes failed to reach significance (CYP1A2, $r=0.27$, $p=0.32$; CYP2A6, $r=0.47$, $p=0.07$; CYP2C19, $r=0.16$, $p=0.55$; CYP2D6, $r=0.41$, $p=0.11$; CYP2E1, $r=0.40$, $p=0.13$; CYP4A11, $r=0.33$, $p=0.21$).

### 2.3.5 Metabolism with single cDNA-expressed human CYP enzymes

Trazodone incubations with microsomes expressing only CYP3A4 resulted in mCPP production (0.3903 +/- 0.0631 nmol/min/mg protein, n=4), whereas incubations with microsomes expressing only CYP1A1, CYP1A2, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, or CYP2D6 did not result in detectable mCPP formation.

### 2.3.6 Inhibition with ketoconazole

The CYP3A4 inhibitor ketoconazole resulted in a concentration-dependent inhibition of mCPP production in both human liver microsomes and microsomes expressing human CYP3A4, whereas incubations with quinidine did not inhibit mCPP formation (Figure 2.7).

### 2.3.7 Interactions with SSRIs

Interactions with each of the SSRIs FVX, FLU, and NFLU, resulted in statistically significant decreases in mCPP production, whereas incubations with PRX did not (Figure 2.8, and Table 2.1). The overall ANOVA revealed significant differences between the groups ($F=17.36$, $df=4$, $p=0.0002$). *Post hoc* analysis revealed that mCPP production was significantly decreased in the incubations with FVX, FLU, and NFLU ($p<0.01$), but not with PRX ($p>0.05$).

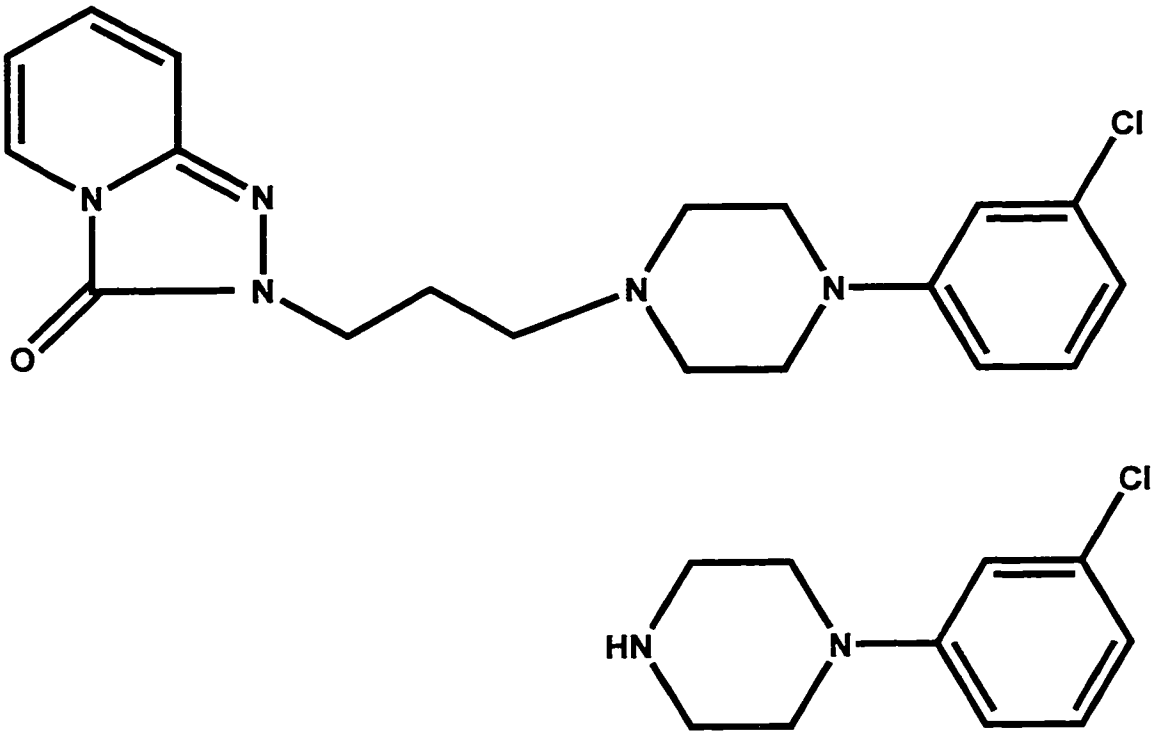



FIGURE 2.1 The chemical structures of trazodone (top) and mCPP (bottom).

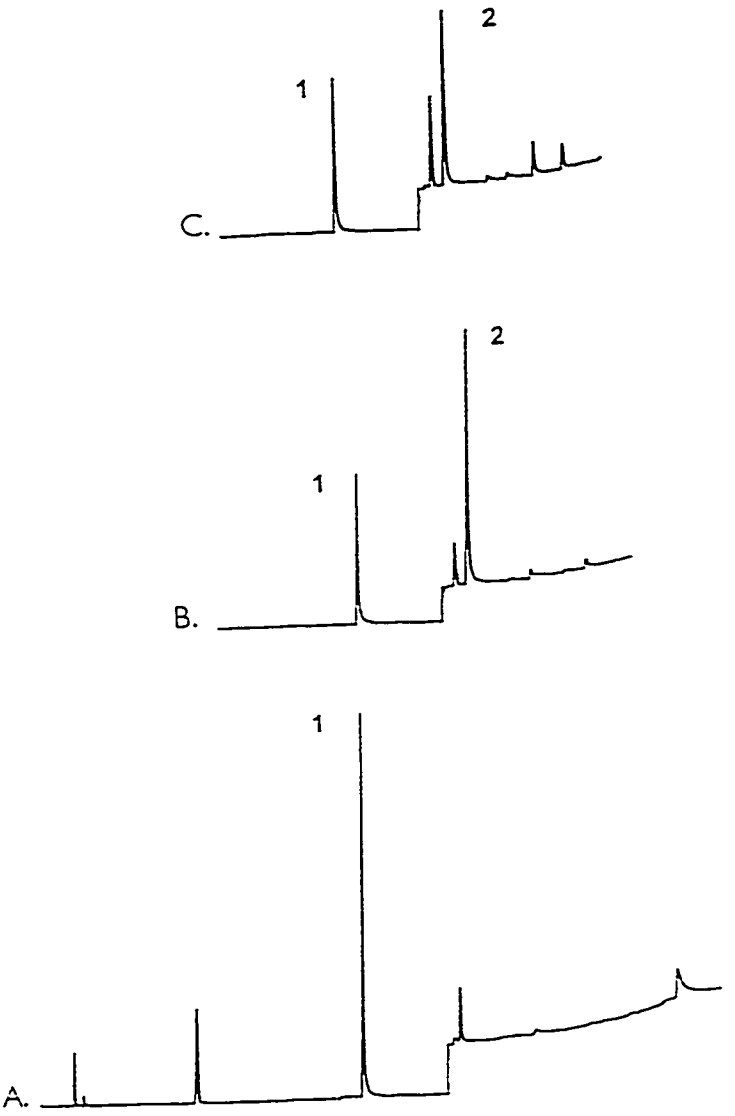



FIGURE 2.2 Typical gas chromatographs from derivatized incubation media of trazodone incubated with cells expressing human CYP3A4. A) Chromatograph from incubation medium spiked with 1000 ng of internal standard p-chlorophenylethylamine [p-CPEA; retention time (RT) 8.81 min; peak 1]. B) Chromatograph from medium spiked with 0.325 nmole mCPP (RT 11.52 min, peak 2) and 1000 ng p-CPEA (peak 1). C) Chromatograph from medium containing trazodone and CYP3A4 showing a peak for derivatized mCPP (peak 2) and derivatized p-CPEA (peak 1).

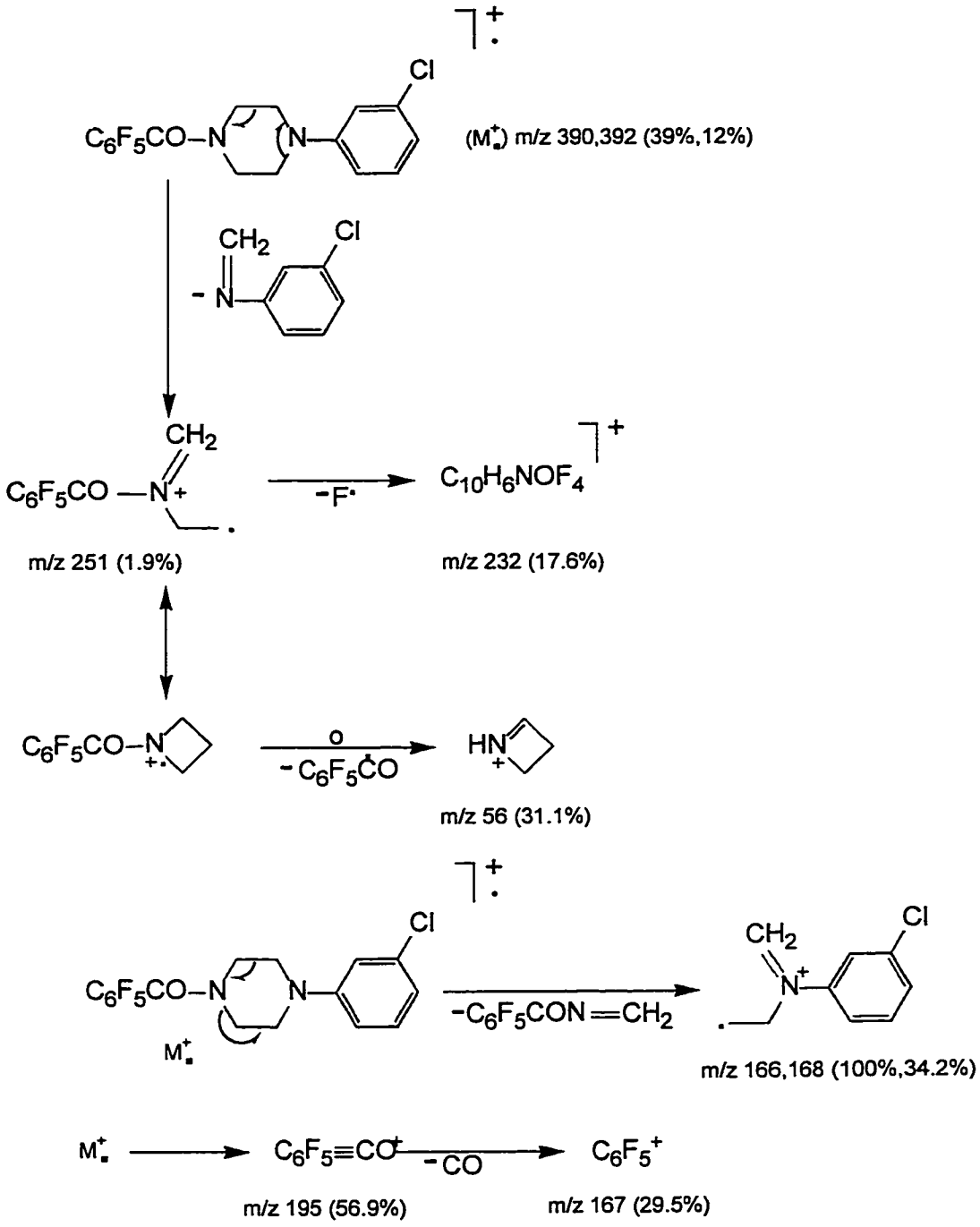



FIGURE 2.3 Proposed mass fragmentation (electron impact mode) of mCPP derivatized with pentafluorobenzoyl chloride (PFBC).

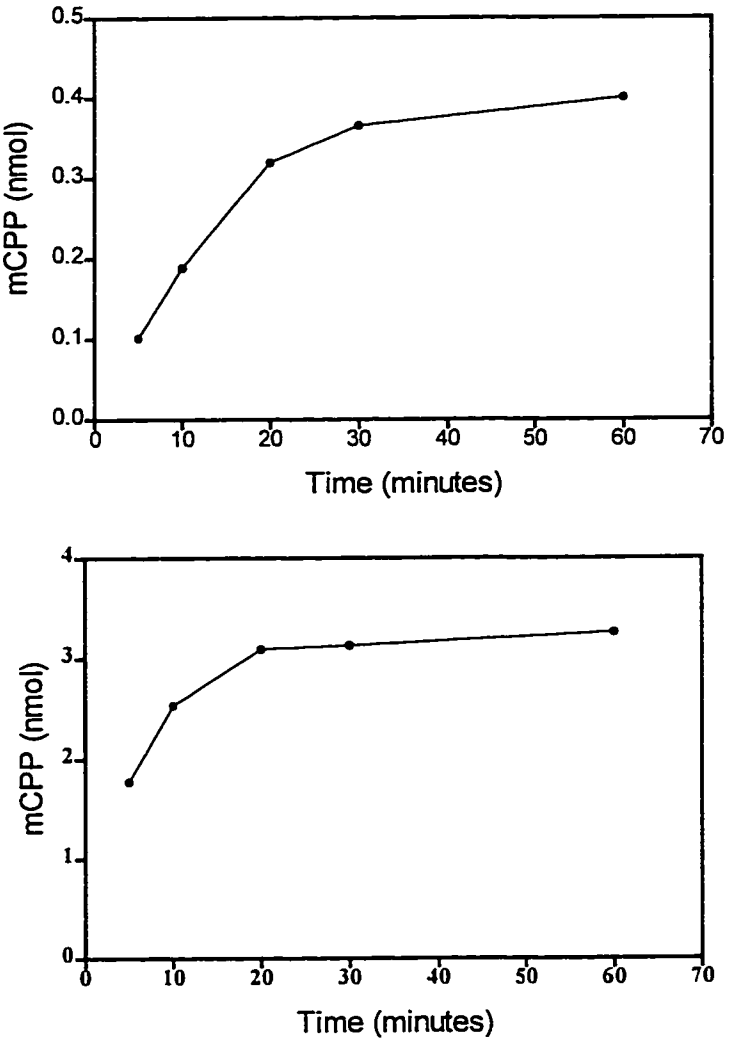



FIGURE 2.4 Time course of the production of mCPP from trazodone (100 μM) in human liver microsomes (top) and in microsomes prepared from cells expressing human CYP3A4 only (bottom).

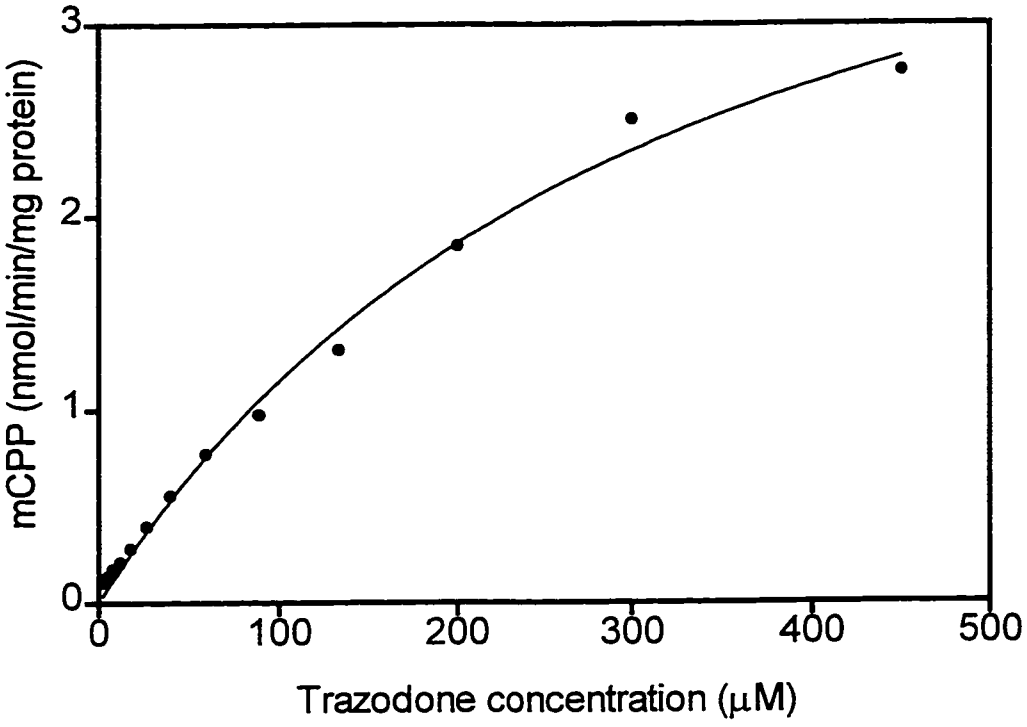



FIGURE 2.5 Formation of mCPP (y-axis, nmol/min/mg protein) at various concentrations of the substrate trazodone (x-axis, μM). The solid line was determined by nonlinear least squares regression analysis.

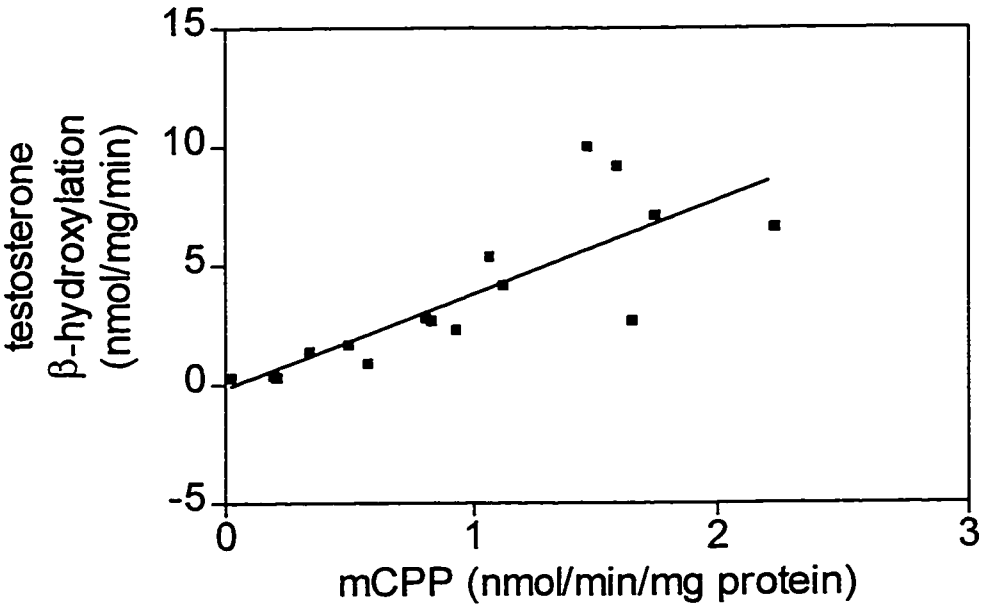



FIGURE 2.6 Correlation ($r=0.81$, $p<0.0001$) of the activity of CYP3A4 (rate of testosterone β-hydroxylation, nmol/mg/min; y-axis) with the rate of mCPP production from trazodone (100 μM, 10 min incubation, x-axis) in 16 individual human liver samples. Correlations with rates of reaction for other CYP enzymes failed to reach significance.

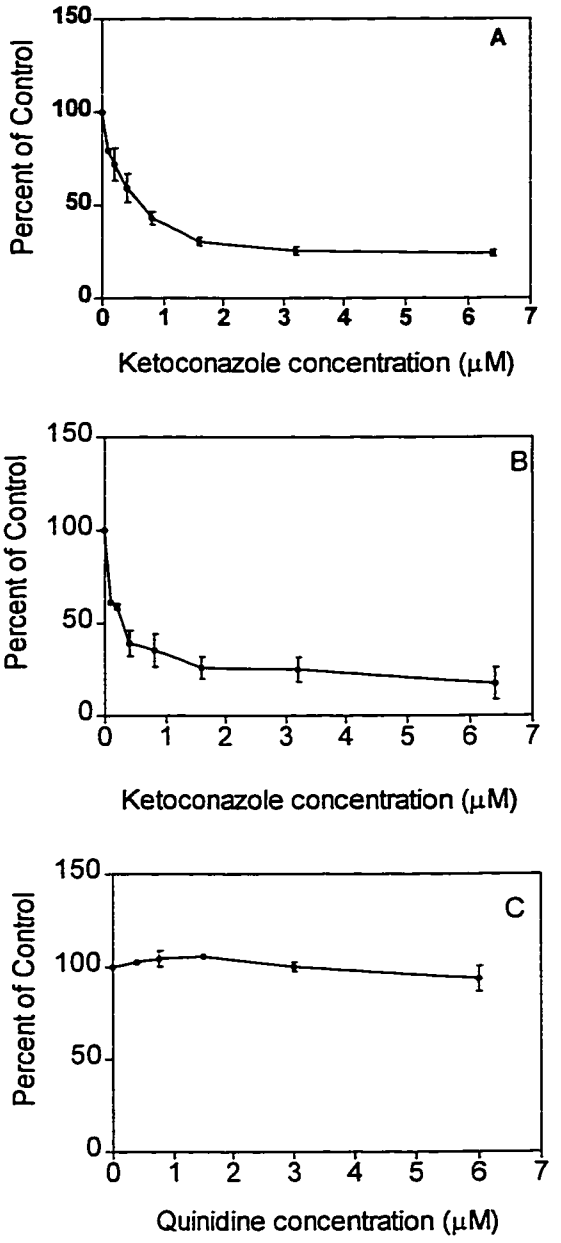



FIGURE 2.7 Inhibition of mCPP production (expressed as % of control activity; y-axis) from trazodone (100 μM) by ketoconazole with human liver microsomes (A), cells expressing human CYP3A4 (B), and by quinidine with human liver microsomes (C). Each point represents the mean ± SEM of three separate experiments.

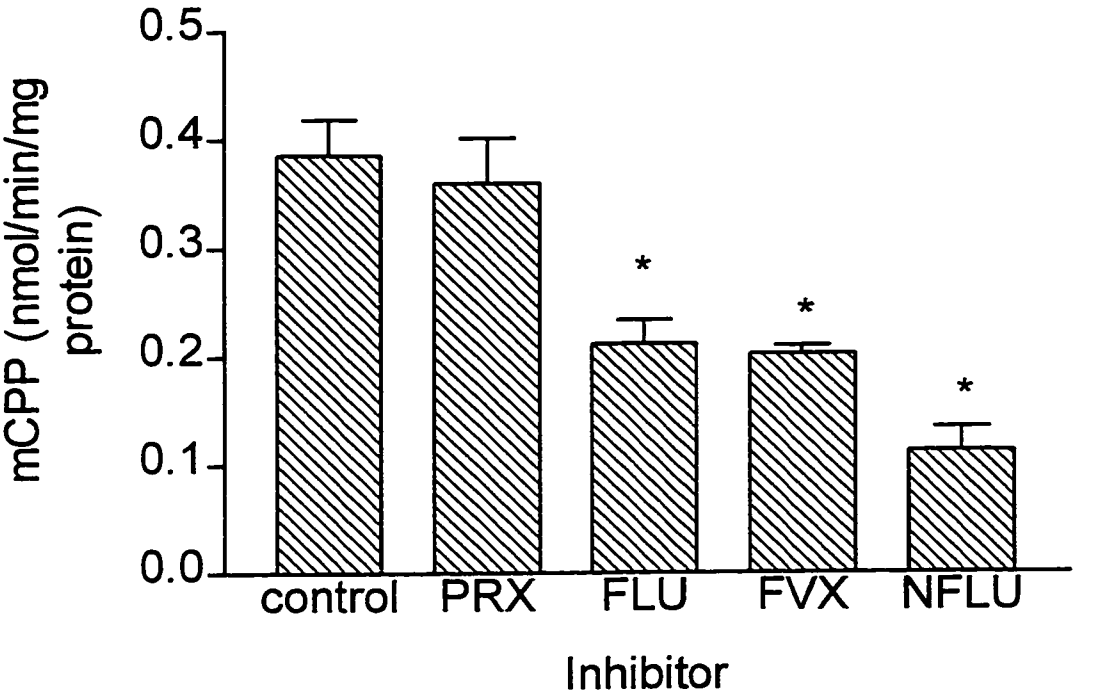



FIGURE 2.8 The production of mCPP from trazodone incubated with microsomes from cells expressing human CYP3A4 in the presence of no inhibitor (control), or one of the SSRIs FVX, PRX, FLU, or NFLU (100 μM) [* = significantly different from control, $p<0.01$]. Results are the means ± SEM of three separate experiments.

| *Inhibitor* | *Mean mCPP (nmol/min/mg)* | *S.E.M* | *Percent of Control* |
|---|---|---|---|
| Control | 0.386 | 0.033 | 100 |
| PRX | 0.360 | 0.042 | 93.00 |
| FLU | 0.212 | 0.022 | 54.93 |
| FVX | 0.202 | 0.008 | 52.44 |
| NFLU | 0.113 | 0.02 | 29.25 |

TABLE 2.1 Mean mCPP production from trazodone following incubations with SSRIs (100 μM). Results are from three separate experiments.

## 2.4 DISCUSSION

The present experiments showed that mCPP production from trazodone is correlated with CYP3A4 activity in human liver microsomes, is formed from incubations with cells expressing CYP3A4 only, and is diminished in the presence of an inhibitor of CYP3A4. Furthermore, the SSRIs FLU, NFLU and FVX, which all inhibit CYP3A4 (Richelson, 1997), inhibited the formation of mCPP from trazodone in the presence of cells expressing only CYP3A4. Taken together, these results indicate strongly that trazodone is a substrate of CYP3A4, and that the metabolite mCPP is formed through this pathway. Therefore, there is the potential for drug-drug interactions with this drug and other substrates, inhibitors or inducers of CYP3A4.

The inhibition studies with the SSRIs revealed a rank order of potency of inhibition of mCPP production with PRX being the weakest inhibitor, followed by FLU, FVX, and NFLU. This is in slight contrast to other studies which have reported rank order CYP3A4 inhibition potencies with FLU being the weakest, followed by PRX, NFLU, and FVX (Richelson, 1997). However, the present studies were carried out in cells expressing only CYP3A4, which may account for some variability, as might the use of trazodone as the substrate.

Plasma levels of trazodone show wide inter-individual differences, and typically range from about 0.38 - 5.8 μM (Vatassery et al., 1997). However, it is not the plasma concentration which is relevant, but rather the concentration at the enzyme site, or the hepatic concentration, which is of importance with

respect to drug interactions (Harvey and Preskorn, 1995; Preskorn, 1996; von Moltke et al., 1996). Lipophilic drugs partition extensively into the liver, and liver/water partition ratios are typically used to estimate liver drug concentrations (Greenblatt et al., 1996). The hepatic extraction ratio for trazodone is not currently known, but if it is assumed to have a partition ratio similar to the SSRIs, which are also highly lipophilic, and have hepatic extraction ratios of 12-26 (Harvey and Preskorn, 1995; Schmider et al., 1996), then hepatic concentrations of trazodone can be expected to range from 60 - 100 μM, at a plasma concentration of 5 μM. Therefore, the concentration of trazodone used in the present experiments can be considered to be clinically relevant.

The clinical significance of potential drug interactions with trazodone depends upon several factors. First, trazodone may affect the metabolism of other CYP3A4 substrates through competitive inhibition of CYP3A4. The consequences of this interaction will depend upon the relative affinities and concentrations of trazodone and the competing drug at the enzyme, as well as the therapeutic index of both drugs. Although trazodone is relatively safe on its own, it does have potentially bothersome or dangerous side effects such as excessive sedation, which could become a problem at higher plasma concentrations (Haria et al., 1994). The therapeutic index of the interacting drug is also important, as clinically significant interactions with a CYP3A4 substrate with a narrow therapeutic index could result, as has occurred with terfenadine (Wilkinson, 1996). Another important consideration with respect to drug

interactions is the effects of co-administered drugs on the ability to maintain therapeutic plasma concentrations. Clinical antidepressant response is significantly correlated with steady-state plasma trazodone concentrations, and a threshold concentration of 650 ng/ml is considered necessary for antidepressant response (Monteleone et al., 1989). Therefore, any factor which results in a lowering of plasma trazodone levels may interfere with the clinical efficacy of the drug. Since CYP3A4 levels vary widely among individuals (Wilkinson, 1996), and since CYP3A4 is inhibited and induced by many commonly encountered drugs and environmental compounds (von Moltke et al., 1995; Wilkinson, 1996), it is important to be aware that trazodone is a substrate of CYP3A4, and thus is subject to many factors which may alter its plasma concentration. This is particularly true since therapeutic concentrations of trazodone are typically below the $K_m$ of trazodone, and thus are subject to first-order kinetics, and are highly sensitive to changes in the concentration of enzyme or substrate (Iwatsubo et al., 1997). The high $K_m$ value found in the present experiments for trazodone transformation is consistent with the linear pharmacokinetics of trazodone and mCPP seen clinically (Nilsen et al., 1993).

The present experiments are a direct examination of trazodone metabolism to mCPP by the CYP enzymes, and provide evidence that CYP3A4 is a major enzyme responsible for this biotransformation. This finding indicates that the potential for drug-drug interactions exists with trazodone and other substrates or inhibitors of CYP3A4.

## 2.5 REFERENCES


Aranow RB, Hudson JI, Harrison GP, Grady TA, Laage TA, Bell IR and Cole JO (1989) Elevated antidepressant levels after addition of fluoxetine. *Am J Psychiatry* **146:** 911-913.

Baiocchi BL, Frigerio A, Giannangeli M and Palazzo G (1974) Basic metabolites of trazodone in humans. *Arzneim.-Forsch. (Drug Res.)* **24:**1699-1706 (1974).

Conn JP and Sanders-Bush E (1987) Relative efficacies of piperazines at the phosphoinositide hydrolysis-linked serotonergic (5-HT-2 and 5-HT-1C) receptors. *J Pharmacol Exp Ther* **242:** 552-557.

Crewe HK, Lennard MS, Tucker GT, Woods FR and Haddock RE (1992) The effect of selective serotonin re-uptake inhibitors on cytochrome P4502D6 (CYP2D6) activity in human liver microsomes. *Br J Pharmacol* **34:** 262-265.

Fabre LF (1990) Trazodone dosing regimen: Experience with single daily administration. *J Clin Psychiatry* **51:** 23-26 (1990).

Fiorella D, Rabin RA and Winter JC (1995) The role of the 5-HT2A and 5-HT2C receptors in the stimulus effects of m-chlorophenylpiperazine. *Psychopharmacol* **119:** 222-230.

Greenblatt DJ, von Moltke LL, Schmider JS, Harmatz JS and Shader RI (1996) Inhibition of human cytochrome P4503A isoforms by fluoxetine and norfluoxetine: *In vitro* and *in vivo* studies. *J Clin Pharmacol* **36:** 792-798.

Guengerich FP (1996) In vitro techniques for studying drug metabolism. *J Pharmacokinet Biopharmaceut* **24**:521-533.

Haria M, Fitton A and McTavish D (1994) Trazodone: A review of its pharmacology, therapeutic use in depression and therapeutic potential in other disorders. *Drugs & Aging* **4:** 331-355.

Harvey AT and Preskorn SH (1995) Interactions of serotonin reuptake inhibitors with tricyclic antidepressants [letter]. *Arch Gen Psychiatry* **52**:783-784.

Ishida M, Otani K, Kaneko S, Ohkubo T, Osanai T, Yasui N, Mihara K, Higuchi H and Sugawara K (1995) Effects of various factors on steady state plasma concentrations of trazodone and its active metabolite *m*-chlorophenylpiperazine. *Int Clin Psychopharmacol* **10:**143-146.

Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA and Sugiyama Y (1997) Prediction of *in vivo* drug metabolism in the human liver from in vitro metabolism data. *Pharmacol Ther* **73**:147-171.

Jacobsen FM (1990) Low-dose trazodone as a hypnotic in patients treated with MAOIs and other psychotropics: A pilot study. *J Clin Psychiatry:* **51,** 298-302.

Kahn RS and Wetzler S (1991) m-Chlorophenylpiperazine as a probe of serotonin function. *Biol Psychiatry* **30:**1139-1166.

Maes M, Westenberg H, Vandoolaeghe E, Demedts P, Wauters A, Neels H and Metzler HY (1997) Effects of trazodone and fluoxetine in the treatment of major depression: Therapeutic pharmacokinetic and pharmacodynamic interactions through formation of meta-chlorophenylpiperazine. *J Clin Psychopharmacol* **17**:358-364.

Melzacka M, Boksa J and Maj J (1979) 1-(*m*-Chlorophenyl)piperazine: a metabolite of trazodone isolated from rat urine. *J Pharm Pharmacol* **31**: 855-856.

Metz A and Shader RI (1990) Adverse interactions encountered when using trazodone to treat insomnia associated with fluoxetine. *Int Clin Psychopharmacol* **5:**191-194.

Monteleone P, Gnocchi G and Delrio G (1989) Plasma trazodone concentrations and clinical response in elderly depressed patients: A preliminary study. *J Clin Psychopharmacol* **9**:284-287.

Nierenberg AA, Cole JO and Glass L (1992) Possible trazodone potentiation of fluoxetine: A case series. *J Clin Psychiatry* **53:** 83-85.

Nierenberg AA, Adler LA, Peslow E, Zornberg G and Rosenthal M (1994) Trazodone for antidepressant-associated insomnia. *Am J Psychiatry* **151:**1069-1072.

Nilsen OG, Dale O and Husebo B (1993) Pharmacokinetics of trazodone during multiple dosing to psychiatric patients. *Pharmacol & Toxicol* **72**:286-289.

Otani K, Ishida M, Kaneko S, Mihara K, Ohkubo T, Osanai T and Sugawaara K (1996) Effects of carbamazepine coadministration on plasma concentrations of trazodone and its active metabolite, m-chlorophenylpiperazine. *Ther Drug Monit* **18:**164-167.

Preskorn S (1996) *Clinical Pharmacology of Selective Serotonin Reuptake Inhibitors.* (Appendix), Professional Communications, Inc., Cado, OK

Schmider J, Greenblatt DJ, von Moltke LL, Harmatz JS and Shader RI (1996) Inhibition of cytochrome P450 by nefazodone in vitro: studies of dextromethorphan O- and N-demethylation. *Br J Clin Pharmacol* **41**:339-343.

Vatassery GT, Holden LA, Hazel DK and Dysken MW (1997) Determination of trazodone and its metabolite, 1-m-chlorophenylpiperazine, in human plasma and red blood cell samples by HPLC. *Clin Biochem* **30**:149-153.

von Moltke LL, Greenblatt DJ, Harmatz JS, Duan SX, Harrel LM, Cotreau-Bibbo MM, Pritchard GA, Wright CE and Shader RI (1996) Triazolam biotransformation by human liver microsomes in vitro: Effects of metabolic inhibitors and clinical confirmation of a predicted interaction with ketoconazole. *J Pharmacol Exp Ther* **276**:370-379.

von Moltke LL, Greenblatt DJ, Schmider J, Harmatz JS and Shader RI (1995) Metabolism of drugs by cytochrome P450 3A isoforms. Implications for drug interactions in psychopharmacology. *Clin Pharmacokinet* **29(Suppl. 1)**:33-44.

Wilkinson GR (1996) Cytochrome P4503A (CYP3A) Metabolism: Prediction of *in vivo* activity in humans. *J Pharmacokinet Biopharmaceut* **24**:475-489.

Yamato C, Takahashi T and Fujita T (1974a) Studies on the metabolism of trazodone, I. Metabolic fate of [$^{14}$C]trazodone hydrochloride in rats. *Xenobiotica* **4**:313-326.

Yamato C, Takahashi T, Fujita T , Kuriyama S and Hirose N (1974b) Studies on the metabolism of trazodone, II. Metabolic fate after intravenous administration and effects on liver microsomal drug-metabolizing enzymes in rats. *Xenobiotica* **4**:765-777.

Yasui N, Otani K, Kaneko S, Ohkubo T, Osanai T, Ishida M, Mihara K, Kondo T, Sugawara K and Fukushima Y (1995) Inhibition of trazodone metabolism by thioridazine in humans. *Ther Drug Monit* **17**: 333-335.

# Chapter 3

## Human CYP2D6 and the metabolism of m-chlorophenylpiperazine (mCPP)

A version of this chapter is currently in press (Rotzinger et al., *Biol Psychiatry,* 1998)

## 3.1 INTRODUCTION

The present experiments were designed to study the metabolism of mCPP, the pharmacologically active metabolite of the antidepressants trazodone, nefazodone (NEF) and etoperidone, and of the minor tranquilizer mepiprazole (Melzacka et al., 1979; Fong et al., 1982; Caccia et al., 1981; Mayol et al., 1994b). This metabolite is a 5-$HT_{2A}$ antagonist and 5-$HT_{2C}$ agonist (Conn & Sanders-Bush, 1987; Fiorella et al., 1995) which causes a dose-dependent elevation of adrenocorticotropic hormone (ACTH), cortisol, and prolactin (Kahn & Wetzler, 1991; Cowen et al., 1995). mCPP also decreases food intake and increases measures of anxiety in both animals (Samanin et al., 1979; Gibson et al., 1994; Kahn & Wetzler, 1991) and humans (Krystal et al., 1993; Walsh et al., 1994; Cowen et al., 1995). In addition, it can worsen the core symptoms of many psychiatric disorders such as the positive symptoms of schizophrenia (Krystal et al., 1993) and panic symptoms in patients with panic disorder (Kahn et al., 1988; Germine et al., 1994).

These behavioral effects have been noted to occur at plasma levels of mCPP of 10 to 40 ng/ml (Murphy et al., 1989; Kahn et al., 1990; Kalus et al., 1992), levels which are readily achieved clinically following therapeutic doses of trazodone (Ishida et al., 1995; Otani et al., 1997) and NEF (Kaul et al., 1995; Barbhaiya et al., 1996b, 1996c). Although trazodone and NEF both possess 5-$HT_{2C}$ antagonistic activity (Marek et al., 1992; Ellingrod & Perry, 1995; Taylor et al., 1995), which might normally counteract the effects of mCPP, a drug-drug

interaction which results in increased mCPP levels may have the potential to offset this inhibition, resulting in adverse effects.

mCPP is known to be eliminated by oxidation to p-hydroxy-mCPP (OH-mCPP) [Figure 3.1] followed by rapid conjugation to both glucuronide and sulfate conjugates (Mayol et al., 1994a). The enzymes involved in this reaction are not presently known, although some clinical studies have suggested the involvement of CYP2D6. For example, mCPP clearance is decreased in poor metabolizers of dextromethorphan, who are genetically deficient in CYP2D6, which suggests that CYP2D6 may be the enzyme responsible for the hydroxylation of mCPP (Buch et al., 1993; Barbhaiya et al., 1996a). mCPP levels are also increased when trazodone is co-administered with thioridazine, an inhibitor of CYP2D6 (Yasui et al., 1995). Similarly, mCPP levels are increased when NEF is co-administered with propranolol (Ellingrod & Perry, 1995) and FLU (Marino et al., 1996), both of which are competitive inhibitors of CYP2D6 (Bertz & Granneman, 1997). However, it is not possible to identify the cause of these interactions with certainty, since several possible enzymes, metabolites, and metabolic pathways may be involved. No definitive studies of mCPP metabolism *in vitro,* which are necessary to clearly identify the enzymes responsible for the production of OH-mCPP from mCPP, have yet been reported.

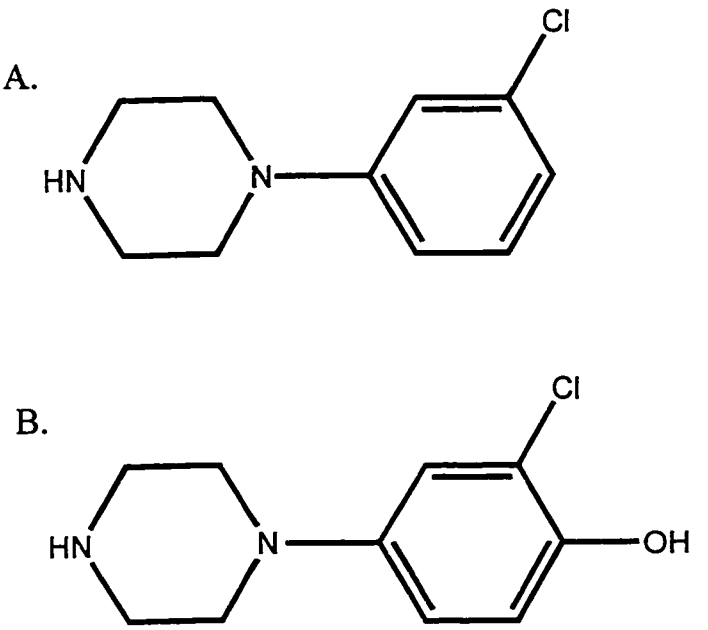



FIGURE 3.1 Chemical structures of mCPP (A) and its metabolite OH-mCPP (B).

## 3.2 METHODS

### 3.2.1 Incubation conditions

Preliminary experiments indicated that the production of OH-mCPP was linear for up to 20 minutes of incubation time; therefore, an incubation time of 10 minutes was chosen for the following experiments. Incubations were conducted as indicated in Chapter 1, Section 1.4.3, and as indicated below. Following the incubation period, the tubes were placed on ice, 50 µl of ice-cold acetonitrile was added, and the tubes were allowed to sit for 10 min to terminate metabolism and precipitate the proteins. The tubes were then centrifuged in a Beckman microfuge for 5 min, and the supernatant was removed for HPLC analysis.

### 3.2.2 Instrumental analysis

A 30 µl aliquot of the incubation supernatant was injected into a high pressure liquid chromatographic (HPLC) system with a Waters model 510 pump linked to a Waters Intelligent Sample Processor model 710B. Detection was performed using a Waters Lambda Max Model 481 variable-wavelength UV detector linked to a Hewlett Packard model 3392A integrator.

The HPLC mobile phase consisted of acetonitrile and ammonium acetate buffer [100:98:2 ml (v/v) acetonitrile: distilled $H_2O$: 1M ammonium acetate] adjusted to pH 5.4 with acetic acid. The flow-rate was 1.5 ml/min. A Phenomenex Hypersil CN (250 x 4.6 mm I.D., 5 µm particle size) column was used and was coupled to a guard column which was packed with the same

material as the analytical column. Detection was performed using UV absorbance at 254 nm.

### 3.2.3 Correlation with human liver microsome enzyme activities

mCPP (100 μM) was incubated individually with microsomes prepared from 16 different human livers, and the rate of production of OH-mCPP (nmol/min/mg) was correlated with the catalytic activities of the human livers (GraphPad™ Prism).

### 3.2.4 Metabolism with single cDNA-expressed human CYP enzymes

mCPP (100 μM, added in a volume of 50 μl) was incubated with 10 μl (equivalent to 0.1 mg microsomal protein/100 μl incubation mixture) of commercially-prepared microsomes expressing human cDNA for one of the following enzymes: CYP1A2, CYP2D6, CYP3A4, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, or CYP2C19; these cells were characterised for protein and P450 content by the manufacturer.

### 3.2.5 Inhibition of CYP2D6 by quinidine.

Quinidine (6, 3, 1.5, 0.75, 0.375, or 0 μM, added in a volume of 10 μl) was pre-incubated for 10 min with 10 μl of microsomes [either human liver microsomes (0.04 mg microsomal protein/100 μl incubation medium) or microsomes expressing human CYP2D6 (0.1 mg protein/ 100 μl incubation

medium)], 25 µl of the NADPH-generating system, and 5 µl of buffer. Following pre-incubation, mCPP (100 µM final concentration, in a volume of 50 µl) was added and incubation continued for a further 10 min. An inhibition constant ($K_i$) for quinidine was determined using a range of concentrations of mCPP (5, 10, 20, 40, 80, 160, and 320 µM) and a fixed concentration of quinidine (4 µM). The data were analyzed by nonlinear regression analysis fit to the equation:

$$V = (V_{max} \cdot S) / [S + K_m \cdot (1 + [I]/K_i)]$$

where V = reaction velocity, $V_{max}$ = maximum velocity, S = substrate concentration, $K_m$ = substrate concentration at which reaction velocity equals 50% of $V_{max}$, and [I] = concentration of inhibitor.

## 3.3 RESULTS

### 3.3.1 Correlation with human liver microsome enzyme activities

The rate of formation of OH-mCPP (nmol/min/mg protein) in human liver microsomes correlated significantly (r=0.69, p=0.0034) with CYP2D6 activity (Figure 3.2). Correlations with all other CYP enzymes failed to reach significance: (CYP1A2 (r=0, p=0.9579), CYP2A6 (r=0.21, p=0.4390), CYP2C19 (r=0.18, p=0.5156), CYP2E1 (r=0.06, p=0.8081, CYP3A4 (r=0.27, p=0.3119), CYP4A11 (r=0.19, p=0.4858).

### 3.3.2 Metabolism with single cDNA-expressed human CYP enzymes

Incubations with single cDNA-expressed human CYP enzymes resulted in OH-mCPP production following incubations with CYP2D6 only (1.78 ± 0.06 nmol/min·mg protein, n=3). Figure 3.3 shows representative chromatographs from incubations of mCPP with CYP2D6.

### 3.3.3 Inhibition of CYP2D6 by quinidine

The results of the mCPP incubations in the presence of the CYP2D6 inhibitor quinidine indicate a concentration-dependent inhibition of OH-mCPP production in both human liver microsomes and cells expressing CYP2D6 (Figure 3.4). The apparent $K_i$ was calculated to be 0.33 ± 0.10 μM (n=3). Incubations with the CYP3A4 inhibitor ketoconazole did not decrease the production of OH-mCPP (data not presented).

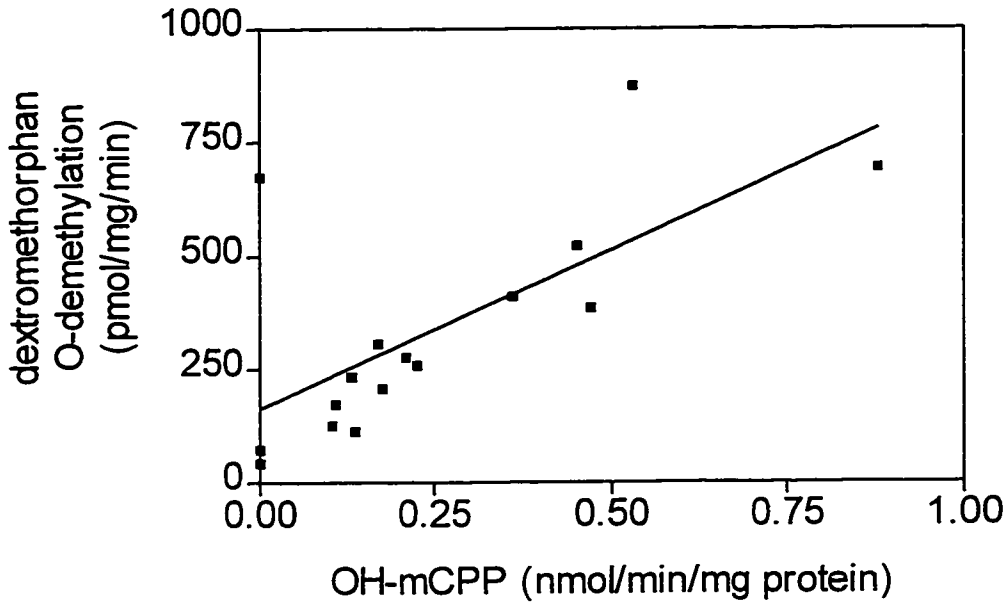



FIGURE 3.2. Correlation of OH-mCPP production with CYP2D6 activity in microsomes from 16 human livers (r=0.69, p=0.0034).

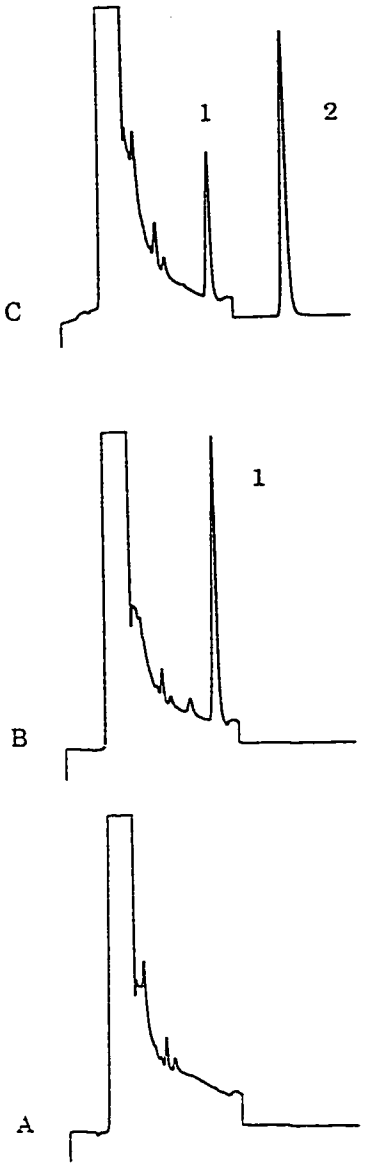



FIGURE 3.3. Representative chromatographs from microsomal incubations with cells expressing human CYP2D6. A) Blank incubation medium. B) Medium spiked with 20 μM OH-mCPP [approximately 570 ng in 100 μl incubation; peak 1, retention time (RT) 5.19 min]. C) Medium from microsomes incubated with 100 μM mCPP (approximately 2.33 μg in 100μl incubation) showing a peak for mCPP (peak 2, RT 7.77 min), and OH-mCPP (peak 1, RT 5.19 min).

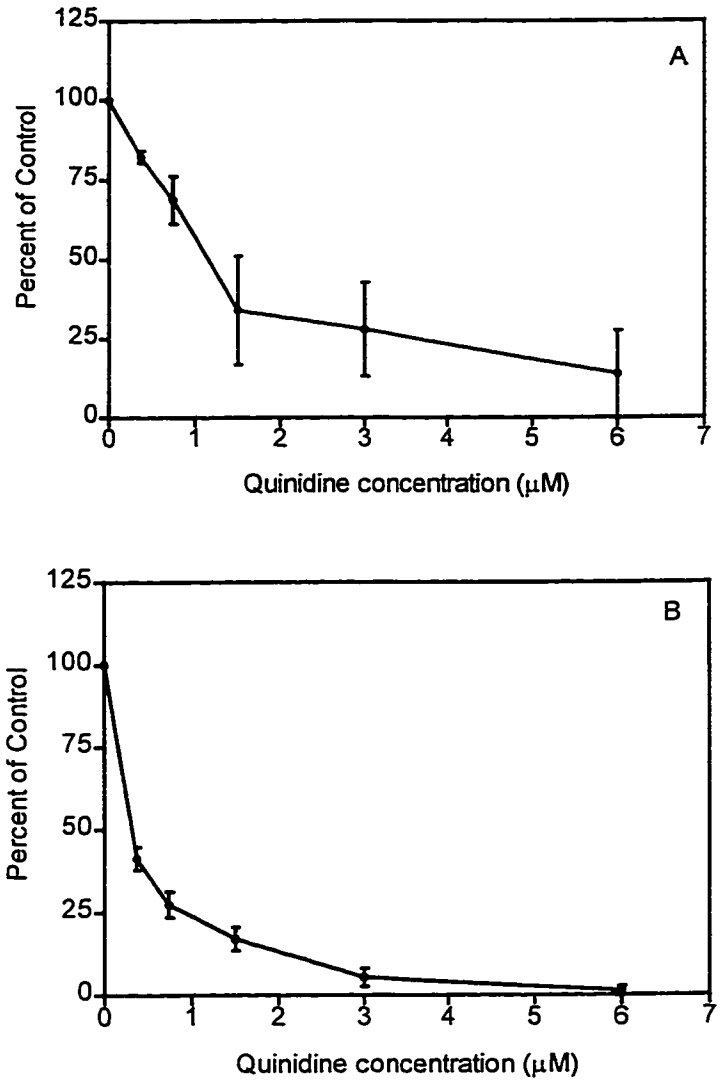



FIGURE 3.4 Metabolism of mCPP to OH-mCPP in the presence of the specific CYP2D6 inhibitor quinidine. (A) Incubations with human liver microsomes. (B) Incubations with microsomes expressing human CYP2D6 only. Inhibition is expressed as per cent of control activity, and each point represents the average of three separate experiments (mean ± SEM).

## 3.4 DISCUSSION

The present experiments employed human liver microsomes and microsomes expressing individual human CYP enzymes to identify the major CYP enzyme involved in the production of OH-mCPP from mCPP. First, mCPP was incubated with a panel of human liver microsomes prepared from 16 different donors and characterized for the activities of several CYP isoforms. The rate of OH-mCPP formation correlated with CYP2D6 activity only, indicating that CYP2D6 was the most likely isoform involved in this reaction. The studies in human microsomes were corroborated using recombinant human CYP enzymes, and mCPP metabolism to OH-mCPP was found to be catalyzed by CYP2D6 only. Finally, the selective CYP2D6 inhibitor quinidine was used to assess its effects on OH-mCPP production in both human liver microsomes and cDNA-expressed CYP2D6. In both cases, quinidine resulted in a concentration-dependent inhibition of OH-mCPP production, with a $K_i$ within the range of 0.06 - 0.40 μM, which has been reported in the literature for quinidine inhibition of CYP2D6 (Brosen & Gram, 1989; Bourrie et al., 1996).

The present results confirm that CYP2D6 is the major isoform involved in the p-hydroxylation of mCPP to OH-mCPP, and support clinical findings of decreased mCPP metabolism in poor metabolizers of dextromethorphan (Barbhaiya et al., 1996), and increased mCPP levels when its parent compounds are co-administered with inhibitors of CYP2D6 (Ellingrod & Perry, 1995; Yasui et al., 1995; Marino et al., 1996). Considering the potential psychopharmacological

consequences of increased mCPP levels, these results indicate that caution should be exercised in combining drugs that have mCPP as a metabolite with other substrates and/or inhibitors of CYP2D6. A wide variety of drugs from several different classes are CYP2D6 substrates or inhibitors (Ereshefsky et al., 1995; Shen 1995). Common CYP2D6 substrates include cardiovascular and psychoactive agents, which often have narrow therapeutic windows, and codeine, which lacks analgesic effects if it cannot be converted to morphine *via* CYP2D6 (Cholerton, 1992; Desmeules et al., 1991). The potential for drug interactions through competitive inhibition of CYP2D6 exists with CYP2D6 substrates and drugs which have mCPP as a metabolite. Furthermore, patients who are PMs of dextromethorphan may have higher than expected plasma levels of mCPP due to slowed metabolism. The present results indicate the need to be aware of potential interactions with CYP2D6 when prescribing nefazodone, trazodone, etoperidone, or mepiprazole.

## 3.5 REFERENCES


Barbhaiya, R.H., Buch, A.B., Greene, D.S. (1996a). Single and multiple dose pharmacokinetics of nefazodone in subjects classified as extensive and poor metabolizers of dextromethorphan. *Br J Clin Pharmacol* **42:**573-581.

Barbhaiya RH, Dandekar KA, Greene DS (1996b) Pharmacokinetics, absolute bioavailability, and disposition of [$^{14}$C]nefazodone in humans. *Drug Metab Dispos* **24**:91-95.

Barbhaiya RH, Shukla UA, Chaikin P, Greene DS, Marathe PH (1996c) Nefazodone pharmacokinetics: assessment of nonlinearity, intra-subject variability and time to attain steady-state plasma concentrations after dose escalation and de-escalation. *Eur J Clin Pharmacol* **50**:101-107.

Bertz RJ, Granneman GR (1997) Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet* **32**:210-258.

Bourrie M, Meunier V, Berger Y, Fabre G (1996) Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *J Pharmacol Exp Ther* **277**:321-332.

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

Buch AB, Shukla UA, Pittman K, Barbhaiya RH (1993) Pharmacokinetics of nefazodone in extensive and poor metabolizers of dextromethorphan. *Pharm Res* **10**:S314.

Caccia S, Ballabio M, Samanin R, Zanini MG, Garattini S (1981) (-)-m-Chlorophenylpiperazine, a central 5-hydroxytryptamine agonist, is a metabolite of trazodone. *J Pharm Pharmacol* **33**:477-478.

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

Conn JP, Sanders-Bush E (1987) Relative efficacies of piperazines at the phosphoinositide hydrolysis-linked serotonergic (5-HT-2 and 5-HT-1C) receptors. *J Pharmacol Exp Ther* **242:**552-557.

Cowen PJ, Sargent PA, Williams C, Goodall EM, Orlikov AB (1995) Hypophagic, endocrine and subjective responses to m-chlorophenylpiperazine in healthy men and women. *Hum Psychopharmacol* **10**:385-391.

Desmeules J, Gascon MP, Dayer P, Magistris M (1991). Impact of environmental and genetic factors on codeine analgesia. *Eur J Clin Pharmacol* **41**:23-26.

Ellingrod VL, Perry PJ (1995) Nefazodone: A new antidepressant. *Am J Health Syst Pharm* **52**:2799-2812.

Ereshefsky L, Riesenman C, Lam YMF (1995) Antidepressant drug interactions and the cytochrome P450 system. The role of cytochrome P450 2D6. *Clin Pharmacokinet* **29(suppl 1)**:10-19.

Fiorella D, Rabin RA, Winter JC (1995) The role of the 5-HT2A and 5-HT2C receptors in the stimulus effects of m-chlorophenylpiperazine. *Psychopharmacol* **119**:222-230.

Fong MH, Garattini S, Caccia S (1982) 1-m-Chlorophenylpiperazine is an active metabolite common to the psychotropic drugs trazodone, etoperidone, and mepiprazole. *J Pharm Pharmacol* **34**:674-675.

Germine M, Goddard AW, Sholomskas DE, Woods SW, Charney DS, Heninger GR (1994) Response to meta-chlorophenylpiperazine in panic disorder patients an healthy subjects: Influence of reduction in intravenous dosage. *Psychiatry Res* **54**:115-133.

Gibson EL, Barnfield AMC, Curzon G (1994) Evidence that mCPP-induced anxiety in the plus-maze is mediated by postsynaptic 5-HT(2C) receptors but not by sympathomimetic effects. *Neuropharmacol* **33**:3-4.

Ishida M, Otani, K., Kaneko, S., Ohkubo, T., Osanai, T., Yasui, N., Mihara, K., Higuchi, H., Sugawara, K. (1995). Effects of various factors on steady state plasma concentrations of trazodone and its active metabolite *m*-chlorophenylpiperazine. *Int Clin Psychopharmacol* **10**:143-146.

Kahn RS, Asnis GM, Wetzler S, van Praag HM (1988) Neuroendocrine evidence for serotonin receptor hypersensitivity in panic disorder. *Psychopharmacol* **96**:360-364.

Kahn RS, Wetzler S (1991) m-Chlorophenylpiperazine as a probe of serotonin function. *Biol Psychiatry* **30:**1139-1166.

Kalus O, Wetzler S, Kahn RS, Asnis GM, van Praag HM (1992) A dose-response study of intravenous m-chlorophenylpiperazine in normal subjects. *Psychopharmacol* **106**:388-290.

Kaul S, Shukla UA, Barbhaiya RH (1995) Nonlinear pharmacokinetics of nefazodone after escalating single and multiple oral doses. *J Clin Pharmacol* **35**:830-839.

Krystal JH, Seibyl JP, Price LH, Woods SW, Heninger GR, Aghajanian GK, Charney DS (1993) m-Chlorophenylpiperazine effects in neuroleptic-free schizophrenic patients. *Arch Gen Psychiatry* **50**:624-635.

Maes M, Westenberg H, Vandoolaeghe E, Demedts P, Wauters A, Neels H, Meltzer HY (1997) Effects of trazodone and fluoxetine in the treatment of major depression: Therapeutic pharmacokinetic and pharmacodynamic interactions through formation of meta-chlorophenylpiperazine. *J Clin Psychopharmacol* **17**:358-364.

Marek GJ, McDougle CJ, Price LH, Seiden LS (1992) A comparison of trazodone and fluoxetine: implications for a serotonergic mechanism of antidepressant action. *Psychopharmacol* **109:** 2-11.

Marino MR, Langenbacher KM, Uderman HD (1996) Interaction of nefazodone (N) and fluoxetine (F) [abstract]. *Clin Pharmacol Ther* **59**:180.

Mayol RF, Cole CA, Colson KE, Kerns EH (1994a) Isolation and identification of the major urinary metabolite of *m*-chlorophenylpiperazine in the rat. *Drug Metab Dispos* **22**:171-174.

Mayol RF, Cole CA, Luke GM, Colson KL, Kerns EH (1994b) Characterization of the metabolites of the antidepressant drug nefazodone in human urine and plasma. *Drug Metab Dispos* **22**:304-311.

Melzacka M, Boksa J, Maj J (1979) 1-(*m*-Chlorophenyl)piperazine: a metabolite of trazodone isolated from rat urine. *J Pharm Pharmacol* **31**:855-856.

Murphy DL, Mueller EA, Hill JL, Tolliver TJ, Jacobsen FM (1989): Comparative anxiogenic, neuroendocrine, and other physiologic effects of m-chlorophenylpiperazine given intravenously or orally to healthy volunteers. *Psychopharmacol* **98:**275-282.

Otani K, Mihara K, Yasui N, Ishida M, Kondo T, Tokinaga N, Ohkubo T, Osanai T, Sugawara K, Kaneko S (1997) Plasma concentrations of trazodone and m-chlorophenylpiperazine at steady state can be predicted from those after an initial dose of trazodone. *Prog Neuropsychopharmacol Biol Psychiatry* **21**:239-244.

Samanin R, Mennini T, Ferraris A, Bendotti C, Borsini F, Garattini S (1979) m-Chlorophenylpiperazine: A central serotonin agonist causing powerful anorexia in rats. *Naunyn Schmiedeberg's Arch Pharmacol* **308**:159-163.

Shen WW (1995) Cytochrome P450 monooxygenase and interactions of psychotropic drugs: A five year update. *Int J Psychiatry Med* **25**:277-290.

Taylor DP, Carter RB, Eison AS, Mullins UL, Smith HL, Torrente JR, Wright RN, Yocca FD (1995) Pharmacology and neurochemistry of nefazodone, a novel antidepressant drug. *J Clin Psychiatry* **56 (suppl 6)**:3-11.

Walsh AES, Smith KA, Oldman AD, Williams C, Goodall EM, Cowen PJ (1994) m-Chlorophenylpiperazine decreases food intake in a test meal. *Psychopharmacol* **116**:120-122.

# Chapter 4

## The metabolism of NEF and the active metabolite OH-NEF

## 4.1 INTRODUCTION

NEF was rationally designed after the success of trazodone to be a 5-$HT_{2A}$ antagonist, but without the $\alpha_1$-adrenergic antagonism of trazodone, which contributes to its sedative effects (Taylor et al., 1986; 1995). NEF also inhibits the uptake of 5-HT and NE, but has very little effect on other neurotransmitter receptors, and thus a very low incidence of side effects (Taylor et al., 1995; Marcus, 1996; Robinson et al., 1996). It is as effective as imipramine (IMI), fluoxetine (FLU), and paroxetine (PRX) in placebo-controlled clinical trials in the treatment of all types of depression (Montgomery, 1996), and appears to have a particular therapeutic advantage in treating anxiety and agitation associated with depression (Fawcett et al., 1995; Malik, 1996; Montgomery, 1996; Nutt, 1996).

The present experiments were designed to investigate the metabolism of NEF to its major active metabolites, hydroxy-nefazodone (OH-NEF), triazoledione (TD) and m-chlorophenylpiperazine (mCPP). A proposed metabolic scheme for the formation of these metabolites is shown in Figure 4.1. The formation of OH-NEF (Figure 4.1B) is through hydroxylation of the ethyl side chain of the triazolone nucleus. It is present in the plasma at levels approximately one-third of those of NEF (Barbhaiya et al., 1995; Preskorn et al., 1995), but is considered to have similar clinical efficacy to NEF (Ellingrod & Perry, 1995), and, like NEF, is a 5-$HT_{2A}$ and 5-$HT_{2C}$ antagonist (Taylor et al., 1995).

Another of NEF's major metabolites, TD (Figure 4.1C), is formed from OH-NEF, but the mechanism of this formation is currently unknown (Mayol et al., 1994b). TD inhibits the 5-$HT_{2A}$ receptor more selectively, but with only 15-30% of the potency of NEF (Mayol et al., 1994b; Taylor et al., 1995), and does not block the uptake of 5-HT (Ellingrod & Perry, 1995). However, TD does reach plasma levels approximately 10 times those of NEF (Preskorn et al., 1995; Barbhaiya et al., 1996a), and therefore probably also contributes to NEF's efficacy.

The third of NEF's main metabolites, mCPP, is formed directly from N-dealkylation of the piperazinyl nitrogen of either NEF or OH-NEF (Mayol et al., 1994b; see Figure 4.1D). It is then further hydroxylated to p-OH-mCPP (Figure 4.1E) and excreted in the urine as glucuronide and sulfate conjugates (Yamato et al., 1974b; Melzacka et al., 1979; Mayol et al., 1994a). Although mCPP is a 5-$HT_{2C}$ agonist and 5-$HT_{2A}$ antagonist (Conn & Sanders-Bush, 1987; Fiorella et al., 1995), it may not have any clinically significant effects with NEF treatment since mCPP normally accumulates at levels only one-tenth to one one-hundredth those of NEF, and has a half life of only 4-9 h (Barbhaiya et al., 1995, 1996a; Kaul et al, 1995; Preskorn et al., 1995). In addition, NEF and OH-NEF are antagonists at the 5-$HT_{2C}$ receptor, and thus probably oppose the effects of mCPP under normal circumstances (Preskorn et al., 1995).

Clinical data suggest that NEF may be an inhibitor of CYP3A4 to a significant extent (Richelson, 1997). Pharmacokinetic interactions with CYP3A4 substrates such as triazolam, alprazolam (Greene et al., 1995; Barbhaiya et al.,

1995; Kroboth et al., 1995), carbamazepine (Ashton et al., 1996), cyclosporine (Helms-Smith et al., 1996), terfenadine and astemizole (Ellingrod & Perry, 1995; Robinson et al., 1996) have been reported.

Current *in vitro* evidence also suggests that NEF is a strong inhibitor of CYP3A4 (Ellingrod & Perry, 1995; Marcus, 1996; von Moltke et al., 1996a; 1996b) and a very weak *in vitro* inhibitor of CYP1A2 (von Moltke et al., 1996c) and CYP2D6 (Schmider et al., 1996), as measured by the ability of NEF to inhibit marker reactions for these enzymes. The active metabolite OH-NEF is also a potent *in vitro* inhibitor of CYP3A4, as measured by the ability to inhibit the formation of 4-OH-alprazolam (von Moltke et al., 1996a). In addition, ketoconazole and antibodies against rat cytochrome P450-3A1 (analog of human CYP3A4) inhibit the formation of all metabolites of NEF *in vitro,* suggesting that NEF is also a substrate for CYP3A4 (von Moltke et al., 1996b).

However, several key experiments in the elucidation of the metabolism of NEF and its metabolites have not been completed, and the individual metabolic pathways leading to the various metabolites have not been investigated in detail. Given the importance of NEF's metabolites in contributing to its clinical efficacy, such an investigation is of interest. Identification of the isoforms involved in a drug's metabolism is best accomplished using several lines of evidence, and key methods for the elucidation of the roles of the CYP enzymes in the oxidation of a compound *in vitro* have been identified (Guengerich, 1996). These methods include correlations of drug oxidation with marker reactions in human liver microsomes, attenuation of drug oxidation by selective chemical inhibitors or

specific antibodies, and oxidation by recombinant P450 vector systems (Guengerich, 1996; Iwatsubo et al., 1997). Therefore, the present experiments were designed to investigate the *in vitro* metabolism of NEF to its active metabolites using several convergent lines of evidence. Since the metabolites may play a role in the clinical efficacy of NEF, the metabolism of OH-NEF was also investigated. An HPLC method for the quantification of NEF and its metabolites in the microsomal incubation medium was developed. Finally, possible drug-drug interactions of NEF with pindolol were investigated, since the combination of antidepressants with pindolol is currently showing promise as a new way to improve antidepressant efficacy (Artigas et al., 1996).

Pindolol, a 5-$HT_{1A}$/β-adrenergic antagonist, has been shown to accelerate the onset of efficacy of certain antidepressant drugs (Artigas et al., 1996; Tome et al., 1997). The rationale behind its use comes from studies which have found a decrease in the firing rate of dorsal raphé 5-HT neurons during the initial days of antidepressant therapy, which is thought to be due to the activation of inhibitory 5-$HT_{1A}$ somatodendritic autoreceptors by the antidepressant-induced increase in extracellular 5-HT (Blier et al., 1987; 1990; Briley & Moret, 1993; Artigas et al., 1996). This attenuation of firing is temporary, and normal firing rates recover within approximately 2 weeks, which is thought to reflect autoreceptor desensitization, and interestingly, coincides with the onset of antidepressant efficacy (Blier et al., 1987; 1990; Briley & Moret, 1993; Artigas et al., 1996). It was theorized that if this initial attenuation in firing could be

prevented by a pre-synaptic 5-$HT_{1A}$ antagonist, then the onset of antidepressant efficacy could be accelerated (Artigas et al., 1996). Therefore, pindolol has often been co-prescribed, particularly with the SSRIs, to increase the therapeutic efficacy of these drugs.

Recently, an open-label trial of NEF combination therapy with pindolol in twenty outpatients showed dramatic rates of remission, and improvement of depressive symptomology after only 2-4 days of treatment (Bakish et al., 1997). The combination of NEF with pindolol and tryptophan has also been shown to be effective in the treatment of recurrent major depressive disorder, within one week of initiation (Dursun et al., 1997). Since the combination of NEF and pindolol shows promise for the improved treatment of depression, it is important to assess the risks of drug-drug interactions occurring with this drug combination. Little is known about the CYP-mediated metabolism of pindolol, except that it is a competitive inhibitor of CYP2D6 *in vitro* with a $K_i$ value of 60 μM (Otton et al., 1984). Clinically, it has been found to increase plasma levels of thioridazine, but not haloperidol, phenytoin, or phenobarbital (Greendyke and Guyla, 1988). Therefore, considering the potential therapeutic usefulness of the combination of NEF and pindolol, the effect of this drug combination on NEF metabolites was investigated *in vitro*.

## 4.2 METHODS

### 4.2.1 Development of an HPLC method for the detection of NEF, OH-NEF, TD, and mCPP in incubation medium

A 30 μl aliquot of the incubation medium (following the addition of acetonitrile and centrifugation, as described in Chapter 1, section 1.4.3) was injected into a high pressure liquid chromatographic (HPLC) system with a Waters model 510 pump linked to a Waters Intelligent Sample Processor model 710B. Detection was performed using a Waters Lambda Max Model 481 variable-wavelength UV detector linked to a Hewlett Packard model 3392A integrator.

The HPLC mobile phase consisted of acetonitrile and ammonium acetate buffer [100:98:2 ml (v/v) of acetonitrile: distilled $H_2O$: 1M ammonium acetate] adjusted to pH 5.4 with acetic acid. The flow-rate was 1.5 ml/min. A Phenomenex Hypersil CN (250 x 4.6 mm I.D., 5 μm particle size) column was used and was coupled to a guard column which was packed with the same material as the analytical column. Detection was performed using UV absorbance at 254 nm.

### 4.2.2 Nefazodone Metabolism

*4.2.2.1 Enzyme concentration curves*

The formation of OH-NEF, TD, mCPP and OH-mCPP from NEF (100 μM) was assessed using different concentrations of human liver microsomes in the

incubation. NEF was incubated with either 210, 105, 52.5, or 26.25 μg microsomal protein per 100 μl of incubation mixture.

#### *4.2.2.2 Time course of NEF metabolite formation*

NEF (500 μM) was incubated with human liver microsomes (0.02 mg microsomal protein/100 μl incubation mixture) under the conditions described in Chapter 1, Section 1.4.3. Incubations were terminated after either 5, 10, 20, 30 or 60 min of incubation time, and the incubation medium was assayed for the formation of mCPP and OH-NEF.

#### *4.2.2.3 Nefazodone concentration curves*

Varying concentrations of NEF (125, 250, 500, or 1000 μM) were incubated with human liver microsomes (0.02 mg microsomal protein / 100 μl incubation mixture) for 10 min to assess the effect of NEF concentration on the rate of metabolite formation and to determine appropriate substrate concentrations for the inhibition and correlation studies.

#### *4.2.2.4 Correlation with human liver microsome enzyme activities*

NEF (500 μM) was incubated with microsomes (0.02 mg microsomal protein / 100 μl incubation mixture) prepared from a panel of 16 human livers characterized for their catalytic activity as described in General Methods. Correlations were calculated using GraphPad™ Prism.

#### *4.2.2.5 Metabolism with single cDNA-expressed human CYP enzymes*

NEF (100 μM, added in a volume of 50 μl) was incubated with 10μl (equivalent to 0.1 mg microsomal protein / 100 μl incubation mixture) of commercially-prepared microsomes expressing human cDNA for one of the following enzymes: CYP1A2, CYP2A6, CYP2D6, CYP3A4, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, or CYP2C19; these microsomes were also characterised for protein and CYP content.

#### *4.2.2.6 Studies on the inhibition of metabolism of NEF*

Human liver microsomes were pre-incubated for 10 min with either ketoconazole (25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, or 0.0 μM), pindolol (240, 120, 60, 30, 15, 0 μM), or quinidine (8, 4, 2, 1, 0.5, 0 μM). NEF (500 μM) was added and the mixture was incubated for a further 10 min.

### 4.2.3 OH-Nefazodone Metabolism

#### *4.2.3.1 OH-NEF concentration curves*

Varying concentrations of OH-NEF (31.25, 62.5, 125, 250, 500 μM) were incubated with human liver microsomes (0.02 mg microsomal protein/100 μl incubation mixture) for 10 min to assess the rate of metabolite formation at different substrate concentrations.

#### 4.2.3.2 *Correlations with human liver microsome enzyme activities.*

OH-NEF (500 μM) was incubated with microsomes (0.02 mg microsomal protein / 100 μl incubation mixture) prepared from a panel of 16 human livers characterized for their catalytic activity as described in General Methods. Correlations for metabolite formation and enzyme activity were calculated using GraphPad™ Prism.

#### 4.2.3.3 *Metabolism with single cDNA-expressed human CYP enzymes.*

OH-NEF (100 μM, added in a volume of 50 μl) was incubated with 10μl (equivalent to 0.1 mg microsomal protein / 100 μl incubation mixture) of commercially-prepared microsomes expressing human cDNA for one of the following enzymes: CYP1A2, CYP2D6, CYP3A4, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, or CYP2C19; these microsomes were also characterised for protein and P450 content.

#### 4.2.3.4 *Studies on inhibition of metabolism of OH-NEF.*

OH-NEF (200 μM) was incubated with human liver microsomes and varying concentrations of either the specific CYP3A4 inhibitor ketoconazole (25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, or 0.0 μM) or the specific CYP2D6 inhibitor quinidine (8, 4, 2, 1, 0.5, 0.0 μM). The microsomes were pre-incubated with the inhibitor for 10 min, after which OH-NEF was added and the incubation continued for a further 10 min.

## 4.3 RESULTS

### 4.3.1 HPLC method

The HPLC method described resulted in the rapid and sensitive detection of NEF, OH-NEF, TD, mCPP, and OH-mCPP in the incubation medium. Typical chromatographs from spiked incubation media and incubations of NEF with human liver microsomes are shown in Figure 4.2. Inter-assay coefficients of variation (CV) were determined by comparing spiked standards across five consecutive assays, and calculating the CV by dividing the standard deviation by the mean. The interassay CVs for OH-mCPP, TD, mCPP, and OH-NEF were 8.19, 10.78, 1.72, and 9.38% for 10 μM standards, and 7.90, 9.48, 1.34, and 10.17% for 5 μM standards, respectively. As NEF was not quantified in the present experiments, no CV was calculated for NEF.

Intra-assay coefficients of variation were determined by carrying five separate standards through the assay procedure, and comparing variation within a single assay. CVs for intra-assay variations were 1.52, 1.88, 1.19, 5.16, and 7.70% for OH-mCPP, TD, mCPP, OH-NEF, and NEF, respectively for 10 μM standards, and 1.53, 5.41, 6.37, 4.99, and 5.65% for 5 μM standards.

The recovery of metabolites from the incubation media was calculated by comparing spiked samples in incubation medium taken through the assay procedure to spiked samples in buffer which were directly injected. Recoveries were virtually quantitative.

The sensitivity of the method was 31, 32, 14, 30, and 17 pg "on column" for NEF, OH-NEF, mCPP, TD, and OH-mCPP, respectively, at a 3:1 signal to noise ratio.

### 4.3.2 Nefazodone metabolism

#### *4.3.2.1 Enzyme concentration curves*

The effects of varying the concentrations of human liver microsomes on metabolite formation from NEF are presented in Figure 4.3. The first observation to note from this graph is that, as expected, there is less formation of the secondary metabolites TD and OH-mCPP with decreasing enzyme concentration. Thus, to enable a more specific investigation of the primary metabolic pathways to OH-NEF and mCPP, without potential interferences of further metabolism to secondary metabolites, a low enzyme concentration (approximately 0.021 mg protein/100 μl incubation) was chosen for subsequent investigations.

The second point to note is that the more enzyme that is present, the less OH-NEF is present. This is due to the fact that OH-NEF is formed directly from NEF, and is then further metabolized to mCPP and TD (see Figure 4.1). However, at lower enzyme concentrations, OH-NEF is not metabolized as quickly, and therefore, there is less formation of the secondary metabolites, mCPP and TD.

The third point is that there is considerable formation of mCPP at all enzyme concentrations, and that this formation increases with increasing enzyme concentration. This is likely due to mCPP formation occurring through several possible pathways, from NEF, OH-NEF, and possibly also triazoledione (see Figure 4.1).

Therefore, since different enzymes may be involved in the formation of the various metabolites of NEF and OH-NEF, and since secondary metabolism of the primary metabolites OH-NEF and mCPP might interfere with the interpretation of the experimental results, a low enzyme concentration was chosen for the experiments to minimize further metabolism of OH-NEF and mCPP.

#### *4.3.2.2 Time course of metabolite formation from NEF*

The production of OH-NEF and mCPP from NEF after varying incubation times is shown in Figure 4.4. It can be seen that metabolite formation is relatively linear up to 30 min incubation time, and therefore, a 10 min incubation period was used in all subsequent studies. The 10 min incubation was also chosen to avoid depletion of the substrate, which might occur at longer incubation periods.

#### *4.3.2.3 NEF concentration curves*

The effects of varying concentrations of NEF on metabolite formation are presented in Figure 4.5. Increasing concentrations of metabolites were seen

with increased substrate concentrations, which appeared to level off after approximately 500 μM. Therefore, a concentration of 500 μM was chosen for the correlation analysis, to avoid substrate depletion, yet not be at a saturating concentration.

#### *4.3.2.4 Correlation with human liver microsome enzyme activities*

At the concentrations of NEF (500 μM) and microsomes used, there was insignificant formation of TD or OH-mCPP, therefore, correlations were not calculated for these metabolites. The formation of **OH-NEF** correlated significantly with the human liver microsome activities of **CYP1A2** (r=0.5144, p=0.0415), **CYP2A6** (r=0.5308, p=0.0344), and **CYP3A4** (r=0.7922, p=0.0003) [Figure 4.6]. The formation of **mCPP** correlated significantly with the human liver microsome activities of **CYP2A6** (r=0.5385, p=0.0314), and **CYP3A4** (r=0.8425, p<0.0001) [Figure 4.7].

#### *4.3.2.5 Metabolism with single cDNA-expressed human CYP enzymes*

When NEF was incubated with microsomes expressing only CYP1A1, CYP1A2, CYP2A6, CYP2D6, CYP3A4, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, or CYP2C19, metabolite formation was seen with the CYP3A4 incubations only. Only OH-NEF (0.44 ± 0.12 nmol/min/mg protein) and mCPP (0.17 ± 0.01 nmol/min/mg protein) were formed.

#### *4.3.2.6 NEF Inhibition experiments*

Incubations of NEF with the CYP3A4 inhibitor ketoconazole resulted in a concentration-dependent inhibition of the formation of mCPP and OH-NEF (Figure 4.8) which was complete at a concentration of 25.6 μM ketoconazole for mCPP formation, and almost complete at 25.6 μM ketoconazole for OH-NEF formation.

Incubations with the CYP2D6 inhibitors quinidine (Figure 4.9) and pindolol (Figure 4.10) did not affect the production of OH-NEF and mCPP from NEF.

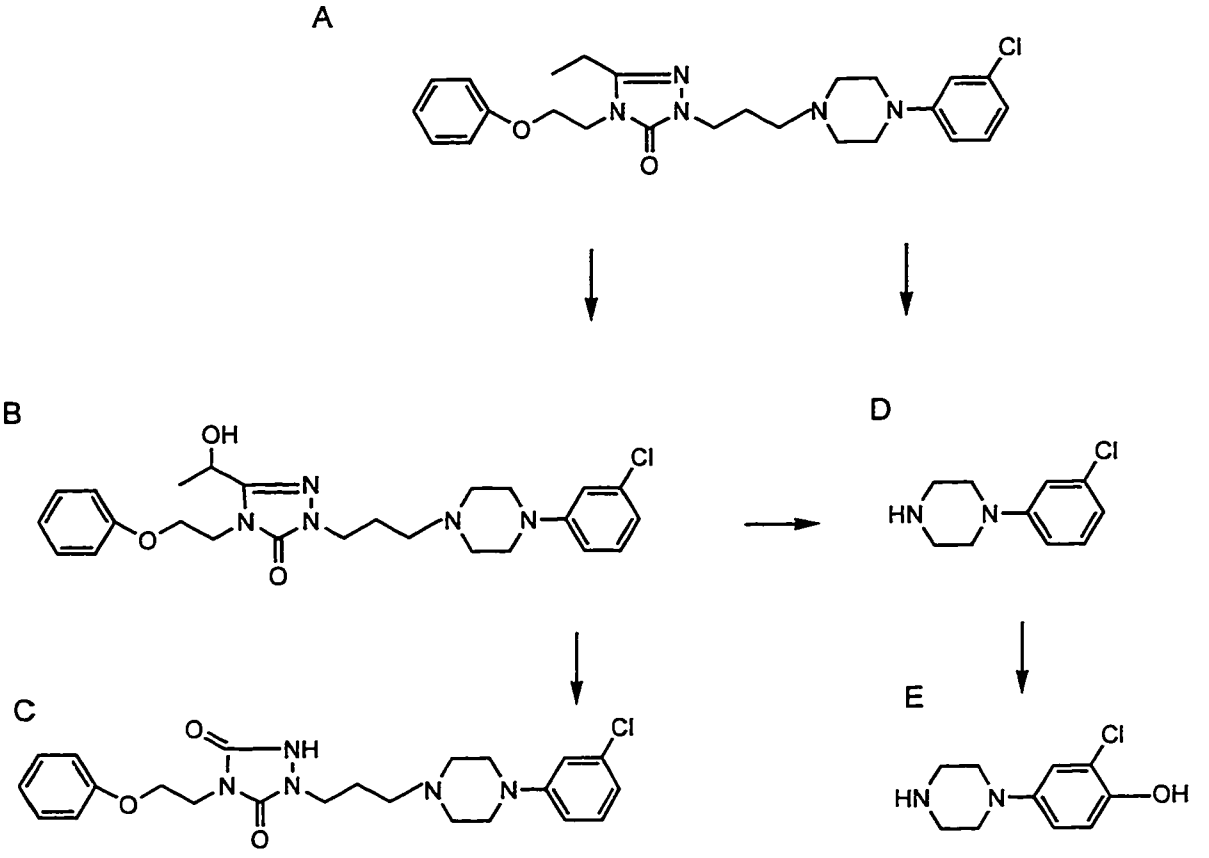



FIGURE 4.1 Chemical structures of NEF (A) and the metabolites OH-NEF (B), TD (C), mCPP (D) and OH-mCPP (E).

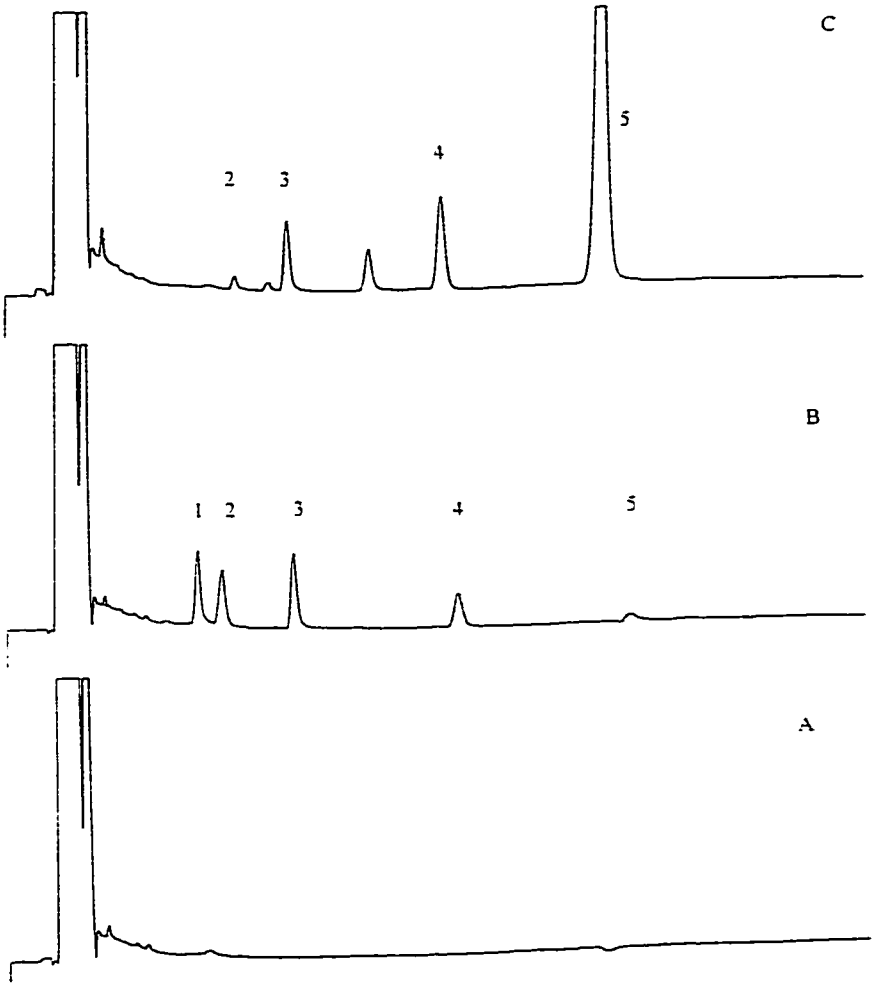



FIGURE 4.2 Typical HPLC chromatographs from microsomal incubations with human liver microsomes. A) Blank incubation medium. B) Incubation medium spiked with standard solutions of NEF and metabolites equivalent to 5 μM final concentration. The peaks and retention times (RT) are: NEF (253 pg, peak 5, RT 23.94 min), OH-NEF (261 pg, peak 4, RT 17.37), mCPP (116 pg, peak 3, RT 11.13), TD (247 pg, peak 2, RT 8.31) and OH-MCPP (142 pg, peak 1, RT 7.33). C) Incubation medium from samples of NEF (500 μM; approximately 25 ng/100μl incubation) incubated with human liver microsomes, showing peaks for the metabolites OH-NEF (peak 4), mCPP (peak 3), and TD (peak 2).

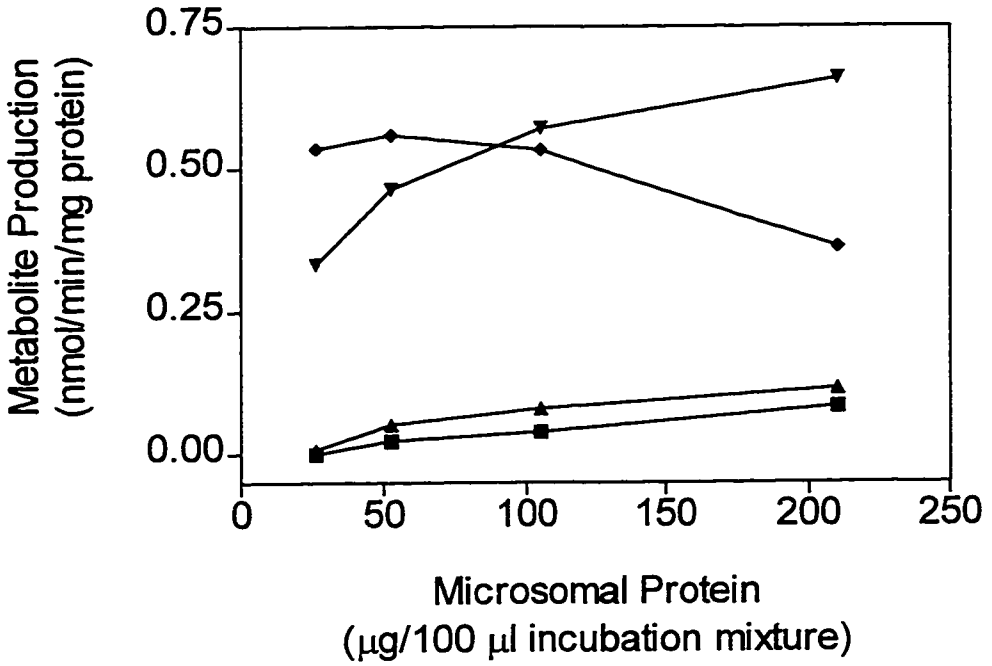



FIGURE 4.3. Effect of varying enzyme concentrations (x-axis) on the production of metabolites (y-axis, nmol/min/mg protein) from NEF (100 μM; n=1; ◆-OH-NEF; ▲-TD; ▼-mCPP; ■-OH-mCPP).

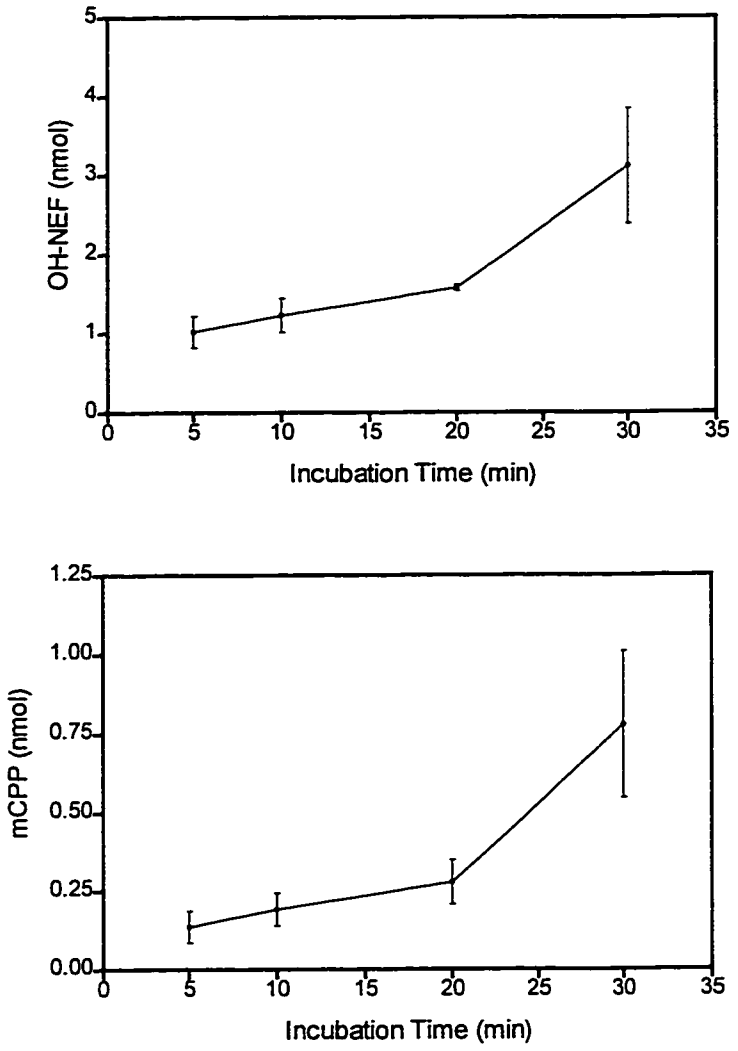



FIGURE 4.4. Effect of incubation time on the formation of OH-NEF (top) and mCPP (bottom) from NEF (500 μM) in human liver microsomes. Each point represents the mean ± SEM of three separate experiments.

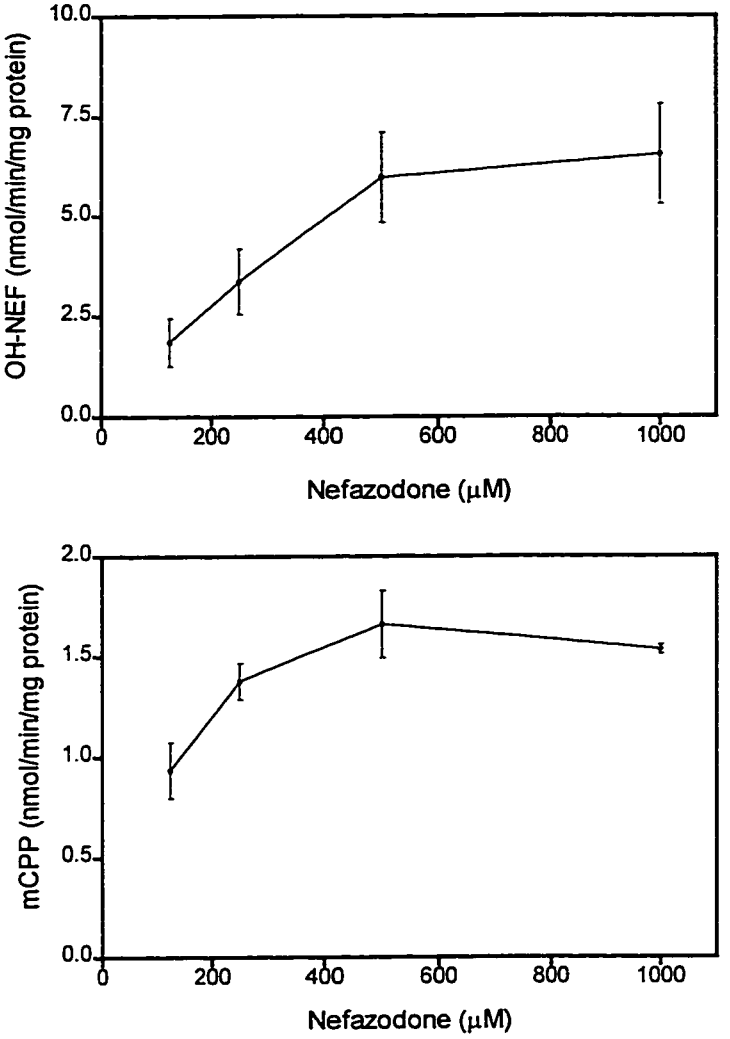



FIGURE 4.5 Effect of varying concentrations of NEF on the formation of OH-NEF (top) and mCPP (bottom) in human liver microsomes. Each point represents the mean ± SEM of three separate experiments.

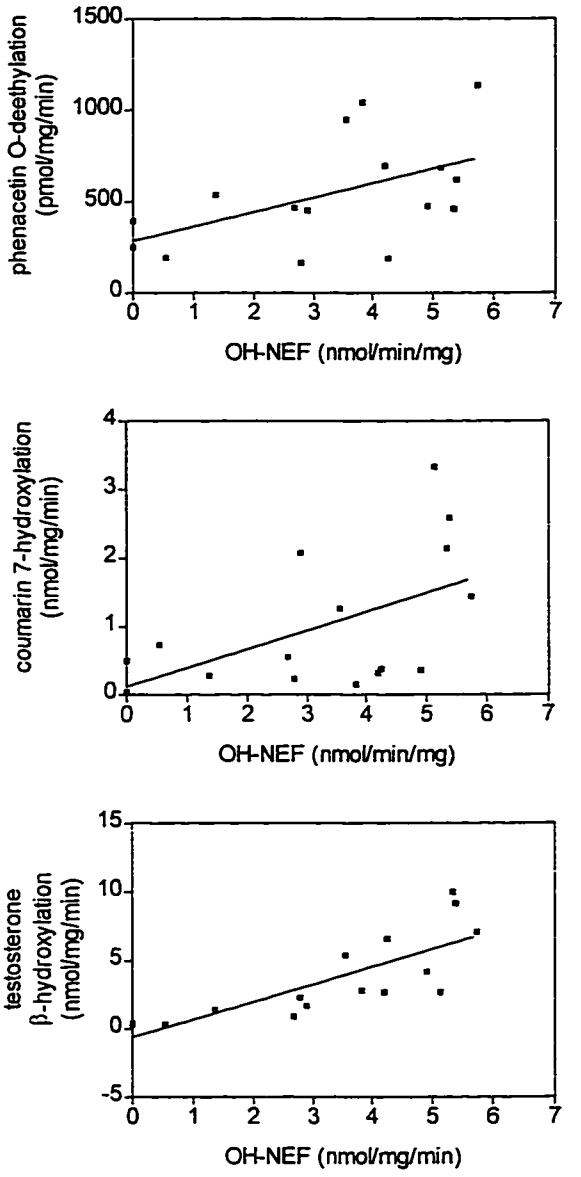



FIGURE 4.6 Significant correlation of NEF metabolism to OH-NEF with the activities of CYP1A2 (r=0.51, p=0.04, top), CYP2A6 (r=0.53, p=0.03, middle), and CYP3A4 (r=0.79, p=0.0003, bottom) in a panel of 16 human liver microsomes.

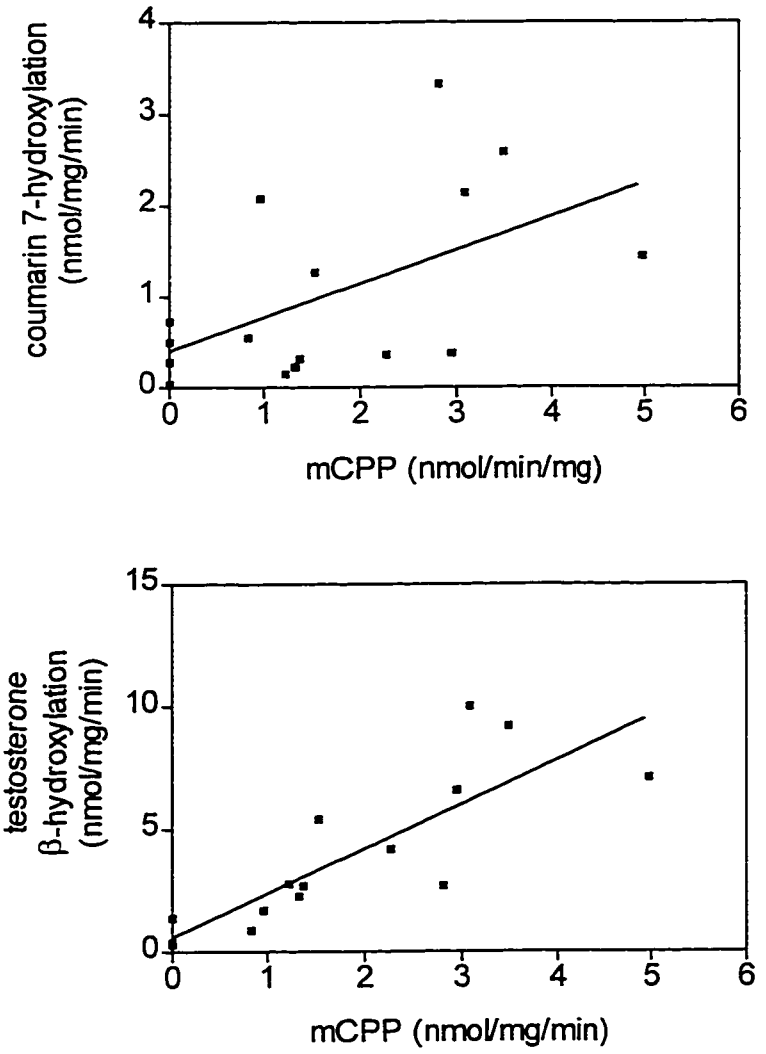



FIGURE 4.7 Significant correlation of NEF metabolism to mCPP with the activities of CYP2A6 (r=0.54, p=0.03, top) and CYP3A4 (r=0.84, p<0.0001, bottom) in a panel of 16 human liver microsomes.

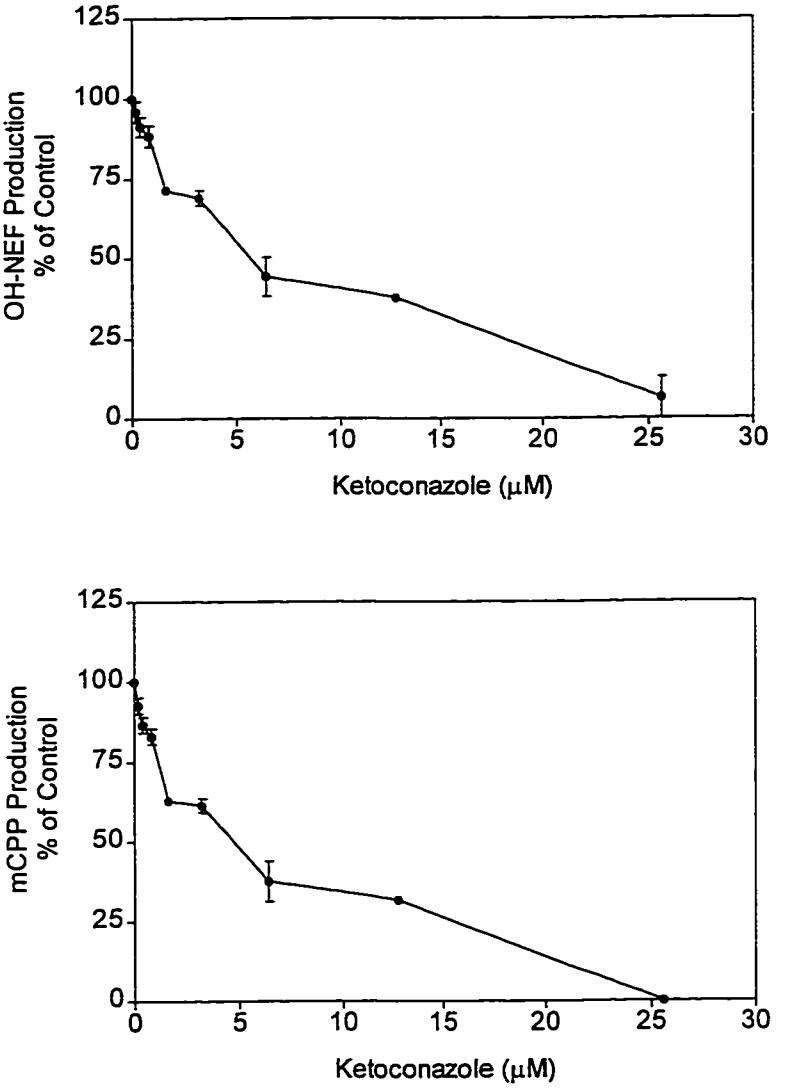



FIGURE 4.8 Effect of the specific CYP3A4 inhibitor ketoconazole on the metabolism of NEF (500 μM) to OH-NEF (top) and mCPP (bottom) in human liver microsomes. Each point represents the mean ± SEM for three separate experiments.

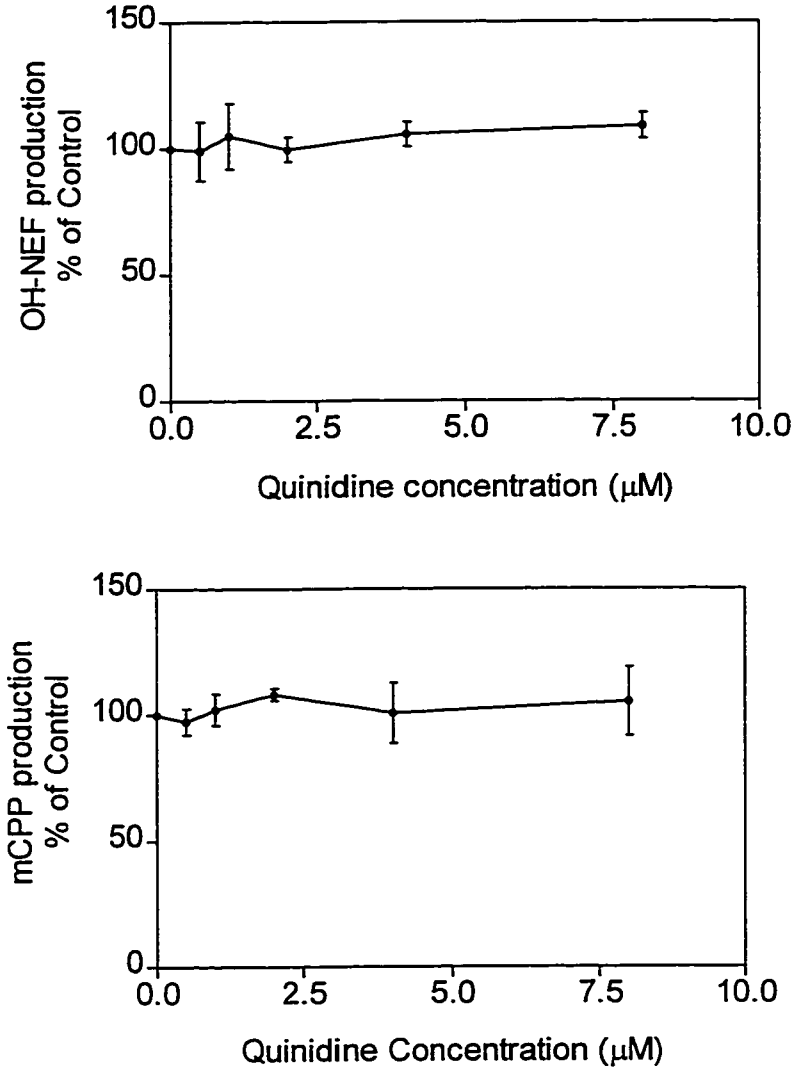



FIGURE 4.9 Effect of the specific CYP2D6 inhibitor quinidine on the metabolism of NEF (500 μM) to OH-NEF (top) and mCPP (bottom) in human liver microsomes. Each point represents the mean ± SEM for three separate experiments.

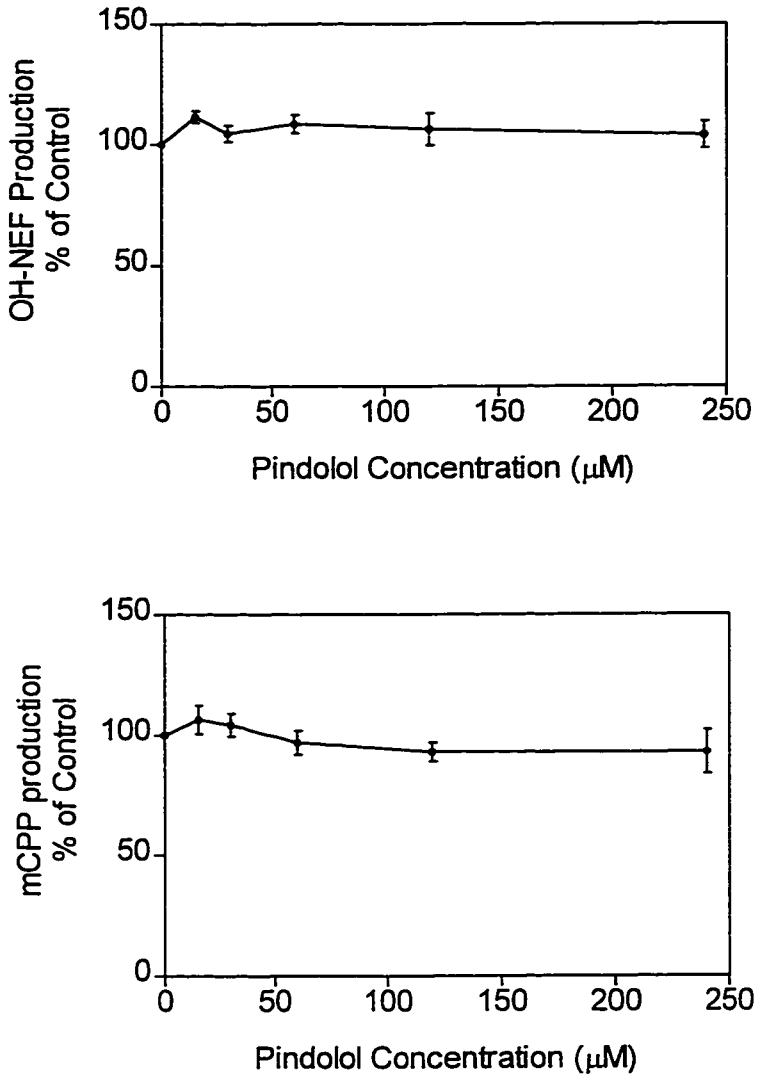



FIGURE 4.10 Effect of pindolol on the metabolism of NEF (500 μM) to OH-NEF (top) and mCPP (bottom) in human liver microsomes. Each point represents the mean ± SEM for three separate experiments.

### 4.3.3 OH-Nefazodone Metabolism

#### 4.3.3.1 *OH-NEF concentration curves*

The effects of varying concentrations of OH-NEF on substrate formation are presented in Figure 4.11. Increasing concentrations of metabolites were seen with increasing substrate concentrations, and this effect appeared to reach a plateau at approximately 500 μM.

#### *4.3.3.2 Correlations with human liver microsome enzyme activities*

The formation of TD and mCPP from OH-NEF (500 μM) were compared with CYP enzyme activities from 16 different human livers. The formation of **TD** correlated significantly with the human liver microsome activity of **CYP3A4** only ($r=0.876$, $p<0.0001$) [Figure 4.12]. The formation of **mCPP** correlated significantly with the human liver microsome activities of **CYP2A6** ($r=0.5253$, $p=0.0367$), and **CYP3A4** ($r=.8855$, $p<0.0001$) [Figure 4.13].

#### *4.3.3.3 Metabolism with single cDNA-expressed human CYP enzymes*

When OH-NEF was incubated with microsomes from cells expressing only one of the CYP enzymes CYP1A1, CYP1A2, CYP2A6, CYP2D6, CYP3A4, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, or CYP2C19, metabolite formation was seen with the CYP3A4 incubations only. Only TD (0.14 ± 0.04 nmol/min/mg protein) and mCPP (0.12 ± 0.02 nmol/min/mg protein) were formed.

#### *4.3.3.4 OH-NEF Inhibition experiments*

Incubations of OH-NEF with the specific CYP3A4 inhibitor ketoconazole resulted in a concentration-dependent inhibition of the formation of mCPP and OH-NEF (Figure 4.14) which was complete at a concentration of 25.6 μM ketoconazole. Incubations with quinidine did not result in a concentration-dependent inhibition of metabolite formation (Figure 4.15).

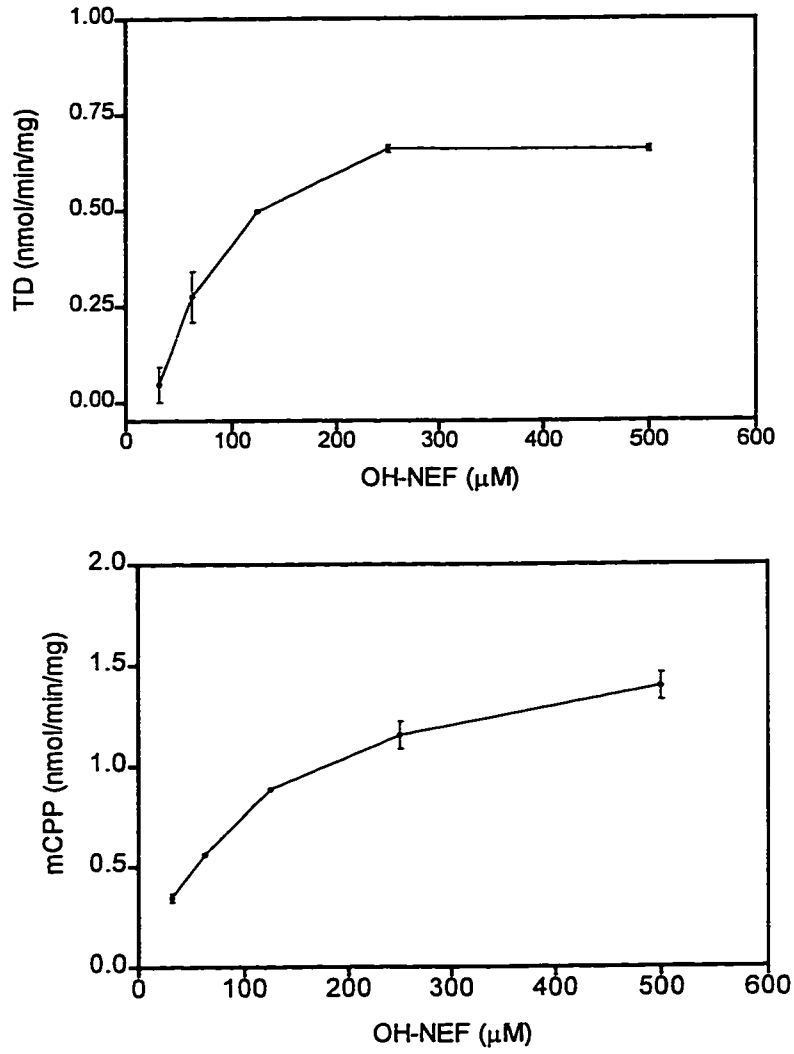



FIGURE 4.11 Effect of varying concentrations of OH-NEF on the formation of TD (top) and mCPP (bottom) in human liver microsomes. Each point represents the mean ± SEM for three separate experiments.

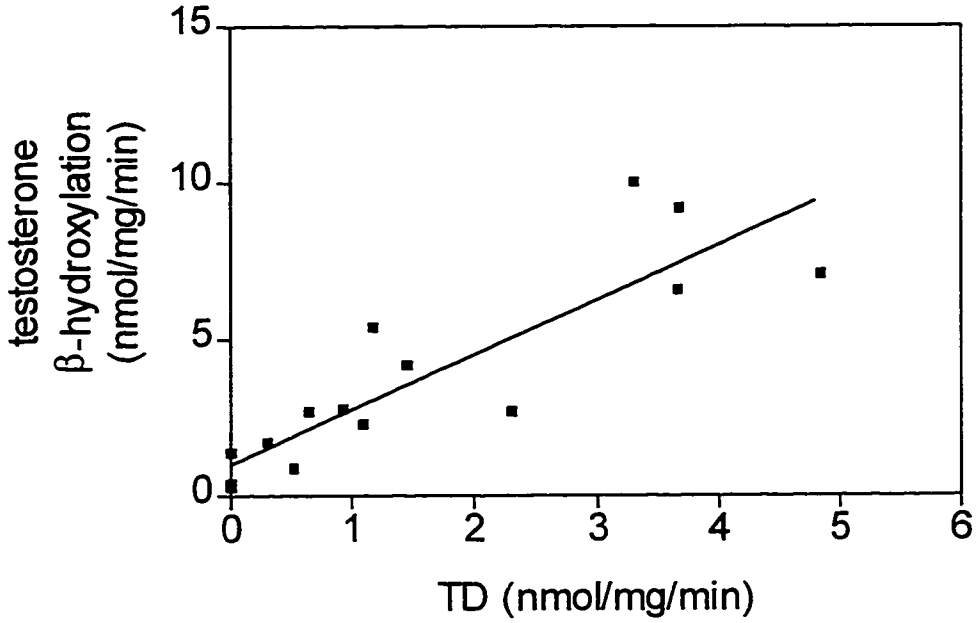



FIGURE 4.12 Correlation of the production of TD from OH-NEF (500 μM) with the activity of CYP3A4 in 16 human livers ($r=0.88$, $p<0.0001$).

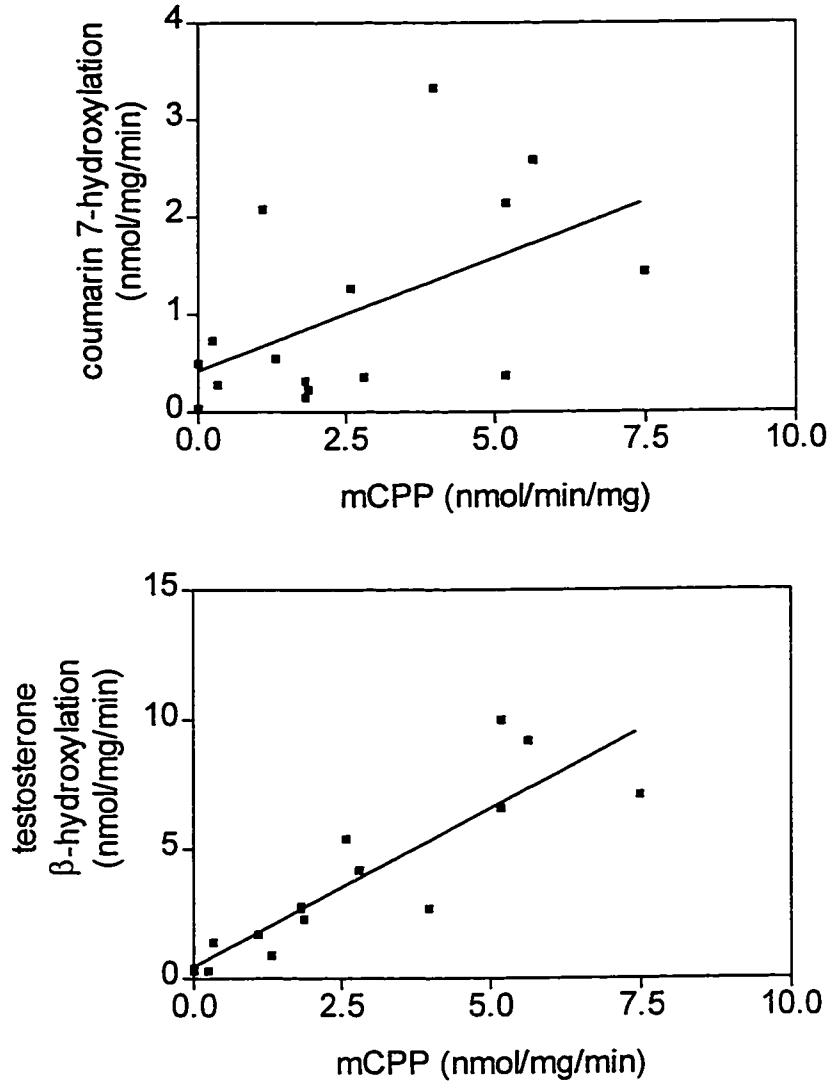



FIGURE 4.13 Correlations of the production of mCPP from OH-NEF (500 μM) with the activity of CYP2A6 (r=0.53, p=0.04, top) and CYP3A4 (r=0.89, p<0.0001, bottom) in 16 human livers.

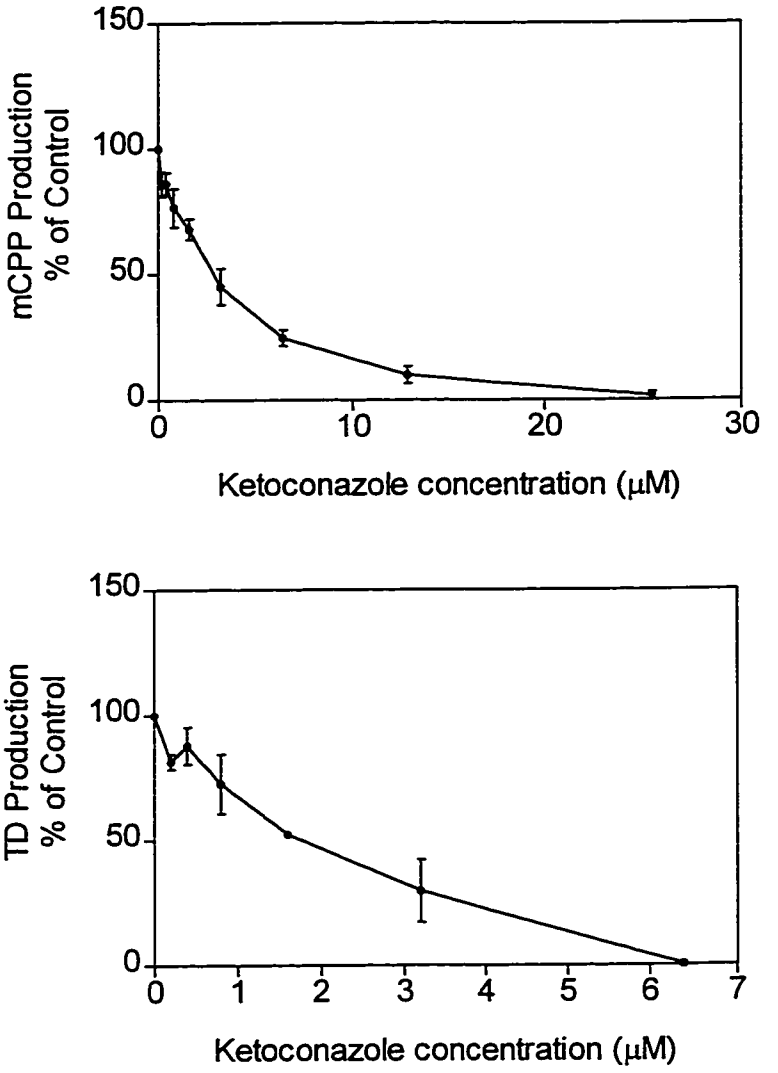



FIGURE 4.14 Effect of the specific CYP3A4 inhibitor ketoconazole on the formation of mCPP (top) and TD (bottom) from OH-NEF (500 μM) in human liver microsomes. Each point represents the mean ± SEM for three separate experiments.

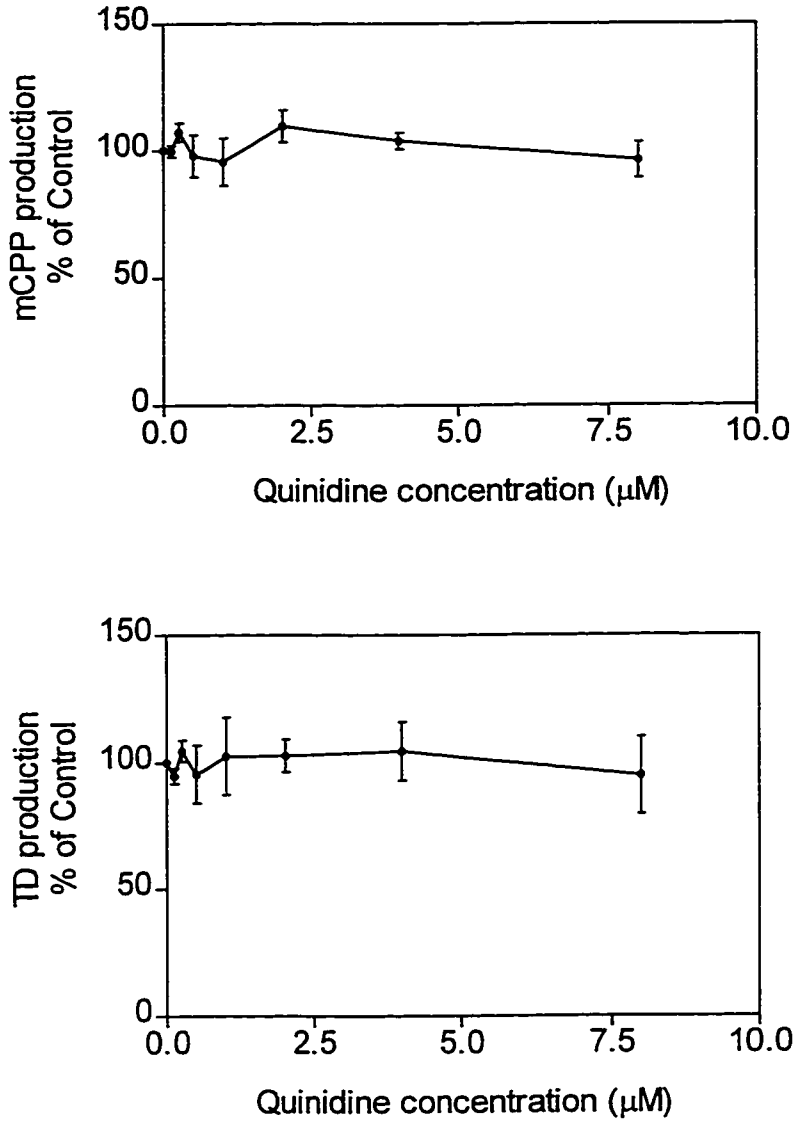



FIGURE 4.15 Effect of the specific CYP2D6 inhibitor quinidine on the formation of mCPP (top) and TD (bottom) from OH-NEF (500 μM) in human liver microsomes. Each point represents the mean ± SEM for three separate experiments.

## 4.4 DISCUSSION

The present experiments examined the metabolism of NEF to its primary metabolites OH-NEF and mCPP, as well as the metabolism of OH-NEF to its primary metabolites TD and mCPP. Using a combination of several different *in vitro* methods, evidence was obtained to suggest that the formation of OH-NEF and mCPP from NEF is mediated primarily by CYP3A4, and that the metabolism of OH-NEF to TD, and of OH-NEF to mCPP, are also mediated by CYP3A4. These findings are consistent with reports in the literature that NEF is a substrate and inhibitor of CYP3A4 *in vitro* (von Moltke et al., 1996a, 1996b), and an inhibitor of CYP3A4 *in vivo* (Barbhaiya et al., 1995; Greene et al., 1995; Kroboth et al., 1995).

The analyses of NEF and OH-NEF metabolism were conducted at a concentration of enzyme (approximately 0.021 mg protein/ 100 µl incubation mixture) which resulted in the formation of the primary metabolites OH-NEF and mCPP from NEF, and TD and mCPP from OH-NEF, but minimal further metabolism to secondary metabolites. This enabled a more specific study of these metabolic pathways without potential interference from secondary metabolic pathways.

The substrate concentrations chosen for the present experiments were based on the findings from the substrate concentration curves, and were conducted at concentrations at which metabolite formation was within the linear portion of the curve. As long as linearity of metabolite formation is maintained,

the kinetic parameters should be constant, and thus, the *in vitro* conditions are comparable to the *in vivo* situation (Iwatsubo et al., 1997). As a further note to the clinical relevance of the concentrations used in the present studies, typical plasma concentrations of NEF range from 1- 5 μM, depending on the dose, the duration of treatment, and individual differences (Franc et al., 1991; Ferry et al., 1994; Salazar et al., 1995; van Laar et al., 1995; Barbhaiya et al., 1995, 1996a, 1996b). Very large intra- and inter-individual differences in plasma levels are seen following NEF administration (Davis et al., 1997), which may occur due to non-linear kinetics, pre-hepatic extraction, or differences in levels of CYP3A4 due to induction, inhibition, or genetics (von Moltke et al., 1995; Iwatsubo et al., 1997).

Nevertheless, it is not the plasma concentration which is important for drug interactions, but rather the concentration at the site of the enzyme, which is primarily in the liver (Harvey and Preskorn, 1995; Preskorn, 1996; von Moltke et al., 1996a). Although there is not yet a clearly established model for determining hepatic concentrations, liver/water partition ratios are typically used to estimate liver concentrations (Greenblatt et al., 1996). The liver/water partition ratio of NEF has not yet been reported; however, since it is a highly lipophilic drug, it can be expected to have a partition ratio similar to that of other lipophilic drugs such as the SSRIs, which have hepatic extraction ratios of 12-26 (Harvey & Preskorn, 1995; Schmider et al., 1996). Many lipophilic psychotropic drugs partition extensively into the liver tissue, even despite high levels of plasma protein

binding, with liver/plasma partition ratios of 6-15 for benzodiazepines, 36-46 for amitriptyline and nortriptyline, 22-35 for sertraline and desmethylsertraline, and 53-151 for fluphenazine and fluphenazine sulphoxide, respectively (Greenblatt et al., 1996). Therefore, even with a NEF plasma concentration of 5 μM and a conservative estimate of a partition ratio of 20, hepatic levels can be expected to be at least 100 μM. The present experiments were conducted at substrate levels of 100-500 μM.

The correlational analyses with NEF as a substrate revealed strong correlations of CYP3A4 activity with the formation of both OH-NEF ($r=0.79$, $p=0.0003$) and mCPP ($r=0.84$, $p<0.0001$) from NEF. The activities of CYP1A2 and CYP2A6 also correlated significantly with OH-NEF formation, but with lower correlation coefficients (CYP1A2, $r=0.51$, $p=0.04$; CYP2A6, $r=0.53$, $p=0.03$). Therefore, more of the variation in the production of OH-NEF from the 16 different human liver microsomal samples can be explained by differences in the activities of CYP3A4 than by differences in the activities of CYP1A2 or CYP2A6. This does not mean that CYP1A2 or CYP2A6 are not involved in the formation of OH-NEF from NEF, only that these enzymes are probably involved to a lesser extent than CYP3A4. This finding is supported by the studies using singly-expressed human enzymes which showed OH-NEF production following incubations with CYP3A4-expressing microsomes, but not following incubations with microsomes expressing only CYP1A2 or CYP2A6. If metabolism does occur *via* these enzyme pathways, it is below the levels of detection of the

current assay, and is therefore likely to be clinically insignificant with respect to drug-drug interactions.

Similarly, the formation of mCPP from NEF correlated with the activities of both CYP2A6 ($r=0.54$, $p=0.03$) and CYP3A4 ($r=0.84$, $p<0.0001$), but with a higher correlation with CYP3A4 activity than with CYP2A6 activity, indicating that CYP3A4 is the major pathway catalyzing the formation of mCPP. Furthermore, mCPP was formed following incubations with CYP3A4, but not with CYP2A6. Therefore, although CYP2A6 may contribute to the formation of mCPP, it is not likely to be to a clinically significant extent.

When OH-NEF was used as the substrate, mCPP formation also correlated with CYP2A6 ($r=0.52$, $p=0.04$) and CYP3A4 ($r=0.89$, $p<0.0001$) activities, and was formed following incubations with OH-NEF and microsomes expressing CYP3A4 only. This pattern of results is the same as was seen for the formation of mCPP from NEF, which would be expected since in both cases mCPP is formed by N-dealkylation at the piperazinyl N (see Figure 4.1). Again, these results indicate a major role for CYP3A4 and a minor role for CYP2A6 in the formation of mCPP.

The formation of TD from OH-NEF correlated significantly with the activity of CYP3A4 only ($r=0.88$, $p<0.0001$), and was present following incubations only with CYP3A4-expressing microsomes, indicating that this was the major pathway for TD formation. The exact mechanism for the formation of TD is not presently known, but has been suggested to occur through a keto-NEF intermediate (Mayol, 1994b). It is interesting to note that TD was not seen following

incubations of NEF with cells expressing only CYP3A4, but was present following incubations of OH-NEF with CYP3A4-expressing cells.

The almost complete inhibition of metabolite formation from NEF and OH-NEF by ketoconazole confirms that CYP3A4 is the major isoform involved in these metabolic pathways. When the specific CYP2D6 inhibitor quinidine was incubated with NEF or OH-NEF, no inhibition of metabolism was seen. This confirms previous findings of a lack of effect of quinidine on NEF metabolism *in vitro* (von Moltke et al., 1996), and lack of *in vivo* interactions with CYP2D6 metabolic phenotypes (Barbhaiya et al., 1996; Ferry et al., 1994).

Pindolol similarly failed to affect NEF metabolism, which would be expected since pindolol is approximately 1000-fold weaker an inhibitor of CYP2D6 than is quinidine, with a $K_i$ of 60 μM as compared to 0.06 μM for quinidine (Otton et al., 1984). Since NEF does not appear to be a substrate for CYP2D6, an interaction with pindolol is not expected. Furthermore, typical plasma concentrations of pindolol are about 20 ng/ml following a 5 mg dose (Meier, 1982), which is approximately 0.08 μM. Hepatic concentrations of pindolol are not expected to be much higher, since pindolol is only of intermediate lipophilicity, and has a partition coefficient of 0.82 (McDevitt, 1987). Therefore, since no inhibition of NEF metabolism was seen in the present experiments at pindolol concentrations of 15-240 μM, interactions *in vivo* would not be expected either.

The effect of NEF and its metabolites on pindolol metabolism was not investigated in the present experiments. The metabolism of pindolol has not been thoroughly investigated, but a significant clinical interaction with NEF is probably unlikely since NEF's major effect on the CYP enzymes appears to be the inhibition of CYP3A4 (Schmider et al., 1996; von Moltke et al., 1996a, 1996b, 1996c). Pindolol is not likely to be a substrate for CYP3A4 since its metabolism shows very little inter-individual variation, and since it is cleared only partly by the liver and partly by the kidney (McDevitt, 1987).

Overall, the current results suggest that the metabolism of NEF to its metabolites OH-NEF and mCPP, as well as the metabolism of OH-NEF to its metabolites mCPP and TD, are mediated primarily by CYP3A4. These findings are consistent with reports in the literature that NEF may be a substrate and inhibitor of CYP3A4 *in vitro* and *in vivo*, and indicate the potential for drug-drug interactions with NEF and other CYP3A4 substrates or inhibitors.

## 4.5 REFERENCES


Artigas F, Romero L, de Montigny C, Blier P (1996) Acceleration of the effect of selected antidepressant drugs in major depression by 5-$HT_{1A}$ antagonists. *Trends Neurosci* **19**:378-383.

Ashton AK, Wolin RE (1996) Nefazodone-induced carbamazepine toxicity [letter]. *Am J Psychiatry* **153**:733.

Bakish D, Hooper CL, Thornton, MD, Wiens A, Miller CA, Thibaudeau TA (1997) Fast onset: An open study of the treatment of major depressive disorder with nefazodone and pindolol combination therapy. *Int Clin Psychopharmacol* **12**:91-97.

Barbhaiya RH, Marathe PH, Greene DS, Mayol RF, Shukla UA, Gammans RR, Pittman KA, Robinson D (1995) Safety, tolerance, and preliminary pharmacokinetics of nefazodone after administration of single and multiple oral doses to healthy adult male volunteers: A double-blind, Phase I study. *J Clin Pharmacol* **35**: 974-984.

Barbhaiya RH, Shukla UA, Chaikin P, Greene DS, Marathe PH (1996a) Nefazodone pharmacokinetics: assessment of nonlinearity, intra-subject variability and time to attain steady-state plasma concentrations after dose escalation and de-escalation. *Eur J Clin Pharmacol* **50**:101-107.

Barbhaiya RH, Dandekar KA, Greene DS (1996b) Pharmacokinetics, absolute bioavailability, and disposition of [$^{14}$C]nefazodone in humans. *Drug Metab Dispos* **24**:91-95.

Blier P, de Montigny C, Chaput Y (1987) Modifications of the serotonin system by antidepressant treatments: implications for the therapeutic response in major depression. *J Clin Psychopharmacol* **7**(suppl 6):24S-35S.

Blier P, de Montigny C, Chaput Y (1990) A role for the serotonin system in the mechanism of action of antidepressant treatments: preclinical evidence. *J Clin Psychiatry* **51**(suppl 4):14-20.

Briley M, Moret C (1993) Neurobiological mechanisms involved in antidepressant therapies. *Clin Neuropharmacol* **16**:387-400.

Conn JP, Sanders-Bush E (1987) Relative efficacies of piperazines at the phosphoinositide hydrolysis-linked serotonergic (5-HT-2 and 5-HT-1C) receptors. *J Pharmacol Exp Ther* **242**:552-557.

Dursun SM, Devarajan S, Kutcher SP (1997) "Dalhousie serotonin cocktail" treatment of ECT resistant recurrent major depressive disorder (RMDD). *J Psychopharmacol* **11**(suppl):A30 [abstract].

Ellingrod VL, Perry PJ (1995) Nefazodone: A new antidepressant. *Am J Health-Syst Pharm* **52**:2799-2812.

Fawcett J, Marcus RN, Anton SF, O'Brien K, Schwiderski U (1995) Response of anxiety and agitation symptoms during nefazodone treatment of major depression. *J Clin Psychiatry* **56(suppl 6)**:37-42.

Fiorella D, Rabin RA, Winter JC (1995) The role of the 5-HT2A and 5-HT2C receptors in the stimulus effects of m-chlorophenylpiperazine. *Psychopharmacol* **119**:222-230.

Greendyke RM, Guyla A (1988) Effect of pindolol administration on serum levels of thioridazine, haloperidol, phenytoin and phenobarbital. *J Clin Psychiatry* **49:**105-107.

Greene DS, Salazar DE, Dockens RC, Kroboth P, Barbhaiya RH (1995) Coadministration of nefazodone and benzodiazepines: III. A pharmacokinetic interaction study with alprazolam. *J Clin Psychopharmacol* **15**:399-408.

Guengerich FP (1996) In vitro techniques for studying drug metabolism. *J Pharmacokinet Biopharmaceut* **24**:521-533.

Harvey AT, Preskorn SH (1995) Interactions of serotonin reuptake inhibitors with tricyclic antidepressants [letter]. *Arch Gen Psychiatry* **52**:783-784.

Helms-Smith KM, Curtis SL, Hatton RC (1996) Apparent interaction between nefazodone and cyclosporine [letter]. *Ann Intern Med* **125**:424.

Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, Sugiyama Y (1997) Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacol Ther* **73**:147-171.

Kaul S, Shukla UA, Barbhaiya RH (1995) Nonlinear pharmacokinetics of nefazodone after escalating single and multiple oral doses. *J Clin Pharmacol* **35**:830-839.

Kroboth PD, Folan MM, Lush RM, Chaikin PC, Shukla UA, Barbhaiya RH, Salazar DE (1995) Coadministration of nefazodone and benzodiazepines: I. Pharmacodynamic assessment. *J Clin Psychopharmacol* **15**:306-319.

Malik K (1996) Nefazodone: structure, mode of action and pharmacokinetics. *J Psychopharmacol* **10 (Suppl. 1)**: 1-4.

Marcus RN (1996) Safety and tolerability profile of nefazodone. *J Psychopharmacol* **10 (suppl 1)**:11-17.

Mayol RF, Cole CA, Colson KE, Kerns EH (1994a) Isolation and identification of the major urinary metabolite of m-chlorophenylpiperazine in the rat. *Drug Metab Dispos* **22**:171-174.

Mayol RF, Cole CA, Luke GM, Colson KL, Kerns EH (1994b) Characterization of the metabolites of the antidepressant drug nefazodone in human urine and plasma. *Drug Metab Dispos* **22**:304-311.

Melzacka M, Boksa J, Maj J (1979) 1-(m-Chlorophenyl)piperazine: a metabolite of trazodone isolated from rat urine. *J Pharm Pharmacol* **31**:855-856.

Montgomery SA (1996) Efficacy of nefazodone in the treatment of depression. *J Psychopharmacol* **10 (suppl 1)**:5-10.

Nutt D (1996) Early action of nefazodone in anxiety associated with depression. *J Psychopharmacol* **10 (suppl 1)**:18-21.

Otton SV, Inaba T, Kalow W (1984) Competitive inhibition of sparteine oxidation in human liver by β-adrenoceptor antagonists and other cardiovascular drugs. *Life Sci* **34**:73-80.

Preskorn SH, Janicak PG, Davis JM, Ayd FJ (1995) Advances in the pharmacotherapy of depressive disorders. In Janicak, PG (Ed.), *Principles and Practice of Psychopharmacotherapy* **1**:1-24.

Richelson E (1997) Pharmacokinetic drug interactions of new antidepressants: A review of the effects on the metabolism of other drugs. *Mayo Clin Proc* **72**:835:847.

Robinson DS, Roberts DL, Smith JM, Stringfellow JC, Kaplita SB, Seminara JA, Marcus RN (1996) The safety profile of nefazodone. *J Clin Psychiatry* **57(suppl 2)**: 31-38.

Schmider J, Greenblatt DJ, von Moltke LL, Harmatz JS, Shader RI (1996) Inhibition of cytochrome P450 by nefazodone *in vitro:* studies of dextromethorphan *O*- and *N*-demethylation. *Br J Clin Pharmacol* **41**:339-343.

Taylor DP, Carter RB, Eison AS, Mullins UL, Smith HL, Torrente JR, Wright RN, Yocca FD (1995) Pharmacology and neurochemistry of nefazodone, a novel antidepressant drug. *J Clin Psychiatry* **56 (suppl 6)**:3-11.

Taylor DP, Smith DW, Hyslop DK, Riblet LA, Temple DL (1986) Receptor binding and atypical antidepressant drug discovery. In O'Brien RA (Ed) *Receptor Binding in Drug Research.* New York: Marcel Dekker, pp. 151-165.

Tome MB, Isaac MT, Harte R, Holland C (1997) Paroxetine and pindolol: a randomized trial of serotonergic autoreceptor blockade in the reduction of antidepressant latency. *Int Clin Psychopharmacol* **12**:81-89.

von Moltke LL, Greenblatt DJ, Duan SX, Schmider J, Kudchadker L, Fogelman SM, Harmatz JS, Shader RI (1996c) Phenacetin *O*-deethylation by human liver microsomes in vitro: inhibition by chemical probes, SSRI antidepressants, nefazodone and venlafaxine. *Psychopharmacol* **128**:398-407.

von Moltke LL, Greenblatt DJ, Harmatz JS, Duan SX, Harrel LM, Cotreau-Bibbo MM, Pritchard GA, Wright CE, Shader RI (1996a) Triazolam biotransformation by human liver microsomes *in vitro*: Effects of metabolic inhibitors and clinical confirmation of a predicted interaction with ketoconazole. *J Pharmacol Exp Ther* **276**:370-379.

von Moltke LL, Greenblatt DJ, Schmider J (1996b) Nefazodone *in vitro*: Metabolic conversions, and inhibition of P450-3A isoforms [abstract]. *Clin Pharmacol Ther* **59**:176.

Yamato C, Takahashi T, Fujita T, Kuriyama S, Hirose N (1974) Studies on metabolism of trazodone II. Metabolic fate after intravenous administration and effects on liver microsomal drug-metabolizing enzymes in rats. *Xenobiotica* **4**:765-777.

# Chapter 5

## A Rapid Electron-Capture Gas Chromatographic Method for the Quantification of Fluvoxamine in Brain Tissue

A version of this chapter has been published (Rotzinger et al., J Pharm Toxicol Meth, 1997, 37, 129-133).

## 5.1 INTRODUCTION

Fluvoxamine (FVX), a selective serotonin reuptake inhibitor (SSRI), has been reported to be effective in the treatment of major depressive disorder, obsessive-compulsive disorder, panic disorder and social phobia (Gasperini et al., 1992; Black et al., 1993; Leonard, 1993; McDougle et al., 1993; Freeman et al., 1994; Ottevanger, 1994; Palmer and Benfield, 1994; van Vliet et al., 1994; Denboer et al., 1995; Dewulf et al., 1995; Kaspar et al., 1995). Several high performance liquid chromatography (HPLC) assays for FVX in plasma have been developed (De Jong, 1980; De Bree et al., 1983; Benfield and Ward, 1986; Schweitzer et al., 1986; Foglia et al., 1989; Pommery and Lhermitte, 1989; Nathan et al., 1990; Van der Meersch-Mougeot and Diquet, 1991; De Vries et al., 1992; Gupta, 1992; Hartter et al., 1992; Wong et al., 1994; Belmadani et al., 1995). A gas chromatographic-mass spectrometric assay procedure for FVX in human plasma has recently been reported by Eap et al. (1996). Hurst et al. (1981) have described an assay for FVX using gas chromatography with electron-capture detection (GC-ECD), but the procedure involves a lengthy sample preparation with several extraction steps, and again is for analysis in plasma. The present method is a sensitive, relatively simple method for the rapid determination of FVX in biological samples (mouse brain homogenate) using simultaneous extraction and derivatization followed by analysis using GC-ECD. This method represents an advantage over previous methods in that it can be used to analyze FVX levels in animal models, and is sensitive enough to

detect 18 ng FVX/ml homogenate, a level well below those that will be routinely encountered in experimental models.

This method was developed for possible applications in the study of drug-drug interactions in animal models.

## 5.2 METHODS

### *5.2.1 Drugs*

FVX maleate was prepared in a 1 mg/ml stock solution in double-distilled water, aliquoted and stored at -80°C. The internal standard norfluoxetine (NFLU) was similarly prepared and stored.

### *5.2.2 Animals*

Male Swiss mice weighing 18-20 g were injected intraperitoneally with 4 or 8 mg/kg of FVX maleate dissolved in double-distilled water or with the vehicle. The animals were sacrificed 1 h later by cervical dislocation followed by decapitation. Whole brains were quickly removed and frozen at -80°C until time of analysis.

### *5.2.3 Sample preparation and extraction*

Brain tissue was weighed and homogenized in five volumes of ice-cold double-distilled water, and a 1 ml portion was removed and used in the analytical procedure. NFLU (1000 ng) was added as internal standard to the samples,

which were basified (pH 8.5) by the addition of solid potassium bicarbonate (approximately 0.5 g). Four ml of a solution of ethyl acetate:acetonitrile:PFBC (9:1:0.01) were added to each sample tube. The tubes were then capped and shaken vigorously on an Ika Vibrex VXR vortex mixer for 15 min and centrifuged at 1000 g for 5 min in a benchtop centrifuge. The organic phase was pipetted into 100 mm x 13 mm screw cap culture tubes and taken to dryness in a Savant evaporator. The residue was reconstituted in 150 μl of toluene and washed with 150 μl of 1 N ammonium hydroxide. The samples were centrifuged for 1 min and the top layer removed for GC analysis (1 μl was injected). Calibration curves were prepared with each assay run by carrying through the procedure in parallel a series of tubes containing known, varying amounts of FVX and the same fixed amount of the internal standard as was added to the brain extracts. These calibration samples were prepared using homogenates of brain from drug-naive mice. The peak height ratios of FVX to internal standard in the brain extracts of interest were compared to the values from the calibration curve.

### *5.2.4 Instrumental analysis*

All samples were analyzed using a Hewlett Packard (HP) Model 5890 gas chromatograph equipped with a $^{63}$Ni electron-capture detector and linked to a HP 3392A integrator/printer. A fused silica capillary column (25 m x 0.32 mm) coated with 1.05 μm film thickness of 5% phenylmethylsilicone was employed. The carrier gas was pure helium at a flow rate of 3 ml/min. The make-up gas

was argon-methane (95:5) at a flow rate of 35 ml/min. The oven temperature was set at an initial temperature of 105°C for 0.5 min. The temperature was then increased by 10°/min to 255°, followed by a 1°/min increase to 270°C. Injection port and detector temperatures were 270°C and 325°C, respectively. All injections were carried out using the splitless mode of injection with a purge off time of 0.5 min.

## 5.3 RESULTS AND DISCUSSION

The procedure resulted in the formation of a derivative of FVX with excellent chromatographic properties (Figure 5.1). The structure of the derivative was confirmed using combined GC-mass spectrometry in the electron-impact mode; the proposed mass fragmentation is shown in Figure 5.2.

Calibration curves (usually 50-1000 ng FVX) were linear, with correlation coefficient values of > 0.99 obtained routinely. The maximum sensitivity of the assay, based on a 3-to-1 signal-to-noise ratio, was less than 120 pg "on-column", or 18 ng/ml homogenate. The mean intra-assay coefficient of variation for 500 ng samples carried through the procedure was 8.0% (n=6). Inter-assay coefficients of variation for 100, 500, and 1000 ng standards (n=4) were 7.12%, 5.04%, and 7.37%, respectively.

Levels of FVX were determined in whole brains of mice treated intraperitoneally with either vehicle or 4 or 8 mg/kg of FVX maleate one h before sacrifice. Typical chromatograms of derivatized mouse brain extracts from drug-

and vehicle-treated animals are shown in Figure 5.1. The brain levels of FVX were 1578 ± 342 ng/g tissue (mean ± SEM) for mice (n=10) treated with 4 mg/kg and 2620 ± 250 ng/g tissue (mean ±SEM) for mice treated with 8 mg/kg. No FVX was detected in vehicle-treated mice and no chromatographic peaks interfering with the internal standard were noted.

The procedure described here is a rapid, simple and reproducible assay for the analysis of FVX in brain homogenate. The method involves extractive derivatization of FVX with PFBC, followed by quantitation by GC-ECD. Such extractive derivatization with PFBC under aqueous conditions has been shown previously to be particularly useful for analysis of drugs and neurochemicals containing primary or secondary amines (Cristofoli, 1982; Nazarali et al., 1986, 1987; Rao et al., 1987).

The method described in the present report will be useful for assaying tissue levels of FVX in future studies of the pharmacodynamics and pharmacokinetics of this SSRI. Such studies are particularly important given recent reports of metabolic drug-drug interactions occurring between FVX and concomitantly administered medications (Bonnet et al., 1992; Thomson et al., 1992; van Harten, 1993; Hartter et al., 1993; Maskall and Lam, 1993; Spina et al., 1993; Daniel et al., 1994; Devane, 1994; Hiemke et al., 1994; Preskorn et al., 1996). The assay is sensitive enough to detect 18 ng/ml homogenate, levels far below those seen in the low dose (4 mg/kg) FVX group (average of 263 ng/ml). Typical doses (ip) in experimental studies of FVX are 10-40 mg/kg (Maj et al.,

1982; Lapierre et al., 1983; Ortiz & Artigas, 1992). Therefore, the method described in the present report will be useful in studies of FVX in animal models.

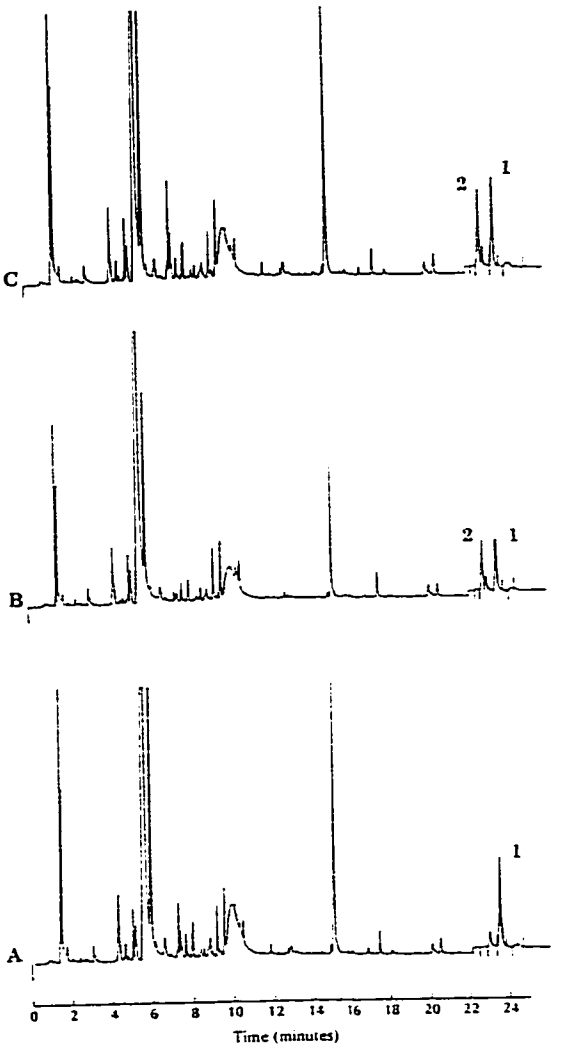



FIGURE 5.1 Typical gas chromatographs from derivatized extracts of mouse brain. (A) Homogenate from drug-naive brain with 1000 ng of internal standard norfluoxetine (NFLU) added (peak 1; no peak was present at the retention time for derivatized NFLU in unspiked homogenates). (B) Homogenate from drug-naive brain with 500 ng FVX (peak 2) and 1000 ng NFLU (peak 1) added. (C) Homogenate from mouse treated 1 h previously with FVX (8 mg/kg ip) [peak 2]; 1000 ng NFLU was added as the internal standard (peak 1). Retention times for peaks 1 and 2 were 23.43 and 22.69 min, respectively.

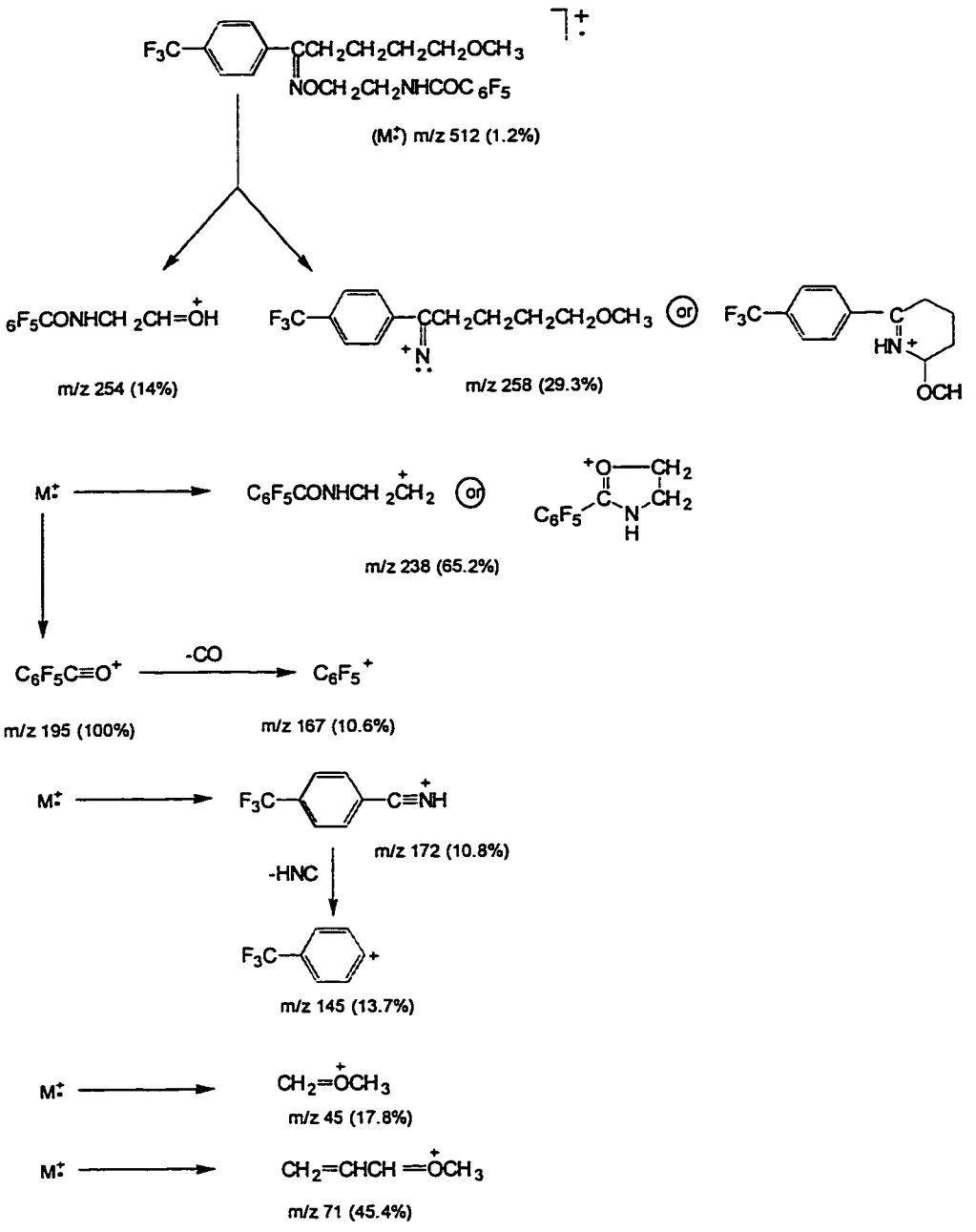



FIGURE 5.2 Proposed mass fragmentation (electron impact mode) of FVX derivatized with pentafluorobenzoyl chloride (PFBC). The per cent relative abundances are indicated in parentheses.

## 5.4 REFERENCES


Belmadani A, Combourieu I, Bonini M, Creppy EE (1995) High performance liquid chromatography with ultraviolet detection used for laboratory routine determination of fluvoxamine in human plasma. *Hum Exp Toxicol* 14:34-37.

Benfield P, Ward A (1986) Fluvoxamine: A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in depressive illness. *Drugs* 32:313-334.

Bertschy G, Vandel S, Vandel B, Allers G, Volmat R (1991) Fluvoxamine-tricyclic antidepressant interaction. *Eur J Clin Pharmacol* 40:119-120.

Black DW, Wesner R, Bowers W, Gabel J (1993) A comparison of fluvoxamine, cognitive therapy, and placebo in the treatment of panic disorder. *Arch Gen Psychiatry* 50:44-50.

Bonnet P, Vandel S, Nezelof S, Sechter D, Bizouoard P (1992) Carbamazepine, fluvoxamine. Is there a pharmacokinetic interaction? [letter] *Therapie* 47:165.

Cristofoli WA, Baker GB, Coutts RT, Benderly A (1982) Analysis of a monofluorinated analogue of amphetamine in brain tissue using gas chromatography with electron-capture detection. *Prog Neuro-Psychopharmacol Biol Psychiatry* 6:373.

Daniel DG, Randolph C, Jaskiw G, Handel S, Williams T, Abidargham A, Shoaf S, Egan M, Elkashef A, Liboff S, Linnoila M (1994) Coadministration of fluvoxamine increases serum concentrations of haloperidol. *J Clin Psychopharmacol* 14:340-343.

Denboer JA, Westenberg HGM, Deleeuw AS, Van Vliet IM (1995) Biological dissection of anxiety disorders: The clinical role of selective serotonin uptake inhibitors with particular reference to fluvoxamine. *Int Clin Psychopharmacol* 9:47-52.

De Bree H, Van Der Schoot JB, Post LC (1983) Fluvoxamine maleate: Disposition in man. *Eur J Drug Metabol Pharmacokinet* 8:175-179.

DeJong GJ (1980) The use of a pre-column for the direct high performance liquid chromatographic determination of the antidepressants clovoxamine and fluvoxamine in plasma. *J Chromatogr* 183:203-211.

DeVane CL (1994) Pharmacogenetics and drug metabolism of newer antidepressant agents. *J Clin Psychiatry* 55:38-45.

De Vries MH, Raghoebar M, Mathlener IS, vanHarten J (1992) Single and multiple oral dose fluvoxamine kinetics in young and elderly subjects. *Ther Drug Monit* 14:493-498.

Dewulf L, Hendrickx B, Lesaffre E (1995) Epidemiological data of patients treated with fluvoxamine: Results from a 12 week noncomparative multicentre study. *Int Clin Psychopharmacol* 9:67-72.

Foglia JP, Birder LA, Perel JM (1989) Determination of fluvoxamine in human plasma by high performance liquid chromatography with ultraviolet detection. *J Chromatogr* 495:295-302.

Eap CB, Gaillard N, Powell K, Baumann P (1996) Simultaneous determination of plasma levels of fluvoxamine and of the enantiomers of fluoxetine and norfluoxetine by gas chromatography-mass spectrometry. *J Chromatogr Biomed Appl* 682:265-272.

Freeman CPL, Trimble MR, Deakin JFW, Stokes TM, Ashford JJ (1994) Fluvoxamine versus clomipramine in the treatment of obsessive-compulsive disorder: A multicenter, randomized double blind, parallel study. *J Clin Psychiatry* 55:301.

Gasperini M, Gatti F, Bellini L, Anniverno R, Smeraldi E (1992) Perspectives in clinical psychopharmacology of amitriptyline and fluvoxamine. A double blind study in depressed inpatients. *Neuropsychobiol* 26:186-192.

Gupta RN (1992) Drug level monitoring: antidepressants. *J Chromatogr Biomed Anal* 576:183-211.

Hartter S, Wetzel H, Hammes E, Hiemke C (1993) Inhibition of antidepressant demethylation and hydroxylation by fluvoxamine in depressed patients. *Psychopharmacol* 110:302-308.

Hartter S, Wetzel H, Hiemke C (1992) Automated determination of fluvoxamine in plasma by column-switching high performance liquid chromatography. *Clin Chem* 38:2082-2086.

Hiemke C, Weigman H, Hartter S, Dahmen N, Wetzel H, Muller H (1994) Elevated levels of clozapine in serum after addition of fluvoxamine. *J Clin Psychopharmacol* 14:279-281.

Hurst HE, Jones DR, Jarboe CH, deBree H (1981) Determination of clovoxamine concentration in human plasma by electron capture gas chromatography. *Clin Chem* 27:1210-1212.

Kaspar S, Miller H-J, Montgomery SA, Zondag E (1995) Antidepressant efficacy in relation to item analysis and severity of depression: a placebo-controlled trial of fluvoxamine. *Int Clin Psychopharmacol* 9(Suppl 4):3-12.

Lapierre YD, Rastogi TB, Singhal RL (1983) Fluvoxamine influences serotonergic system in the brain: Neurochemical evidence. *Neuropsychobiol* 10:213-216.

Leonard BE (1993) The comparative pharmacology of new antidepressants. *J Clin Psychiatry* 54:3-15.

Maj J, Rogoz Z, Skuza G (1982) Fluvoxamine, a new antidepressant drug, fails to show antiserotonin activity. *Eur J Pharmacol* 81:287-292.

Maskall DD, Lam RW (1993) Increased plasma concentration of imipramine following augmentation with fluvoxamine [letter]. *Am J Psychiat* ry150:1566.

McDougle CJ, Goodman WK, Leckman JF, Barr LC, Heninger GR, Price LH (1993) The efficacy of fluvoxamine in obsessive-compulsive disorder - Effects of comorbid chronic tic disorder. *J Clin Psychopharmacol* 13:354-358.

Nathan RS, Perel JM, Pollock BG, Kupfer, DJ (1990) The role of neuropharmacologic selectivity in antidepressant action: fluvoxamine versus desipramine. *J Clin Psychiatry* 51:367-372.

Nazarali AJ, Baker GB, Boisvert DP (1986) Metabolism and disposition of N-(2-cyanoethyl)amphetamine (fenproporex) and amphetamine: study in the rat brain. *J Chromatogr Biomed Appl* 380:393-400.

Nazarali AJ, Baker GBB, Coutts RT, Yeung JM, Rao TS (1987) Rapid analysis of 2-phenylethylamine in tissues and body fluids utilizing pentafluoro-benzoylation followed by electron-capture gas chromatography. *Prog Neuro-Psychopharmacol Biol Psychiatry* 11:251-258.

Ortiz J, Artigas F (1992) Effects of monoamine uptake inhibitors on extracellular and platelet 5-hydroxytryptamine in rat blood: Different effects of clomipramine and fluoxetine. *Br J Pharmacol* 105:941-946.

Ottevanger EA (1994) The efficacy of fluvoxamine in patients with severe depression. *Prog Neuro-Psychopharmacol Biol Psychiatry* 18:731:740.

Palmer KJ, Benfield P (1994) Fluvoxamine - An overview of its pharmacological properties and review of its therapeutic potential in non-depressive disorders. *CNS Drugs* 1:57-87.

Pommery J, Lhermitte M (1989) High performance liquid chromatographic determination of fluvoxamine in human plasma. *Biomed Chromatogr* 3:177-179.

Preskorn, SH (1996) *Clinical pharmacology of selective serotonin reuptake inhibitors*. Cado, OK: Professional Communications, Inc. pp.152-153.

Pullen RH, Fatmi AA (1992) Determination of fluvoxamine in human plasma by high-performance liquid chromatography with fluorescence detection. *J Chromatogr* 574:101-107.

Rao TS, Baker GB, Coutts RT, Yeung JM, McIntosh GJA, Torok-Both GA (1987) Analysis of the antidepressant phenelzine in brain tissue and urine using electron-capture gas chromatography. *J Pharmacol Methods*, 17:297-304.

Schweitzer C, Spahn H, Mutschler E (1986) Fluorimetric determination of fluvoxamine or clovoxamine in human plasma after thin-layer chromatographic or high performance liquid chromatographic separation. *J Chromatogr* 382:405-411.

Seifritz E, Holsboertrachsler E, Hemmeter U, Eap CB and Baumann P (1994) Increased trimipramine plasma levels during fluvoxamine comedication. *Eur Neuropsychopharmacol* 4:15-20.

Spina E, Pollicino AM, Avenoso A, Campo GM, Perucca E, Caputi AP (1993) Effect of fluvoxamine on the pharmacokinetics of imipramine and desipramine in healthy subjects. *Ther Drug Monit* 15:243-246.

Thomson AH, McGovern EM, Bennie P, Caldwell G, Smith M (1992) Interaction between fluvoxamine and theophylline. *Can Pharmaceut J* 249:137.

Van Harten J (1993) Clinical pharmacokinetics of selective serotonin reuptake inhibitors. *Clin Pharmacokinet* 24:203-220.

Van der Meersch-Mougeot V, Diquet B (1991) Sensitive one-step extraction procedure for column liquid chromatographic determination of fluvoxamine in human and rat plasma. *J Chromatogr* 567:441-449.

Van Vliet IM, Denboer JA, Westenberg HGM (1994) Psychopharmacological treatment of social phobia; a double blind placebo controlled study with fluvoxamine. *Psychopharmacol* 115:128-134.

Wong SHY, Kranzler HR, DellaFera S, Fernandes R (1994) Determination of fluvoxamine concentration in plasma by reversed-phase liquid chromatography. *Biomed Chromatogr* 8:278-282.

# Chapter 6

# General Discussion

## 6.1 Summary of the present work

The studies that comprise the present thesis were designed to identify the major CYP enzymes involved in the metabolism of two related antidepressants, trazodone and nefazodone, to their active metabolites. The methodology employed was the *in vitro* incubation of the drug substrates with human liver microsomes or microsomes expressing specific human CYP enzymes, and using a set of experiments that has recently become accepted as part of a general strategy for identifying the CYP enzymes involved in the biotransformation of a drug (Guengerich, 1996; Maurel, 1996). The present work adds to this body of data, and identifies previously uncharacterized metabolic pathways of trazodone and nefazodone, which it is hoped will increase their safe and effective use. Finally, novel analytical methods were developed for future studies of the metabolism and interactions of these drugs. The major findings of the present thesis can be summarized as follows:

1. The metabolism of trazodone to the active metabolite mCPP was shown to be mediated by CYP3A4 under physiologically relevant conditions. This pathway has not previously been investigated *in vitro*, and the finding is consistent with literature reports of trazodone interactions with other CYP3A4 substrates and inhibitors *in vivo* (Otani et al., 1996).

2. The metabolism of mCPP to OH-mCPP was shown to be mediated by CYP2D6. This novel finding is consistent with literature reports of interactions of mCPP with CYP2D6 inhibitors and/or substrates *in vivo* (Yasui et al., 1995; Marino et al., 1996; Maes et al., 1997), and with delayed metabolism of mCPP in PMs of CYP2D6 (Buch et al., 1993; Barbhaiya et al., 1996).

3. The metabolism of NEF to OH-NEF, and of NEF to mCPP was found to be mediated by CYP3A4. This finding is consistent with reports in the literature of clinical interactions of NEF with CYP3A4 substrates and/or inhibitors (Barbhaiya et al. 1995; Kroboth et al., 1995; Ashton et al., 1996; Helms-Smith et al., 1996; Robinson et al., 1996), and with *in vitro* findings that inhibitors of CYP3A4 can inhibit the formation of all metabolites of NEF *in vitro* (von Moltke et al., 1996b).

4. The metabolism of OH-NEF to TD and mCPP was also found to be mediated by CYP3A4, which is consistent with reports that inhibitors of CYP3A4 inhibit the formation of these metabolites of NEF *in vitro* (von Moltke et al., 1996b).

5. A novel GC method was developed for the analysis of mCPP following microsomal incubations, which would be useful for further studies of the metabolism of its parent compounds trazodone, NEF, etoperidone, or mepiprazole.

6. A novel HPLC method was developed for the simultaneous detection of NEF, OH-NEF, TD, mCPP and OH-mCPP following microsomal incubations. This is a rapid, reliable method which will be useful in further studying NEF and mCPP metabolism and drug-drug interactions.

7. In looking at the interactions of SSRIs with the metabolism of trazodone to mCPP, it was discovered that FVX produced a peak that was well resolved from mCPP. Since a rapid GC method for the analysis of FVX was not currently available, a novel GC method was developed for the detection of FVX. This assay should be useful in studying drug-drug interactions of trazodone or NEF with FVX *in vivo* in the future.

## 6.2 Limitations of the present work

There are certain limitations of the present work that must be taken into consideration. First, no *in vitro* preparation can ever completely model the complex interaction of processes that occurs *in vivo*, or the dynamic cellular environment provided by constant blood flow (Maurel, 1996). However, certain systems have proven adequate for studying defined steps in the drug metabolism process, with the type of system used depending upon the questions being asked (Skett et al., 1995). Liver slices and hepatocytes most closely model the *in vivo* situation, and are needed for generating complete metabolic profiles; however, there are difficulties in obtaining and maintaining suitable

samples which makes the use of these systems impractical in some cases (Skett et al., 1995; Maurel, 1996). The experiments in the present thesis were carried out in human liver microsomes and microsomes expressing single human CYP enzymes. Although these systems lack Phase 2 and cytosolic enzymes, the goal of the present work was to identify the CYP enzymes involved in the metabolism of trazodone and nefazodone, and therefore, the systems used were appropriate for this purpose. In fact, it is generally agreed that human microsomes are useful in studies of drug-drug interactions, and in identifying the major CYP enzymes involved in a drug's metabolism (Skett et al., 1995; Guengerich, 1996; Maurel, 1996; Iwatsubo et al., 1997).

A limitation of working with any *in vitro* preparation is that it is difficult to account for the wide inter-individual differences in enzyme levels seen in the population (Bertz & Granneman, 1997). For this reason, it is important to note that *qualitative* predictions about drug metabolism and interactions are much more likely to be successful than *quantitative* predictions, and that the absolute values of kinetic parameters such as $K_m$, $V_{max}$, and $K_i$ are not necessarily important or even meaningful (Bertz & Granneman, 1997). Different $K_i$ values may be obtained for different index drugs, and the rates of reaction are highly dependent upon factors such as pH, protein and phosopholipid content of the incubation medium, which may vary considerably from one laboratory to another (Bertz & Granneman, 1997). Kinetic parameters can vary 3- to 30-fold, depending upon the CYP enzyme involved (Skett et al., 1995). It has even been argued that the large number of individual microsomes required for the

determination of $K_i$ values may not be justifiable for these reasons (Newton et al., 1995; Bertz & Granneman, 1997). Therefore, in identifying the enzymes involved in a particular metabolic pathway, it is not so much the numerical quantity of the kinetic parameters which is important, but rather the ability to provide evidence of an enzyme's involvement using several converging lines of investigation (Guengerich, 1996; Maurel, 1996).

Many mathematical models of drug metabolism and drug interactions have been put forth, and some have proven quite useful in predicting drug-drug interactions (von Moltke et al., 1996a; Iwatsubo et al., 1997). However, these models are dependent upon many variables, and must be interpreted with caution. Accurate quantitative predictions require consideration of factors such as liver/plasma partition ratios, the components of total clearance of a drug, protein binding, systemic bioavailability, absorption, pre-systemic extraction, rate of blood flow, and the presence of active metabolites (Bertz & Granneman, 1997; Iwatsubo et al., 1997). Therefore, the important point about the present studies is not in predicting precise drug plasma levels of interacting compounds, but rather to point out situations in which potentials for interactions exist.

## 6.3 Implications of the present work

The finding that both trazodone and nefazodone are substrates of CYP3A4 is significant for several reasons. CYP3A4 is the most abundant of the CYP enzymes in the liver, and is involved in the oxidation of a wide array of compounds. There are large individual differences in CYP3A4 activity, and its presence in the intestinal epithelium leads to prehepatic extraction, and large individual differences in metabolism (Wilkinson, 1996). These are important factors to be aware of so that individual responses to trazodone and nefazodone can be monitored, and potential interfering factors controlled.

CYP3A4 is involved in a wide variety of metabolic processes, as can be seen from the present studies. CYP3A4 was found to mediate the N-dealkylation of NEF, OH-NEF, and trazodone, to mCPP, as well as the hydroxylation of NEF to OH-NEF, and the formation of TD from OH-NEF, by an as yet unknown mechanism (Mayol et al., 1994). These diverse metabolic pathways are consistent with reports in the literature that CYP3A4 is prominently involved in many N-dealkylation reactions (Coutts et al., 1994), as well as a wide variety of other oxidations such as aromatisation, N- and O-demethylations, N-oxidations, N-deethylations, methyl hydroxylations, and aromatic hydroxylations of both endogenous and exogenous compounds (Smith & Jones, 1992; Coutts et al., 1994; Wilkinson, 1996).

The finding that mCPP is a substrate for CYP2D6 is also consistent with typical CYP2D6 substrates, which possess a basic N which is protonated at physiological pH, and a hydrophobic region 0.5-0.7 nm from this basic N which

can attach itself to an anionic region in the enzyme active site (Guengerich et al., 1986; Coutts et al., 1994; Maurel,1996). Provided that the hydrophobic site is appropriately distanced from the basic N, CYP2D6 will catalyse a wide range of reactions, including aromatic and aliphatic hydroxylations, and N-dealkylations (Coutts et al., 1994; Maurel,1996).

It is particularly important to identify drugs which are substrates for CYP3A4 and CYP2D6 because of the wide range of potentially interacting substances. There are substrates and inhibitors of CYP3A4 and CYP2D6 in virtually every drug class (Bertz & Granneman, 1997), as well as readily available compounds such as grapefruit juice for CYP3A4, and codeine and dextromethorphan for CYP2D6 (Richelson, 1997). This problem is even more pronounced in psychiatry, where the drugs used often have many dose-dependent adverse side effects, which can be harmful, or at the very least interfere with compliance and quality of life.

Another point that is brought to light by the present work is the importance of accounting for the metabolism of metabolites, as well as that of the parent compounds. Although CYP3A4 is predominantly involved in the formation of mCPP from trazodone and NEF, CYP2D6 is responsible for the breakdown of mCPP. Therefore, trazodone and NEF have the potential to interact with substrates and/or inhibitors of CYP2D6, as well as having altered metabolism in genetically poor metabolizers of CYP2D6. This is particularly important information since mCPP is a psychopharmacologically active metabolite, which could interfere with the desired drug therapy. In addition, since the formation

and breakdown of mCPP are mediated by different enzymes, its plasma levels are highly susceptible to alterations in enzyme levels. For example, an induction of CYP3A4 would result in an increase in the formation of mCPP from its parent compounds, but its rate of metabolism by CYP2D6 would not necessarily increase, which could lead to an accumulation of mCPP and potential adverse effects. Similarly, if CYP2D6 was inhibited by a co-administered compound, the rate of formation of mCPP by CYP3A4 would remain constant, but its rate of breakdown would be decreased, again leading to an accumulation of mCPP. Thus it can be seen that it is important to know the enzymes involved in the formation *and* the metabolism of a drug, as well as to know the enzymes involved not only in the metabolism of the parent compound, but also in the metabolism of their metabolites as well.

There are basically two types of interactions to consider resulting from the present work. One is the effect of co-administered compounds on the metabolism of trazodone or nefazodone, and the second is the effect of trazodone, nefazodone, or mCPP on the metabolism of other compounds. The effect of co-administered compounds will depend upon the relative affinities and concentrations of the drugs at the enzyme site, individual levels of the enzyme, and the relative importance of the affected enzyme in overall drug metabolism. The consequences of the interaction will depend upon the therapeutic indices and side effects of the interacting drugs, as well as upon which particular metabolic pathways are affected, and whether parent compounds, active metabolites, or inactive metabolites are involved.

The effect of co-administered compounds on the metabolism of trazodone and NEF is also important. Attaining and maintaining therapeutic plasma drug levels is critical in psychiatry, and is particularly important for antidepressants, since clinical improvement may take weeks to show up. Furthermore, since not all antidepressants are effective in all individuals, it is important to know if the lack of clinical improvement is due to a resistance to the particular drug, or if it is due to an inadequate plasma level.

It can be seen, therefore, that there is no simple *a priori* method of precisely predicting the extent of a particular interaction. However, the importance of such studies is that they indicate potentials for interactions, so that adverse interactions can be avoided.

## 6.4 Future directions

It has been proposed (Maurel, 1996) that before any new drug is administered to humans, answers to the following questions should be obtained:

1) What is the metabolic pathway of the drug and what are the main metabolites?

2) Which enzyme system is involved in the metabolism of the drug?

3) Is the drug an inducer or inhibitor of drug metabolizing enzymes?

4) What are the possible drug interactions?

5) Can the drug be activated to cytotoxic or genotoxic metabolites?

Although trazodone and NEF are already on the market, these are still important questions to answer. Question 1 has been investigated for trazodone and NEF, and progress toward the answering of Questions 2 and 4 was made with the present thesis. The next questions to answer relate to the inducing and inhibiting capabilities of these drugs, as well as further characterizing possible drug interactions. The question of cytotoxic or genotoxic metabolites is also certainly vital, but is well beyond the scope of the present thesis.

Studies of enzyme induction are experimentally difficult, and pose many theoretical problems (Guengerich, 1996). For example, although animal tissue is the most readily available, and many CYP inducers have been characterized in animals, species differences do exist which make animal models questionable (Maurel, 1996). Human hepatocytes are acceptable models, but are often difficult to obtain (Maurel, 1996). *In vivo* approaches require the use of a marker drug, and have ethical considerations (Guengerich, 1996). Therefore, this is one area of investigation which requires the development of better models, and which will be interesting in future studies.

Further studies of the ability of trazodone and nefazodone to inhibit enzyme activity are also necessary to fully characterize their interaction potential *in vivo*. Although the fact that they are substrates of CYP3A4 makes them potential competitive inhibitors of this enzyme, it is also possible that they possess the ability to inhibit this and other CYP enzymes through another mechanism, as well as through other metabolites and metabolic pathways. For example, drugs can bind to an enzyme and block its activity without themselves

being substrates (non-competitive inhibition), they may have metabolites which bind to and inactivate an enzyme (suicide inhibitors), or they may interfere with the accumulation of an enzyme by interfering with the gene expression or protein processing of the enzyme (Maurel, 1996). These studies can be carried out *in vitro* by assessing the ability of these drugs to inhibit marker reactions for specific enzymes, and can be carried out in cell-free systems such as microsomes, facilitating this process.

Finally, it would be very useful if a procedure for determining individual levels of CYP3A4 activity existed. There is currently no known non-invasive and routinely available phenotyping procedure for CYP3A activity (Wilkinson, 1996). Such a test would be a great advantage for testing individual differences in CYP3A4 activity caused by both genetic and environmental factors, and for assisting in the adequate and appropriate dosing of CYP3A4 substrates.

As we learn more about drug metabolism and drug-drug interactions, it is becoming clear that a better system of informing physicians, pharmacists, and the general population about the risks of drug interactions is necessary. This is especially true in psychiatry, where polypharmacy is the norm, rather than the exception. Furthermore, with more drugs coming on to the market, there is an ever-increasing opportunity for drug interactions (Vargas et al., 1997). Coupled with this is an aging population with an increased life expectancy, which has resulted in an increase in multipathology and polypharmacy (Beers and Ouslander, 1989; Cardieux, 1989). A recent study of cardiac inpatients found that 25.4% of the patients were administered at least two potentially interacting

drugs simultaneously *while in hospital* (Vargas et al., 1997). Of all adverse drug reactions reported in this study, 20.2% were due to the joint action of two or more drugs. Clearly there is a need for increased education regarding the risks involved in polypharmacy.

As ever increasing amounts of time and resources are being dedicated to the continuing search for better and more effective antidepressant treatments, it would seem that the logical first step would be to improve the efficacy of our currently available antidepressants, by taking steps to ensure therapeutic plasma levels and to avoid drug-drug interactions.

## 6.5 REFERENCES


Ashton AK, Wolin RE (1996) Nefazodone-induced carbamazepine toxicity [letter]. *Am J Psychiatry* **153**:733.

Barbhaiya RH, Buch AB Greene DS (1996) A study of the effect of age and gender on the pharmacokinetics of nefazodone after single and multiple doses. *J Clin Psychopharmacol* **16**:19-25.

Barbhaiya RH, Shukla UA, Kroboth PD, Greene DS (1995) Coadministration of nefazodone and benzodiazepines: II. A pharmacokinetic interaction study with triazolam. *J Clin Psychopharmacol* **15**:320-326.

Beers MH, Ouslander JG (1989) Risk factors in geriatric drug prescribing, A practical guide to avoiding problems. *Drugs* **37**:105-112.

Bertz RJ, Granneman GR (1997) Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet* **32**:210-258.

Buch AB, Shukla UA, Pittman K, Barbhaiya RH (1993) Pharmacokinetics of nefazodone in extensive and poor metabolizers of dextromethorphan. *Pharm Res* **10**:S314.

Cardieux RJ (1989) Drug interactions in the elderly. How multiple drugs usage increases risk exponentially. *Postgrad Med* **86:**179-186.

Coutts RT, Su P, Baker GB (1994) Involvement of CYP2D6, CYP3A4, and other cytochrome P-450 isozymes in N-dealkylation reactions. *J Pharm Toxicol Meth* **31**:177-186.

Guengerich FP (1996) In vitro techniques for studying drug metabolism. *J Pharmacokinet Biopharmaceut* **24**:512-533.

Helms-Smith KM, Curtis SL, Hatton RC (1996) Apparent interaction between nefazodone and cyclosporine [letter]. *Ann Intern Med* **125:**424.

Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, Sugiyama Y (1997) Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacol Ther* **73**:147-171.

Kroboth PD, Folan MM, Lush RM, Chaikin PC, Shukla UA, Barbhaiya RH, Salazar DE (1995) Coadministration of nefazodone and benzodiazepines: I. Pharmacodynamic assessment. *J Clin Psychopharmacol* **15**:306-319.

Maes M, Westenberg H, Vandoolaeghe E, Demedts P, Wauters A, Neels H, Meltzer HY (1997) Effects of trazodone and fluoxetine in the treatment of major depression: Therapeutic pharmacokinetic and pharmacodynamic interactions through formation of meta-chlorophenylpiperazine. *J Clin Psychopharmacol* **17**:358-364.

Marino MR, Langenbacher KM, Uderman HD (1996) Interaction of nefazodone (N) and fluoxetine (F) [abstract]. *Clin Pharmacol Ther* **59**:180.

Maurel P (1996) The use of adult human hepatocytes in primary culture and other in vitro systems to investigate drug metabolism in man. *Adv Drug Deliv Rev* **22:**105-132.

Mayol RF, Cole CA, Luke GM, Colson KL, Kerns EH (1994b) Characterization of the metabolites of the antidepressant drug nefazodone in human urine and plasma. *Drug Metab Dispos* **22**:304-311.

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

Otani K, Ishida M, Kaneko S, Mihara K, Ohkubo T, Osanai T, Sugawaara K (1996) Effects of carbamazepine coadministration on plasma concentrations of trazodone and its active metabolite, m-chlorophenylpiperazine. *Ther Drug Monit* **18**:164-167.

Richelson E (1997) Pharmacokinetic drug interactions of new antidepressants: A review of the effects on the metabolism of other drugs. *Mayo Clinic Proc* **72**: 835-847.

Robinson DS, Roberts DL, Smith JM, Stringfellow JC, Kapilta SB, Seminara JA, Marcus RN (1996) The safety profile of nefazodone. *J Clin Psychiatry* **57**(suppl 2):31-38.

Skett, P, Tyson C, Guillouzo A, Maier P (1995) Report on the international workshop on the use of human in vitro liver preparations to study drug metabolism in drug development. *Biochem Pharmacol* **50**:280-285.

Smith DA, Jones BC (1992) Speculations on the substrate structure-activity relationship (SSAR) of cytochrome P450 enzymes. *Biochem Pharmacol* **44**:2089-2098.

Vargas E, Navarro MI, Laredo L, Garcia-Arenillas M, Garcia-Mateos M, Moreno A (1997) Effect of drug interactions on the development of adverse drug reactions. *Clin Drug Invest* **13**:282-289.

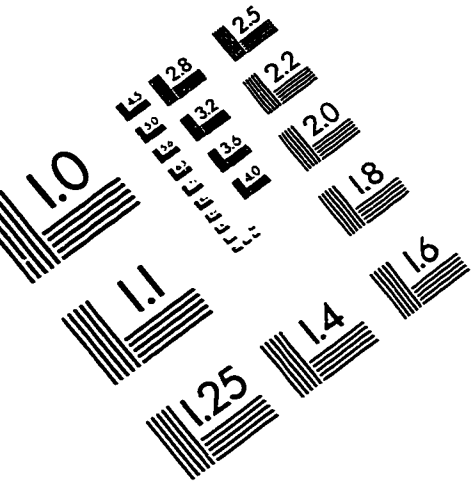
von Moltke LL, Greenblatt DJ, Harmatz JS, Duan SX, Harrel LM, Cotreau-Bibbo MM, Pritchard GA, Wright CE, Shader RI (1996a) Triazolam biotransformation by human liver microsomes in vitro: Effects of metabolic inhibitors and clinical confirmations of a predicted interaction with ketoconazole. *J Pharmacol Exp Ther* **276**:370-379.

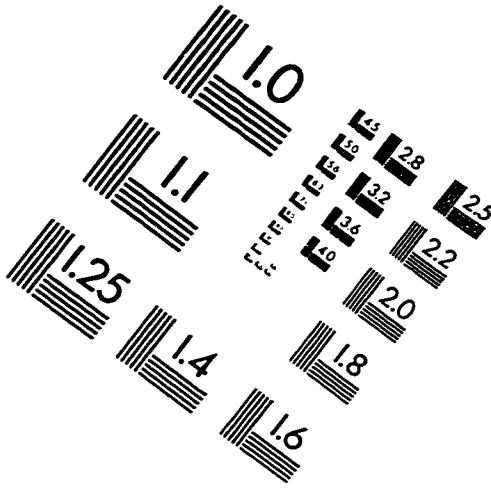
von Moltke LL, Greenblatt DJ, Schmider J (1996b) Nefazodone in vitro: Metabolic conversions, and inhibition of P450-3A isoforms [abstract]. *Clin Pharmacol Ther* **59**:176.

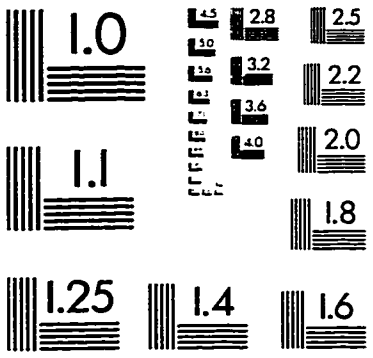
Wilkinson GR (1996) Cytochrome P4503A (CYP3A) metabolism: Prediction of an in vivo activity in humans. *J Pharmacokinet Biopharmaceut* **24**:475-489.

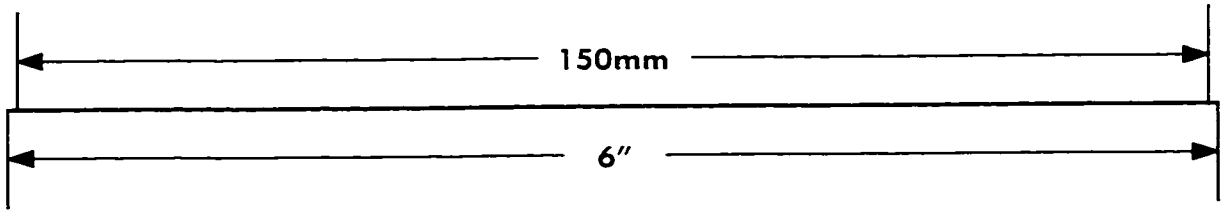
Yasui N, Otani K, Kaneko S, Ohkubo T, Osanai T, Ishida M, Mihara K, Kondo T, Sugawara K, Fukushima Y (1995) Inhibition of trazodone metabolism by thioridazine in humans. *Ther Drug Monit* **17**:333-335.

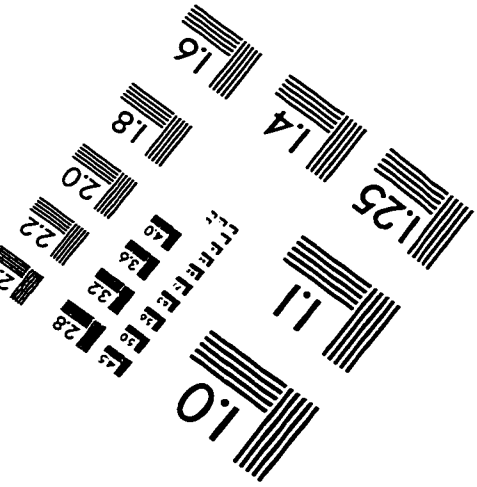
# IMAGE EVALUATION
# TEST TARGET (QA-3)

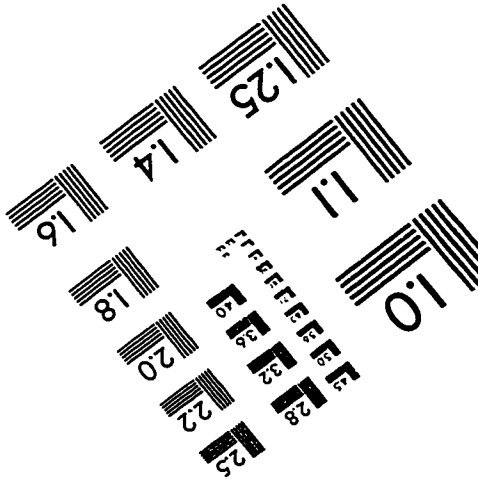



APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989