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THE METABOLISM OF AMITRIPTYLINE AND SOME ANALOGS OF AMPHETAMINE

by

MIMI VU BACH ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of DOCTOR OF PHILOSOPHY

in

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **THE METABOLISM OF AMITRIPTYLINE AND SOME ANALOGS OF AMPHETAMINE** submitted by **MIMI VU BACH** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL SCIENCES (DRUG METABOLISM AND ANALYSIS).**

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To my husband, Quang and my children, Tammy and Phil.

ABSTRACT

It is well recognized that *in vivo* drug biotransformation, an essential process in humans, can influence the clinical efficacy of a drug and may cause side effects and/or toxicity. Currently, *in vitro* models of human drug metabolism aid significantly in the determination of the enzyme systems that are involved in the biotransformation of a given drug and in the identification of metabolites and of compounds (drugs and xenobiotics) which might inhibit drug metabolism. These *in vitro* drug enzyme studies can also be used in the screening of new potential drugs and can provide helpful data on drug efficiency and toxicity. Either human liver microsomes or specific drug-metabolizing enzymes expressed in various cell culture systems are used for this purpose.

Evidence available from many studies suggests that most basic N-alkylated drugs, including antidepressant drugs and amphetamines, undergo extensive N-dealkylation to form N-dealkylated metabolites (nor-compounds) which may or may not possess pharmacological activities. For many N-alkylated drugs, the enzymes involved in this metabolic pathway have not been elucidated.

The major topic of interest in the studies presented in this thesis, therefore, was to identify the cytochrome P-450 (CYP) enzymes that catalyze the *in vitro* metabolic N-dealkylation of amitriptyline (AT). Related research was also conducted to determine CYP2D6's abilities to catalyze the N-dealkylation and ring C-oxidation of amphetamines.

The metabolism of AT was conducted *in vitro* with microsomes isolated from human liver and with CYP1A2, CYP2D6 and CYP3A4 enzymes expressed in human

cells to determine the metabolic profile of AT. Results from these studies indicated that CYP1A2, CYP2A6, CYP2D6, CYP3A4 and CYP4A were involved to different extents in the N-demethylation of AT to nortriptyline (NT). A published claim that CYP2C19 was involved in this metabolic pathway could not be confirmed.

In vitro metabolic studies of N-alkylated amphetamines, 4-methoxy-N-alkylated amphetamines and some N,N-dialkylated amphetamines, namely Deprenyl®, N-allyl-N-methylamphetamine, N,N-diallylamphetamine and N-methyl-N-propylamphetamine, were performed with CYP2D6 expressed in human cells. The N,N-dialkylated amphetamines were designed and synthesized to further investigate the role of CYP2D6 in metabolic N-dealkylation. GC analytical procedures were established to identify and quantify all metabolites formed.

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LIST OF ABBREVIATIONS

CH_3CN	Acetonitrile
NAA	N-Allylamphetamine
NAMA	N-Allyl-N-methylamphetamine
BPA	Benzphetamine
NAAA	N,N-Diallylamphetamine
AT	Amitriptyline
AM	Amphetamine
NBA	N-(n-Butyl)amphetamine
CNS	Central nervous system
CMI	Clomipramine
cDNA	Complementary deoxyribonucleic acid
J	Coupling constant
CYP	Cytochrome P450
DBQ	Debrisoquine
°C	Degree Celsius
DPR	Deprenyl
DMI	Desipramine
DXM	Dextromethorphan
ddAT	10,11-Didehydroamitriptyline
ddNT	10,11-Didehydronortriptyline
DLPC	L-α-Dilauroylphosphatidylcholine
DOPC	L-α-Dioleoylphosphatidylcholine
NEA	N-Ethylamphetamine
EM	Extensive metabolizer
FLU	Fluoxetine
FX	Fluvoxamine
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry

GC/MS	Gas chromatography/mass spectrometry
G6P	D-Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
Hz	Hertz
h	Hour
CYP	Human cytochrome P450
E-10-HO-AT	E-10-Hydroxyamitriptyline
Z-10-HO-AT	Z-10-Hydroxyamitriptyline
HI	Hydroxylation index
E-10-HO-NT	E-10-Hydroxynortriptyline
Z-10-HO-NT	Z-10-Hydroxynortriptyline
5-HT	5-Hydroxytryptamine (serotonin)
IMI	Imipramine
IS	Internal standard
KE	Ketoconazole
l	Litre
$MgCl_2$	Magnesium chloride
nmol	Nanomole
MT	Maprotiline
MS	Mass spectrometry
V_{max}	Maximal reaction velocity
m.p.	Melting point
MPT	Mephenytoin
M-AM	4-Methoxyamphetamine
M-NBA	4-Methoxy-N-(n-butyl)amphetamine
M-NEA	4-Methoxy-N-ethylamphetamine
M-NMA	p-Methoxy-N-methylamphetamine
NMA	N-Methylamphetamine
NMPA	N-Methyl-N-propylamphetamine
K_m	Michaelis constant

$NADP^+$	Nicotinamide adenine dinucleotide phosphate
μM	Micromole
ml	Millilitre
min	Minute
NPD	Nitrogen-phosphorus detector
M	Molar
MAO	Monoamine oxidase
MAOIs	Monoamine oxidase inhibitors
NE	Norepinephrine
NT	Nortriptyline
NMR	Nuclear magnetic resonance
PS	L-α-Phosphatidylserine
PD	Pondinil [(N-3-chloropropyl)amphetamine]
PM	Poor metabolizer
K_2CO_3	Potassium carbonate
$KHCO_3$	Potassium hydrogen carbonate
NPA	N-(n-Propyl)amphetamine
PT	Protriptyline
QND	Quinidine
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
SSRI	Selective serotonin reuptake inhibitor
$(CF_3CO)_2O$	Trifluoroacetic anhydride
TCAs	Tri-and tetra-cyclic antidepressants
URMs	Ultra-rapid metabolizers
vs	Versus

Chapter 1

Introduction

1.1 PREAMBLE

The major topic of interest of the research that is described in this thesis is the human metabolism of basic N-alkylated drugs. Initially, it was of prime interest:

1 to identify the CYP enzymes that catalyzed the metabolic N-dealkylation of these drugs;

2 and to determine what effect(s), if any, the N-alkyl group(s) had on metabolic oxidation at positions in the drug structure that were remote from the N-alkyl group;

While research studies were in progress, it also became of interest to include a preliminary study on a third topic:

3 to determine whether any simple pharmacokinetic relationships existed between the *in vitro* data (e.g. intrinsic clearance) that could be obtained from the *in vitro* studies with expressed human CYP enzymes (points 1 and 2 above), and literature *in vitro* data collected in drug metabolism experiments with human liver enzyme preparations.

1.2 DRUG METABOLISM

That drugs are metabolized in all cellular organisms, from single cell species to mammals, has been known for many years, but a thorough examination of drug metabolism (biotransformation) did not begin until the second half of the 20th century when biochemical and analytical techniques, as well as sophisticated instrumentation, became available to researchers interested in how the body rids itself of drugs, dietary organic compounds and environmental compounds (collectively known as xenobiotics). In the last forty to fifty years, great strides have been made in the identification of the biochemical systems involved in drug metabolism, and the types of chemical reactions that are commonly observed, which in general are predictable. Many excellent reviews are available on these topics (Testa and Jenner, 1976; Eichelbaum, 1982; Eichelbaum and

Gross, 1990; Brosen, 1990; Guengerich, 1990, 1995; Gonzalez, 1992; Wrighton and Stevens, 1992; Bertilsson, 1995; Bertilsson and Dahl, 1996; Nemeroff *et al.* 1996).

Most drugs and xenobiotics are lipophilic compounds, and, therefore, are not well excreted in the urine. They are reabsorbed in the kidneys and their actions would be prolonged if the body did not have mechanisms to terminate them. An unidentified speaker at a recent conference on drug metabolism estimated that if renal excretion was the only mechanism of terminating the actions of highly lipophilic drugs and xenobiotics, the action of drugs such as pentobarbital would continue for many years. The most important mechanism of ridding the body of drugs is their enzymatic conversion to more polar products which are relatively rapidly excreted in the urine. The body has enzymes that are distinct from those involved in protein, carbohydrate and lipid metabolism. They catalyze the biotransformation of drugs and xenobiotics by four general reactions: oxidation, reduction, hydrolysis and conjugation. The first three are known collectively as phase I reactions, and conjugation is termed a phase II reaction. Oxidation is by far the most important metabolic reaction. It occurs mainly in the liver, but also occurs in other organs and tissues such as gastrointestinal tract, lungs, and brain.

1.2.1 Common metabolic pathways of basic drugs

Many drugs with varying pharmacological properties possess common structural features – they are basic compounds or salts of bases. Examples of drugs that fall into this category are to be found in various drug groups, including β-adrenoceptor blocking agents, analgesics, local anesthetics, antiarrhythmic agents, antiepileptics, antihistamines, sympathomimetics, antidepressants, neuroleptics, CNS stimulants and anorectics. Basic drugs can be primary, secondary or tertiary amines. Quaternary amines are not relevant in the present study. If a drug is a tertiary amine, it is capable of being metabolized to a secondary amine which, in turn, may be further metabolized to a primary amine. Immediately, one is aware that members of different drug groups can be metabolized by an identical pathway, N-dealkylation. If the drug's structure incorporates an aromatic ring, and many do, the drug, regardless of its pharmacological classification, will most likely be metabolically oxidized at a site in the aromatic ring. In addition, if a basic drug

has an aromatic ring which possesses a methoxy (CH_3O) group, as codeine does, it is likely that that drug will be O-demethylated to the corresponding phenol. These three metabolic pathways, N-dealkylation, ring hydroxylation, and O-dealkylation are common, and often major routes of metabolism of drugs, regardless of their pharmacological class (Guengerich, 1990).

1.2.2 Drugs of major interest

Drugs of particular interest in the present work are those that are used in the treatment of depression and others that are capable of stimulating the central nervous system (CNS). Of some interest also are drugs used in the treatment of schizophrenia. Most such drugs are salts of basic compounds. Many of the tri- and tetra- cyclic antidepressants (TCAs) are salts of tertiary amines [e.g. amitriptyline (AT), imipramine (IMI), clomipramine (CMI), trimipramine, doxepin], or of secondary amines [e.g. desipramine (DMI), nortriptyline (NT), protriptyline (PT), maprotiline (MT)] (figure 1.1). DMI and NT are secondary amines and are active antidepressants, but they are also N-monodemethylated metabolites of IMI and AT, respectively. Secondary amines with one N-alkyl group, e.g. PT, MT, and the N-monodealkylated metabolites of the tertiary amines, or cyclic tertiary amines with one N-methyl group, e.g. mianserin, also undergo metabolic N-dealkylation. Only one available TCA, amoxapine, cannot be metabolically N-dealkylated. All of the selective serotonin reuptake inhibitor (SSRI) group of antidepressants that are in clinical use are also basic drugs (figure 1.2). Citalopram has an N,N-dialkylated structure and fluoxetine (FLU) and sertraline have a single N-alkyl group, so all three can undergo N-dealkylation. In contrast, neither fluvoxamine (FX) nor paroxetine can be metabolically N-dealkylated.

In all the drug examples that have been mentioned to this point the N-alkyl group is an N-methyl moiety. This is a reminder that the majority of secondary and tertiary drug amines that are used throughout the world are N-methylated or N,N-dimethylated, but it is not always the case. The anorectic, fenfluramine, for example contains an N-ethyl group, and both of the monoamine oxidase (MAO) inhibitors (MAOIs), pargyline and deprenyl (figure 1.3), as indicated below, have an N-propargyl (N-2-propynyl) group.

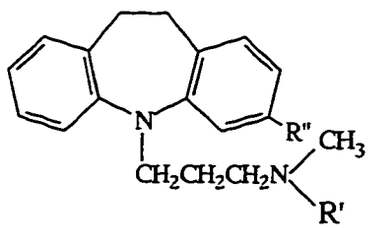
A few monoamine oxidase (MAO) inhibitors (MAOIs) are still prescribed as antidepressants. Phenelzine and tranylcypromine have been available for many years, and moclobemide was recently introduced. None of these three drugs has an N-alkyl group (figure 1.3), but they all possess an aromatic ring which, in most cases, is the likely target for biotransformation. Two other MAOIs are also used clinically, but not as antidepressants. They are deprenyl (an antiparkinson drug) and pargyline (an antihypertensive agent), both of which possess an N-methyl and an N-propargyl group and would be expected to undergo mono- and di-N-dealkylation as well as ring oxidation.

All of the important antipsychotic drugs (neuroleptics) are basic compounds. Some contain simple N-alkyl groups, but many have complex N-substituents (figures 1.4a;1.4b). Examples of those with simple N-substituents are chlorpromazine (N,N-dimethylated); thioridazine, thiothixene, clozapine and olanzapine (N-methylated); and fluphenazine (N-2-hydroxyethylated). In contrast, haloperidol, pimozide and risperidone have complex N-substituents; nevertheless, N-dealkylation is also a major metabolic route for these drugs. The biotransformation of risperidone by this mechanism (He and Richardson, 1995) is illustrated (figure 1.5).

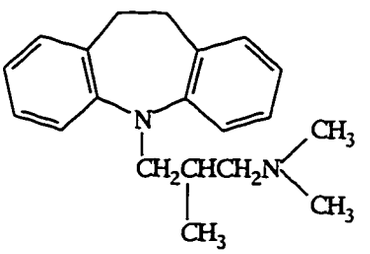
Many of the psychoactive drugs to which reference has been made also possess aromatic rings, and metabolic oxidation often occurs in such rings, unless the ring contains a deactivating group, such as the chlorine atom in CMI. However, the presence of an aromatic ring does not guarantee that metabolic ring oxidation will occur. A comparison of the metabolic pathways of amitriptyline with those of imipramine reveals that the major site of oxidation in the former is at C_{10} in the alicyclic ring, whereas in imipramine the site of oxidation is at C_2 in the aromatic ring (figure 1.6). The only difference in the structures of these two antidepressants is the attachment of the N,N-dimethylamino alkyl group to the ring system. This observation suggests that ring attachments remote from the site of metabolic oxidation influence the oxidative mechanism.

Relatively few drugs contain an aromatic ring methoxyl group. An example that is often quoted is the psychotropic drug, codeine, which has to be metabolically O-

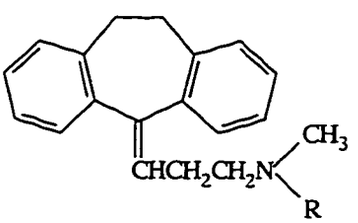
demethylated *in vivo* to morphine before its analgesic effect is apparent (Desmeules *et al.* 1991). An individual who is incapable of performing this biotransformation can ingest excessive doses of codeine and still not respond to the drug. This lack of response could mistakenly be ascribed to noncompliance, or worse, the individual could be wrongly identified as an abuser of the drug because he doesn't respond to large drug doses, but, in fact, the therapeutic failure is due to an inability to metabolically activate the drug. Two examples of psychoactive drugs that contain a methoxyl group are the "designer" drug of abuse, p-methoxymethylamphetamine (M-NMA) (Kitchen *et al.* 1979) and the MAOI brofaromine (Feifel *et al.* 1993). A major human *in vivo* metabolite of ring-methoxylated drugs is often the product of O-demethylation.



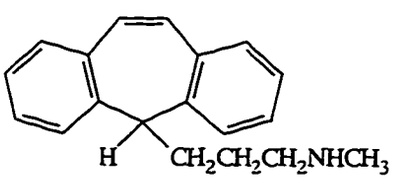
Imipramine $R' = CH_3$ $R'' = H$
Desipramine $R' = R'' = H$
Clomipramine $R' = CH_3$ $R'' = Cl$



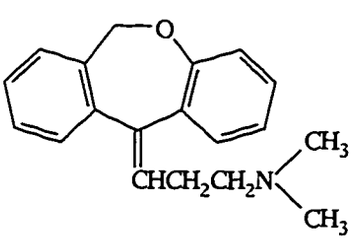
Trimipramine



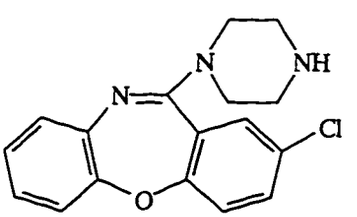
Amitriptyline $R = CH_3$
Nortriptyline $R = H$



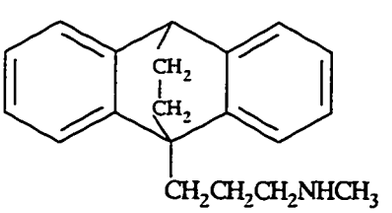
Protriptyline



Doxepin

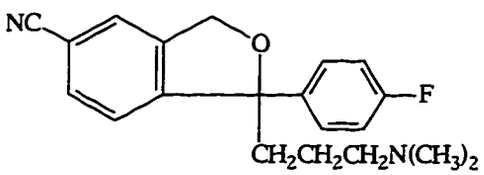


Amoxapine

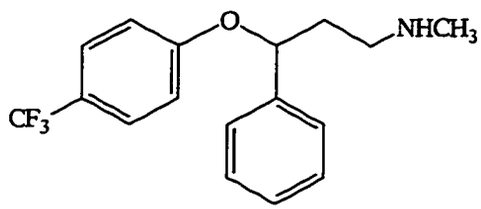


Maprotiline

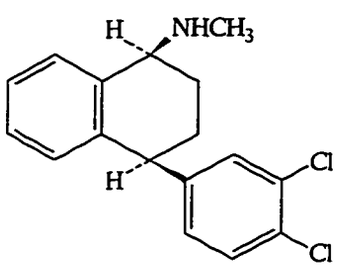
Figure 1.1 Tri-and Tetra-cyclic Antidepressants (TCAs)



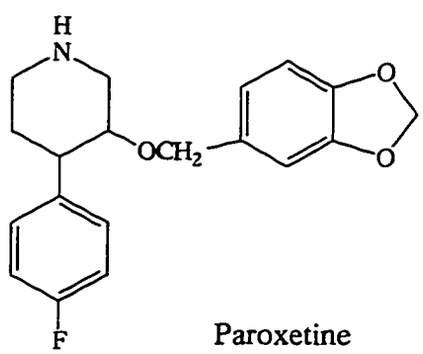
Citalopram



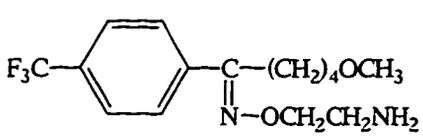
Fluoxetine



Sertraline

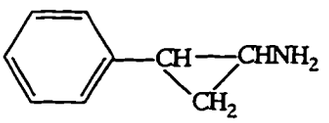


Paroxetine



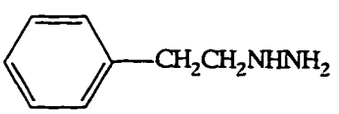
Fluvoxamine

Figure 1.2 Selective Serotonin Reuptake Inhibitors [SSRIs]

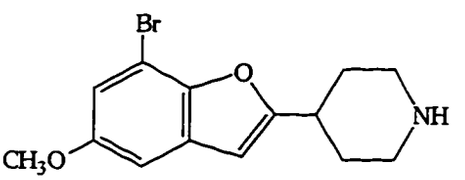


Tranylcypromine

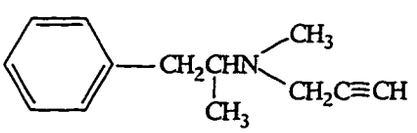
Moclobemide



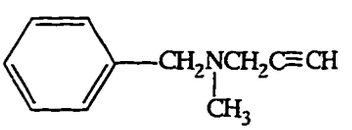
Phenelzine



Brofaromine

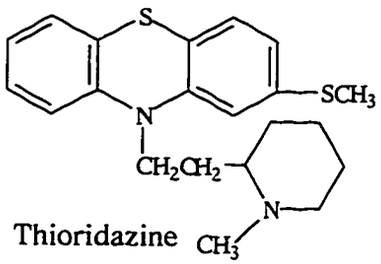


Deprenyl

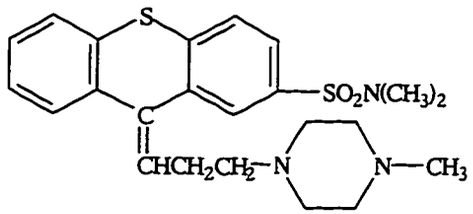


Pargyline

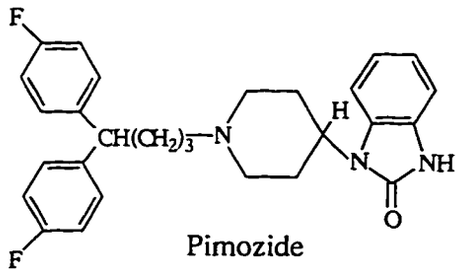
Figure 1.3 Monoamine Oxidase Inhibitors [MAOIs]



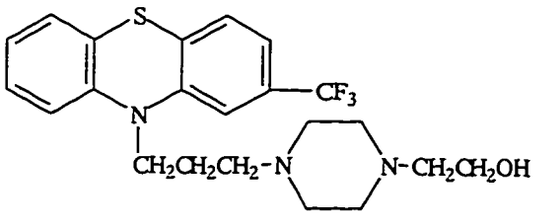
Thioridazine



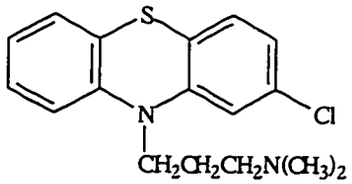
Thiothixene



Pimozide

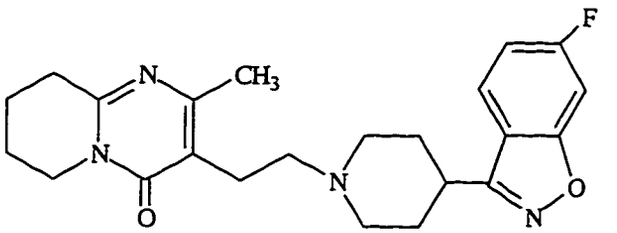


Fluphenazine

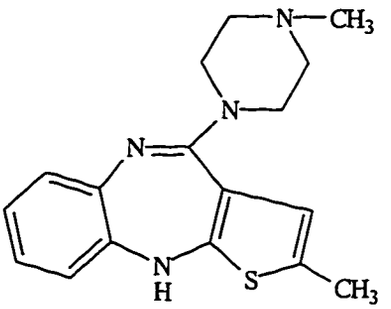


Chlorpromazine

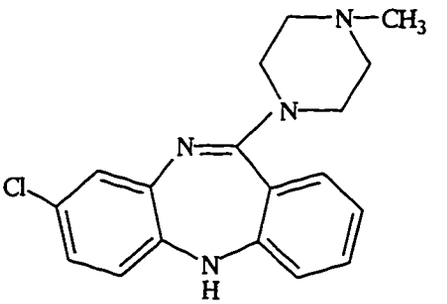
Figure 1.4a Neuroleptics



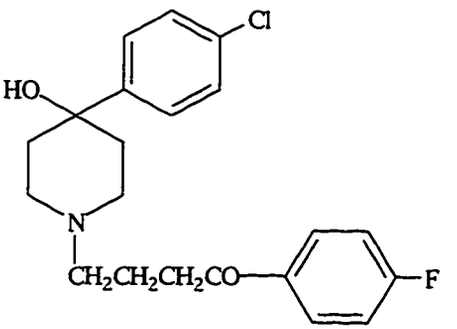
Risperidone



Olanzapine



Clozapine



Haloperidol

Figure 1.4b Neuroleptics

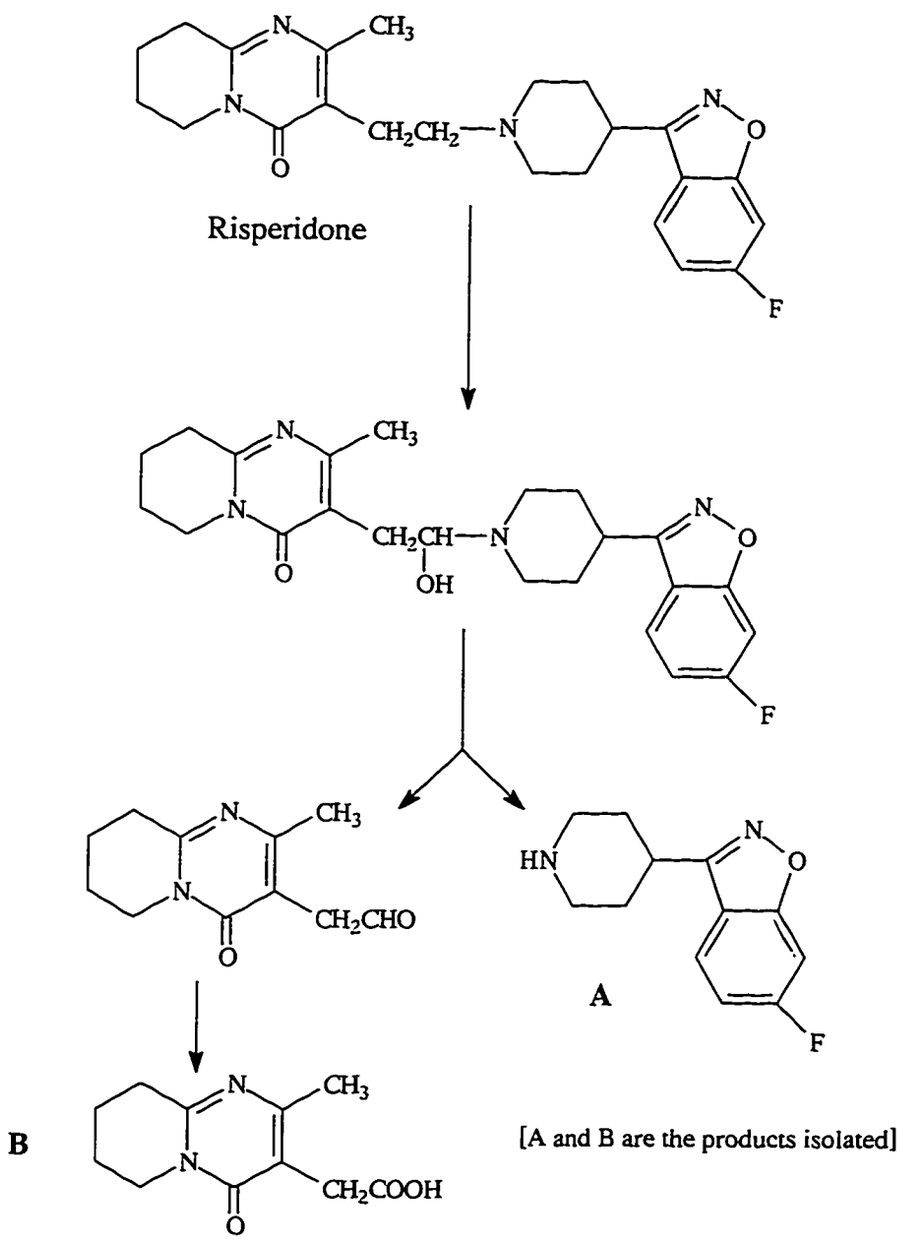


Figure 1.5 N-Dealkylation of Risperidone

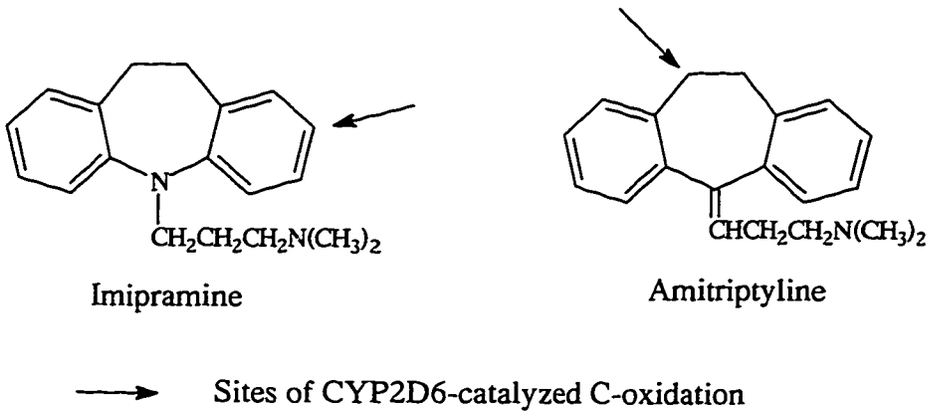


Figure 1.6 The major sites of ring hydroxylation

1.2.3 Human cytochrome P450 (CYP) enzymes

1.2.3.1 Characteristics of CYP enzymes

Cytochrome P450 is the term used to identify a large group of intrinsic membrane-bound enzymes or isozymes containing a single molecule of heme. Cytochrome P450 enzymes, now called CYP enzymes, vary in size from 45 to 60 kDa. The heme portion is an iron-containing porphyrin called protoporphyrin IX, and the protein portion is called the apoprotein. The name cytochrome P450 is derived from the fact that the reduced (Fe^{2+}) form of this enzyme binds with carbon monoxide to form a complex that has a distinguishing spectroscopic absorption maximum at 450 nm (Omura and Sato, 1964).

The human genome contains at least 31 CYP genes and 3 pseudogenes (Nelson *et al.* 1993). Each CYP isozyme is encoded by a separate gene. Thus, each CYP isozyme

consists of a different protein structure which is responsible for the different substrate specificities.

1.2.3.2 Function of CYP enzymes

The cytochrome P450-dependent monooxygenases (P450s or CYP enzymes) constitute a superfamily of metabolic enzymes (isozymes) that catalyze the oxygenation of endogenous (e.g. steroids) and exogenous chemicals (e.g. basic drugs) by the insertion into that chemical of one atom of oxygen derived from atmospheric O_2 (Guengerich, 1988). The CYP enzymes defend the body against environmental pollutants, usually they detoxify drugs, and they are involved in numerous endogenous oxidations. The overall metabolic oxidation reaction that is catalyzed by CYP enzymes can be depicted as follows:

$$\underset{\text{(drug)}}{RH} + O_2 + NADPH + H^+ \xrightarrow{\text{(CYP system)}} \underset{\text{(oxidized drug)}}{R\text{-}OH} + NADP^+ + H_2O$$

O_2 = atmospheric oxygen; NADPH = reduced form of nicotinamide adenosine dinucleotide phosphate; CYP system = CYP450, NADPH-cytochrome P450 reductase and a NADH-linked cytochrome b_5.

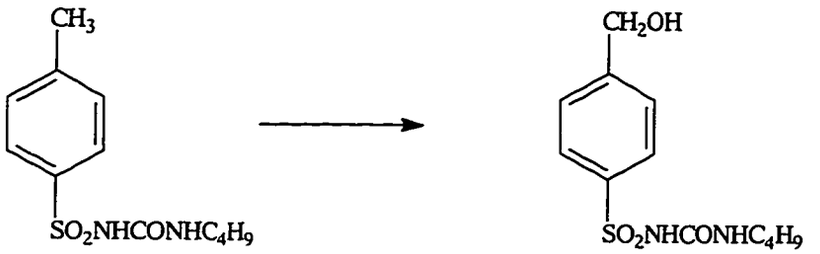
The CYP enzyme determines the substrate specificity of the overall system and structure(s) of product(s) formed during substrate metabolism. NADPH-cytochrome P450 reductase is responsible for the transfer of electrons from NADPH to CYP enzymes.

Examples of oxidation reactions most often seen in drug metabolism are:

Aromatic hydroxylation:

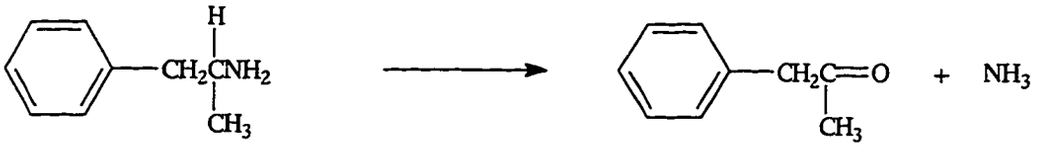
Phentermine

Aliphatic hydroxylation:



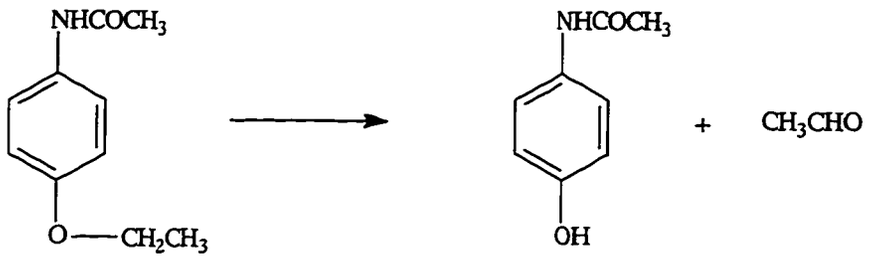
Tolbutamide

Oxidative deamination:



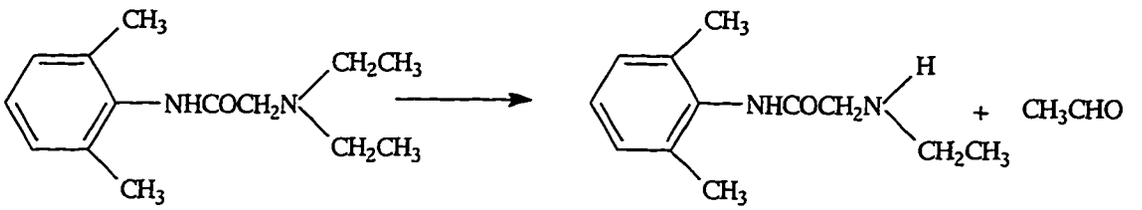
Amphetamine

O-Dealkylation:



Phenacetin

N-Dealkylation:



Lidocaine

1.2.3.3 Classification and nomenclature

Human P450 (CYP) isozymes have been allocated to 14 different gene families (1, 2, 3, 4, 5, 7, 8, 11, 17, 19, 21, 24, 27 and 51) based on the degree of similarity in the amino acid sequences of the CYP proteins. Most of these CYP enzymes families are listed in table 1.1 (Gonzalez, 1992, Nelson *et al.* 1996).

Table 1.1 Human CYP families (Gonzalez, 1992).

CYP family	No. of subfamilies	Major isozymes	Functions
CYP1	1	2	Xenobiotic and steroid metabolism
CYP2	6	12	Xenobiotic metabolism
CYP3	1	4	Xenobiotic and steroid metabolism
CYP4	1	1	Fatty acid hydroxylations
CYP7	1	1	Cholesterol 7α-hydroxylation
CYP11	2	3	Steroid 11β-hydroxylation
CYP17	1	1	Steroid 17α-hydroxylation
CYP19	1	1	Steroid aromatization
CYP21	1	1	Steroid 21-hydroxylation
CYP27	1	1	Cholesterol 27-hydroxylation

Each distinctive gene family displays less than 40% amino acid sequence similarity with all other families. All members of the same family have 40% or greater amino acid sequence identity (homology). Some of the gene families, especially family 2, contain subfamilies, each of which is designated a different capital letter. Members of the same subfamily have greater than 55% amino acid sequence similarity, and individual CYPs within a subfamily are distinguished by a terminal Arabic number (Nebert *et al.* 1989, 1991; Nelson *et al.* 1993). Although many CYP isozymes are found in human liver, only 15 specific forms have been identified as being involved in drug metabolism, and they are members of three families, CYP1, CYP2 and CYP3 (Wrighton and Stevens, 1992). Isoforms in these families have individual substrate characteristics which are related to the size and lipophilicity of the substrate, substrate spatial configuration and substrate ionization at physiological pH. Some enzymes, e.g. CYP2E1, oxidize a

relatively small number of substrates (Guengerich, 1995). Fortunately, relatively few human CYP isozymes are involved in most of the important transformations of drugs and chemicals. They are CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19 (CYP_{MP}), CYP2D6, CYP2E1 and CYP3A4. Of these, CYP2D6 and CYP3A4 are dominant in the metabolism of basic drugs (Guengerich, 1995), despite the fact that CYP2D6 is present in human liver and other tissues in relatively small amounts (Shimada *et al.* 1994). About 70% of liver cytochrome P450 can be accounted for by CYP1A2 (~12% of total), CYP2A6 (~4%), CYP2B6 (<1%), CYP2C (~20%), CYP2D6 (~4%), CYP2E1 (~7%) and CYP3A (~30%) proteins. These percentages are approximate means. Neonatal liver samples tend to have lower amounts of CYP1A2, CYP2A6, and CYP2E1.

DeVane (1994), Guengerich (1995), Spatzenegger and Jaeger (1995) and Daniel *et al.* (1996) identify many substrates, inducers and inhibitors of the most important CYP enzymes, but their lists are not identical and are incomplete. A combined list of the CYP substrates provided by these investigators is provided (table 1.2). At the time the lists of substrates in table 1.2 were constructed, many CYP2C substrates had not been identified. The situation is improving, but complete lists of specific substrates for CYP2C8, CYP2C9, CYP2C18 and CYP2C19 are not yet available.

1.2.3.4 Amino acid sequences of CYP1A2, CYP2D6 and CYP3A4

The amino acid sequences of some human CYP enzymes are available. A WorldWide Web server entitled "The Directory of P450-containing Systems" is located at <http://www.icgeb.trieste.it/p450/>. It contains the most up-to-date lists of mRNA base sequences, and the amino acid sequences in most CYP proteins, and is readily accessible. The directory is well referenced and it provides accession numbers and cross-links to relevant sequence data banks (Degtyarenko and Fabian, 1996). The amino acid (AA) sequences provided in table 1.3 were obtained from this Directory.

Table 1.2 Common substrates of various cytochrome P450 enzymes.

CYP1A1:

Polycyclic aromatic hydrocarbons, e.g. benzo[*a*]pyrene 7,8-epoxidation, and some of the substrates listed for CYP1A2. The CYP1A1 enzyme is normally absent from mature human livers unless its production is induced.

CYP1A2:

Acetaminophen (paracetamol), antipyrine, bufuralol, caffeine, chlorotrianisene, chlorzoxazone, dantrolene, diethylstilbestrol, estradiol, flutamide, lidocaine, ondasetron paraxanthine, phenacetin, procarbazine, propafenone, prostaglandins, tacrine, tamoxifen, theobromine, theophylline, toltrazuril, verapamil, warfarin, zoxazoleamine, N-demethylation of TCAs.

CYP2A:

Coumarin, testosterone.

CYP2B:

Adriamycin, benzphetamine, cocaine, cyclophosphamide, diazepam, ethosuximide, ethylmorphine, ifosfamide, procarbazine, trimethadone, warfarin.

CYP2C: [Specific isozyme not identified]:

Benzphetamine, clomipramine, cyclophosphamide, dapsone, diazepam, diclofenac, ethosuximide, ibuprofen, mephenytoin, losartan, naproxen, nifedipine, oxyphenazole, phenylbutazone, phenytoin, piroxicam, progesterone, proguanil, sulfaphenazole, sulfinpyrazone, tamoxifen, taxol, tenoxicam, testosterone, tetrahydrocannabinol, toltrazuril, trimethadone, warfarin.

CYP2C8:

Retinoic acid, retinol, tienilic acid (also 2C9 and 2C18), tolbutamide (also 2C9 and 2C18).

CYP2C9:

Diclofenac, hexobarbital, ibuprofen, mefanamic acid, phenytoin, piroxicam, tenoxicam, tienilic acid (also 2C8 and 2C18); tolbutamide (also 2C8 and 2C18), trimethadone, S-warfarin.

CYP2C18:

Tienilic acid (also 2C8 and 2C9); tolbutamide (also 2C8 and 2C9).

CYP2C19:

Amitriptyline, clomipramine, citalopram, desmethyldiazepam, diazepam, (-)-hexobarbital, imipramine, S-mephenytoin, R-mephobarbital, methylphenytoin, moclobemide, nirvanol, omeprazole, proguanil, propranolol.

[continued]

CYP2D6:

Ajmaline, amiflamine, amitriptyline, apridine, brofaromine, bufuralol, bunitrolol, bupranolol, CGP 15210G, captopril, citalopram, CMI, clozapine, codeine, debrisoquine, deprenyl, desipramine, dextromethorphan, encainide, ethylmorphine, flecainide, fluoxetine, fluperlapine, fluphenazine, guanoxan, haloperidol, hydrocodone, imipramine, indoramine, lidocaine, MDL 73005, MT, p-methoxyamphetamine, methoxyphenamine, metoprolol, mexiletine, minaprine, norfluoxetine, nortriptyline, omeprazole, ondansetron, paroxetine, perhexiline, perphenazine, phenformin, propafenone, propranolol, N-propylajmaline, remoxipride, risperidone, sparteine, thebaine, thioridazine, timolol, tomoxetine, trifluperidol, trimipramine.

CYP2E1:

Acetaminophen (paracetamol), caffeine, chlorzoxazone, diethyl ether, enflurane, ethanol, ethosuximide, halothane, isoflurane, methoxyflurane, sevoflurane, theophylline.

CYP3A4:

[Many of these drugs are also substrates for CYP3A3. N-de-Me and N-de-Et refer to N-demethylation and N-deethylation, respectively].

Acetaminophen (paracetamol); alfentanil (N-dealkylation), alpidem, alprazolam, amiodarone (N-de-Et), Bayer R4407 [(+)K8644], Bayer R5417 [(-)K8644], benzphetamine (N-de-Me), budesonide, caffeine, cannabidiol, canrenoate potassium, carbamazepine, cocaine, codeine (N-de-Me); cyclophosphamide, cyclosporin A, cyclosporin G, dantrolene, dapsone, dehydroepiandrosterone, dextromethorphan (N-de-Me), diazepam, digitoxin, dihydroergotamine, diltiazem, ebastine, enalapril, ergot CQA 206-291, erythromycin (N-de-Me), 17β-estradiol, 17α-ethinyl estradiol, etoposide, ethosuximide, ethylmorphine, felodipine, FK 506, flutamide, gestodene, glyceryl trinitrate, ifosfamide, imipramine (N-de-Me), ketoconazole, lidocaine (N-de-Et), losartan, lovastatin, MDL 73005, mephenytoin, miconazole, midazolam, nifedipine, niludipine, nimodipine, nisoldipine, nitrendipine, omeprazole, oxodipine, prednisone, propafenone, progesterone, quinidine, rapamycin, retinoic acid, RU 486, sertindole (N-dealkylation), sulfamethoxazole, sulfentanil, tamoxifen (N-de-Me), taxol, TCAs (N-de-Me), teniposide, terfenadine (N-dealkylation), testosterone, tetrahydrocannabinol, theophylline, toltrazuril, triacetyloleandomycin (≡ troleandomycin), triazolam, trimethadone (N-de-Me), verapamil, vinblastine, warfarin, zatosetron, zonisamide.

Table 1.3 CYP1A2, CYP2D6 and CYP3A4 Amino Acid Sequences

CYP2D6: Sequence: 497 AA; MW: 55801; Heme-binding amino acid residue = 443.

```
MGLEALVPLA VIVAIFLLLV DLMHRRQRWA ARYPPGPLPL PGLGNLLHVD    50
FQNTPYCFDQ LRRRFGDVFS LQLAWTPVVV LNGLAAVREA LVTHGEDTAD   100
RPPVPITQIL GFGPRSQGVF LARYGPAWRE QRRFSVSTLR NLGLGKKSLE   150
QWVTEEAACL CAAFANHSGR PFRPNGLLDK AVSNVIASLT CGRRFEYDDP   200
RFLRLLDLAQ EGLKEESGFL REVLNAVPVL LHIPALAGKV LRFQKAFLTQ   250
LDELLTEHRM TWDPAQPPRD LTEAFLAEME KAKGNPESSF NDENLRIVVA   300
DLFSAGMVTT STTLAWGLLL MILHPDVQRR VQQEIDDVIG QVRRPEMGDQ   350
AHMPYTTAVI HEVQRFGDIV PLGMTHMTSR DIEVQGFRIP KGTTLITNLS   400
SVLKDEAVWE KPFRFHPEHF LDAQGHFVKP EAFLPFSAGR RACLGEPLAR   450
MELFLFFTSL LQHFSFSVPT GQPRPSHHGV FAFLVSPSPY ELCAVPR      497
```

CYP2D6-Val: 374 Met in the structure above is replaced with Val.

CYP1A2: Sequence: 515 AA; MW: 58294; Heme-binding amino acid residue = 458.

```
MALSQSVPFS ATELLLASAI FCLVFWVLKG LRPRVPKGLK SPPEPWGWPL    50
LGHVLTLGKN PHLALSRMSQ RYGDVLQIRI GSTPVLVLSR LDTIRQALVR   100
QGDDFKGRPD LYTSTLITDG QSLTFSTDSG PVWAARRRLA QNALNTFSIA   150
SDPASSSSCY LEEHVSKEAK ALISRLQELM AGPGHFDPYN QVVVSVANVI   200
GAMCFGQHFP ESSDEMLSLV KNTHEFVETA SSGNPLDFFP ILRYLPNPAL   250
QRFKAFNQRF LWFLQKTVQE HYQDFDKNSV RDITGALFKH SKKGPRASG    300
LIPQEKIVNL VNDIFGAGFD TVTTAISWSL MYLVTKPEIQ RKIQKELDTV   350
IGRERRPRLS DRPQLPYLEA FILETFRHSS FLPFTIPHST TRDTTLNGFY   400
IPKKCCVFVN QWQVNHDPEL WEDPSEFRPE RFLTADGTAI NKPLSEKMML   450
FGMGKRRCIG EVLAKWEIFL FLAILLQQLE FSVPPGVKVD LTPIYGLTMK   500
HARCEHVQAR RFSIN                                         515
```

CYP3A4: Sequence: 503 AA; MW: 57343; Heme-binding amino acid residue = 442.

```
MALIPDLAME TWLLLAVSLV LLYLYGTHSH GLFKKLGIPG PTPLPFLGNI    50
LSYHKGFCMF DMECHKKYGK VWGFYDGQQP VLAITDPDMI KTVLVKECYS   100
VFTNRRPFGP VGFMKSAISI AEDEEWKRLR SLLSPTFTSG KLKEMVPIIA   150
QYGDVLVRNL RREAETGKPV TLKDVFGAYS MDVITSTSFG VNIDSLNNPQ   200
DPFVENTKKL LRFDFLDPFF LSITVFPFLI PILEVLNICV FPREVTNFLR   250
KSVKRMKESR LEDTQKHRVD FLQLMIDSQN SKETESHKAL SDLELVAQSI   300
IFIFAGYETT SSVLSFIMYE LATHPDVQQK LQEEIDAVLP NKAPPTYDTV   350
LQMEYLDMVV NETLRLFPIA MRLERVCKKD VEINGMFIPK GVVVMIPSYA   400
LHRDPKYWTE PEKFLPERFS KKNKDNIDPY IYTPFGSGPR NCIGMRFALM   450
NMKLALIRVL QNFSFKPCKE TQIPLKLSLG GLLQPEKPVV LKVESRDGTV SG 503
```

[A=Ala; C=Cys; D=Asp; E=Glu; F=Phe; G=Gly; H=His; I=Ile; K=Lys; L=Leu; M=Met; N=Asn; P=Pro; Q=Gln; R=Arg; S=Ser; T=Thr; V=Val; W=Trp; Y=Tyr]

1.2.3.5 Enzyme polymorphism

The metabolism of many clinically important drugs in human is dominantly under genetic control and exhibits genetic polymorphism in the population (Daly *et al.* 1993). An individual with deficient metabolic capacity for a drug catalyzed by a polymorphic enzyme(s) is called a poor metabolizer (PM) whereas the individual who has a normal metabolic capacity is an extensive metabolizer (EM). Polymorphism in CYP genes results from changes in nucleotide base sequences of an mRNA which generally produce changes in amino acid sequences of CYP enzyme proteins. Two main types of changes in DNA that cause genetic disorders are major abnormalities (due to deletions, insertions or rearrangements of genes) and single nucleotide abnormalities (point mutation that results from the substitution of a single nucleotide base) in a critical region of a gene. These mutations normally lead to the formation of nonfunctional CYP enzymes. To date, only two polymorphic CYP enzymes have been identified, CYP2D6 and CYP2C19. Other CYP enzymes, including CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2E1 and CYP3A4 are sometimes claimed to be polymorphic, but definite genetic information about these enzymes is not yet available.

1.2.3.5.1 CYP2D6 polymorphism

CYP2D6 polymorphism is known as debrisoquine/sparteine hydroxylation polymorphism and is the best understood example of polymorphic drug oxidation (Mahgoub *et al.* 1977; Eichelbaum *et al.* 1979). The gene encoding the CYP2D6 enzyme is located on chromosome 22q13.1 (Eichelbaum *et al.* 1987; Guengerich, 1995). This enzyme is expressed in human liver and other tissues (intestine, kidney and human brain). The complete amino acid sequence of wild type (wt, nonmutated) CYP2D6 is listed in table 1.3.

Several mutant alleles of CYP2D6 gene that cause the CYP2D6 PM phenotype have been identified. All known mutant CYP2D6 alleles, CYP2D6A, B, C, D, E, F, H, J, L, T and Z, result from different types of mutations. These mutations include:

(a) single base deletion [CYP2D6A (Gough *et al.* 1990)],

(b) single point mutations [CYP2DE (Daly *et al.* 1995; Evert *et al.* 1994) and CYP2D6H (Marez *et al.* 1996)],

(c) association of multiple point mutations [CYP2D6L (Agúndez *et al.* 1995; Dahl *et al.* 1995a) and CYP2D6J (Johansson *et al.* 1991; Johansson *et al.* 1994; Dahl *et al.* 1995b)],

(d) splice-site mutation associated with several point mutations [CYP2D6B (Gonzalez and Idle 1994; Lin *et al.* 1996) and CYP2D6F (Gonzalez and Idle 1994; Marez *et al.* 1996)],

(e) single codon deletions [CYP2D6C (Tyndale *et al.* 1991)],

(f) complete gene deletion [CYP2D6D; Gaedigk *et al.* 1991; Dahl *et al.* 1995b)].

A small proportion of the population possesses extremely high CYP2D6 activity (Bertilsson *et al.* 1985). This group is called "ultra-rapid metabolizers" (URMs). These subjects have funtionally active CYP2D6(L_1 and L_2) genes which cause extra CYP2D6 enzyme to be expressed (Johansson *et al.* 1993; Agúndez *et al.* 1995; Dahl *et al.* 1995a). The majority of the population lies somewhere between PMs and URMs and are called EMs.

The lack of functional CYP2D6 enzyme in PMs will lead to an accumulation of the parent drug in the blood and tissues and may result in concentration-dependent side effects. In contrast, the URMs will have extremely low plasma concentrations of a pharmacological active agent, which may lead to therapeutic failure or delayed response. Ethnic differences in the incidence of the CYP2D6 PM individuals average 6.7% in Caucasian, 2% in Orientals and 1% in Arabics (Gaedigk *et al.* 1991; Gonzalez and Meyer, 1991). An individual's CYP2D6 activity can be predicted by phenotyping or genotyping.

1.2.3.5.2 CYP2C19 polymorphism

Mephenytoin hydroxylation polymorphism was firstly described by Küpfer and Bircher (1979). The enzyme was originally known as P450meph (Meyer *et al.* 1986) and later called P4502C9 (Nebert *et al.* 1989). Recently, the polymorphic enzyme responsible for the aromatic 4'-hydroxylation of S-mephenytoin was identified as

CYP2C19 (Goldstein *et al.* 1994). This CYP enzyme is located on chromosome 10q24.1–24.3 (Guengerich, 1995) and it is the least abundant of the four CYP2C isozymes, CYP2C8, CYP2C9, CYP2C18 and CYP2C19, expressed in liver (Goldstein *et al.* 1994). The two mutations responsible for defects in the CYP2C19 protein are:

(a) mutant m_1 caused by single base change guanine → adenine at position 681 of exon 5 of the CYP2C19 gene. This results in a truncated, nonfunctional protein (De Morais *et al.* 1994a).

(b) mutant m_2 caused by single base change guanine → adenine at position 636 of exon 4 of the CYP2C19 gene which also creates a truncated, nonfunctional protein (De Morais *et al.* 1994b). This mutation has not been detected in Caucasians, but has been in Japanese and other oriental subjects.

Both mutations accounted for 100 percent of the CYP2C19 PM phenotype in Japanese and for 83 percent of PMs in the Caucasian group. There are important ethnic differences in the aromatic 4'-hydroxylation of S-mephenytoin *in vivo* in human. About 3% in Caucasians are CYP2C19 PMs (Alván *et al.* 1990; Bertilsson *et al.* 1992) but between 18 and 23% of Japanese (Nakamura *et al.* 1985; Jurima *et al.* 1985), 15 to 17% of Chinese (Bertilsson *et al.* 1992) and 13% of Koreans (Sohn *et al.* 1992) are PMs. An individual's CYP2C19 activity can be predicted by phenotyping or genotyping.

1.2.3.6 Phenotyping

Phenotyping is a relatively simple method of assessing an individual's ability to metabolize drugs that are substrates of the polymorphic enzymes, CYP2D6 or CYP2C19. It has benefitted older patients who receive psychotropic drugs and has shown that PMs can be treated successfully with lower doses of antidepressants, while a minority of patients (URMs) need drug doses much higher than those provided to most patients. The author is aware of individuals who have 'responded badly' to drugs over their lifetimes and are grateful and relieved when they learn that they are PMs of CYP2D6 substrates. If these PM individuals must take such drugs, adjustments in drug dosage should be made.

In CYP2D6 phenotyping, debrisoquine (DBQ), sparteine or dextromethorphan (DXM) are the most commonly used probe drugs. All are substrates of CYP2D6. A

suitable dose of the probe drug is administered orally and urine is collected for an appropriate period (8 – 24 h) after drug administration. The urine is hydrolyzed to release metabolites from conjugates. The concentrations of the administered drug and the selected metabolite in the urine are determined and a metabolic ratio (MR) or $\log_{10}$MR is calculated.

MR = % of drug excreted unchanged / % of drug excreted as the metabolite.

The DBQ $\log_{10}$MR ranges are approximately -0.2 to -1.0 for URMs; -1.0 to 1.08 for EMs; >1.1 and rising to >2.0 for PMs. In the EM group, $\log_{10}$MR is -1.0 to +0.5 in most homozygous EMs and >+0.5 in most heterozygous EMs, but there is overlap.

The DXM $\log_{10}$MR values are generally in the range 0.0030 → 5.27 (Henthorn *et al.* 1989), although a $\log_{10}$MR of 9.62 has been recorded for one very poor metabolizer of DXM (Coutts, 1994). There is close correlation between DXM and DBQ phenotypes (Perault *et al.* 1991).

In CYP2C19 phenotyping, the procedure has to be modified because very little nonmetabolized mephenytoin (MPT) is excreted in urine; in some instances, no drug is detected. Also taken into account is the observation that the elimination of the metabolite, 4'-OH-MPT, is stereospecific for the S-enantiomer (Küpfer and Preisig, 1984). Racemic MPT is administered and a 0 – 8 h urine is collected. An hydroxylation index (HI) is determined

HI = dose of the MPT S-enantiomer (μmol) / amount of S-4'-OH-MPT (μmol)

All EMs of MPT have an HI value of 5.6 or less, whereas PMs have an HI well in excess of 5.6 and usually in the range >20 to >700.

There are advantages to the phenotyping technique. It is a relatively simple, rapid, inexpensive, noninvasive and reproducible procedure, and it normally has to be performed only once in a person's lifetime. It could easily be conducted routinely on psychiatric patients in a hospital setting. The major criticism of the procedure is that the

individuals being phenotyped must be completely drug-free. In many instances, a patient's phenotype is required while that patient is taking drugs, or perhaps herbal products that contain flavonoids or other naturally occurring organic chemicals which are likely to interfere in the assessment of phenotype. A patient who is receiving another drug that is a substrate of CYP2D6 would be an apparent PM if the phenotyping was conducted at that time. It is important to remember that the PM phenotype most often is genetically derived, but it also may be drug-induced.

1.2.4 Structural features and physical properties of CYP2D6 substrates

All the drugs identified in table 1.2 as substrates of CYP2D6 possess common structural features and physical properties. They are lipophilic basic compounds that are protonated (quaternized) at physiological pH, and they possess a planar, usually aromatic, ring system. The aromatic ring and the positively charged N atom are both necessary to orientate the drug correctly within the CYP2D6 protein active site where metabolic oxidation occurs. Guengerich *et al.* (1986) proposed a model of the active site of human cytochrome CYP2D6. At this site, the drug molecule is seen to adopt a conformation that orientates the N^+ atom towards an anionic location (a COO^- group) on the CYP protein, while the aromatic ring aligns itself to a relatively planar region of the protein. When this concept is applied to amitriptyline, the model of CYP2D6's active site is as shown in figure 1.7 in which the location in the drug where metabolic oxidation occurs is correctly positioned. The distance between the site of metabolic oxidation (the heme location) and the N^+ atom is always between 0.5 to 0.7 nm (Guengerich *et al.* 1986; Meyer *et al.* 1986).

Islam *et al.* (1991) generated another 3-dimensional molecular template for substrates of CYP2D6, based on information obtained from X-ray crystallographic studies. This template defines the stereochemical requirements for appropriate substrates in terms of molecular volume and position of key atoms. An important feature of this model is that the optimum distance from the drug's N^+ atom to the protein's anionic site is calculated to be in the 0.25 – 0.45 nm range. Both models (N^+ to anionic site distance,

and N^+ to metabolic site distance) are compatible. The information supplied by them is useful in the prediction of possible metabolic products of novel basic drugs. This model can be used, for example, to explain the extensive metabolism of IMI to 2-HO-IMI, the biotransformation of AT to 10-HO-AT, and many other related ring-hydroxylations catalyzed by CYP2D6.

The location of the anionic site (the COO^- group) on the CYP2D6 protein to which substrates bind was identified recently (Mackman *et al.* 1996) as the Asp-301 amino acid residue (see table 1.3). Its replacement with Glu, which preserves the carboxylate side chain, results in only minor changes in enzyme catalytic activity, but when Asp-301 is replaced by Asn or Gly, the carboxylate moiety is eliminated and enzyme's catalytic activity is virtually abolished.

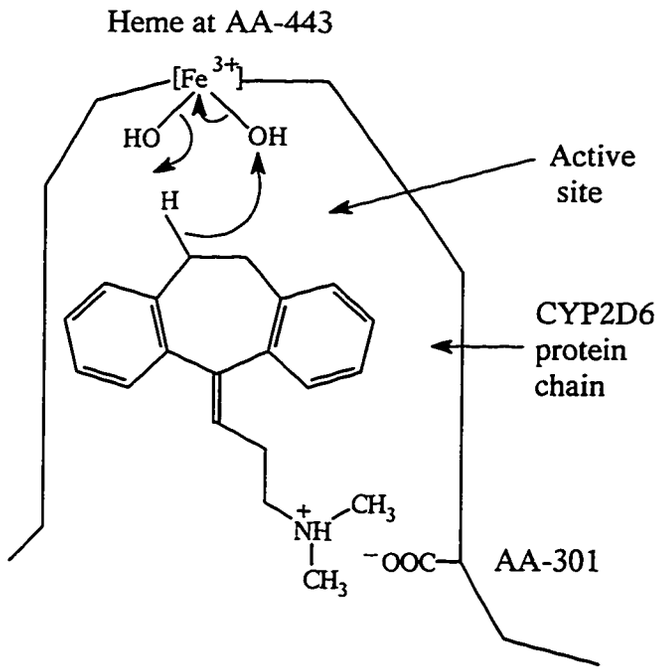


Figure 1.7 Diagram of the active site in CYP2D6 in which AT is suitably oriented for hydroxylation to occur at C-10. [AA-301: the 301-amino acid residue is aspartic acid; AA-443: the 443-amino acid residue is cysteine to which heme binds].

1.2.5 Structural features and physical properties of CYP1A2 and CYP3A4 substrates

Substrates of CYP1A2 and CYP3A4 can be either acidic or basic compounds. As shown in table 1.2, more than 80 drugs are oxidized by CYP3A4 and about 25 drugs are oxidized by CYP1A2. Clearly, the involvement of the CYP3A4 enzyme in drug metabolism is great. It oxidizes a wide range of substrates with diversity in chemical structures and molecular weight. Very often CYP3A4 and CYP1A2 are involved in the N-dealkylation of basic substrates whereas CYP2D6 is involved mainly in the C-oxidation. However, the catalytic specificities of human cytochromes CYP3A4 and CYP1A2 have not been reported. Knowledge of structure-activity relationships that underlie the catalytic specificity of these enzymes will be useful in predicting the substrates for these enzymes.

1.2.6 Metabolic N-dealkylation

Which CYP enzymes are involved in the N-dealkylation of basic drugs in humans? It follows from the conformational studies described earlier that metabolic N-dealkylation and deamination reactions should *not* involve CYP2D6 since the distance between the protonated N atom and the adjacent C atom (the site of oxidation during the dealkylation reaction) is only about 0.15 nm, and not within the optimal 0.5 to 0.7 nm range. Nevertheless, that CYP2D6 can catalyze certain N-dealkylations has been confirmed. The published literature on this subject up until 1993 was reviewed by Coutts *et al.* (1994) and those drugs that underwent *in vivo* N-dealkylation, together with the human CYP enzymes involved in this reaction, were identified. In summary, CYP3A4 and CYP1A2 were most often involved, especially the former, but CYP2D6 was shown to contribute in the *in vivo* N-demethylation of three psychoactive drugs (amiflamine, IMI, desmethylcitalopram) and possibly in the *in vivo* N-demethylation of a fourth drug (AT). It was also apparent that CYP_{MP} catalyzed the *in vivo* N-demethylation of four CNS-active drugs, CMI (Nielsen *et al.* 1994), citalopram (Sindrup *et al.* 1993), diazepam (Bertilsson *et al.* 1989) and IMI (Skjelbo *et al.* 1991), at least to some extent. These observations prompted an investigation to confirm that CYP2D6, CYP3A4 and CYP1A2

were all capable of catalyzing the N-demethylation of one of these substrates, AT, using a recently developed and relatively simple *in vitro* procedure. CYP_{MP} was not selected for study, because, at the time this project began, CYP_{MP}, now known as CYP2C19 (Goldstein *et al.* 1994), had not been purified.

All four drugs that have just been identified as capable of undergoing CYP2D6-catalyzed N-dealkylation are N-methylated compounds, but while the present study was in progress, another drug, L-(-)-deprenyl [(-)-N-methyl-N-propargylamphetamine], an irreversible inhibitor of of monoamine oxidase type B, was reported to be a substrate for CYP2D6. Using expressed CYP2D6 enzyme and a specific inhibitor, Grace *et al.* (1994) found that deprenyl and its (+)-enantiomer underwent what was described as an 'atypical' CYP2D6-catalyzed N-demethylation and N-depropargylation to N-propargylamphetamine and N-methylamphetamine, respectively, and N-demethylation was favored 13-fold over N-depropargylation.

This report by Grace *et al.* (1994), and previously reported studies by Coutts *et al.* (1976) on the *in vivo* metabolism of N-alkylated (methyl, ethyl, propyl, butyl) amphetamines prompted an additional study on the ability of CYP2D6 to catalyze the N-dealkylation of longer chained N-alkyl and N,N-dialkylated amphetamines.

1.2.7 Metabolic formation of phenols

The CYP2D6 enzyme plays a prominent role in the metabolism of many basic TCAs. Those listed in figure 1.1, for example, are ring-C-hydroxylated to phenolic products that are major metabolites, under the catalytic influence of CYP2D6, and the ring location adopted by the entering OH group is such that it can orientate itself to lie within the optimal distance of 0.5 to 0.7 nm from the drug's quaternized N atom in the active site of the enzyme. Another good example of the involvement of CYP2D6 in mediating ring hydroxylation is seen in the metabolism of paroxetine (figure 1.2) from a methylenedioxy compound to a catechol which is further metabolized under the catalytic control of the enzyme, catechol-O-methyltransferase (Kaye *et al.* 1989).

CYP2D6 also catalyzes the metabolism of other basic drugs containing aromatic ether moieties to phenolic products. This metabolic oxidation is known as O-dealkylation.

For example, morphine is the metabolic product resulting from the O-demethylation of codeine, and 4-hydroxyamphetamine is the O-demethylation product of 4-methoxyamphetamine. In addition to O-dealkylation, substrates like methoxyphenamine and 2-methoxyamphetamine, also undergo ring oxidation to phenolic metabolites. If both pathways occur, the ring substitutent(s) will determine the location of the ring oxidation and the amount of product(s) formed.

Clearly, the polymorphic CYP2D6 enzyme mediates aromatic C-oxidation and the O-dealkylation of many drugs to produce phenolic metabolites. A lack of CYP2D6 enzyme may increase the risk of having side effects and therapeutic failure. Therefore, it was of interest to identify the role of CYP2D6 in the ring C-oxidation and the O-dealkylation of these substrates.

1.3 DRUGS STUDIED

1.3.1 Amitriptyline

The TCA drug, amitriptyline (AT) has been used clinically for over 30 years. Despite its unfavourable side effect profile and the availability of so many alternative antidepressants, AT is still a very popular drug under the trade names Deprex (Beecham), Elavil (MSD) or Novotriptyn (Novopharm) and currently it is on the list of the 200 drugs which were the most frequently prescribed in 1996 (*anon*, 1996). In addition to being an effective antidepressant drug, AT is also very useful in the treatment of chronic pain (Max, 1995).

1.3.1.1 Chemistry

Chemically, AT is known as 5-(3-dimethylaminopropylidine)dibenzo[a,d][1,4]-cycloheptadiene and was first synthesized by Schindler and Hafliger in the late 1940s. AT.HCl is a colorless, crystalline substance that melts at 196°C to 197°C. It is soluble in water, dilute acid, and 95% ethanol. In alkaline solution the base is precipitated.

1.3.1.2 Clinical efficacy

AT was orginally introduced as a antidepressant drug for the treatment of major depression and for this it has proved to be effective. The efficacy of this drug was also demonstrated in the treatment of chronic pain. It can be given alone or in combination with other drugs such as SSRIs, neuroleptics and opioids.

In depressed patients, the onset of action of AT usually appears after 2 to 3 weeks of multiple daily dosing. At the same time, 1 to 2 weeks are needed to reach steady state concentration (C_{ss}). However, the anticholinergic and antihistaminic side effects may appear within a few days of the start of treatment. Analysis of available information indicates that plasma concentrations (AT plus NT) of 100 – 300 ng/ml are usually associated with theurapeutic responses (Baumann *et al.* 1986; Preskom *et al.* 1988; Furlanut *et al.* 1993) and the average daily dose is 100 – 150 mg. Decreased efficacy is associated with plasma concentrations (AT plus NT) below 100 ng/ml. After administration of AT, NT is formed in great quantity; the latter's effects as a norepinephrine (NE)-uptake inhibitor at steady state reduces the 5-HT uptake inhibiting properties and the anticholinergic side effects of AT. Thus, the metabolism of AT to NT has an important role that may influence the clinical outcome of AT therapy.

In multidrug therapy for the treatment of depression by coadministration of AT and FLU, a lower dose of AT is required because the plasma concentration of AT and NT are increased. A two-fold increase in C_{ss} of AT and nine fold increase in C_{ss} of NT are observed (El-Yazigi *et al.* 1995). The increase in AT and NT plasma concentrations resulted from the inhibitory effects of FLU on CYP2D6 catalytic activity (Vandel *et al.* 1992). Other drugs like perphenazine (Linnoila *et al.* 1982), valpromide (Bertschy *et al.* 1990) and cimetidine (Curry *et al.* 1985), when given in conjuntion with AT, also increase the plasma level of AT. Therefore, AT's dose has to be adjusted to prevent potentially serious adverse side effects when CYP2D6 inhibitors are co-ingested.

AT has been shown to be more effective than placebo in the treatment of chronic pain (McQuay *et al.* 1992), including postherpetic neuralgia (Bowsher, 1992; Gonzales, 1992), diabetic neuropathy (Max, 1994), musculoskeletal and vascular pain after trauma

(Benoliel *et al.* 1994), and chronic tension-type headache (Gobel *et al.* 1994). Its analgesic efficacy is often evident at a relatively low dose (25 mg – 75 mg/day), and at these doses the side effects of AT are significantly reduced.

1.3.1.3 General pharmacology

AT has a wide variety of pharmacologic actions, but its predominant effect is on the CNS. It possesses tranquilizing as well as antidepressant properties. Based on the biogenic amine deficiency theory of depression, AT remedies the depression in one of two ways:

(a) it blocks the reuptake of norepinephrine (NE) and/or serotonin (5-HT) into the presynaptic neurons, thus increasing intrasynaptic levels, and

(b) it blocks the presynaptic α_2-receptor, thus prolonging the release of NE from the presynaptic neurone into the synapse. A similar presynaptic receptor for a feedback mechanism probably exists to contol 5-HT release. These increases in the synaptic levels of NE and 5-HT alleviate the neurotransmitter deficiency and hence relieve the depression.

However, this theory cannot explain the fact that some effective antidepressants such as iprindole neither inhibit the reuptake nor inhibit the metabolism of neurotransmitters (Stahl and Palazidou, 1986). Other drugs which are reuptake amine blockers, namely cocaine and amphetamine, are not effective antidepressants. Furthermore, the neurotransmitter deficiency theory also fails to explain the delay in therapeutic onset of antidepressants. Thus, the exact mechanism of action of AT and other TCAs is still not clear.

The pain relief effects seem partially to come from an increase or potentiation of endogenous opioids (endorphins) in the brain (Getto *et al.* 1987; Sacerdote *et al.* 1987). However, Max (1994) also suggested that blockade of NE reuptake was the most important action accounting for pain relief found in the treatment of post-therapeutic neuralgia.

1.3.1.4 Pharmacokinetics

When given orally, AT is well absorbed (> 95%) from the gastrointestinal tract, but undergoes an important first pass metabolism (F = 0.45 ± 0.08; Rollins *et al.* 1980; Schulz *et al.* 1983) to NT and 10-HO-AT, both of which are pharmacologically active (Nordin and Bertilsson, 1995). The elimination half life ($t_{1/2}$) of AT is about 16.2 ± 6.1 hours (Schulz *et al.* 1983). Protein binding is high and the apparent volume of distribution is relatively large (14.1 ± 2.0 l/kg). Interindividual variability in the disposition of AT is significant; 5- to 10-fold differences in AT plasma concentrations are normally found among patients who are given the same AT dose. This variability is clinically important and primarily determined by polymorphic CYP2D6 enzymes involved in the hydroxylation of AT and its major metabolite, NT. Age (elderly subjects, more than 65 years) usually decreases the metabolism of AT, leading to higher AT plasma levels and longer $t_{1/2}$ (Schulz *et al.* 1985).

1.3.1.5 Metabolism

Although AT is an old antidepressant and its biotranformation *in vivo* in humans is known, the metabolism of this drug has not been a popular subject of research compared with IMI. This may be because of difficulties associated with its analysis. AT and its metabolites are adsorbed onto glass (Gupta, 1992).

In man *in vivo*, AT biotransformation is catalyzed by a number of hepatic cytochrome P450 isozymes. Its two major routes of biotransformation are:

(a) Monodemethylation of the side chain group $-N(CH_3)_2$ to yield NT, itself a powerful antidepressant.

(b) Ring hydroxylation at C-10 of both AT and NT to both (Z)- and (E)-isomers.

Minor biotransformation pathways, including the formation of the primary amine, aromatic hydroxylation at C-2 of AT and NT, rupture of the ethylene bridge at C-5, and oxidative deamination may also occur:

$$>C{=}CHCH_2CH_2NR^1R^2 \rightarrow >C{=}O$$

$$>C{=}CHCH_2CH_2NR^1R^2 \rightarrow >C{=}CHCH_2CHO \rightarrow >C{=}CHCH_2COOH.$$

Bertilsson *et al.* (1981) reported that 3 – 5% of an AT dose is excreted in human urine as 10-OH-AT, and around 40% is excreted as 10-OH-NT. The formation of 10-OH-NT from the primary metabolite,10-OH-AT, occurs rapidly. Vandel *et al.* (1982) similarly concluded that 10-OH-NT, mainly conjugated in the urine, accounted for 55% of an oral dose of AT. Later, Prox and Breyer–Pfaff (1987) carefully quantified human urinary metabolites of AT, including many minor ones, in 3 patients and showed that 10-OH-AT was excreted almost exclusively as its conjugated E-isomer (3.91 – 6.70%), whereas 10-OH-NT was excreted both as E-10-OH-NT (15.1 – 26.0%; about 50% conjugated) and Z-10-OH-NT (1.83 – 4.40%; about 33% conjugated), and 10-hydroxy-N,N-didemethylated AT (10-OH-diDM-AT) was excreted both as free and conjugated E- and Z-10-OH-diDM-AT (0.96 – 3.45% and 1.83 – 4.40%, respectively). In this study, however, total recoveries in urine only accounted for 28.6 – 60.2% of the administered AT. An explanation for such low recoveries was provided by Rudorfer and Potter (1985) who pointed out that both TCAs and their metabolites undergo enterohepatic circulation, and failure to account for drug and metabolites excreted *via* bile and feces will result in apparently low recoveries (60 – 70%) of administered TCAs.

Mellström and von Bahr (1981) concluded that AT is readily metabolized *in vivo* in human by N-demethylation to NT and by ring oxidation to 10-OH-AT. Subsequently, NT is oxidized, and 10-OH-AT is N-demethylated, to 10-OH-NT. Since N-demethylation proceeds at a faster rate than ring oxidation, the major urinary metabolites are NT and 10-OH-NT. These authors used microsomes prepared from human adult livers in their study of AT metabolism and found that rates of AT N-demethylation (and hydroxylation) depended on substrate concentration. When substrate concentration was 5 μM, 96 – 570 pmol/mg protein/10 min of NT was formed; but when substrate concentration was 100 μM, 1750 – 9230 pmol/mg protein/10 min of NT was produced. The corresponding rates for the hydroxylations were 43 – 146 and 305 – 871 pmol/mg protein/10 min, respectively. These authors also point out that AT hydroxylation by human liver microsomes is inhibited by NT. This inhibition is accompanied by an increase in N-demethylation.

It is also claimed, however, by Mellström *et al.* (1983) that plasma clearance of AT by N-demethylation does not correlate with debrisoquine 4-hydroxylation, so another isozyme must be involved in the N-demethylation reaction. The investigators concluded that 10-hydroxylation of AT and N-demethylation of AT to NT in human liver microsomes were regulated by different enzymatic mechanisms. They studied the *in vivo* metabolism of AT in nine healthy humans, four of whom were cigarette smokers, who had been phenotyped with debrisoquine. Clearance of AT by N-demethylation to NT initially did not correlate with debrisoquine's oxidation to 4-hydroxydebrisoquine, but when the data from the four smokers were omitted from the calculation, a correlation between AT N-demethylation and debrisoquine oxidation in the remaining five subjects was observed. Again the authors speculated that AT N-demethylation was mediated by at least two CYP isozymes, one of which was CYP2D6, and the other was unidentified but was induced in smokers. In a later study, Mellström *et al.* (1986) showed that in nonsmokers (five from the previous study and an additional six), clearance of AT by hydroxylation *and* by N-demethylation both correlated with debrisoquine 4-hydroxylation, i.e. both pathways were catalyzed by CYP2D6 in non-smokers, but AT N-demethylation did *not* correlate with debrisoquine 4-hydroxylation in smokers. Thus AT demethylation is metabolized by at least 2 CYP isozymes, one of which is induced by smoking (presumably CYP1A2 which is so induced). It was concluded that several CYP enzymes, including CYP2D6, could catalyze the N-demethylation of AT, at least in nonsmokers, but only CYP2D6 was involved in the C-oxidation. However, another investigation did not provide strong support of this conclusion. The metabolism of AT was investigated over a period of eight days in 26 hospitalized depressed patients who had been phenotyped for both CYP2D6 and CYP_{MP} status (Breyer–Pfaff *et al.* 1992). Twenty-four hour urinary metabolites were isolated and quantified by a thin layer chromatographic (tlc) procedure on treatment day 8. Metabolites, excreted free and as conjugates, were:

(–)-E-10-OH-NT (41.8%);	(+)-E-10-OH-NT (11.5%);
(–)-Z-10-OH-NT (2.0%);	(+)-Z-10-OH-NT (2.4%);

E-10-OH-diDM-AT (7.7%);	Z-10-OH-diDM-AT (2.8%);
(–)-E-10-OH-AT (8.4%);	(+)-E-10-OH-AT (2.3%);
(+)- and (–)-Z-10-OH-AT (1.0%);	NT (1.2%); 2-OH-NT (0.8%);
AT+AT-N-glucuronide (15.9%);	AT-N-oxide (2.2%).

[% values are in terms of total recovery; diDM = N,N-didemethylated]

The formation of (+)- and (–)-E-10-OH-NT, and (–)-E-10-OH-AT (3 major metabolites) depended upon CYP2D6, but the formation of (+)- and (–)-Z-10-OH-NT and (+)-E-10-OH-AT was not CYP2D6-catalyzed. CYP2C19, however, was deduced to play a major role in N,N-didemethylation, but not in the N-demethylation of AT to NT, because the mephenytoin $\log_{10}$ metabolic ratio only correlated negatively with the relative quantity of E-10-OH-diDM-AT produced. That CYP2D6 played an important role in AT demethylation in nonsmokers was also not confirmed in this study.

Finally, Zhang *et al.* (1993) studied the metabolism of AT in 7 healthy Chinese, six of whom were debrisoquine EMs and one was a PM. Each patient received a single oral dose of AT (100 mg) and rates of AT 10-hydroxylation and AT N-demethylation were determined. The debrisoquine metabolic ratio correlated significantly with the rate of AT 10-hydroxylation, but not with the rate of AT N-demethylation. From this observation, it was concluded that AT hydroxylation and AT demethylation are regulated by different enzymes.

It can be concluded from the discussions immediately above that the contribution of CYP2D6 to the N-demethylation of AT is a controversial subject. Enzyme(s) involved in the formation of NT from AT have not been unequivocally identified.

1.3.1.6 Adverse reactions

AT not only blocks the reuptake of 5-HT and, to a lesser extent, of NE, it also has a high affinity for several receptors such as muscarinic (Snyder and Yamamura, 1977), histaminergic (Green and Maayani, 1977), α_1-adrenergic (U'Prichard *et al.* 1978) and serotonergic receptors (Snyder and Peroutka, 1982; Richelson and Nelson, 1984). AT is a very potent antagonist of muscarinic, histaminic and α_1 receptors (Potter *et al.* 1984),

resulting in side effects. The clinical effects include: blurred vision, constipation, dry mouth, sinus tachycardia, urinary retention and memory dysfunction (Frazer and Conway, 1984).

AT has a relatively low therapeutic index and is frequently prescribed. Thus, AT was by far the most frequently reported cyclic antidepressant to cause severe adverse side effects (Hebb *et al.* 1982; Osselton *et al.* 1984). A seven-year (1979 – 1985) investigation of fatal poisoning by TCAs revealed that 151 cases were reported. Fifty-nine percent of all cases involved AT, either alone or in combination with other drugs, including alcohol (Worm and Steentoft, 1990).

1.3.2 Amphetamines

Amphetamine (AM) is β-phenylisopropylamine, a simple structure, and was synthesized in 1927. Chemical modifications of the AM molecule have resulted in the synthesis of many derivatives such as ephedrine, methamphetamine, 4-methoxyamphetamine, 3,4-methylenedioxyamphetamine, phenmetrazine and others.

1.3.2.1 General pharmacology

AM and several related drugs enhance the activity of neurotransmitter systems by two major mechanisms:

a) they promote release of dopamine and norepinephrine from the presynaptic neurons

b) they block the re-uptake of catecholamines by the presynaptic neuron. These processes result in an increase in catecholamine levels at the postsynaptic site and they influence the behavioral effects of the amphetamines (Azzaro *et al.* 1974; Chiueh and Moore, 1975).

The most marked and consistent central effect is the production of a state of arousal or wakefulness. This effect is used therapeutically in the treatment of narcolepsy. Amphetamines are also used for the improvement of performance and endurance by offsetting fatigue and sleepiness. Other uses of amphetamines are found in the treatment of obesity and in the treatment of some types of depression (Abramson, 1974).

1.3.2.2 Metabolism

The metabolism of amphetamines and congeners generally involves six pathways, namely aromatic hydroxylation, aliphatic hydroxylation, N-dealkylation, oxidative deamination, perhaps followed by further side chain biotransformations, N-oxidation, and conjugation of the amphetamine and/or its metabolites. Amphetamines are also excreted unchanged to some extent in the urine.

N-Dealkylation is the most important reaction. It commonly gives rise to primary or secondary amine metabolites that retain pharmacological activity. For instance, AM is a metabolite of many N-alkylated amphetamines, and norephedrine is derived from ephedrine. Aromatic hydroxylation provides phenolic amines that may also contribute pharmacological activity. p-Hydroxyamphetamine, for example, is three times more potent than AM as an inhibitor of noradrenaline reuptake (Iversen, 1967) and is a potent pressor amine (Gill *et al.* 1967).

Although data are limited, a few studies on the metabolism of N-alkylated amphetamines have shown that both N-dealkylation and ring hydroxylation occur. *In vivo* ring hydroxylation of AM to 4-hydroxyamphetamine (HO-AM), for example, is a minor metabolic pathway in man, whereas ring oxidative metabolism of NMA is more extensive and yields 4-hydroxy-N-methylamphetamine (HO-NMA) and HO-AM. This topic is expanded in Chapter 4. Ring hydroxylation of pondinil [(N-3-chloropropyl)amphetamine; PD] is even more pronounced; the quantities of 4-hydroxy-PD and HO-AM excreted in 0 – 24 h urine are ~30% and ~6%, respectively (Williams *et al.* 1973; Caldwell, 1976). N-Dealkylation of D-(+)-NMA gives 10% AM, while D-(+)-N-ethylamphetamine produces 30% AM. From these data, it is apparent that N-alkylated amphetamines have higher lipophilicity than AM and this enhances the extent of metabolic ring hydroxylation and N-dealkylation.

In vivo and *in vitro* studies on methoxyamphetamines have revealed that they undergo O-demethylation, ring hydroxylation and N-dealkylation to various extents. Some *in vitro* studies have suggested that metabolic O-demethylations of methoxyamphetamines are mediated by CYP2D6 in humans and by related CYP2D enzymes in

rats, but it is also claimed that N-dealkylation of some analogs of NMA is catalyzed by different CYP isozymes. It is also well known that CYP2D6 catalyzes the ring hydroxylation of many amine compounds, and may also be involved in N-dealkylation. These topics are discussed in detail in Chapters 4 and 5.

The role of CYP2D6 in N-dealkylation of amphetamines is a controversial subject and needs to be clarified.

1.4 SELECTION OF CYP ENZYMES USED IN THE METABOLISM STUDIES REPORTED IN THIS THESIS

The information provided in table 1.2 indicates that various CYP enzymes may be involved in catalyzing the metabolic N-dealkylation of basic drugs. The most likely ones are CYP1A2, CYP2D6, CYP3A4, and one or more of the CYP2C enzymes. That CYP3A4 plays a significant role is clear. In table 1.2, those substrates whose N-dealkylation, especially N-demethylation (N-de-Me), was controlled by CYP3A4 are identified. When the research now reported began, three CYP enzymes, CYP1A2, CYP2D6 and CYP3A4 were initially selected for the study, because purified samples of all three enzymes, expressed in a human cell line, were commercially available. A CYP2C isozyme was not selected for the proposed studies because no pure samples of the individual CYP2C isozymes were available. All four purified CYP2C isozymes (CYP2C8, CYP2C9, CYP2C18 and CYP2C19), also expressed in human cell lines, can now be purchased. At present, there is no positive evidence to confirm the involvement of CYP2C19 in the *in vivo* N-demethylation of AT to NT (Breyer–Pfaff *et al.* 1992), or in *in vitro* studies using human microsomes (Schmider *et al.* 1995).

1.5 *IN VIVO* AND *IN VITRO* DRUG METABOLISM CORRELATIONS

Drug metabolic studies are commonly performed using two different approaches. One utilizes *in vivo* conditions where drug metabolism take place in a natural environment. The other employs *in vitro* conditions, where drug metabolism is conducted in an

enzyme system isolated from a tissue or organ that has been removed from a living body. The latter procedure often provides information that is essential to explain the clinical outcomes of the *in vivo* situation. Another important application of *in vitro* drug studies is to aid in the selection of potential new drugs.

In vitro metabolic studies are usually performed to allow the identification of the enzymes involved in the biotransformation of a drug, the elucidation of metabolic pathways by which the metabolites are formed and the prediction of potential drug-drug interactions. Either human liver microsomes or specific human enzymes expressed in various cell culture systems or primary cultures of human hepatocytes are used for this purpose. In addition, selective enzyme inhibitors (antibodies or chemical inhibitors) are used in conjunction with the enzyme sources to confirm not only which enzymes are involved in a metabolic pathway but also the extent to which each enzyme contributes to enzymatic activity.

What is the relationship between *in vitro* and *in vivo* data? The basis of this relationship is to use *in vitro* intrinsic clearance as a predictor of *in vivo* intrinsic clearance and also the *in vitro* inhibition constant (K_i) to predict potential drug-drug interactions *in vivo*. The *in vitro* intrinsic clearance, which is a measurement only of enzyme activity toward a drug and is not influenced by other physiological determinants of clearance such as hepatic blood flow or blood protein binding, corresponds to the V_{max}/K_m ratio. Retrospective application of this approach has found success for some drugs [lidocaine (deethylation), cortisol (6β-hydroxylation) and chlorzoxazone (6-hydroxylation)] (Hoener, 1994) but not others like warfarin, or theophylline (Thummel and Shen, 1996).

Failure to accurately predict the *in vivo* metabolic elimination of a drug using an *in vitro* procedure can possibly be because *in vivo* variability in drug metabolism is influenced by many factors. These factors include age, genetic or hereditary factors, gender, enzyme induction and enzyme inhibition. This variability might not be accounted for by the *in vitro* estimates. However, if a drug is metabolized by a given pathway by the same enzyme to a number of metabolites which can account for more than 50 % of the dose, then anything that affects that enzyme's activity is likely to be of

clinical significance. Drug metabolism information obtained from *in vitro* studies is useful, but only *in vivo* studies can ultimately establish the quantitative importance of different metabolic pathways under therapeutic conditions.

1.6 OBJECTIVES OF THE RESEARCH

Specific objectives of this thesis were:

(1) To determine the involvement of CYP2D6 in ring hydroxylation and N-demethylation of different substrates.

(2) To identify what enzymes are involved in the metabolic N-demethylation of AT.

(3) To determine the relative contribution of each enzyme that catalyzes the N-demethylation of AT.

(4) To develop suitable methods of extraction and derivatization of basic drugs and their metabolites for GC analysis.

(5) To develop specific and sensitive GC assays for quantitative determinations of drugs and their metabolites.

(6) To evaluate the effects of N-substituent groups on ring hydroxylation and N-dealkylation of AT and amphetamine analogues mediated by CYP2D6.

(7) To synthesize required metabolites and potential CYP2D6 substrates.

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Chapter 2

Metabolism of amitriptyline with CYP2D6 expressed in a human cell line

This chapter has been published (Coutts *et al.* 1997. *Xenobiotica* **27**:33-47).

2.1 INTRODUCTION

The metabolism of the tricyclic antidepressant imipramine (IMI) has been extensively studied *in vivo* in humans and *in vitro* with human cytochrome P450 (CYP) preparations. *In vivo* it is initially metabolized by N-demethylation to desipramine (DMI) and by hydroxylation to 2-hydroxyimipramine(2-HO-IMI), and both primary metabolites are metabolized further to 2-hydroxydesipramine (2-HO-DMI), which is a major metabolite (Brøsen *et al.* 1991). Ring oxidation (2-hydroxylation) is catalyzed by CYP2D6, but the N-demethylation reaction is mediated mainly by other CYP enzymes, although CYP2D6 is known to be involved, but only to a small extent and at a slow rate (Coutts *et al.* 1994). Recent investigations have suggested that CYP1A2 mediates the N-demethylation of IMI because this biotransformation reaction is induced by smoking (Madsen *et al.* 1995), a practice known to induce the formation of CYP1A2 (Pasco *et al.* 1993). IMI N-demethylation is also efficiently inhibited by fluvoxamine, a known potent inhibitor of CYP1A2 (Skjelbo and Brøsen 1992; Hartter *et al.* 1993; Spina *et al.* 1993). Experiments with inducers, inhibitors and antibodies have revealed that an additional isozyme, CYP3A4, contributes to the N-demethylation of IMI (Lemoine *et al.* 1993).

In contrast to the extensive studies that have been conducted on IMI, the structurally related antidepressant, amitriptyline (AT), has received much less attention. This may be due, at least in part, to analytical difficulties which were also experienced in the present study and are described below.

Amitriptyline is extensively metabolized *in vivo* in humans mainly to 10-hydroxy-amitriptyline (10-HO-AT), nortriptyline (NT) and 10-hydroxynortriptyline (10-HO-NT). Early studies on AT metabolism (Mellström and von Bahr, 1981), in which rates of formation of metabolites were determined, suggested that the N-dealkylation and 10-hydroxylation transformations of AT were catalyzed by different CYP enzymes. Further investigations included smokers and nonsmokers (Mellström *et al.* 1983, 1986). These latter studies showed that in nonsmokers, significant relationships existed between the 4-hydroxylation of debrisoquine and both the N-demethylation of AT and its ring oxidation to 10-HO-AT, and thereby confirmed that CYP2D6 was capable of catalyzing both

metabolic reactions, at least to some extent. Also revealed was the fact that AT N-demethylation was induced in smokers and, therefore, apparently involved at least one CYP enzyme other than CYP2D6, since CYP2D6 is not induced by smoking (Smith, 1991). The involvement of CYP2D6 as well as an inducible demethylase in the formation of NT from AT in humans has also been suggested by Muller and coworkers (1991). In contrast, another investigation of AT metabolism *in vivo* in humans provided evidence that CYP2C19 was the enzyme that catalyzed the N-dealkylation reaction and that CYP2D6 played no important role in this pathway (Breyer–Pfaff *et al.* 1992). In addition, these investigators concluded that smoking had no effect on N-demethylation rates.

In the current study on the metabolism of AT by CYP enzymes, we have attempted to clarify these conflicting results on the mechanism of the N-demethylation of AT and now report our studies on the *in vitro* metabolism of amitriptyline by CYP2D6 enzyme expressed in human lymphoblastoid cells. Although *in vivo* kinetic parameters cannot be reproduced *in vitro*, the *in vitro* technique can identify the metabolic roles played by specific CYP enzymes. A knowledge of these roles is important in clinical practice where variability in clinical reponses to antidepressant therapy do occur when other drugs are coadministered (Meyer *et al.* 1996).

2.2 EXPERIMENTAL

2.2.1 Chemicals and reagents

AT.HCl, NT.HCl, maprotiline.HCl (MT.HCl; internal standard), NADP sodium salt from yeast ($NADP^+$), D-glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase Type XII from Torula yeast (G6PD) were purchased from Sigma Chemical Co. (St. Louis, MO). E- and Z-10-HO-AT and E- and Z-10-HO-NT were gifts from Dr. G. McKay, College of Pharmacy and Nutrition, University of Saskatchewan. Trifluoroacetic anhydride [$(CF_3CO)_2O$], magnesium chloride ($MgCl_2.6H_2O$), and dichlorodimethylsilane (99%) were purchased from Aldrich Chemical Co. (Milwaukee, WI); $KHCO_3$, K_2CO_3, quinidine sulfate, HPLC grade methanol, n-hexane, acetonitrile

(CH_3CN), isopropanol and toluene were purchased from BDH (Toronto, Canada). The last two solvents were distilled before use. The buffer solution used in the experiments was 100 mM potassium phosphate (pH = 7.4).

2.2.2 Analysis of kinetic data

The Michaelis Menten parameters, K_m and V_{max}, were obtained by fitting the reaction velocities and substrate concentrations to the simple Michaelis Menten equation (E_{max} model), and the computer program PCNONLIN (version 4; SCI, Lexington, KY) was utilized to estimate the values of apparent K_m and V_{max}.

2.2.3 *In vitro* enzymatic studies

2.2.3.1 Microsomal protein

Human CYP2D6 microsomal protein and control microsomal protein were purchased from Gentest Corporation (Woburn, MA, U.S.A.). The CYP2D6 microsomal protein was expressed in a human AHH-1TK +/- cell line transfected with complementary DNA that encoded human CYP2D6. Control microsomal protein that was not transfected was also prepared from the same human cell line. Control microsomes contained a low level of CYP1A1 activity but lacked CYP2D6. The commercial products (10 mg protein/ ml) were used as supplied. The CYP2D6 content was 170 pmol CYP2D6/mg microsomal protein.

2.2.3.2 NADPH-generating system components

The NADPH generating system was prepared by mixing fresh stock solutions of $NADP^+$(1.3 mM; 1 mg/ml), G6P (3.3 mM; 1 mg/ml) and G6PD (50 U/ml buffer) in a 5:5:2 volume ratio.

2.2.3.3 Reaction tubes

KIMAX glass culture tubes (16 mm overall diam. x 100 mm) for metabolism and analytical studies were purchased from Fisher Scientific (Ottawa, Canada) and silanized

before use. The silanization procedure described by Kristinsson (1981) was followed with some modification. Culture tubes were filled with an 8% solution of dimethyl-dichlorosilane (99%; Aldrich Chemical Co.) in toluene and left to stand at room temperature in a fume hood with Parafilm® cover for 3 days. After rinsing twice with n-hexane and once with methanol, the tubes were dried at 110° C for 1 h. Just prior to use, tubes were rinsed with the same solvent mixture that was used for extraction of metabolites.

2.2.3.4 Metabolism of amitriptyline

In vitro metabolism studies were performed in KIMAX glass culture tubes by adding CYP2D6 microsomal protein (0.50 mg protein) to a prewarmed (5 min at 37°C) incubation mixture (450 μl) consisting of AT (9.07 nmol), $MgCl_2$ (1.64 μmol), and NADPH generating system (60 μl) in potassium phosphate buffer. After incubation at 37°C for 1.5 h, the mixture was cooled in an ice bath and the reaction terminated by the addition of 25% K_2CO_3 (50 μl). Internal standard (MT; 4.84 nmol) was added and the solution extracted into organic solvent (2% isopropanol in hexane; 3ml x 3) by vortex mixing for 5 min and centifuging for 6 min. The organic phase was separated and transferred to a silylated KIMAX tube. The combined organic phases were concentrated to dryness under a stream of nitrogen. This procedure provided a nonderivatized metabolic residue which contained substrate, substrate metabolites and internal standard.

2.2.3.4.1 Time course of AT metabolism

Incubation mixtures (720 μl) contained AT (47.72 nmol), $MgCl_2$ (2.62 μmol), NADPH generating system (176 μl) and phosphate buffer. After 5 min preincubation at 37°C in a Magni Whirl® constant temperature shaking bath, (Blue M Electric Co., Blue Island, IL), the reaction was started by the addition of CYP2D6 microsomal protein (0.80 mg). Samples (125 μl) were removed at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h. The metabolic reaction was stopped by cooling the container in an ice bath and adding a solution of K_2CO_3 (25%; 50 μl). All samples were stored at -38°C until analyzed. Each solution was allowed to reach room temperature and internal standard (MT; 1.21 nmol) and buffer

(115 μl) were added. Each solution was extracted with organic solvent (2% isopropanol in hexane; 1.5 ml x 3) by vortex mixing for 5 min and centifugation for 6 min. The organic phases were separated and transferred to a silylated KIMAX tube. The combined organic phases were concentrated to dryness under a stream of nitrogen and trifluoro-acetylated as described below in the "Anhydrous acylation" section 2.2.4.1.2. The derivatized sample was then analyzed by GC as described in section 2.2.4.2.

2.2.3.4.2 Kinetic studies on the formation of AT metabolites

The "Metabolism of amitriptyline" reaction in section 2.2.3.4 was repeated at various substrate concentrations (3.73, 6.21, 9.07, 12.42, 17.39, 22.38, 29.82, 44.15 and 59.75 nmol/0.5 ml). The "Anhydrous acylation" reaction described in section 2.2.4.1.2 was performed on each residue and the trifluoroacetylated products were quantitatively analyzed by the procedure described in the "GC analysis of derivatized metabolites" section 2.2.4.2.

2.2.3.4.3 Inhibition study

The "Metabolism of amitriptyline" reaction in section 2.2.3.4 was repeated except that quinidine sulfate (4.55 nmol) was added to the incubation mixture (500 μl) at the same time as the AT (9.07 nmol), and incubation was continued for 2.5 h. The "Anhydrous acylation" reaction described in section 2.2.4.1.2 was performed on each residue and products were analyzed by the procedure described in section 2.2.4.2.

2.2.3.4.4 Control study

The "Metabolism of amitriptyline" procedure described above in section 2.2.3.4 was repeated except that the CYP2D6 microsomal protein was replaced by control microsomal protein, and the incubation period was 2.5 h. The final residue was trifluoro-acetylated and analyzed as described in section 2.2.4.

2.2.3.5 Metabolism of E-10-hydroxyamitriptyline and Z-10-hydroxyamitriptyline

The "Metabolism of amitriptyline" procedure described in section 2.2.3.4 was applied to individual solutions of E- and Z-10-hydroxy-AT (6.81 nmol). The final residue was trifluoroacetylated and analyzed as described in section 2.2.4.

2.2.3.6 Metabolism of nortriptyline

The "Metabolism of amitriptyline" procedure described in section 2.2.3.4 was applied to a solution of NT (6.45 nmol). The final residue was trifluoroacetylated and analyzed as described in section 2.2.4.

2.2.3.7 Metabolism of E-10-hydroxynortriptyline and Z-10-hydroxynortriptyline

The "Metabolism of amitriptyline" procedure described in section 2.2.3.4 for AT was performed on separate solutions of E- and Z-10-HO-NT (1.44 nmol). The final residue was trifluoroacetylated and quantitated as described in section 2.2.4.

2.2.4 GC assay procedure

2.2.4.1 Derivatization procedures

2.2.4.1.1 Aqueous acylation

When the incubation of AT was terminated and cooled on ice, $KHCO_3$ (450 mg) and internal standard (MT; 4.84 nmol) were added and the mixture allowed to come to room temperature. After the addition of acetic anhydride (300 µl), the mixture was left at room temperature for 30 min at which time release of CO_2 was complete. The acetylated solution was extracted into organic solution (2% isopropanol in hexane; 3 ml x 3) by vortex mixing for 5 min and centifugation for 6 min. The organic phase was separated and transferred to a silylated KIMAX tube. The combined organic phases were concentrated to dryness under a stream of nitrogen. The residue from the acetylated mixture was reconstituted in toluene (100 µl) and a 2 µl aliquot was used for GC analysis.

2.2.4.1.2 Anhydrous acylation

Each nonderivatized metabolic residue, obtained as described in the "Metabolism of amitriptyline" section, was dissolved in a 1:1 mixture of CH_3CN-$(CF_3CO)_2O$ (100 μl) by vortex mixing for 30 sec and then heating for 8 min at 95°C. The reaction mixture was cooled to room temperature and excess reagent was removed under a stream of nitrogen. The residue was reconstituted in toluene (100 μl) prior to GC analysis using a 2 μl aliquot.

2.2.4.2 GC analysis of derivatized metabolites

A portion (2 μl) of the toluene solution of each acetylated and trifluoroacetylated metabolism mixture, prepared as described above, was injected splitless onto a 15m x 0.25 mm ID, 0.25 μm film thickness DB-17 fused silica capillary column (J and W Scientific, CA) in a Hewlett-Packard 5730A gas chromatograph equipped with a nitrogen-phosphorus detector. The flow rate of the carrier gas (helium) was adjusted to maintain a column head pressure of 10 psi. Make-up gas at the detector was a mixture of hydrogen (3 ml/min) and air (80 ml/min). The injector and detector temperatures were 256°C and 308°C, respectively. An oven temperature program was used to elute analytes. The initial oven temperature (200°C) was held for 1 min for acetylated products and for 2 min for trifluoroacetylated derivatives, then, in both instances it was increased linearly to 280°C at a rate of 4°C/min and held at that temperature for 2 min.

Acetylation products: Under these conditions, AT (underivatized); E- and Z-10-HO-AT (underivatized); N-acetylated NT, and N-acetylated MT eluted at 8.12, 11.55, 11.85, 18.29, 20.78 min, respectively. N-Acetylated E and Z-10-HO-NT coeluted at 22.17 min.

Trifluoroacetylated products: Under these conditions, AT (underivatized); 10,11-dehydrated E- and Z-10-HO-AT; N-trifluoroacetylated NT; 10,11-dehydrated-N-trifluoroacetylated E and Z-10-HO-NT; and N-trifluoroacetylated MT eluted at 9.81, 11.23, 13.79, 14.95 and 16.27 min, respectively. Retention times of AT, derivatized

metabolites and the internal standard differed slightly in later studies due to GC column shortening to clean up the column.

Quantitation of these analytes was achieved in the following manner.

Standard curves for AT and each of its trifluoroacetylated metabolites were prepared by adding 4.84 nmol of internal standard (MT) and varying amounts of analytes (1.25 – 44.81 nmol of AT; 0.85 – 8.54 nmol of 10-HO-AT; 0.27 – 6.73 nmol of NT; and 0.36 – 5.38 nmol of 10-HO-NT) to separate 500 μl volumes of incubation mixture, prepared as described in the "Metabolism of amitriptyline" section above. Each analyte containing incubation mixture was heated to 37°C, retained at this temperature for 5 min, then cooled in an ice bath to room temperature prior to the addition of 25% K_2CO_3 (50 μl) and control microsomal protein (0.50 mg). Each mixture was then extracted and trifluoroacetylated in the manner described for the incubation samples. The ratio of the area of each final analyte peak to that of the internal standard was calculated and plotted against the concentration of the drug or metabolite added. The intra-assay and inter-assay precision was consistently ≤ 10%.

2.3 RESULTS

The *in vitro* metabolism of AT by human CYP2D6 microsomes produced mainly E- and Z-10-HO-AT, and lesser amounts of NT and E- and Z-10-HO-NT (figure 2.1, table 2.1). Attempts to separate and quantify the recovered drug and its metabolites (underivatized) by GC presented problems. GC traces (not shown) contained peaks which had retention times identical to those of authentic samples of NT, E- and Z-10-hydroxyamitriptyline (10-HO-AT), and E- and Z-10-hydroxynortriptyline (10-HO-NT), but the peaks were broad and overlapping, and many contaminant peaks, presumably originating from the incubation mixture, were present in the GC trace and made quantitation of the metabolites impossible. In addition, AT and some of its metabolites were highly adsorbed to test tube surfaces and reproducible GC results could not be obtained even when test tubes were silanized regularly.

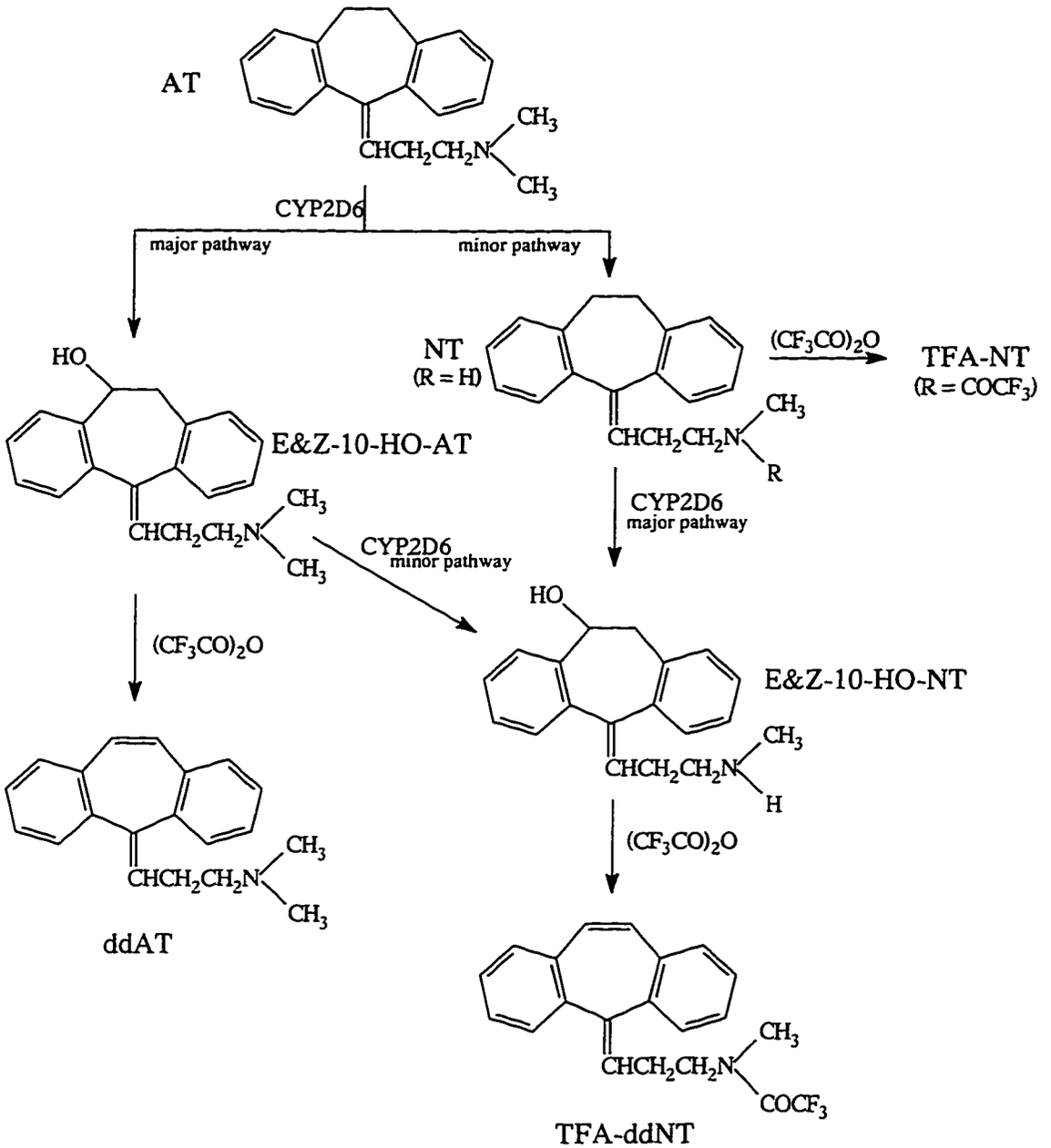

Figure 2.1 Metabolites of AT mediated by human expressed CYP2D6 *in vitro* and their trifluoroacetylated derivatives.

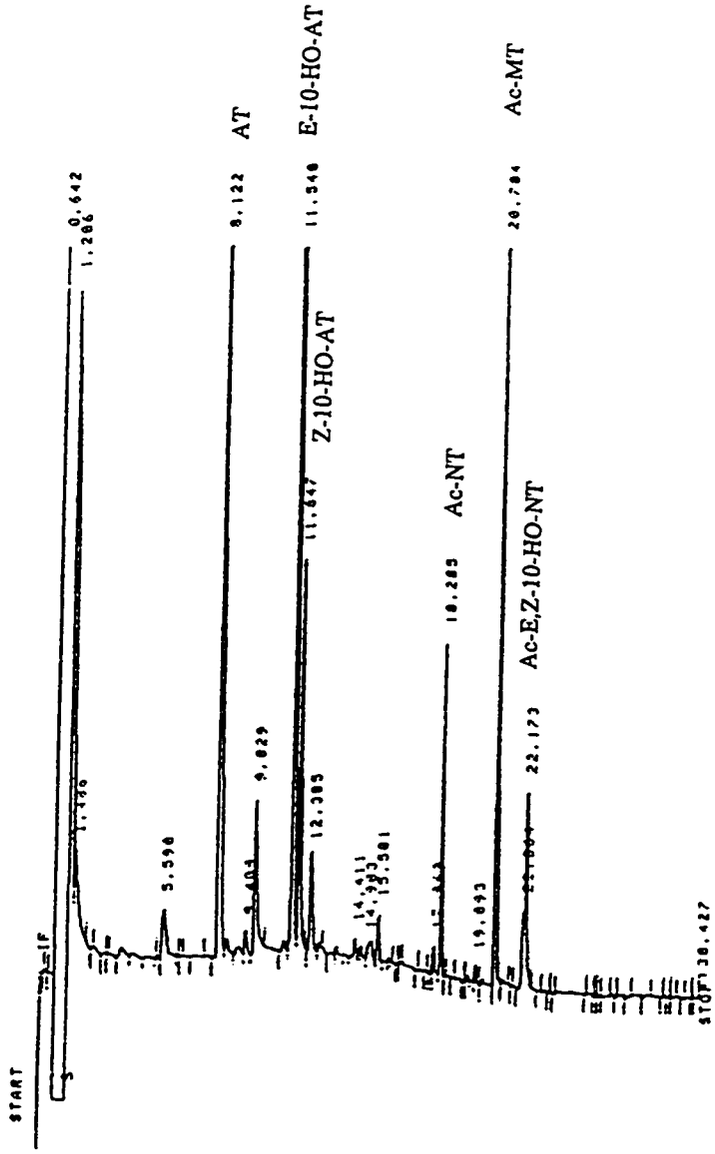

Figure 2.2 GC trace of an aqueous acetylated extract of an 0.5 ml incubation of AT (9.07 nmol) with CYP2D6 protein preparation (0.50 mg). Incubation time: 1.5 h. For aqueous acetylation conditions, refer to the experimental section 2.2.4.1.1. Numbers refer to the retention times (min): AT, 8.122; E-10-HO-AT, 11.548; Z-10-HO-AT, 11.847; Ac-NT, 18.285; Ac-MT, 20.784; Ac-E,Z-10-HO-NT, 22.173

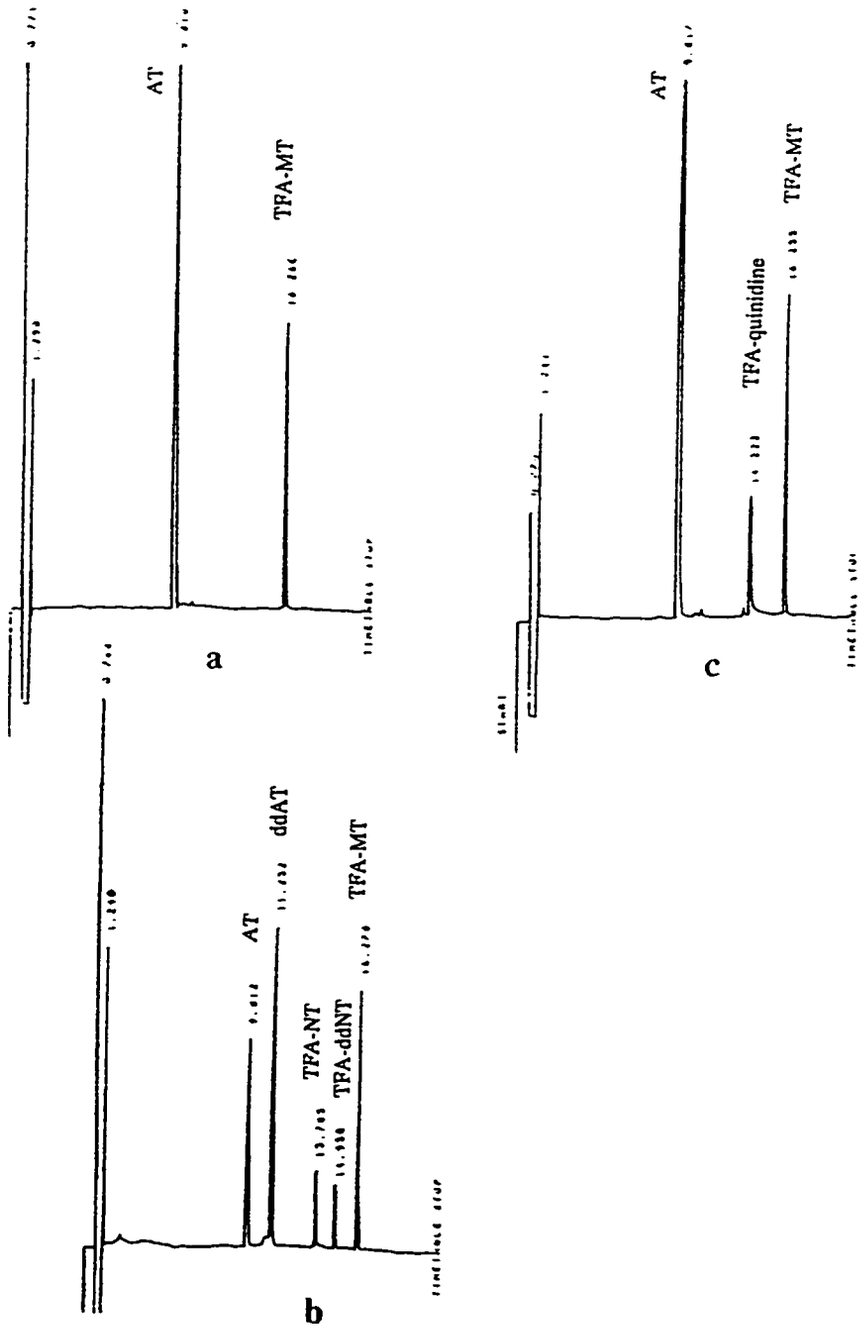

Figure 2.3 GC traces of trifluoroacetylated dried extracts of AT (9.07 nmol) incubation with (a) control microsomal protein; (b) CYP2D6 protein preparation; and (c) CYP2D6 protein preparation in the presence of quinidine (4.55 nmol). Incubation time: 2.5 h. All incubation mixtures were performed under identical conditions. ddAT, 10,11-didehydro-AT; TFA-ddNT, N-trifluoroacetyl-10,11-didehydro-NT; TFA-NT, N-trifluoroacetyl-NT; TFA-quinidine, O-trifluoroacetyl-quinidine; TFA-MT, N-trifluoroacetyl-MT. Numbers refer to the retention times (min): AT, 9.812; ddAT, 11.232; TFA-quinidine, 14.223; TFA-NT, 13.785; TFA-ddNT, 14.950; TFA-MT, 16.270.

Two derivatization procedures were then applied to each incubation solution that contained metabolites. First, it was acetylated before extraction of AT and metabolites. The extract was examined by GC (figure 2.2) and was shown to contain AT, E-10-HO-AT, Z-10-HO-AT, N-acetylated NT, N-acetylated MT and N-acetylated 10-HO-NT (presumably overlapping E- and Z-10-HO-NT). Retention times of these products were the same as those of authentic reference compounds that had been treated identically. The gas chromatogram (figure 2.2), however, was not reproducible. Large variations in metabolite peak heights were repeatedly observed and the chromatogram also contained contaminating peaks. For these reasons, this procedure was of no value in the quantitation of metabolites. In the second procedure, the residue obtained by evaporation of the metabolism extract was reacted with trifluoroacetic anhydride in freshly silanized test tubes prior to GC examination. This procedure resulted in reproducible chromatograms which were free of contaminants, and analyte peaks were narrow and completely resolved (figure 2.3b).

For quantitative analyses, calibration curves were constructed using varying amounts of authentic samples of AT, NT, E- and Z-10-OH-AT, E- and Z-10-HO-NT and a constant amount of MT as internal standard. Mixtures were trifluoroacetylated prior to GC examination and peak heights of analytes (recovered AT and derivatized metabolites) were measured. Plots of the ratio of analyte peak height to N-trifluoroacetyl-MT peak height *versus* the quantity of analyte added were constructed. Each calibration was linear over the selected concentration range; all r^2 values were always >0.99.

The time course of AT metabolism is shown in figure 2.4. AT and CYP2D6 microsomal protein concentrations are both constant. The rate of formation of 10-HO-NT increases linearly with increasing incubation time; the rate of formation of 10-HO-AT deviated from linearity after 2 h of incubation (r^2 over the 0 – 2 h period = 0.991), and the rate of formation of NT began to decrease after 1 h of incubation and continued to decrease as the incubation reaction was prolonged.

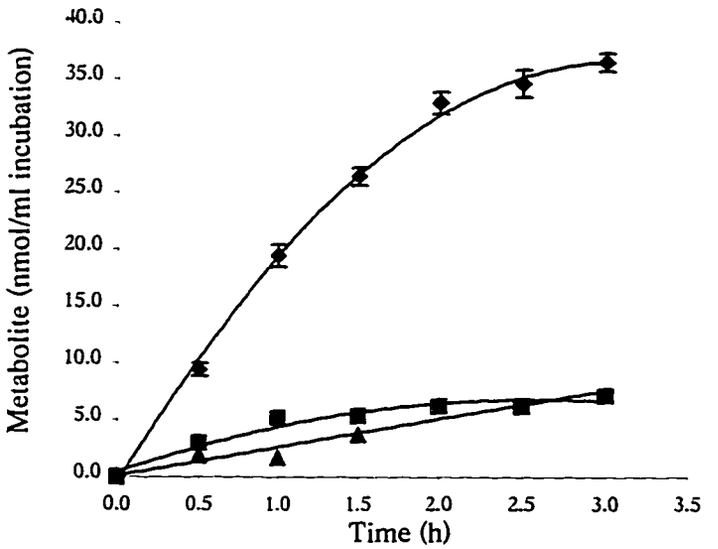

Figure 2.4 Time course study of AT metabolism by CYP2D6. Substrate, 60 nmol/ml incubation; protein, 1.0 mg/ml incubation. 10-HO-AT (◆); NT (■); and 10-HO-NT (▲). Results are means of three determinations. NT and 10-HO-NT points overlap at 2.0, 2.5 and 3.0 h.

Table 2.1 Metabolic ring hydroxylation and N-demethylation of AT by CYP2D6. Protein: 1mg/ml incubation. Incubation time: 1.5 h. The results represent means ± SD (n=3).

AT (nmol/ml incubation)	Metabolite	Yield of metabolite (nmol/ml incubation)
	combined E&Z 10-HO-AT	8.23 ± 0.12
18.14	NT	1.05 ± 0.10
	combined E&Z 10-HO-NT	1.41 ± 0.28
	combined E&Z 10-HO-AT	26.45 ± 2.32
60.00	NT	5.34 ± 1.06
	combined E&Z 10-HO-NT	3.69 ± 0.80

Table 2.2 Aparent K_m and V_{max} values* for AT metabolism by human expressed CYP2D6.

Metabolic reaction	Apparent K_m	V_{max} (nmol/h/mg protein)
AT → E&Z 10-HO-AT	10.70 ± 0.36	8.99 ± 0.81
AT → NT	47.48 ± 2.27	3.95 ± 0.19

* The apparent K_m and V_{max} values were calculated from raw data obtained from three experiments. These data were fitted into an appropriate program (see section 2.2.2) which provided mean values ± SD.

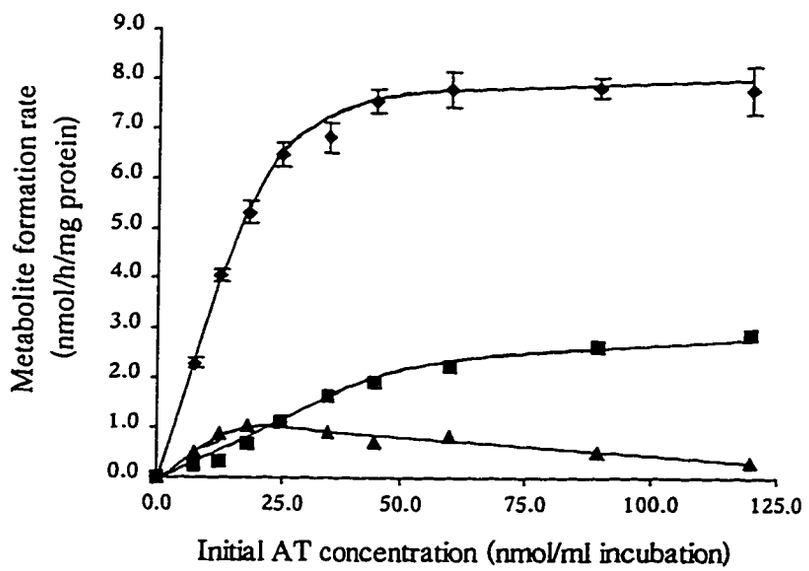

Figure 2.5 Effects of substrate concentration on the yields of AT metabolites. Incubation mixture: AT range: 3.73 – 119.5 nmol/ml incubation.
Protein: 1.0 mg/ml incubation. Incubation time: 1.5 h. The results represent the means of three experiments. 10-HO-AT (◆); NT (■); and 10-HO-NT (▲).

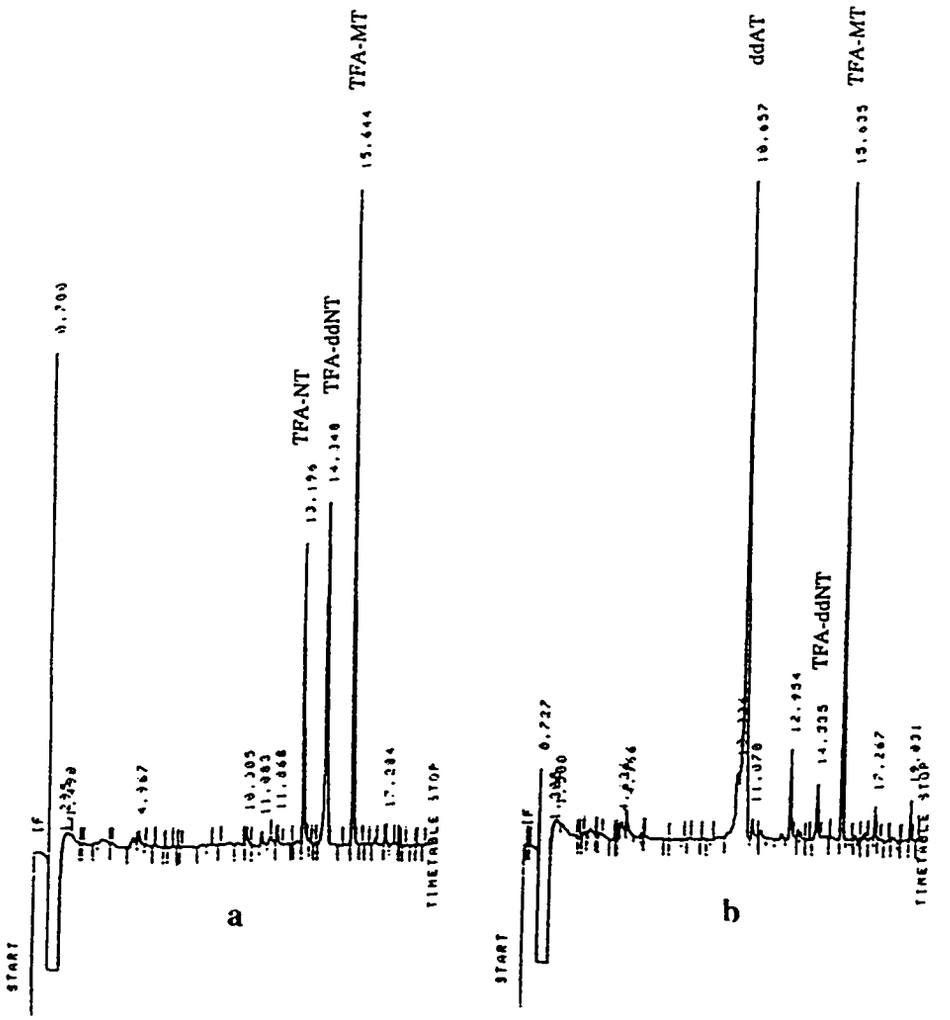

Figure 2.6 GC traces of trifluoroacetylated dried extracts of (a) NT (6.45 nmol) incubation with CYP2D6; and (b) E-10-HO-AT (6.81 nmol) with CYP2D6. Protein: 0.50 mg. Incubation time: 1.5 h. Incubation mixtures were performed under identical conditions to each other. Incubation volume: 0.5 ml. Numbers refer to the retention times (min): ddAT, 10.657; TFA-NT, 13.196; TFA-ddNT, 14.348; TFA-MT, 15.644.

The kinetics of AT metabolite formation were studied; yields and rates of formation of two metabolites, 10-HO-AT (Z + E) and NT, were determined. The maximum velocity of formation (V_{max}) and Michaelis constant (K_m) for each metabolite

are shown in table 2.2. It was interesting to note (figure 2.5) that when the same quantity of CYP2D6 isozyme was incubated with increasing amounts of AT, both 10-HO-AT and NT formation reached saturation. The value of V_{max} for 10-HO-AT was 2.28-fold higher than that for NT, showing that AT is metabolically hydroxylated by CYP2D6 more rapidly to 10-HO-AT than it is demethylated to NT. Rates of formation of both 10-HO-AT and NT increase linearly at low substrate concentrations and plateau at higher concentrations. The apparent K_m and V_{max} values of 10-HO-NT formation could not be calculated because quantities of this metabolite were highest when low concentrations of AT were metabolized, but were reduced when higher amounts of AT were subjected to metabolism.

To verify that the major pathway of formation of 10-HO-NT was *via* NT, the two primary AT metabolites, 10-HO-AT and NT, were separately metabolized *in vitro* with the CYP2D6 enzyme system (figure 2.6). Nortriptyline readily underwent ring hydroxylation to E- and Z-10-HO-NT. In contrast, both E- and Z-10-HO-AT proved to be poor substrates for this isozyme; only small amounts of the N-demethylated metabolites, E- and Z-10-HO-NT, were detected when E and Z-10-HO-AT were separately metabolized. The Z isomer was the preferred substrate (table 2.3). Attempts to metabolize E- and Z-10-HO-NT using CYP2D6 were unsuccessful (figure 2.7).

Table 2.3 Formation of 10-HO-NT from the metabolism of NT, E and Z-10-HO-AT by CYP2D6. Protein: 1.0 mg/ml incubation. Incubation time: 1.5 h. The results represent the means of three experiments ± SD.

Metabolic reaction	Substrate (nmol/ml incubation)	Yield of metabolite (nmol /ml incubation)
NT → 10-HO-NT	12.90	6.09 ± 0.23
Z-10-HO-AT → Z-10-HO-NT	13.61	1.57 ± 0.07
E-10-HO-AT → E-10-HO-NT	13.61	0.62 ± 0.09

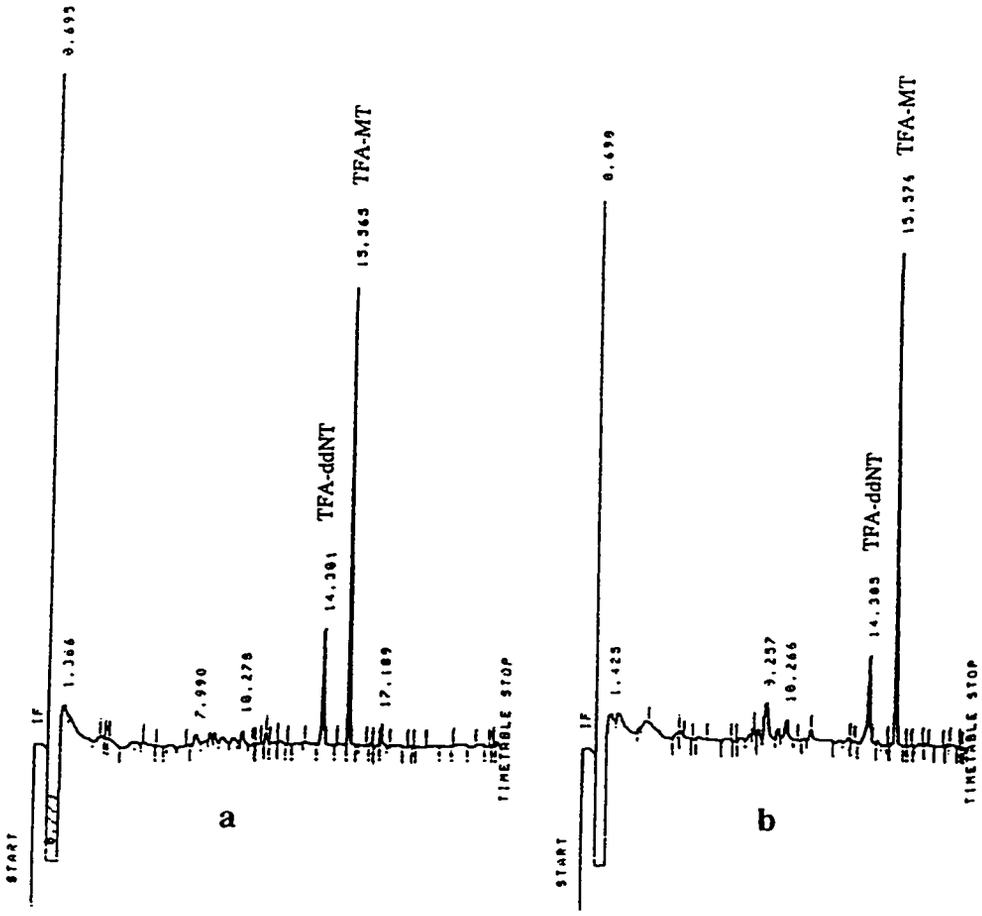

Figure 2.7 (a) GC trace of trifluoroacetylated dried extract of E-10-HO-NT incubation with CYP2D6. (b) GC trace of trifluoroacetylated dried extract of Z-10-HO-NT incubation with CYP2D6. Incubation mixtures were carried out under identical conditions to each other. Substrate amount: 1.435 nmol. Protein: 0.50 mg. Incubation time: 1.5 h. Incubation volume: 0.5 ml. Numbers refer to the retention times (min): TFA-ddNT, 14.301; TFA-MT, 15.565.

2.4 DISCUSSION

In a previous investigation, Breyer–Pfaff and coworkers (1992) concluded that human CYP2D6 enzyme catalyzed the ring hydroxylation of AT whereas others (Nusser *et al.* 1990; Zhang *et al.* 1993) have found that the conversion of AT to NT was mediated by a different CYP enzyme. The present study has shown that the *in vitro* metabolism of AT by human CYP2D6 microsomes produced E- and Z-10-HO-AT in significant quantities, and lesser amounts of NT, and E- and Z-10-HO-NT (figure 2.1 and table 2.1). The involvement of CYP2D6 in AT metabolism was confirmed when it was observed that incubations of AT with control microsomal protein (which lacked CYP2D6) or with CYP2D6 microsomal protein to which quinidine, a well known selective inhibitor of CYP2D6 (Kobayashi *et al.* 1989) had been added failed to produce any AT metabolites even when incubations were continued for up to 2.5 h (figures 2.3a and 2.3c). A concentration of quinidine as low as 4.55 nmol completely inhibited both ring hydroxylation and N-dealkylation of AT even when the concentration of AT was significantly higher (9.07 nmol) than that of the quinidine (figure 2.3c). In all of these studies, incubations were performed at least in duplicate.

In the initial metabolic study, attempts were made to separate and quantify underivatized metabolites of AT by a gas chromatographic procedure, but problems were encountered. Underivatized extracts containing AT, E- and Z-10-HO-AT, NT,and E- and Z-10-HO-NT gave tailing peaks and the E- and Z-isomers were poorly resolved. In addition, AT and some of its metabolites were highly adsorbed to test tube surfaces and reproducible GC results could not be obtained even when test tubes and other glassware were silanized regularly.

The problem of poor analyte separation was solved by acylation of metabolite mixtures prior to GC analysis. When acetylation with acetic anhydride in an aqueous medium is used, secondary amines and phenolic compounds are converted to acetamides and acetates, respectively, but tertiary amines and alcohols do not acetylate (Baker *et al.* 1981). Thus, AT and any E- and Z-10-HO-AT that were produced would not be derivatized, whereas secondary amines such as NT and E- and Z-hydroxy-NT, if formed,

would be N-acetylated. The GC trace of the acetylated metabolism mixture is provided (figure 2.2). The peaks were identified by comparisons of retention times with those of authentic samples that had been acetylated in identical fashion. In this way, it was possible to identify the analytes in figure 2.2 as AT, E-10-HO-AT, Z-10-HO-AT, N-acetyl-NT, N-acetyl-MT (internal standard) and N-acetyl-10-HO-NT. The last of these peaks probably consists of very small amounts of the isomers, E- and Z-10-HO-NT. Important qualitative deductions can be made from this GC trace. It is clear that CYP2D6-mediated 10-hydroxylation is a dominant metabolic reaction. The production of 10-HO-AT and 10-HO-NT result from this metabolic pathway. It is also apparent that both E- and Z-10-hydroxylation of AT occurs, and the former reaction predominates. Preferential production of E-10-HO-AT was also observed *in vivo* in humans (Breyer–Pfaff *et al.* 1992), but these latter authors suggest that only the E-10-hydroxylation of AT was mediated by CYP2D6. Attempts were made to quantify the metabolites in the solution that had undergone acetylation, but this could not be done. Ratios of E-10-HO-AT:Z-10-HO-AT were inconsistent in identically treated extracts, and large differences were observed in N-acetyl-metabolite:N-acetyl-MT ratios of equivalent extracts. In some extracts, the N-acetyl-NT was absent. This lack of consistency was attributed to unavoidable adsorption of analytes to glass surfaces. Even when tubes and glassware had been freshly silylated, inconsistent analytical results were always obtained.

When trifluoroacetylation of extracts was performed prior to GC analysis, reproducible quantitative data were obtained but this derivatizing treatment made it impossible to quantify separately the E- and Z-10-hydroxylated enantiomers of AT and NT. C_{10}-C_{11} dehydration occurred during trifluoroacetylation reaction so that E- and Z-10-HO-AT were both converted to the same product (10,11-didehydro-AT; ddAT). Similarly, E- and Z-10-HO-NT both yielded N-trifluoroacetyl-10,11-didehydro-NT (TFA-ddNT). The structures of ddAT and TFA-ddNT were confirmed by electron-impact (EI) mass spectrometry. The GC-mass spectrum of ddAT contained a weak molecular ion of m/z 275 (0.40%) and fragment ions of m/z 216 (4.9%), 215 (16.1%), 213 (4.2%), 202 (6.1%), 189 (3.6%) and 58 (100%). It was virtually identical to the reported (Pfleger *et al.* 1985) mass spectrum of ddAT. The EI mass spectrum of TFA-

ddNT had a molecular ion of m/z 357 (13.1%), and fragment ions of m/z 230 (71.0%), 229 (42.5%), 217 (73.3%), 216 (29.5%), 215 (100%), 202 (62.9%), 189 (11.4%), 140 (16.7%) and 69 (14.9%). This spectrum was reminiscent of the reported EI mass spectrum of N-acetyl-10,11-didehydroNT (Pfleger *et al.* 1985). Both spectra have ions in common of m/z 230, 229, 217, 216, 215, 202 and 189. Characteristic ions present only in the spectrum of TFA-ddNT are those of m/z 357 (molecular ion), 140 $[CH_2{=}N(CH_3)COCF_3]^+$ and 69 $[CF_3]^+$. Solutions containing recovered AMI and derivatized metabolites (TFA-NT, ddAMI and TFA-ddNT) were stable at -4°C for at least 10 weeks and reproducible quantitative analyses of total 10-HO-AT (Z + E) and total 10-HO-NT (Z + E) were achieved in this way. The GC trace of a trifluoroacetylated extract is provided (figure 2.3).

In kinetic studies, an incubation time of 1.5 h was selected because the rates of formation of 10-HO-AT and 10-HO-NT from AT were linear over this period. The deviation from linearity of NT formation suggested that NT was an intermediate metabolite from which 10-HO-NT formed. However, the formation of 10-HO-NT from AT could proceed *via* two intermediates, 10-HO-AT or NT, so both of these intermediates were separately metabolized *in vitro*. The data that resulted (table 2.3) permitted the conclusion that the metabolic conversion of AT to E- and Z-10-HO-NT by CYP2D6 proceeds mainly *via* NT. In contrast to the *in vitro* results of the present study, large quantities of E and Z-10-HO-NT are detected in human urine of AT-treated patients or in the *in vitro* incubation of AT with human liver microsomes. Their generation must proceed *via* NT, as previously suggested by Bertilsson and colleagues (1979), and be catalyzed by one or more isozymes in addition to CYP2D6.

It was also observed that at low substrate concentrations, the rate of formation of 10-HO-NT was slightly greater than the rate of formation of NT, but the rate of formation of 10-HO-NT slowly declined as AT substrate concentration was gradually increased (figure 2.5). This observation suggested that 10-HO-NT was produced in lesser amounts or it was further metabolized to an unknown metabolite. The latter hypothesis was excluded when attempts to metabolize both E- and Z-10-HO-NT with CYP2D6 revealed that both 10-HO-NT isomers were very poor substrates for CYP2D6 (figure 2.7).

The present study confirms that human CYP2D6 not only catalyzes the ring hydroxylation of AT to 10-HO-AT and NT to 10-HO-NT, but also mediates the N-dealkylation of AT to NT to a significant extent (up to 25% of total AT metabolites). Our findings suggest either that CYP2D6 has two distinct binding sites, one which orientates AT and NT for 10-hydroxylation and the other which is responsible for N-demethylation of AT to NT, or that CYP2D6 can bind to two different locations on the AT as proposed by Islam *et al.* (1991). At high substrate concentrations which saturated the CYP2D6 enzyme, the rate of formation of 10-HO-AT was constant and very little conversion of NT to 10-HO-NT occurred. This is consistent with there being a competition by AT and NT for enzyme binding sites. Consistent results were also obtained when the incubation of AT at a concentration which just saturated enzyme capacity to hydroxylate AT, was prolonged (figure 2.4). The decline in the amount of NT detected can be associated with the ability of CYP2D6 to hydroxylate the metabolically formed NT to 10-HO-NT.

This study has clearly shown that CYP2D6 is capable of catalyzing the N-demethylation of AT to NT. Very few drugs undergo N-demethylations catalyzed by this enzyme. Those that have been identified to do so, at least to some extent, are amiflamine, desmethylcitalopram, imipramine (Coutts *et al.* 1994) and deprenyl (Grace *et al.* 1994). Further *in vitro* studies to identify other CYP enzymes that may be involved in the N-demethylation of AT are described in Chapter 3.

2.5 REFERENCES

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Chapter 3

The N-demethylation of amitriptyline

3.1 INTRODUCTION

The tricyclic antidepressant amitriptyline (AT) is still widely prescribed in clinical practice for the treatment of major depression (Baker, 1996) and chronic pain (Bowshers, 1993; Max, 1995), either as a single drug or in combination with other antidepressants. The effects of AT treatment vary greatly from patient to patient and depend largely on its rate of elimination from the body (Vandel *et al.* 1982; Sjoqvist and Bertilsson, 1986). According to the records maintained by the Saskatchewan Prescription Drug Plan of antidepressants used by eligible residents in 1995, 30.35% of the 268,145 prescriptions for antidepressants were for AT (Baker, 1996). When AT is coadministered with antidepressants that are selective serotonin reuptake inhibitors (SSRIs), for example fluoxetine (Brøsen, 1993; El-Yazigi *et al.* 1995; Vandel *et al.* 1992) and fluvoxamine (FX) (Hartter *et al.* 1993), drug-drug interactions occur and AT's pharmacokinetic parameters are altered. The likelihood of the occurrence of drug-drug interactions could be predicted more accurately if the cytochrome P450 (CYP) isozymes involved in the biotransformation of AT to its major metabolites (Breyer-Pfaff *et al.* 1992), by N-demethylation and/or ring hydroxylation to nortriptyline (NT), 10-hydroxyamitriptyline (10-OH-AT) and 10-hydroxynortriptyline (10-OH-NT), were known.

AT is extensively metabolized by ring hydroxylation to 10-HO-AT and by N-demethylation to nortriptyline (NT), the major metabolite of AT (Ereshefshy *et al.* 1988). This metabolite subsequently undergoes ring hydroxylation to 10-HO-NT. A number of isozymes have been implicated in the metabolism of AT to NT, but the CYP enzymes involved in this N-demethylation reaction have not yet been unequivocally identified. It has been suggested that CYP2C19 is involved in the formation of the N,N-didemethylated AT (diDM-AT), but this enzyme does not catalyze the mono N-demethylation of AT to NT and neither does CYP2D6 (Breyer-Pfaff *et al.* 1992). Previous *in vitro* metabolic studies by us on AT (Coutts *et al.* 1997; see Chapter 2) that involved the use of purified expressed microsomal CYP2D6 revealed that this human enzyme not only catalyzes ring hydroxylation, but also mediates the N-demethylation of AT to a significant extent, although the N-demethylation reaction never dominates as it does in

patients treated with AT (Vandel *et al.* 1982; Prox and Breyer–Pfaff, 1987). Thus, other CYP enzymes in addition to CYP2D6 must be involved in the N-demethylation reaction.

Since the N-dealkylation of many drugs with diverse chemical structures is mediated by members of the CYP3A subfamily of enzymes, especially CYP3A4 (table 3.1; see also Coutts *et al.* 1994), a cytochrome P450 enzyme abundantly expressed in human liver (Guengerich, 1995), it seemed likely that CYP3A4 could be involved in the N-demethylation of AT, especially in view of the observation that CYP3A4 mediates to some extent the biochemical transformation of imipramine (IMI) to desipramine (DMI) (Ohmori *et al.* 1993; Lemoine *et al.* 1993). To test this hypothesis, the *in vitro* metabolic N-demethylation of AT was investigated using a purified CYP3A4 microsomal preparation. Using this technique, the role of CYP3A4 can be precisely predicted. In a previous study (Chapter 2), we found that an expressed CYP2D6 preparation which did not require augmentation with cytochrome P450 reductase was capable of catalyzing the N-demethylation of AT. When the same conditions were used in a preliminary study in which CYP2D6 was replaced with CYP3A4, no N-demethylation of AT occurred. The difficulties associated with the reconstitution of CYP3A4 catalytic activity in *in vitro* preparations are well documented (Waxman *et al.* 1985; Imaoka *et al.* 1988; Guengerich,1995). Conditions used to modify CYP3A incubation media to attain optimal activity are not constant; they differ with different substrates. For instance, the presence of lipid(s), detergent and cytochrome b_5 is necessary for expressed CYP3A4 to mediate the 6β-hydroxylation of testosterone but the same supplementation was found to inhibit the metabolism of erythromycin, benzphetamine and IMI to various extents (Shet *et al.* 1993). Other studies have indicated that the metabolism of halofantrine (Halliday *et al.* 1995), tropisetrone (Firkusny *et al.* 1995), phenanthrene, 7,8-benzoflavone (Shou *et al.* 1994) and terfenadine (Ling *et al.* 1995) did not require the presence of NADPH-cytochrome P450 reductase, lipid, detergent or cytochrome b_5. However, an addition of NADPH-cytochrome P450 reductase to the CYP3A4 incubation medium often enhances the enzyme's activity (Ling *et al.* 1995). When all these facts are considered, it is apparent that CYP3A4 catalytic activity is generally improved by the addition of NADPH-cytochrome P450 reductase to the reconstituted system.

Recently, CYP3A4 enzyme which was either coexpressed or fortified with NADPH cytochrome P450 reductase was shown to mimic the activity of human liver enzymes in the metabolism of alfentanil (Labroo *et al.* 1995) and testosterone (Lee *et al.* 1995). Also the ability of this CYP enzyme to oxidize nifedipine was enhanced when it was coexpressed with NADPH-cytochrome P450 reductase, and supplemented with cytochrome b_5 (Peyronneau *et al.* 1992). It was appropriate, therefore, to use coexpressed CYP3A4 and NADPH-cytochrome P450 reductase (identified from now as CYP3A4/r) to identify the role played by CYP3A4 in the N-demethylation of AT. While the study was in progress, Schmider *et al.* (1995) reported an *in vitro* study on AT N-demethylation with human liver microsomes in which CYP3A4 antibodies and inhibitors were used. This study revealed that the N-demethylation of AT was mediated with CYP3A4. Our interest in AT N-demethylation expanded to include a determination of optimal conditions for the N-demethylation of AT by fortified, expressed CYP3A4.

Various factors have been found to alter the N-demethylation of AT. Mellström *et al.* (1986) and Edelbroek *et al.* (1987), for instance, reported that cigarette smoking induced the formation of NT in AT-treated patients. Later, Hartter *et al.* (1993) found that fluvoxamine (FX) inhibited N-demethylation of AT, and Baumann and Rochat (1995) obtained a similar result when FX was coadministered with AT. The correlations observed between enzyme induction by cigarette smoke and inhibitory effects caused by FX on the formation of NT strongly suggest that CYP1A2 may be involved in the N-demethylation of AT, in spite of the conclusion by Schmider *et al.* (1995) that AT was not a substrate for CYP1A2.

Our other interests were to identify the role of CYP1A2 in the N-demethylation of AT and to determine the cumulative contributions of three isozymes (CYP2D6, CYP3A4 and CYP1A2) in the N-demethylation of AT to NT. The knowledge gained from these studies may be clinically relevant, particularly when AT is given in conjunction with other drugs (antidepressants, neuroleptics and antiarrythmics). The elimination of many drugs by oxidations in the liver is catalyzed predominantly by the three enzymes, CYP2D6, CYP3A4 and CYP1A2 (Preskorn, 1996).

Oxidations catalyzed by CYP3A4 in a reconstitutive system are greatly affected by the presence of cytochrome b_5 in the incubation medium (Guengerich *et al.* 1986a; Renaud *et al.* 1990; Peyronneau *et al.* 1992; Goldstein *et al.* 1994). The cytochrome b_5 contribution varied with different CYP isoforms (Omata *et al.* 1994; Matsusue *et al.* 1996) and with different substrates (Lu *et al.* 1974). The metabolism of zonisamide to 2-sulfamoylacetylphenol (SMAP) by CYP3A4, for example, was not affected by the presence of cytochrome b_5 (Nakasa *et al.* 1993) whereas the N-demethylation of aminopyrine was increased significantly by its addition to the metabolism mixture (Jansson and Schenckman 1987). The presence of cytochrome b_5 also induced the stimulatory and inhibitory effects of CYP1A1 and CYP2B1, respectively, on the hydroxylation of 2,3',4',5-tetrachorobiphenyl (Omata *et al.* 1994; Matsusue *et al.* 1996).

It was necessary, therefore, to investigate the role of cytochrome b_5 on the oxidative abilities of CYP1A2 and CYP2D6, in addition to CYP3A4, in the AT N-dealkylation pathway. In order to evaluate the ability of individual CYP enzymes to catalyze the conversion of AT to NT, Michaelis-Menten parameters (apparent K_m and V_{max}) were mathematically converted into intrinsic clearance (Cl_{int}) and the Cl_{int} values for the N-demethylation pathway by the three CYP enzymes were then compared.

It was hoped, as mentioned in Chapter 1 in the section 1.5 "*in vivo* and *in vitro* correlation", that the Cl_{int} values obtained from the *in vitro* studies could be used to estimate the *in vivo* Cl_{int} of the drug. This strategy was found to be successful to some extent when liver microsomal protein or hepatocytes were used in metabolic reactions as sources of enzymes. Purified enzymes (from human tissues or expressed human cell line) are generally not considered as appropiate enzymes for this particular area of study. Some believe that in liver microsomal preparations there are many other components that are not present in purified expressed enzymes and consequently, catalytic activities of both enzyme preparations will vary. If this is so, the *in vitro* Cl_{int} of a drug using purified enzymes has no clinical application or value and the assessment of the *in vitro* Cl_{int} as predictor of *in vivo* Cl_{int} may be considered to be absurd. However, it should be stressed that if there is no difference in the ability of liver microsomes and purified enzymes to oxidize drugs to the same product(s) and at similar rates, the use of purified enzymes can

provide some advantages over liver microsomes. They are more specific, commercially available and not expensive. Based on the studies using liver microsomes reported by Schmider *et al.* (1995), CYP3A4 is the only major enzyme that catalyzes the N-demethylation of AT to NT. This pathway accounted for about 60% of the AT dose given to the patient. Thus, AT may be considered to be a good drug model to assess correlations between *in vivo* and *in vitro* Cl_{int} values.

In addition to the aims of the study mentioned above, another interest was to conduct a preliminary investigation on the correlation between results obtained with the two *in vitro* enzyme preparations that are used in AT metabolic studies, liver microsomes and expressed enzymes.

Total Cl_{int} values of the three CYP enzymes were also calculated and compared to data calculated from the apparent K_m and V_{max} values obtained in an *in vitro* study using human microsomal proteins (Schmider *et al.* 1995). That AT oxidations were catalyzed by CYP1A2, CYP2D6 and CYP3A4 was confirmed by inhibition studies using fluvoxamine (FX), quinidine (QND) and ketoconazole (KE), respectively.

3.2 EXPERIMENTAL

3.2.1 Chemicals and reagents

AT.HCl, NT.HCl, maprotiline HCl (MT.HCl), NAPD sodium salt from yeast ($NADP^+$), D-glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase Type XII from Torula yeast (G6PD), L-α-phosphatidylserine sodium salt (PS), L-α-dilauroylphosphatidylcholine (DLPC) and L-α-dioleoylphosphatidylcholine (DOPC) and sodium cholate were purchased from Sigma Chemical Co. (St. Louis, MO). E-10-hydroxyamitriptyline (E-10-HO-AT) was a gift from Dr. G. McKay, College of Pharmacy and Nutrition, University of Saskatchewan. FX malate was a gift from Prof. Michel Bourin (Université de Nantes, France) and KE was purchased from Research Biochemicals International (Natick, MA). Other reagents and solvents were obtained from the following suppliers: trifluoroacetic anhydride [$(CF_3CO)_2O$ analytical grade], magnesium

chloride ($MgCl_2.6H_2O$), and dichlorodimethylsilane (99%), Aldrich Chemical Co. (Milwaukee, WI); and quinidine sulfate (QND sulfate), methanol, n-hexane, acetonitrile (CH_3CN) (HPLC grade), isopropanol and toluene (both distilled before use), BDH (Toronto, Canada). Potassium phosphate solution (100 mM; pH = 7.4) was the buffer used in all experiments.

3.2.2 Microsomal proteins

Expressed human CYP1A2, CYP2D6, CYP3A4 and CYP3A4 coexpressed with NADPH cytochrome P450 reductase (CYP3A4/r) microsomal proteins which were prepared in a human lymphoblastoid cell line (AHH-1TK+/- cells) that had been transfected with cDNAs encoding human CYP1A2, CYP2D6 and CYP3A4, respectively, were purchased from Gentest Corporation (Woburn, MA, U.S.A.). The protein content of each microsomal preparation was 10 mg/ml in 0.1 M potassium phosphate (pH = 7.4) and cytochrome P450 contents were 93, 260, 58 and 60 pmol/mg protein for CYP1A2, CYP2D6, CYP3A4 and CYP3A4/r, repectively. Control microsomes prepared in the same human cell line without the addition of a cDNA, were also purchased from the same supplier. They contained a low level of cytochrome P450 activity which was inducible by pretreatment with polycyclic aromatic hydrocarbons.

Recombinant human cytochrome b_5 (51.5 μM in buffer) and rabbit NADPH-cytochrome P450 reductase (22.0 μM in buffer) were gifts from Dr. K.E. Thummel, University of Washington in Seattle.

Human microsomal proteins from 15 livers were purchased from the International Institute for the Advancement of Medicine (IIAM; Exton, PA). Each preparation was characterized for protein content (measured by Pierce protein assay), total CYP content (determined by the carbon monooxide spectrum) and catalytic activity of the seven CYP enzymes 1A2, 2A6, 2C19, 2D6, 2E, 3A4 and 4A (determined using isoform selective catalytic probes).

Table 3.1 Metabolic N-dealkylation reactions mediated by CYP3A enzymes.

Drug	Enzyme Source	Metabolic Pathways	References
Alfentanil	Human recombinant enzyme	N-de-[2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)]ethylation	Labroo *et al.* 1995
Alpidem	Not available	N-depropylation	Guengerich, 1995
Amiodarone	Human microsomes	N-deethylation	Trivier *et al.* 1993
Benzphetamine	Human microsomes	N-demethylation	Ged *et al.* 1989
Cocaine	Human microsomes	N-demethylation	Pellinen *et al.* 1994
Codeine	Human fetal microsomes	N-demethylation	Ladona *et al.* 1991
Cyclosporin analog (SDZ IMM 125)	Human microsomes and recombinant enzyme	N-demethylation	Vickers *et al.* 1995
Dextromethorphan	Human microsomes	N-demethylation	Gorski *et al.* 1994
Erythromycin	Human recombinant enzyme	N-demethylation	Brian *et al.* 1990
Ethylmorphine	Human microsomes	N-demethylation	Liu *et al.* 1995
Halofantrine	Human microsomes and recombinant enzyme	N-debutylation	Halliday *et al.* 1995
Ifosfamide	Human microsomes	N-de-(2-chloro)ethylation N-de-(3-chloro)ethylation	Walker *et al.* 1994
Imipramine	Human microsomes	N-demethylation	Lemoine *et al.* 1993
Lidocaine	Human microsomes and purified enzyme	N-deethylation	Bargetzi *et al.* 1989 Imaoka *et al.* 1990
Propafenone	Human microsomes and recombinant enzyme	N-depropylation	Botsch *et al.* 1993
Sertindole	Human microsomes	N-de-[2-(2-oxoimidazolin 1-yl)]ethylation	Mulford *et al.* 1993
Tamoxifen	Human microsomes	N-demethylation	Jacolot *et al.* 1991
Terfenadine	Human microsomes	N-de-[4-hydroxy-4-(4-t-butyl)phenyl]butylation	Ling *et al.* 1995
Zatosetron	Rat, human and monkey microsomes.	N-demethylation	Ring *et al.* 1994

3.2.3 NADPH Generating System

This was prepared by mixing fresh stock solutions of $NADP^+$(1.3 mM), G6P (3.3 mM) and G6PD (50 U/ml buffer) in a 5:5:2 volume ratio.

3.2.4 GC assay procedure

3.2.4.1 Instrumental analysis

Analysis of AT metabolites was conducted on a Hewlett Packard 5730A gas chromatograph equipped with nitrogen-phosphorus detector (NPD) and linked to an HP 3392A integrator. A DB-17 fused silica capillary column (J and W Scientific, CA) with 15 m x 0.25 mm ID, 0.25 ml film thickness was employed for separation of analytes. The flow rate of the carrier gas (helium) was adjusted to maintain a column head pressure of 10 psi. Make-up gas at the detector was a mixture of hydrogen (3.4 ml/min) and air (95.2 ml/min) and the combined flow rate of helium and make-up gas was 119 ml/min. The injector and detector temperatures were 250°C and 300°C, respectively. A splitless injection system was used.

3.2.4.2 Extraction and derivatization with trifluoroacetic anhydride

At the conclusion of each enzymatic incubation, 50 μl of a 25% K_2CO_3 solution, internal standard (MT, 2.42 nmol) and phosphate buffer (375 μl) were added and the resulting mixture was extracted with a mixed organic solvent (2% v/v isopropanol in n-hexane; 2 x 3.5 ml). After evaporation of the combined extracts, each residue was trifluoroacetylated by dissolving it in a mixture of trifluoroacetic anhydride (50 μl) and acetonitrile (25 μl) and vortexing for 30 sec, followed by heating for 8 min at 95°C. The reaction was allowed to cool to room temperature and the residual solvent and excess anhydride were removed in a stream of nitrogen. The resulting residues were reconstituted in toluene (200 μl) and an aliquot (1 μl) was used for analysis on the GC system described in section 3.2.4.1. Chromatographic separation was accomplished using the following oven temperature program. The initial temperature was held at 180°C

for 2 min after each injection, then increased linearly to 280°C at a rate of 8°C/min and the final temperature was held for 4 min.

3.2.4.3 Quantification of AT incubation products

For the quantitation of AT and its metabolites, analytes were eluted using the GC temperature program described in section 3.2.4.2. Under these conditions, AT, 10,11-didehydroamitriptyline (ddAT), N-trifluoroacetyl-NT (N-TFA-NT) and N-trifluoroacetyl-MT (N-TFA-MT) eluted at 8.20, 9.01, 10.48 and 11.81 min, respectively. Calibration curves were constructed from GC traces of solutions containing varying quantities of authentic samples of AT (2.38, 12.68, 25.36, 44.38 and 62.93 nmol/125 μl of incubation solution), E-10-HO-AT (0.43, 0.85, 1.71 and 2.56 nmol/125 μl of incubation) and NT (0.34, 1.12, 2.24, 3.36 and 5.04 nmol/125 μl of incubation solution), and a constant amount of internal standard (MT, 4.84 nmol). The final solutions were trifluoroacetylated as described in section 3.2.4.2 and peak area ratios (AT/N-TFA-MT, ddAT/ N-TFA-MT and N-TFA-NT/T-TFA-MT) *vs* concentration of AT, E-10-HO-AT and NT were plotted.

3.2.5 Concentrations of chemical inhibitors

For inhibition studies, QND (4.56 μM) was used as the inhibitor of CYP2D6 (Labroo *et al.* 1995), KE (9.92 μM) as the inhibitor of CYP3A4 (Firkusny *et al.* 1995; Schmider *et al.* 1995) and FLU (2.08 and 4.16 μM) as the CYP1A2 inhibitor (Brøsen *et al.* 1993; Schmider *et al.* 1995). The concentrations of inhibitors were selected on the basis of literature data that have shown these concentrations to inhibit more than 80% of the enzyme activity of each of these three CYP enzymes.

3.2.6 Enzyme kinetic calculations

3.2.6.1 Statistical analyses

Data points consisting of reaction velocities (V) at varying concentrations of the substrate AT (S) were fitted into the equation (1) and the apparent K_m and V_{max} values

were determined by nonlinear least-squares regression analysis of reaction velocities vs substrate concentration curves using the computer program "WinNonLin" (version 1.0; Scientific Consulting, Inc.).

$$V = \frac{V_{max} \cdot S^n}{S_{50}^n + S^n} \qquad (1)$$

S_{50} is the substrate concentration at which the reaction velocity equals 50% of V_{max} and is thus equivalent to the K_m derived by the Michaelis-Menten equation, and n is the Hill coefficient of cooperative substrate binding. The apparent K_m and V_{max} values for CYP1A2 and CYP3A4 determined using equation (1) were favored statistically (the standard error was small).

Correlation of AT metabolism to NT in human microsomes was evaluated based on the r and p values obtained from linear regression analysis of the rate of NT formation vs the quantity of CYP enzyme (it was determined by the rate of enzyme exclusive substrate-metabolite formation) using the program "Prism" (version 2.01; GraphPad Software Inc.; San Diego CA). A value of $p < 0.05$ was accepted as significant.

To determine whether cytochrome b_5 had any effect on the metabolism of AT to NT when mediated by CYP1A2 or CYP2D6, the extent of formation of NT in the absence and presence of cytochrome b_5 in reconstituted CYP1A2 and CYP2D6 systems was compared using paired t tests. The extents of ring hydroxylation of AT mediated by CYP2D6 enzyme in the presence and absence of cytochrome b_5 were similarly compared.

3.2.6.2 Intrinsic and hepatic clearance equations.

Intrinsic clearance of AT (Cl_{int}) can be determined from Michaelis-Menten parameters:

$$Cl_{int} = V_{max}/K_m \qquad (2)$$

When more than one enzyme is involved in the same metabolic pathway, the total Cl_{int} and Cl_H (hepatic clearance) will be:

$$Cl_{int} = \sum_{k=1}^{n} Cl_{int,k} \qquad (3)$$

$$Cl_H = \sum_{k=1}^{n} Cl_{H,k} \quad (4)$$

where k = process identity number and n = total number of enzymatic processes.

Hepatic clearance (Cl_H) can be calculated using the following equations:

$$Cl_H = Q_H \cdot f_u \cdot Cl_{int} / (Q_H + f_u \cdot Cl_{int}) \quad (5a)$$

$$Cl_H = Q_H \cdot E_H \quad (5b)$$

where f_u is the fraction of unbound drug in the blood, E_H is the hepatic extraction ratio, and Q_H represents hepatic blood flow rate.

Hepatic clearance of AT as NT by liver enzymes ($Cl_{H,NT(AT)}$) may be deduced from the following equation:

$$Cl_{H,NT(AT)} = f_{NT(AT)} \cdot Cl_H \quad (6)$$

where $f_{NT(AT)}$ is the fraction of the dose of AT that is converted to NT.

3.2.6.3 Intrinsic and hepatic clearance calculations

In order to calculate the *in vivo* Cl_H of AT from Q_H and E_H values, it is assumed that all the reactions involved in AT metabolism follow first order kinetics. Literature values of Q_H (1.21 ± 0.55 l/min; range from 0.59 – 2.08 l/min), E_H (0.57 ± 0.06; range from 0.49 – 0.65), f_u (0.052 ± 0.006; range from 0.044 – 0.059), and $f_{NT(AT)}$ (0.62 ± 0.22; range from 0.25 – 0.89) were obtained from *in vivo* studies in humans (Rollins *et al.* 1980). *In vitro* Michaelis-Menten parameters were taken from the study by Schmider *et al.* (1995) on human microsomal protein from four livers. Since the V_{max} data are reported as rate per mg of protein, they were recalculated as rate per g of liver. The content of microsomal protein in the average human liver is 265.28 g (Lentner, 1981; Vickers *et al.* 1995). The approximate expression of each isozyme, CYP1A2, CYP2D6 and CYP3A4, in the total hepatic CYP content is 12%, 4% and 28%, respectively (Guengerich, 1995) and the total CYP concentration in liver microsomal protein is 0.53 nmol/mg protein (Guengerich, 1990; Yamazaki *et al.* 1996).

3.2.7 *In vitro* enzymatic reactions of AT with human expressed CYP enzymes

3.2.7.1 CYP3A4-mediated N-demethylation of AT

3.2.7.1.1 Metabolism of AT by CYP3A4 microsomal protein

A typical incubation mixture contained microsomal CYP3A4 (7.5 pmol), $MgCl_2$ (0.41 μmol), AT (7.55 nmol) and pH 7.4 buffer to a final volume of 110 μl. This mixture was preincubated for 5 min at 37°C in a water bath. The metabolic reaction was then initiated by an addition of the NADPH generating system (15 μl). The reaction was allowed to proceed for 1.5 h at 37°C and was terminated by cooling on ice followed by the addition of 50 μl of a 25% K_2CO_3 solution. Internal standard (MT; 2.42 nmol) and phosphate buffer (375 μl) were added and the incubation mixture (now 590 μl) was extracted with an organic solvent (2% isopropanol in n-hexane; 3.5 ml x 2). The combined extract was evaporated to dryness in nitrogen at room temperature. Samples were derivatized and analyzed as described in section 3.2.4.

This entire reaction was repeated after the addition of 10 μg of mixed lipids (DLPC, DOPC, PS) in a 1:1:1 ratio and 100 μg of sodium cholate prior to the preincubation step.

3.2.7.1.2 Metabolism of AT by CYP3A4/r microsomal protein

The metabolic reaction described in the section 3.2.7.1.1 above was repeated except that CYP3A4 was replaced by CYP3A4/r and two different quantities of AT were used (7.55 and 50.34 nmol). Samples were analyzed as described in section 3.2.4.

3.2.7.1.2.1 Effect of adding lipids, NADPH-cytochrome P450 reductase, cytochrome b_5, and sodium cholate

The incubation mixture (220 μl) contained CYP3A4/r (15.0 pmol), NADPH-cytochrome P450 reductase (75.0 pmol), cytochrome b_5 (15.0 pmol), 5 μg of mixed lipids (PS, DLPC and DOPC; 1:1:1 ratio), sodium cholate (50.0 μg), $MgCl_2$ (0.82 μmol), AT (14.91 nmol) and buffer. The first four components were combined and placed in ice

bath for 20 min before the last four components were added in the order indicated here. The reaction mixture was then preincubated for 5 min at 37°C prior to addition of the NADPH generating system (30 μl). Incubation was allowed to proceed for 1.5 h.

Similar experiments were conducted in which the four components (lipids, NADPH-cytochrome P450 reductase, cytochrome b_5 and sodium cholate) in the incubation medium were replaced by different combinations as listed in table 3.2. Incubation samples were then derivatized and quantitated by GC analysis as described in section 3.2.4.

3.2.7.1.2.2 Effect of additional NADPH-cytochrome P450 reductase

Each 110 μl of incubation mixture contained CYP3A4/r (7.5 pmol) and varying quantities of rabbit NADPH-cytochrome P450 reductase (0; 7.5; 15.0 26.3 and 37.5 pmol), $MgCl_2$ (0.41 μmol), AT (50.34 nmol) and buffer. The first two components were combined and placed in ice for 20 min. To this mixture, the last three components were added and the resulting mixture was incubated at 37°C for 5 min. Reaction was initiated by an addition of the NADPH generating system (15 μl). After incubation for 1.5 h at 37°C, the reaction mixture was then extracted, derivatized and quantitated by a GC assay as described in section 3.2.4.

3.2.7.1.2.3 Effect of adding human cytochrome b_5

CYP3A4/r (7.5 pmol) was mixed with varying concentrations of human cytochrome b_5 (0 to 30.0 pmol) and all solutions were placed on ice for 20 min. $MgCl_2$ (0.41 μmol) and AT (50.34 nmol) were added, followed by phosphate buffer (pH = 7.4) to a final volume of 110 μl. The mixture was incubated at 37°C for 5 min, prior to initiation of the metabolic reaction by the addition of the NADPH generating system (15 μl). Incubation and work-up of this mixture were conducted as described in the section 3.2.7.1.1. The final dry extract was trifluoroacetylated and derivatized samples were analyzed by GC as described in section 3.2.4.

Time course study

Microsomal CYP3A4/r solution (45.0 pmol) was mixed with human cytochrome b_5 (135.0 pmol) and the mixture was placed on ice for 20 min. $MgCl_2$ (2.47 μmol), and AT (301.5 nmol) were added and the mixture was diluted to a final volume of 660 μl with phosphate buffer (pH = 7.4). This solution was incubated for 5 min at 37°C prior to the addition of 90 μl of the NADPH generating system which initiated the metabolic reaction. Aliquots (125 μl) were removed at 0, 1, 1.5, 2 and 2.5 h intervals. Samples were extracted, derivatized and analyzed as decribed in section 3.2.4.

Effect of varying the amount of CYP3A4/r enzyme

Each incubation mixture (110 μl) contained 0 to 0.375 mg of protein (≡ 0 to 22.5 pmol of CYP3A4/r), a suitable amount of cytochrome b_5 (the ratio of CYP3A4/r to cytochrome b_5 was always 1:3), $MgCl_2$ (0.41 μmol), AT (50.34 nmol) and potassium phosphate buffer (pH = 7.4). The mixture was incubated for 5 min prior to the addition of the NADPH generating system (15 μl). Reactions were conducted for 1.5 h at 37°C. Samples were analyzed after derivatization for trifluoroacetylated NT by GC as decribed in section 3.2.4.

CYP3A4/r kinetic study

Mixtures were made of CYP3A4/r solution (7.5 pmol), cytochrome b_5 (22.5 pmol), $MgCl_2$ (0.41 μmol), NADPH generating system (15 μl), varying amounts of AT, ranging from 2.3 to 62.9 nmol, and phosphate buffer to 125 μl. Each mixture was incubated for 1.5 h at 37°C. The trifluoroacetylated NT content of each incubation mixture was determined by GC analysis as described in section 3.2.4.

3.2.7.1.3 Control metabolism

The metabolism procedure described in section 3.2.7.1.2.3 above was repeated except that control microsomal protein was used in place of the CYP3A4/r and the amount of cytochrome b_5 added was 22.5 pmol.

Another control experiment was also performed as described in section 3.2.7.1.2.3 above, except the CYP3A4/r was omitted.

3.2.7.2 CYP1A2-mediated N-demethylation

3.2.7.2.1 Metabolism of AT by CYP1A2

Time course study

A typical incubation mixture contained microsomal CYP1A2 (45 pmol), $MgCl_2$ (2.47 μmol), AT (301.5 nmol) and pH 7.4 buffer to a final volume of 660 μl. This solution was incubated for 5 min at 37°C prior to the addition of 90 μl of the NADPH generating system which initiated the metabolic reaction. Aliquots (125 μl) were removed at 0, 1, 1.5, 2 and 2.5 h intervals. Incubation samples were then derivatized and quantitated by GC analysis as described in section 3.2.4.

CYP1A2 kinetic study

The incubations were performed as described in section 3.2.7.1.1 above, except that CYP3A4 was replaced by CYP1A2, the AT concentration was varied from 1.52 nmol to 38.04 nmol/125 μl of incubation and incubation time was 1 h. Analytical procedures were identical to those described in section 3.2.4.

3.2.7.2.2 Comparison of the catalytic activities of CYP1A2, CYP2D6 and CYP3A4/r in the metabolism of AT

3.2.7.2.2.1 In the absence of cytochrome b_5

Separate metabolic studies of AT were conducted in the presence of each of the three CYP enzymes, namely CYP1A2, CYP2D6 and CYP3A4/r. Incubation mixtures contained microsomal protein (7.5 pmol), $MgCl_2$ (0.41 μmol), AT (25.87 nmol) and pH 7.4 buffer to a final volume of 110 μl. Each mixture was preincubated for 5 min at 37°C in a water bath. The metabolic reaction was then initiated by an addition of 15 μl of the

NADPH generating system. The reaction was allowed to proceed for 1.0 h at 37°C and was terminated by cooling on ice. Subsequent derivatized samples were analyzed as described in section 3.2.4.

3.2.7.2.2.2 In the presence of cytochrome b_5

Separate enzyme mixtures containing a single CYP enzyme (7.5 pmol) and cytochrome b_5 (22.5 pmol) were prepared and placed on ice. Twenty minutes later, $MgCl_2$ (0.41 μmol), AT (25.87 nmol) and buffer were added to give each solution a final volume of 110 μl. Each mixture was preincubated for 5 min at 37°C before the addition of the NADPH generating system (15 μl) was made. After 1.0 h of incubation, AT and its metabolites were extracted into organic solvent (2% isopropane-hexane; 2 x 3.5 ml), derivatized and analyzed as described in section 3.2.4.

3.2.7.3 The metabolism of AT by a mixture of three CYP enzymes

3.2.7.3.1 In the absence of cytochrome b_5

From the separate 110 μl mixtures containing the CYP enzymes, CYP1A2, CYP2D6 or CYP3A4/r, AT, $MgCl_2$ and buffer described in section 3.2.7.2.2.1, 36.7 μl of each was withdrawn and combined to give a total volume of 110 μl. This mixture was preincubated for 5 min at 37°C before the addition of the NADPH generating system (15 μl). Incubation was allowed to proceed for 1.0 h at 37°C. At the conclusion of the reaction, the analytical procedure described in section 3.2.4.was applied.

3.2.7.3.2 In the presence of cytochrome b_5

The incubations were performed as described in section 3.2.7.2.2.2, except that the single microsomal protein was replaced with a mixture of CYP1A2, CYP2D6 and CYP3A4/r. From the separate 110 μl mixtures containing these enzymes, AT, $MgCl_2$, cytochrome b_5 and buffer, 36.7 μl of each was withdrawn and combined to give a total volume of 110 μl. The NADPH generating system was added prior to incubation. Dried

extracts were trifluroacetylated and derivatized samples were assayed by GC analysis, as described in section 3.2.4.

3.2.7.4 Inhibition studies

3.2.7.4.1 Fluvoxamine

Individual CYP microsomal proteins (CYP1A2, CYP2D6 and CYP3A4/r), or a mixture of all three CYP enzymes were incubated in the presence of human cytochrome b_5 as decribed in sections 3.2.7.2.2.2 and 3.2.7.3.2, except that FX (2.08 or 4.16 μM) was included in prewarmed mixtures before the addition of the NADPH generating system (15 μl). The analysis of AT metabolites was conducted by GC after derivatization with trifluoroacetic anhydride as described in section 3.2.4.

3.2.7.4.2 Ketoconazole

A portion of KE (9.92 μM) in ethanol was evaporated and to the residue an ice-cold (20 min immersion in ice) mixture of microsomal protein (7.5 pmol) and cytochrome b_5 (22.5 pmol) was added followed by the addition of $MgCl_2$ (0.41 μmol), AT (25.87 nmol) and phosphate buffer to a final volume of 110 μl. This mixture was preincubated for 5 min at 37°C and the metabolism of AT was initiated by the addition of the NADPH generating system (15 μl). Incubation at 37°C was conducted for 1.0 h. Extraction and derivatization procedures and GC analysis, as described in section 3.2.4 were applied.

3.2.7.4.3 Quinidine

The enzymatic reaction described in the section 3.2.7.2.2.2 was repeated except that QND (4.56 μM) was added to each of the three separate incubation mixtures immediately prior to the addition of phosphate buffer to give the final volumes of 110 μl. A portion (36.7 μl) was removed from each incubation mixture and mixed to give a final solution (110 μl) that contained the 3 CYP enzymes, cytochrome b_5, QND, AT, and $MgCl_2$ in buffer. This mixture was preincubated at 37°C for 5 min before the addition of

the NADPH generating system and incubation was conducted for 1.0 h. Extractive derivatization procedures and GC analysis were identical to those described in section 3.2.4.

3.2.8 *In vitro* metabolism of AT by human microsomal proteins

3.2.8.1 Time course study of the AT metabolism

Typically, reaction mixtures containing AT (20.5 nmol), 0.15 mg hepatic microsomal protein, $MgCl_2$ (0.33 μmol), and buffer to a volume of 88 μl were preincubated at 37°C for 5 min before the addition of the NADPH generating system (12 μl). Incubations were terminated at 5, 10, 20, 40 or 60 min time intervals. All incubation samples were extracted, derivatized and analyzed as described in section 3.2.4.

3.2.8.2 Enzymatic reaction of AT with human microsomal proteins

Metabolic reactions of AT with human microsomal proteins as decribed in the section 3.2.8.1 were repeated, except that 15 different human microsomal protein preparations containing known concentrations of CYP enzymes 1A2, 2A6, 2C19, 2D6, 2E, 3A4 and 4A, were used and enzymatic reactions were incubated for 20 min at 37°C.

3.3 RESULTS

AT was metabolized *in vitro* using individual fortified CYP enzyme preparations containing CYP1A2 or CYP2D6 or CYP3A4, or a mixture of the three enzymes. The metabolites were extracted, separated, trifluoroacetylated and identified by a GC procedure that is described elsewhere (Chapter 2). To quantitate the AT metabolites, calibration curves were constructed using known amounts of authentic compounds that were added to incubation mixtures that contained only control CYP enzyme. The resulting solutions were analyzed in a manner that was identical to the treatment of incubation solutions. The GC traces of the calibration curve solutions contained no interfering peaks at the retention times of the metabolites.

3.3.1 The N-demethylation of AT mediated by expressed CYP3A4

An initial attempt was made to metabolize AT with CYP3A4 in a simple incubation medium, but no metabolism of AT occurred, despite the fact that CYP3A4 is known to catalyze N-dealkylations of many drugs *in vivo* in humans (Coutts *et al.* 1994; Guengerich, 1995). Because of this negative result, it was necessary to confirm the viability of the CYP3A4 enzyme. Nifedipine, a dihydropyridine and a known substrate of CYP3A4 (Guengerich *et al.* 1986a) was incubated in the same simple medium (CYP3A4, $MgCl_2$ and an NADPH-generating system). Its metabolism to the corresponding pyridine did occur (figure 3.1). This result showed that viable CYP3A4 in this simple medium was unable to catalyze the metabolism of AT.

When CYP3A4 was replaced with CYP3A4/r, however, some metabolism of AT to NT was observed. Further improvements in CYP3A4/r enzyme catalytic activities were sought. The addition of a 3-lipid mixture and sodium cholate (Imaoka *et al.* 1988; Imaoka *et al.* 1992), with or without cytochrome b_5, dramatically decreased AT metabolism and only trace amounts of NT were detected. However, the effect of supplementing the CYP3A4/r incubation mixture with cytochrome b_5 without lipid was a significant increase in NT production (table 3.2). Addition of more NADPH-cytochrome P450 reductase to the medium resulted in a decrease in AT N-demethylation (figure 3.2). The N-demethylation of AT was not detected in the incubation medium containing control enzyme.

The metabolism of AT was repeated in fortified CYP3A4/r media containing different concentrations of cytochrome b_5 ranging from 60.0 to 240.0 pmol/ml incubation mixture. It was clear (figure 3.3) that the inclusion of cytochrome b_5 in the incubation mixture stimulated the N-demethylation of AT, and an optimal concentration of cytochrome b_5 could be determined.

The time course of the biochemical transformation of AT to NT by CYP3A4/r augmented with cytochrome b_5 is depicted in figure 3.4. The amount of NT produced increased at a linear rate during the initial 2.0 h of incubation (r^2 over the 0 – 2 h period = 0.995).

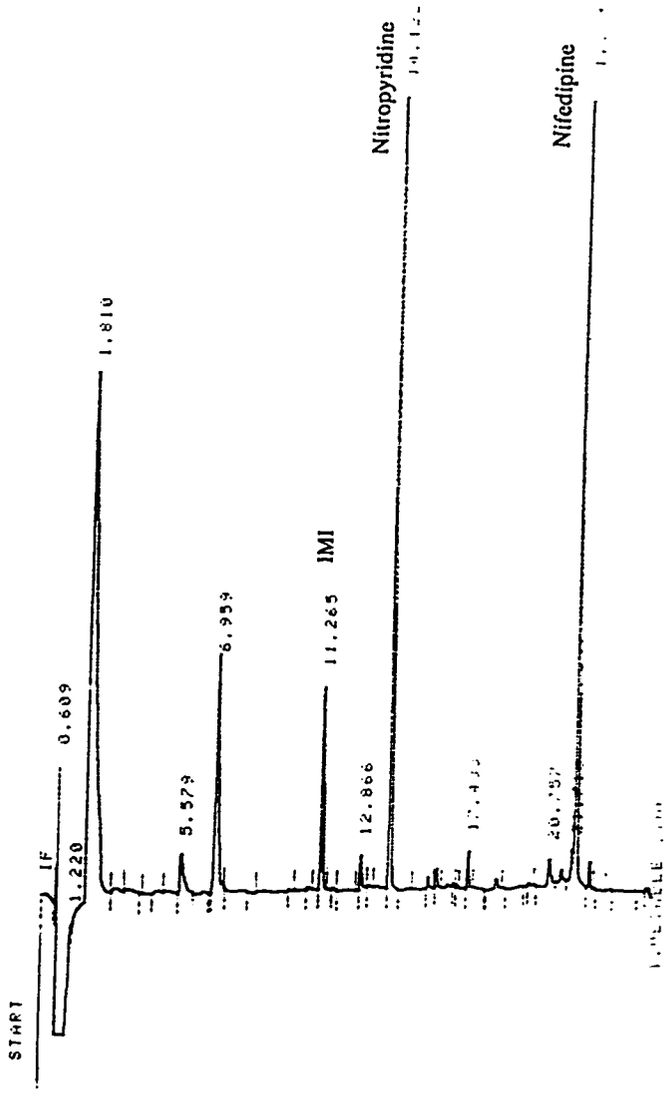

Figure 3.1 GC trace of dried extract of nifedipine incubation with CYP3A4. Internal standard: IMI

Table 3.2 Yield of NT formation mediated by CYP3A4/r which was reconstituted in different incubation media. Results are averages of two values which differed by less than 5%.

Incubation conditions	N-Demethylation of AT (nmol formed per incubation)
CYP3A4/r	0.66
CYP3A4/r + b_5	0.83
CYP3A4/r + reductase[a]	0.56
CYP3A4/r + reductase[a] + b_5	0.66
CYP3A4/r + reductase[a] + b_5 + PS[b]	0.56
CYP3A4/r + lipids[c] + sodium cholate	trace
CYP3A4/r + reductase[a] + b_5 + lipids[c] + sodium cholate	trace

[a] extra rabbit NADPH-cytochrome P450 reductase. [b] Phosphatidylserine.
[c] lipids: a mixture of PS, DLPC and DOPC (1:1:1 ratio).

The effects of cytochrome CYP3A4/r concentration on AT metabolism to NT were also investigated. A linear relationship between the amount of NT formed and concentration of CYP3A4/r employed was observed over the range 0 to 2 mg protein/ml incubation (≡ 0 to 180.0 pmol CYP3A4/ml incubation; $r^2 = 0.992$). Minor departure from linearity occurred when the protein concentration exceeded 2 mg/ml (r^2 over the complete protein range, 0 – 3 mg/ml, was 0.965; figure 3.5).

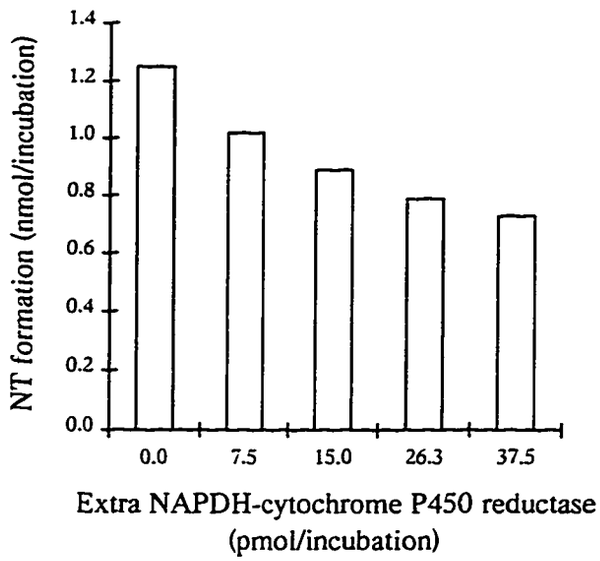

Figure 3.2 Effects of extra rabbit NADPH-cytochrome P450 reductase on AT N-demethylation by CYP3A4/r. Data are average values of two determinations which differed by less than 5%.

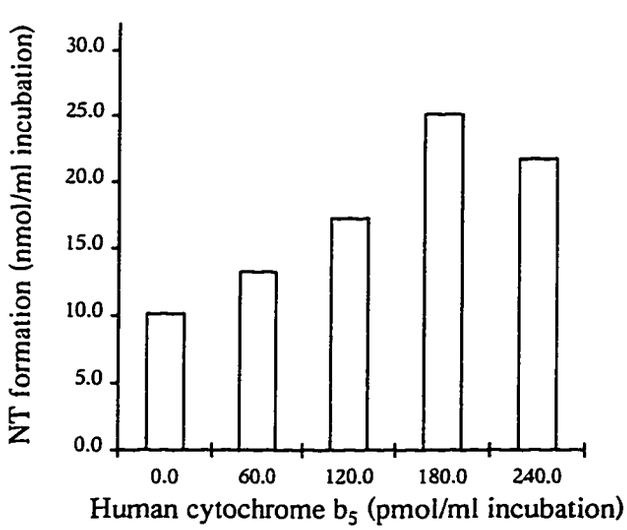

Figure 3.3 The influence of human purified cytochrome b_5 on the N-demethylation of AT by CYP3A4/r. Results were obtained from duplicate experiments; values differed by less than 5% from the average values in all cases.

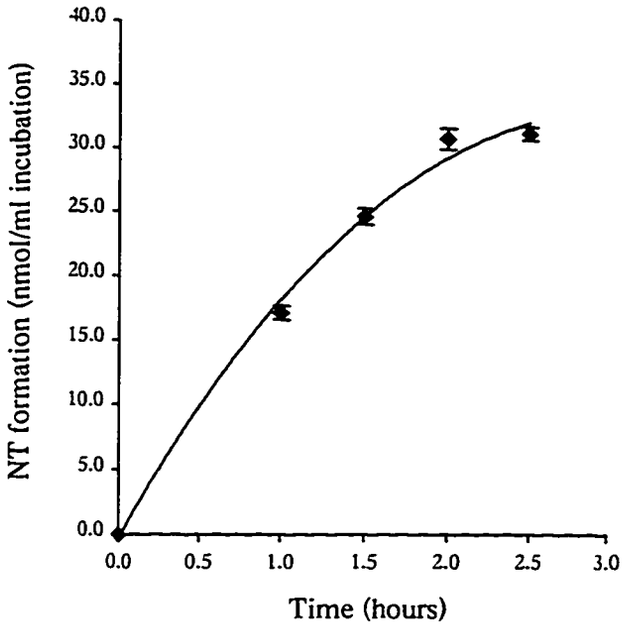

Figure 3.4 Time course of NT formation by CYP3A4/r. Values represent means ± SD (n = 3)

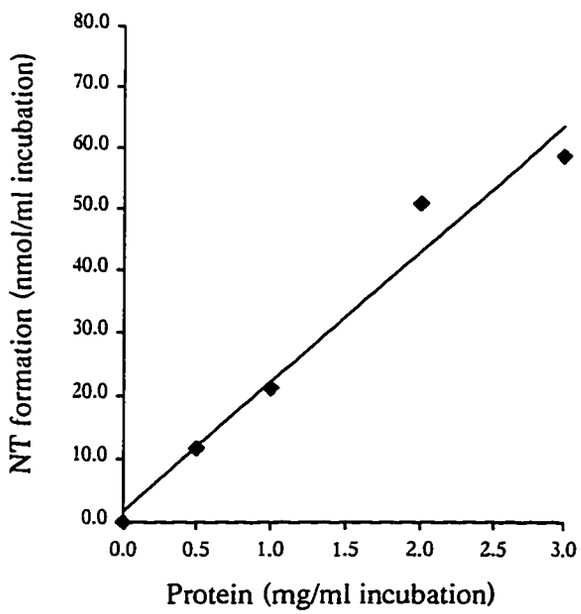

Figure 3.5 Effect of varying the amount of CYP3A4/r on the N-demethylation of AT. Each point represents mean ± SD (n = 3). Values differed by less than 5% of mean in all cases

Experiments were conducted to determine the kinetic parameters of AT metabolism to NT when catalyzed by CYP3A4/r. The AT concentration required to saturate the CYP3A4 is indicated in figure 3.6. The apparent K_m and V_{max} parameters were calculated by fitting the data points to the Hill equivalent equation (1). These values were: K_m = 209.91 ± 18.73 μM and V_{max} = 23.98 ± 1.24 nmol/h/mg protein (n = 3; table 3.3). This calculation also gave a Hill coefficient of 1.40 for CYP3A4.

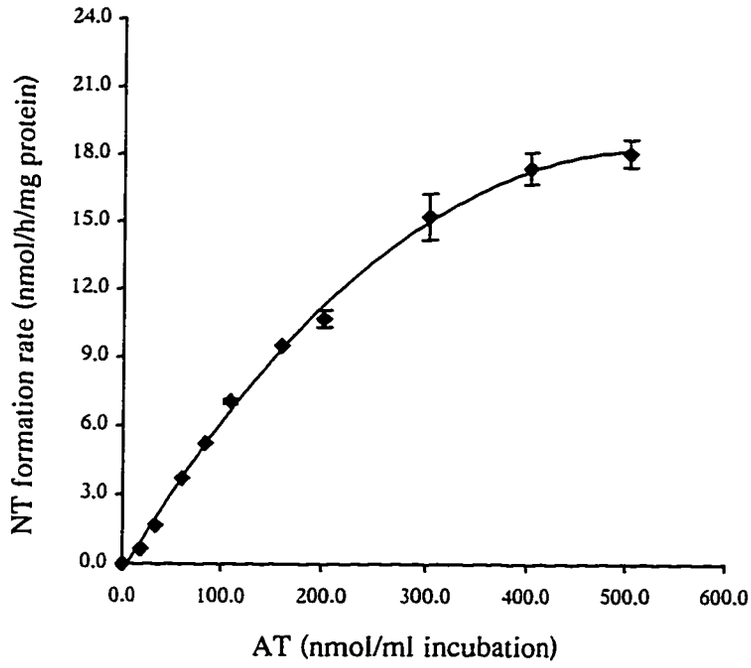

Figure 3.6 Saturation curve for the formation of NT catalyzed by CYP3A4/r. Values are means ± SD (n=3); SD values are shown when they are >5% of the mean value.

3.3.2 The N-demethylation of AT mediated by expressed CYP1A2

3.3.2.1 Kinetic parameters of this metabolic reaction

When AT was metabolized by expressed CYP1A2, it yielded only NT. The formation of NT was linear for 2.0 h at a protein concentration of 1 mg/ml. Data

obtained from 1.0 h incubation reactions with varying concentrations of AT from 1.52 nmol to 38.04 nmol/125 μl of incubation were used to construct the saturation curve for the activity of CYP3A4 (figure 3.7). The results are expressed as mean values ± SD of three observations. The apparent K_m and V_{max} values for the N-demethylation of AT were 64.76 ± 4.65 μM and 7.35 ± 0.34 nmol/h/mg protein respectively (table 3.3). The Hill coefficient for CYP1A2 was 2.06.

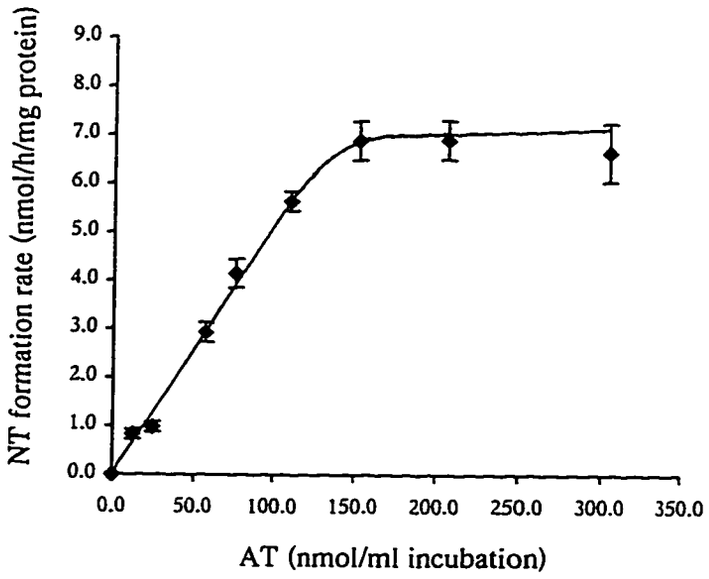

Figure 3.7 Saturation curve of AT metabolism mediated by CYP1A2. Incubation time:1 h. The results represent the means ± SD(n = 3).

3.3.2.2 Comparison of the catalytic activities of CYP1A2, CYP2D6 and CYP3A4/r in the metabolism of AT

3.3.2.2.1 In the absence of cytochrome b_5

The N-demethylation of AT to NT was catalyzed by each of the three CYP enzymes, but only CYP2D6 could mediate the ring hydroxylation of AT to 10-HO-AT. It is shown in table 3.4 that the yield of NT was similar in the presence of CYP1A2

(75.48 ± 5.48 nmol/nmol CYP enzyme) and CYP3A4/r (73.39 ± 3.74 nmol/nmol CYP enzyme). The amount of NT formed (11.00 ± 1.28 nmol/nmol CYP enzyme) was significantly lower when CYP2D6 was the catalyst.

3.3.2.2.2 In the presence of cytochrome b_5

AT metabolism by the three CYP enzymes was reevaluated after the addition of human cytochrome b_5 to the AT incubation medium. A significant increase in NT formation was observed in the incubation of AT with CYP3A4/r, but no stimulation of the CYP1A2 and CYP2D6 catalyic activities was seen (table 3.4).

3.3.2.3 Effects of fluvoxamine on AT metabolism

When the incubation of AT with CYP1A2 was conducted in the presence of FX at a concentration of 4.16 μM, NT formation was not detected. When the concentration of FX was reduced to 2.08 μM, AT metabolism was still lowered by 90%. This inhibitory effect was also observed in the metabolism of AT by CYP2D6 and CYP3A4/r (table 3.5). The formation of NT by CYP2D6 was unaffected by the presence of FX but a 25% decrease in 10-HO-AT formation was observed. When FX was included in the metabolism medium containing CYP3A4/r, NT formation declined by 24%.

3.3.2.4 Effects of ketoconazole on AT metabolism

The metabolism of AT to NT by CYP3A4/r was completely inhibited when KE at a concentration 9.92 μM was included in the incubation medium. The presence of KE also inhibited the abilities of CYP1A2 and CYP2D6 to catalyze AT metabolism (table 3.5). A decrease of 31% was observed in CYP2D6-catalyzed 10-HO-AT formation, and the amount of NT formed in the presence of CYP1A2 was reduced by 32%.

Table 3.3 The kinetic parameters of apparent K_m and V_{max} for the biotransformation of AT to its active metabolite NT by CYP1A2, CYP2D6 and CYP3A4/r. The results represent means ± SD (n =3).

Metabolic reaction	CYP450	Apparent K_m(μM)	V_{max} (nmol/h/ mg protein)	V_{max} (nmol/h/nmol CYP enzyme)
AT → NT	CYP1A2	64.76 ± 4.65	7.35 ± 0.34	79.03
AT → NT	CYP2D6	47.48 ± 1.32[a]	3.95 ± 0.11[a]	23.24[a]
AT → NT	CYP3A4	209.91 ± 18.73	23.98 ± 1.24	399.67

[a] Data obtained from Chapter 2.

Table 3.4 Effects of human cytochrome b_5 on the metabolism of AT mediated by CYP1A2, CYP2D6 and CYP3A4/r. Data represent the means ± SD ($n \geq 3$ as indicated)

AT(nmol/ml)	CYP enzyme	Yield of NT (nmol/ml)	Yield of NT (nmol/nmol CYP enzyme)	Yield of HO-AT (nmol/ml)	Yield of HO-AT (nmol/nmol CYP enzyme)	Number dertermination (n)
206.96	CYP1A2[a]	7.02 ± 0.52	75.48 ± 5.48	0	0	4
	CYP1A2 + b_5[a]	7.01 ± 0.62	75.40 ± 6.58	0	0	4
206.96	CYP2D6[b]	2.86 ± 0.33	11.00 ± 1.28	5.61 ± 0.92	21.57 ± 3.54	5
	CYP2D6 + b_5[b]	3.16 ± 0.30	12.15 ± 1.14	7.84 ± 0.47	30.15 ± 1.86	5
206.96	CYP3A4/r[c]	4.40 ± 0.22	73.39 ± 3.74	0		3
	CYP3A4/r + b_5[c]	9.22 ± 0.96	153.72 ±16.02	0		3

[a] $p = 0.482$; [b] $p = 0.050$ for NT formation and $p = 0.001$ for 10-HO-AT formation.
[c] $p = 0.010$; p value obtained from one-tailed t test.
$p \geq 0.05$, not significant

Table 3.5 Effects of fluvoxamine, ketoconazole and quinidine on the metabolism of AT in the presence of cytochrome b_5 by different P450 isozymes. Data are averages of values that differed by less than 5 % in duplicated experiments.

Inhibitor (μM)	CYP enzyme: pmol/ml	Yield of NT (nmol/ml)	Yield of 10-HO-AT (nmol/ml)
Fluvoxamine 4.16	CYP1A2: 93	0	0
Fluvoxamine 2.08	CYP1A2: 93	0.88 [a]	0
	CYP2D6: 260	3.43	5.91 [a]
	CYP3A4/r: 60	7.03 [a]	0
Ketoconazole 9.92	CYP3A4/r: 60	0	0
	CYP2D6: 260	2.93	5.38[a]
Fluvoxamine 4.16	CYP1A2: 93	4.75[a]	0
	3-CYP system:	2.38[b]	3.06
Quinidine 4.56	3-CYP system:	3.03[b]	0

[a] Partial inhibition when results are compared with those in table 3.4.

[b] Partial inhibition when results are compared with those in table 3.9.

Table 3.6 Calculated Cl_{int} and $Cl_{H.NT(AT)}$ values for the *in vitro* study (Schmider *et al.* 1995) using human liver microsomes.

Liver	K_m (μM)	V_{max} (nmol/min/mg protein)	Cl_{int} (l/min)	$Cl_{H.NT(AT)}$ (ml/min)
1	79.85	3.42	11.36	397.10
2	65.72	1.04	4.20	185.06
3	33.20	0.42	3.36	152.64
4	89 18	3.03	9.01	337.65
Mean ± SD	66.99 ± 24.50	1.97 ± 1.47	6.98 ± 3.32	268.11 ± 102.11

Table 3.7 Observed and calculated $Cl_{H.NT(AT)}$ (ml/min) values for *in vivo* and *in vitro* N-demethylation of AT.

Method of study	Enzymes	$Cl_{H.NT(AT)}$ (ml/min)	Range of $Cl_{H.NT(AT)}$ (ml/min)
in vivo	hepatic CYPs	443.28 ± 249.96[a]	85.55 – 907.09
in vitro	liver microsomes	268.11 ± 102.11[b]	152.64 – 397.10

[a] Value represents the mean ± SD (n = 5); Rollins *et al.* (1980).
[b] Value represents the mean ± SD (n = 4); see table 3.6.

Table 3.8 Calculated Cl_{int} (l/min) values for AT to NT conversion by CYP1A2, CYP2D6 and CYP3A4 from the *in vitro* studies using recombinant CYP enzymes. Data for apparent K_m are means of three observations ± SD.

CYP enzyme	Apparent K_m (μM)	V_{max} (nmol/h/nmol CYP enzyme)	Cl_{int} (l/min.)
1A2	64.76 ± 4.65	79.03	0.34
2D6	47.48 ± 1.32	23.24	0.04
3A4	209.91 ± 18.73	399.67	1.25
Total 3 CYP enzymes			1.63

3.3.2.5 AT clearance values

In order to calculate *in vitro* drug clearance values from our data and compare them with reported human *in vitro* and *in vivo* data, it was necessary to calculate Cl_{int} for the conversion of AT to NT. Rollins *et al.* (1980) provided *in vivo* kinetic data which included the values of Q_H and E_H from which Cl_H was calculated using equation (5b; section 3.2.6.2). This value for Cl_H was incorporated into equation (6) to obtain $Cl_{H,NT(AT)}$ (table 3.6). Schmider *et al.* (1995) provided *in vitro* K_m and V_{max} data for the AT to NT transition in human liver microsomes. These values were substituted into equation (2) (section 3.2.6.2) and the calculated result was then used in equation (5a) (section 3.2.6.2) to obtain Cl_{int} and $Cl_{H,NT(AT)}$ (tables 3.6 and 3.7). The average *in vitro* $Cl_{H,NT(AT)}$ value was 268.11 ± 102.11 ml/min (range: 152.64 to 397.10 ml/min) when human microsomes were used, and the average *in vivo* $Cl_{H,NT(AT)}$ value was 443.28 ± 249.96 ml/min (range: 85.55 – 907.09 ml/min). These wide ranges clearly show interindividual variabilities in the N-

demethylation of AT by liver. In the present study, the Cl_{int} values calculated for each enzyme (table 3.8) differed greatly (CYP3A4>> CYP1A2 >>CYP2D6) and indicated that CYP3A4 catalyzed the N-demethylation of AT much more efficiently than CYP1A2 and CYP2D6 in the reconstituted system.

3.3.3 The metabolism of AT by a mixture of three CYP enzymes

When the same amount of AT was incubated with a mixture of the three CYP enzymes, the quantity of NT isolated appeared to be less than that calculated by summing the amounts of NT formed in the three single isoenzyme systems (table 3.9). In contrast, the quantity of HO-AT was increased. However, results from the t test (see table 3.9) showed that the metabolic conversion of AT to NT by a mixture of the three CYP enzymes was the same when the incubation mixture was augmented with cytochrome b_5, but there was a slight increase in the amount of 10-HO-AT formed.

Table 3.9 Effects of human cytochrome b_5 on the N-demethylation and ring hydroxylation of AT by a mixture of 3-CYP enzymes. Data are expressed as means ± SD (n = 3). Predicted yield is 1/3 of the sum of observed yields obtained with the three individual enzymes (see table 3.4).

AT (nmol/ml)	CYP enzymes	Yield of NT(nmol/ml)		Yield of HO-AT(nmol/ml)	
		Observed	Predicted	Observed	Predicted
206.96	3-CYP system	4.26 ± 0.10[a]	4.76 ± 0.12[a]	2.32 ± 0.19[b]	1.89 ± 0.31[b]
206.96	3-CYP system + b_5	5.85 ± 0.18[c]	6.47 ± 0.53[c]	2.84 ± 0.08[d]	2.59 ± 0.17[d]

[a] $p = 0.013$; [b] $p = 0.040$; [c] $p = 0.168$; [d] $p = 0.042$; p value obtained from one-tailed t test.
$p \geq 0.05$, not significant

3.3.3.1 Effects of fluvoxamine on AT metabolism

The addition of FX to the medium containing all three CYP enzymes resulted in a significant decrease of NT formation, but had no effect on 10-HO-AT formation (compare tables 3.5 and 3.9).

3.3.3.2 Effects of quinidine on AT metabolism

Ring hydroxylation of AT to HO-AT was completely inhibited by the presence of QND in the incubation mixture containing the three CYP enzymes, and there was a significant decrease (48%) in the formation of NT (table 3.5).

3.3.4 The metabolism of AT *in vitro* by human microsomal proteins

Initially, the time course studies of metabolism of AT by human microsomal protein were performed, and these showed that the metabolism of AT was linear up to 40 min. Thus, 20 min was selected as the incubation time at 37°C. The amount of NT formed was correlated significantly with CYP1A2 ($r = 0.604$, $p = 0.017$), CYP2A6 ($r = 0.607$, $p = 0.016$), CYP2D6 ($r = 0.597$, $p = 0.019$), CYP3A4 ($r = 0.828$, $p = 0.0001$) and CYP4A ($r = 0.570$, $p = 0.027$) protein content in 15 human livers. No significant correlation with the catalytic activity of other CYP enzymes, CYP2C19 and CYP2E, was observed (figures 3.8a and 3.8b).

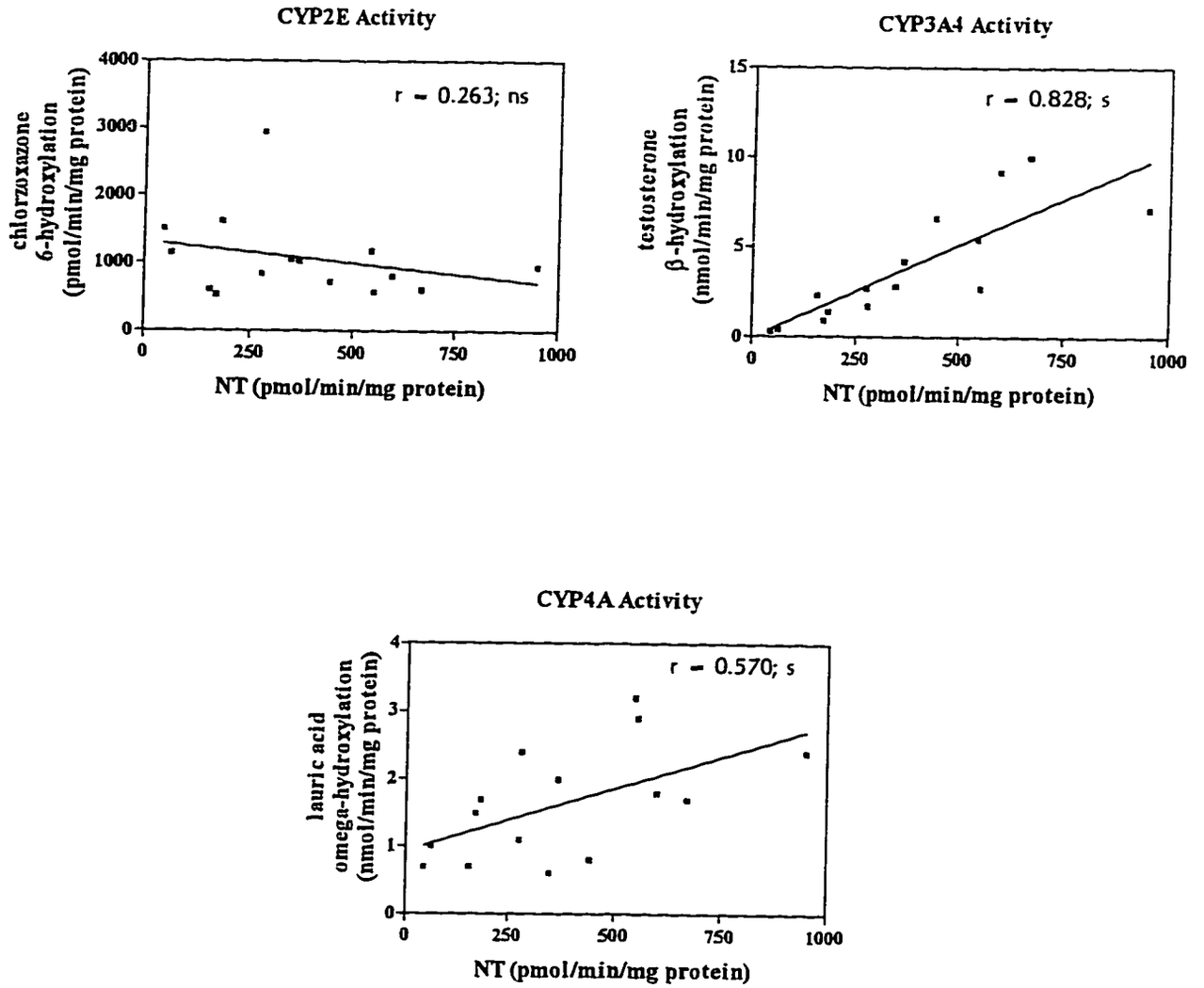

Figure 3.8a Correlation of AT metabolism to NT with different CYP enzymes in liver microsomes (n = 15).

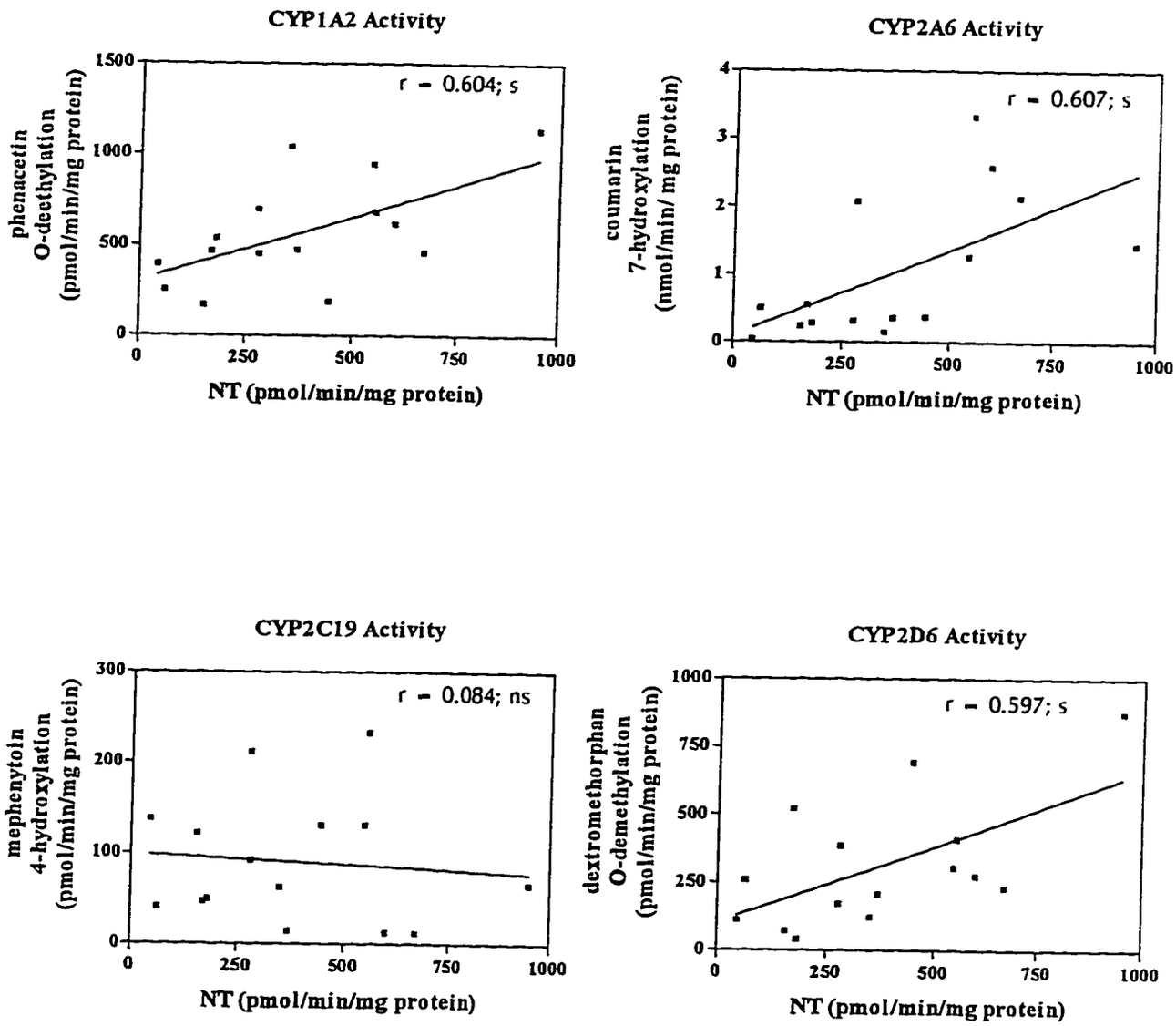

Figure 3.8b Correlation of AT metabolism to NT with different CYP enzymes in liver microsomes (n = 15).

3.4 DISCUSSION

3.4.1 N-demethylation of AT mediated by CYP3A4

CYP3A4 is a very important human cytochrome P450 enzyme that catalyzes the oxidation of a large number of drugs. It can account for up to 30% of total CYP enzymes in the liver (Yamazaki *et al.* 1996). Drugs that are substrates of CYP3A4 have diverse structures (Guengerich, 1995).

Various investigators have observed that reconstituted systems containing purified rat CYP3A or purified human microsomal CYP3A4 (Imaoka *et al.* 1990; Imaoka *et al.* 1992), NADPH, NADPH-cytochrome P450 reductase and DLPC, with or without sodium cholate, do not effectively mediate the N-dealkylation of lidocaine, the 6β-hydroxylation of testosterone, or nifedipine aromatization. When cytochrome b_5 was added to the reconstituted system used by Imaoka *et al.* (1992), the metabolism of lidocaine, testosterone and nifedipine by CYP3A4 was enhanced. Replacement of the DLPC component with a 1:1 mixture of PS and lecithin also increased the extent of testosterone 6β-hydroxylation (Imaoka *et al.* 1988). A modified system that contains various lipids, commonly PS, DLPC and DOPC in a 1:1:1 ratio, sodium cholate, NADPH-cytochrome P450 reductase and cytochrome b_5 in addition to CYP3A4 generally has high catalytic activity (Imaoka *et al.* 1992). It has also been determined (Halvorson *et al.* 1990) that addition of certain detergents, such as Emulgen 911 or Chaps, to the modified system can further stimulate CYP3A4 activity.

Our initial experiments on the *in vitro* metabolism of AT were performed with expressed CYP3A4 in the presence of an NADPH-generating system and $MgCl_2$. We confirmed that this simple enzyme system was capable of metabolizing the dihydropyridine ring of nifedipine to the corresponding pyridine, but it was incapable of mediating the N-demethylation of AT to NT. When the CYP3A4 in the simple medium was replaced with coexpressed CYP3A4 and cytochrome P450 reductase, some metabolism of AT to NT was observed, although yields of NT were always low in these conditions. When a mixture of lipids (DLPC, DOPC and PS) was included in the incubation medium,

the activity of CYP3A4/r was almost completely inhibited (table 3.2). Shet *et al.* (1993) observed a similar result in the metabolic N-demethylation of erythromycin when they included lipid, detergent and cytochrome b_5 in the CYP3A4/r medium, but others have found that the addition of lipid mixtures sometimes improves the catalytic performance of CYP3A4 (Imaoka *et al.* 1990; Guengerich *et al.* 1991; Imaoka *et al.* 1992).

When cytochrome b_5 was subsequently added to a medium that contained CYP3A4/r, it was immediately obvious that its inclusion had a potent stimulatory effect on the N-demethylation of AT (table 3.2). Maximum stimulation was by a factor of 2.5 at a cytochrome b_5 concentration of 180 pmol/ml (figure 3.3). Yamazaki *et al.* (1996) recently proposed a mechanism to explain the role that cytochrome b_5 plays in biotransformations catalyzed by CYP3A4. The same mechanism may be involved in the metabolism of AT catalyzed by CYP3A4. The major feature of this mechanism is the complexation of the initial CYP3A4–substrate reaction product with cytochrome b_5 prior to its reduction and subsequent incorporation of oxygen. The amount of cytochrome b_5 that can be added is limited. When excessive quantities are present, the enhancement of CYP3A4 activity is lowered, presumably because of competition between cytochrome b_5 and CYP3A4 for NADPH electron transfer (Schenkman *et al.* 1994).

This study has confirmed the role played by CYP3A4 in the N-dealkylation of AT to NT, and has revealed that NADPH-cytochrome P450 reductase and cytochrome b_5 are essential components in the *in vitro* metabolism of AT by expressed CYP3A4.

3.4.2 N-Demethylation of AT mediated by CYP1A2 – the contribution of isoenzymes CYP1A2, CYP2D6 and CYP3A4 to this pathway.

The relative catalytic activities of metabolizing enzymes determine the plasma concentrations of a drug, and, therefore, the effects of the drug. It is important to identify the enzymes involved in major pathway(s) of a drug's metabolism in order to predict optimal doses of the drug and its potential involvement in interactions with other drugs or with chemical components in foods.

The objectives of this study were initially: to determine whether CYP1A2 had any role in the metabolism of AT; to investigate the effects of human cytochrome b_5 on

the oxidation of AT with different CYP enzymes; to assess the relative contributions of CYP1A2, CYP2D6 and CYP3A4 in the N-demethylation of AT to NT; and to evaluate the catalytic activities of the two *in vitro* enzyme preparations used in AT metabolic studies, expressed enzymes and liver microsomes. AT metabolism by human liver microsomes was the last strategy used to determine whether additional CYP enzymes participated in NT formation and also to explain the differences in our findings from the results obtained by Schmider *et al.* (1995).

In initial studies (Chapter 2) it was determined that CYP2D6 catalyzed the formation of NT from AT and mediated the ring hydroxylation of AT and NT. These observations were in agreement with the findings of Jerling *et al.* (1994a and 1994b) who observed higher plasma concentrations of NT and AT in poor metabolisers of debrisoquine than in extensive metabolizers. Results from the present study indicate that CYP3A4 and CYP1A2 also catalyzed the N-demethylation of AT. Clearly, the 3 hepatic enzymes, CYP1A2, CYP2D6 and CYP3A4, are all involved in the conversion of AT to NT. That AT is a substrate for CYP1A2 was disclaimed by Schmider *et al.* (1995). In this *in vitro* study of the N-demethylation of AT using liver microsomes it was reported that both CYP2D6 and CYP3A4 catalyzed the formation of NT, but CYP3A4 was the only enzyme with a predominant role in this pathway. Because of this discrepancy, we wished initially to compare the Cl_{int} of AT→NT by expressed 3-CYP enzymes with the Cl_{int} derived from the data published by Schmider *et al.* (1995) for the N-demethylation of AT. We also wished to determine whether one isozyme had any effect on another isozyme's capacity to metabolize AT in reconstituted systems. AT was incubated with a mixture of three CYP enzymes (CYP1A2, CYP2D6 and CYP3A4; the "3-CYP system"). If each enzyme worked independently of the other, the total amount of AT metabolites formed would be expected to be equal to the sum of the amounts of metabolites generated in the three single isozyme systems. However, it was observed that the quantity of 10-HO-AT formed was increased by 23% in the 3-CYP system, and there was a concurrent 11% decrease in NT formation. The reduction in the quantity of NT was quite small and it can be a result of experimental error. The increase in 10-HO-AT formation by the CYP mixture, relative to the amount formed by CYP2D6 alone, is more difficult to explain.

Cytochrome b_5 is a ubiquitous enzyme that is found in many physiological systems involved in the metabolism of xenobiotics (Sugiyama *et al.* 1980; Gruenke *et al.* 1988; Canova-Davis *et al.* 1985), in the biosynthetic reactions of steroidogenesis (Kominami *et al.* 1992), and in the hydroxylation of prostaglandins (Vatsis *et al.* 1982). In *in vitro* studies, cytochrome b_5 may induce stimulatory or inhibitory effects on CYP-catalyzed reactions (Omata *et al.* 1994; Matsusue *et al.* 1996). These variable effects of cytochrome b_5 appear to be substrate- and/or CYP isoform-dependent (Lu *et al.* 1974; Nakasa *et al.* 1993). It was of interest, therefore, to evaluate the role of cytochrome b_5 in oxidations of AT catalyzed by CYP isozymes and compare the contributions of cytochrome b_5 in the efficacy of the three CYP enzymes involved in the formation of NT. Control experiments were also conducted using preparations which contained cytochrome b_5 but did not contain any CYP isozymes. No metabolism of AT to NT occurred in these control experiments. Results from the incubation studies of AT with each CYP isozyme in the presence of cytochrome b_5 indicated that cytochrome b_5 has a profound effect on AT oxidation catalyzed by CYP3A4, and a lesser effect on the ability of CYP2D6 to mediate the ring hydroxylation of AT. Cytochrome b_5 had no significant stimulatory effect on the formation of NT by CYP2D6 and CYP1A2 (table 3.4). When cytochrome b_5 was included in the 3-CYP incubation medium, there was increase in the yield of NT (37%) and HO-AT (22%). It was interesting to observe that only CYP2D6 catalyzed the formation of 10-HO-AT and the quantity of this metabolite was increased by the presence of cytochrome b_5 in the CYP2D6 medium, or by the addition of the two other CYP isoforms, CYP1A2 and CYP3A4, to the CYP2D6 medium. The latter observation suggested that some alteration in the ability of CYP2D6 to bind with AT had occurred (table 3.9).

Activities of CYP1A2 and CYP3A4 in the N-demethylation of AT were inhibited to various extents by FX and KE. The catalytic activity of CYP1A2 was completely inhibited by FX at the concentration 4.16 μM and this activity was still decreased by 90% at a concentration of 2.08 μM. The presence of FX only affected catalytic ring hydroxylation (table 3.5) mediated by CYP2D6; the N-demethylation of AT by CYP2D6 was unaffected. This finding is not in agreement with a previous report (Hartter *et al.*

1993) in which it was stated that the ring hydroxylation of AT was not affected by FX. NT formation induced by CYP3A4 is also affected by the presence of FX, although to a lesser extent (table 3.5). KE is often used as a specific and potent inhibitor of the CYP3A gene subfamily in humans (Maurice *et al.* 1992). A concentration of 9.92 μM not only blocked the entire activity of CYP3A4 but also displayed inhibitory effects on the formation of NT induced by CYP1A2 and on the CYP2D6-catalyzed formation of 10-OH-AT (table 3.5). This observation that KE inhibits both CYP1A2 and CYP3A4 suggests that KE should not be used as selective CYP3A4 inhibitor in metabolic reactions in which both CYP enzymes are involved. Newton *et al.* (1995) and Ono *et al.* (1996) have made similar observations.

The observed significant decrease in NT formation in the presence of QND in the 3-CYP system is attributable to QND's ability to inhibit CYP2D6 and possibly to a reduced availability of CYP3A4 which will also be involved in the metabolism of QND (Guengerich *et al.* 1986b).

To evaluate the involvement of each CYP enzyme on the N-demethylation of AT to NT, it was necessary to derive intrinsic clearance (Cl_{int}) values from measurable kinetic parameters (K_m and V_{max}). By using Cl_{int} values, the contributions of the 3-CYP enzymes in the formation of NT could be directly compared. The total Cl_{int} values with expressed 3-CYP enzymes were also compared with the Cl_{int} values from human liver microsomal data to evaluate the activities of two different enzyme preparations. The comparison becomes useful if the predicted *in vitro* $Cl_{H,NT(AT)}$ value obtained from liver microsomal data is also comparable to the *in vivo* $Cl_{H,NT(AT)}$ value. As shown in table 3.7, the average values of $Cl_{H,NT(AT)}$ derived from *in vivo* and *in vitro* studies using liver microsomal proteins appear to be dissimilar. This discrepancy may be due to a variety of causes, including (a) small sample size (n = 4); (b) the large interindividual variabilily found in AT metabolism as a result of the polymorphism of CYP2D6 (Lennard 1993); (c) enviromental induction of CYP1A2 (Vistisen *et al.* 1992; Schweikl *et al.* 1993); and (d) variable CYP3A4 expression (Guengerich *et al.* 1986a; Guengerich *et al.* 1991; Ling *et al.* 1995). However, the average $Cl_{H,NT(AT)}$ value of 268.11 ± 102.11 ml/min derived from the *in vitro* study is well within the range of $Cl_{H,NT(AT)}$ values determined from *in vivo*

data, and both *in vivo* and *in vitro* $Cl_{H,NT(AT)}$ values are spread over a wide range (table 3.7). Therefore, the apparent dissimilarity in the two average $Cl_{H,NT(AT)}$ values may be expected and may also be acceptable.

When the total Cl_{int} value of 1.63 l/min (table 3.8) derived in the present study was compared with the average value of Cl_{int} (6.99 l/min; table 3.6) obtained with human liver microsomes, it was expected that the two values might not be similar because the catalytic activity of CYP3A4 in the *in vitro* system is known to vary significantly as a result of the differing conditions used to reconstitute the enzyme. These variations are known to be substrate dependent (Waxman *et al.* 1985; Imaoka *et al.* 1988; Guengerich, 1995). Therefore, it was necessary to keep in mind that the catalytic activities of CYP3A4 in the metabolism of AT may not represent its optimal ability to oxidize AT to NT. Possible explanations of the significant difference in the values of $Cl_{H,NT(AT)}$ are: (a) enzyme(s) other than CYP1A2, CYP2D6 and CYP3A4 are involved in the N-demethylation of AT; or (b) the catalytic activity of CYP3A4 in the reconstituted system in the present study is lower than it is in the liver microsomal environment. Since CYP3A4 can account for up to 28% of total CYP enzymes in the liver (Guengerich 1995), it is possible that a significant difference in CYP3A4's catalytic activity in the reconstituted system is the major reason for the large variation between the two *in vitro* Cl_{int} values (tables 3.6 and 3.8).

However, Labroo *et al.* (1995) and Lee *et al.* (1995) had found that recombinant CYP3A4/r enzyme catalyzed the metabolism of alfentanil and testosterone in the same way as human liver microsomes. Therefore, significant discrepancies in the two *in vitro* Cl_{int} values for AT metabolism to NT may be due to the participation of additional unidentified CYP enzyme(s). *In vitro* experiments using human liver microsomal preparations were conducted to investigate the role of other CYP enzymes. Results of this study indicated that at least 5 CYP enzymes are involved in the N-demethylation of AT. They are: CYP1A2, CYP2A6, CYP2D6, CYP3A4 and CYP4A (figures 3.8a and 3.8b). The excellent linear correlation between NT formation and CYP3A4 content ($r = 0.828$; $p = 0.0001$) indicated that CYP3A4 had a major role in the N-demethylation of AT. The linear correlations of NT formation with other CYP enzymes (CYP1A2,

CYP2A6, CYP2D6 and CYP4A) were also significant. That CYP2C19 did not catalyze the N-demethylation was in agreement with the finding of Breyer–Pfaff *et al.* (1992).

The significant difference between the total Cl_{int} value derived in the present study and the Cl_{int} values derived from data publised by Schmider *et al.* (1995) may now can be explained, at least to some extent, by the absence of CYP2A6 and CYP4A contributions in NT formation in the estimation of the total Cl_{int} value and also perhaps by the absence of other CYP enzymes which were not present in the human liver microsomal protein mix used in this study. Further *in vitro* studies using expressed CYP2A6 and CYP4A are appropriate to confirm the role of these two CYP enzymes. Schmider *et al.* (1995) claimed that CYP1A2 did not contribute to the N-demethylation of AT *in vitro* in the human. This apparent discrepancy can perhaps be rationalized by the fact these authors did not confirm the activity of CYP1A2 in their liver microsomal preparations.

In summary, this study provides important qualitative information on the enzymes involved in the metabolism of AT to NT and permits the following conclusions to be made:

3.4.2.1 Metabolism studies with expressed enzymes

(a) CYP1A2 is involved in the N-demethylation of AT to NT in addition to the previously identified CYP2D6 and CYP3A4 isozymes.

(b) The presence of cytochrome b_5 in the metabolism reaction using expressed enzyme does not stimulate the CYP1A2- or CYP2D6-catalyzed formation of NT.

(c) A mixture of the three enzymes identified in (a) (a CYP "cocktail") in the presence of cytochrome b_5, provides the same overall percentage yield of NT as that obtained by summing the yields of NT produced by individual expressed enzyme preparations. The three enzymes do not appear to interfere with each other's efficiency when they are combined.

(d) Of the three CYP enzymes involved in the N-demethylation pathway, CYP3A4 has the most significant role. CYP2D6 contributed little to this metabolic route, in agreement with the *in vitro* observation by Schmider *et al.* (1995).

(e) When AT hepatic clearance [$Cl_{H,NT(AT)}$] was calculated from the *in vitro* human liver microsomal data reported by Schmider *et al.* (1995) and compared with the literature *in vivo* $Cl_{H,NT(AT)}$ value (Rollins *et al.* 1980), they had the same order of magnitude.

(f) Another as yet unidentified CYP enzyme (or enzymes) are apparently involved in the N-demethylation of AT. Therefore, catalytic activity (as indicated by the summation of the Cl_{int} values) of the expressed CYP enzymes used in this study does not approach the *in vitro* activity of liver microsomes reported by Schmider *et al.* (1995).

3.4.2.2 Metabolism studies with human liver microsomal proteins

(a) CYP1A2 is involved in the N-demethylation of AT to NT in addition to the previously identified CYP2D6 and CYP3A4 isozymes. Based only on the positive correlations obtained between the activities of CYP2A6 and of CYP4A and the formation of NT, these two enzymes may also be involved in the N-dealkylation of AT.

(b) CYP3A4 has a major role in the N-demethylation of AT, but other CYP isoenzymes, namely CYP1A2, CYP2A6, CYP2D6 and CYP4A also play a role in the biotransformation of AT to NT.

(c) CYP2C19 is not involved in the metabolism of AT to NT.

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Chapter 4

Involvement of CYP2D6 in the *in vitro* metabolism of amphetamine, two N-alkylamphetamines and their 4-methoxylated derivatives.

4.1 INTRODUCTION

While the investigations described in chapters 2 and 3 were being conducted, parallel *in vitro* studies on the metabolism of amphetamine (AM), two N-alkylated amphetamines and their 4-methoxylated derivatives were performed in an expressed human CYP2D6 enzyme system. Like amitriptyline (AT), these drug substrates possess aromatic rings attached by alkyl chains to an aliphatic amine group and could be expected to undergo metabolic ring oxidation to phenolic products, and possibly N-dealkylation. In addition, the ring methoxylated compounds are good candidates for O-demethylation.

Except for N-methylamphetamine (NMA) and some of its ring-substituted derivatives, few studies on the metabolism of N-alkylated amphetamines in humans have been reported. From those that have, it is apparent that both N-dealkylation and ring hydroxylation of N-alkylamphetamines occur only to a small extent in humans (Smith and Dring, 1970; Gorrod, 1973). *In vivo* ring hydroxylation of AM to 4-hydroxyamphetamine (HO-AM), for example, is a minor metabolic pathway in man (~5% in 0-24 h urine), whereas ring oxidative metabolism of NMA is more extensive and yields 4-hydroxy-N-methylamphetamine (HO-NMA) and HO-AM in yields of 15% and 2-3%, respectively. Ring hydroxylation of pondinil [(N-3-chloropropyl)amphetamine; PD] is even more pronounced; the quantities of 4-hydroxy-PD and HO-AM excreted in 0 – 24 h urine were ~30% and ~6%, respectively (Williams *et al.* 1973; Caldwell, 1976). From these data, it would seem that an increase in lipophilicity as a result of N-substitution results in an increase in the extent of metabolic ring hydroxylation.

The effect of the alkyl chain length in simple N-alkylamphetamines on *in vivo* metabolic ring hydroxylation in rats and man (Coutts *et al.* 1976, 1978; Coutts and Dawson, 1977) was that AM and NMA formed only 4-hydroxylated metabolites whereas N-ethylamphetamine (NEA), N-(n-propyl)amphetamine (NPA) and N-(n-butyl)amphetamine (NBA) in rats, and NPA in man, formed 4-hydroxylated and 3,4-dihydroxylated metabolites. The latter products were further metabolized by COMT-catalyzed O-methylation to the corresponding 3-methoxy-4-hydroxy-N-alkylated amphetamines (Coutts and Jones, 1982). In the rat *in vivo* study (Coutts *et al.* 1978), the ratio of urinary 3-methoxy-

4-hydroxy : 4-hydroxy metabolites isolated increased as the length of the N-alkyl substituent increased in size from ethyl (ratio = 0.139) to n-propyl (ratio = 0.179) to n-butyl (ratio = 0.435).

In the present study, the *in vitro* metabolism of AM, NEA and NBA was performed with a fortified CYP2D6 preparation with four objectives in mind: (a) to determine whether ring hydroxylation of all three drugs occurred and whether the size of the N-substituent influenced the extent of that hydroxylation; (b) in the event that ring hydroxylation did occur, to determine whether any additional ring oxidation to produce catecholamine metabolites was observed; (c) to determine whether the length of the N-substituent had any influence on CYP2D6-catalyzed ring oxidation; and (d) to see whether this CYP enzyme catalyzed the N-dealkylation of NEA and NBA to any extent. Metabolic N-dealkylation is generally not associated with CYP2D6, but some examples of CYP2D6-catalyzed N-dealkylation have been recognized, including amiflamine (Alván *et al.* 1984), AT (Coutts *et al.* 1997), deprenyl (Grace *et al.* 1994), desmethylcitalopram (Sindrup *et al.* 1993), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Coleman *et al.* 1996), mianserin (Dahl *et al.* 1994), and trazodone (Yasui *et al.* 1995). The structures of substrates and putative ring O-demethylated products of the amphetamines examined are provided in figure 4.1.

Various studies on the metabolism of methoxyamphetamines *in vivo* (Kitchen *et al.* 1979; Midha *et al.* 1981; Cho *et al.* 1990; Lin *et al.* 1992; Hiratsuka *et al.* 1995; Chu *et al.* 1996) and *in vitro* (Beckett and Midha, 1974; Coutts *et al.* 1994; Kumagai *et al.* 1994; Geertsens *et al.* 1995) have shown that these compounds can undergo O-demethylation, ring hydroxylation and N-dealkylation to various extents. The site of ring hydroxylation, if it does occur, depends on the ring location of the methoxy group. Whether the nature of the N-alkyl substitutent influences ring hydroxylation is not known.

Many methoxyamphetamines possess hallucinogenic properties; the most potent compounds are 4-methoxylated (Tseng *et al.* 1976). 4-Methoxyamphetamine (M-AM), for example, is five times as potent as mescaline (Shulgin *et al.* 1969). Since abusers of methoxyamphetamines often ingest mixtures of drugs, there is no doubt that drug-drug

interactions resulting in the inhibition of the metabolism of 4-methoxyamphetamines and prolongation of their stimulant effects, are likely to occur. Studies on compounds that will identify the cytochrome P450 (CYP) enzymes involved in O-demethylation, ring hydroxylation and N-dealkylation, therefore, are relevant.

Some *in vitro* studies have suggested that metabolic O-demethylation of methoxy-amphetamines is mediated by CYP2D6 in humans (Coutts *et al.* 1994; Tucker *et al.* 1994; Geertsen *et al.* 1995) and by related CYP2D enzymes in rats (Kumagai *et al.* 1994). It has also been claimed that N-dealkylation of some analogs of NMA is catalyzed by at least two different isozymes, CYP2D6 in man (Kumagai *et al.* 1994; Hiratsuka *et al.* 1995; Geertsen *et al.* 1995) and CYP2B and CYP2D enzymes in the rat (Kumagai *et al.* 1994). CYP2D6 is also known to catalyze the ring hydroxylation of many amine compounds (Coutts *et al.* 1997; Ono *et al.* 1995; Coutts *et al.* 1994; Masubuchi *et al.* 1994; Narimatsu *et al.* 1994; Nielsen *et al.* 1994).

In the present study, the *in vitro* metabolism of M-AM, 4-methoxy-N-ethylamphetamine (M-NEA) and 4-methoxy-N-(n-butyl)amphetamine (M-NBA) was performed with a fortified CYP2D6 preparation with three objectives in mind: (a) to determine whether O-demethylation of all three drugs occurred and whether the size of the N-substituent influenced the extent of O-demethylation; (b) to determine whether any ring oxidation of M-AM, N-NEA and M-NBA occurred prior to or after O-demethylation; and (c) to determine whether CYP2D6 catalyzed N-dealkylation of M-NEA and M-NBA to any extent. The structures of substrates and putative ring hydroxylated products in the present study are provided in figure 4.1.

4.2 EXPERIMENTAL

4.2.1 Chemicals and reagents

All amphetamine derivatives were racemic mixtures.

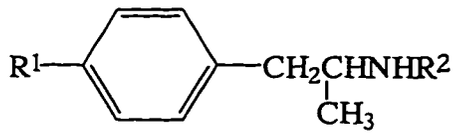
4-Methoxy-N-(n-butyl)amphetamine (M-NBA).HCl. 4-Methoxyphenylacetone (3 g) and a slight excess of n-butylamine were added to 50 ml dry ethanol containing 0.15 g platinum oxide. The mixture was hydrogenated at 35 psi for 18 – 24 h, catalyst was

removed by filtration and solvent was evaporated under vacuum. The residue was dissolved in dry ether and treated with a solution of HCl in ether until no further precipitation occurred. This product was recrystallized from ethanol as a colorless solid, mp. 176 – 177°C. Anal. calcd. for $C_{14}H_{24}ClNO$: C, 65.21%, H, 9.38%, N, 5.43%; found: C, 65.41%, H, 9.24%, N, 5.28%.

4-Methoxy-N-ethylamphetamine (M-NEA).HCl, m.p. 158 – 159°C, [anal. calcd. for $C_{12}H_{20}ClNO$: C, 62.74%, H, 8.77%, N, 6.10%; found: C, 62.79%, H, 8.65%, N, 5.84%], and 4-methoxy-N-methylamphetamine (M-NMA).HCl, m.p. 178 – 179°C, [anal. calcd. for $C_{11}H_{18}ClNO$: C, 61.25%, H, 8.40%, N, 6.49%; found: C, 61.28%, H, 8.37%, N, 6.61%], were obtained by the same procedure except that ethylamine and methylamine, respectively, were substituted for n-butylamine.

4-Hydroxy-N-(n-butyl)amphetamine (HO-NBA).HCl, m.p. 158 – 159°C, 4-hydroxy-N-ethylamphetamine (HO-NEA).HCl, m.p. 159-160°C, and N-(n-butyl)amphetamine (NBA).HCl, m.p 166-168°C were prepared by previously reported methods (Coutts *et al.* 1976, 1978). N-Ethylamphetamine (NEA).HCl, m.p. 148 – 150°C, [anal. calcd. for $C_{11}H_{18}ClN$: C, 66.15%, H, 9.08%, N, 7.01%; found: C, 66.28%, H, 9.00%, N, 6.89%] was prepared in 88% yield by the procedure used for N-(n-butyl)amphetamine hydrochloride except that butylamine was replaced by ethylamine. Amphetamine (AM) sulfate, 4-methoxyamphetamine (M-AM).HCl and 4-hydroxyamphetamine (HO-AM). HBr were gifts from the former Smith, Kline and French Laboratories [Philadelphia]. Other chemicals were obtained from commercial sources: $NADP^+$ sodium salt from yeast, D-glucose 6-phosphate-monosodium salt (G6P) and glucose 6-phosphate dehydrogenase (G6PD) type XII from Torula yeast [Sigma Chemical Co. (St. Louis, MO)]; acetic anhydride and pyridine [Fisher Scientific, Ottawa, Canada]; $KHCO_3$, K_2CO_3 and quinidine sulfate [BDH, Toronto, Canada]. All reagents were of analytical grade. Dichloromethane (CH_2Cl_2) and diethyl ether were distilled before used. K_2CO_3 solution used in this study was a 25% w/v aqueous solution. The buffer used in metabolic reactions was aqueous potassium phosphate buffer (100 mM; pH = 7.4).

Kimax glass culture tubes (16 mm o.d. x 100 mm) purchased from Fisher Scientific (Ottawa, Canada) were used in metabolism studies.



Compound	R^1	R^2
AM	H	H
NEA	H	CH_2CH_3
NBA	H	$(CH_2)_3CH_3$
M-AM	CH_3O	H
M-NMA*	CH_3O	CH_3
M-NEA	CH_3O	CH_2CH_3
M-NBA	CH_3O	$(CH_2)_3CH_3$
HO-AM	HO	H
HO-NEA	HO	CH_2CH_3
HO-NBA	HO	$(CH_2)_3CH_3$

Figure 4.1 Structures of amphetamines and their 4-hydroxylated metabolites. [*M-NMA is the internal standard]

4.2.2 Purified human CYP2D6 isozyme

The human CYP2D6 microsomal product used in this study was derived from a human AHH-1 TK+/- cell line transfected with complementary DNA that encoded human CYP2D6 and was purchased from Gentest Corporation (Woburn, MA, U.S.A.). Total protein content was 10 mg/ml in 100 mM potassium phosphate (pH = 7.4) and CYP2D6 content was 170 pmol/mg protein. Control microsomes that were obtained from the same human cell line but had not be transfected with specific cDNA, were purchased from the same source. These microsomes contained no CYP2D6 but possessed a low level of a cytochrome P450 that was inducible with polycyclic aromatic hydrocarbon.

4.2.3 GC assay procedure

4.2.3.1 Instrumental analysis

The gas chromatograph was an HP 5730A instrument (Hewlett Packard, PA, USA) equipped with a nitrogen-phosphorus detector and an HP-3396A integrator. It contained a DB-5 fused capillary column, 13 m x 0.25 mm ID x 0.25 μm film thickness (J&W Scientific, Folsom, CA). The carrier gas was ultrapure helium (Union Carbide, Edmonton, Canada) and its flow rate was adjusted to maintain a column head pressure of 10 psi. Make-up gas at the detector was a mixture of hydrogen (3 ml/min) and air (80 ml/min). The injector and detector temperatures were 260°C and 310°C respectively. Chromatograms were recorded with an HP 3396A integrator, and peak areas were measured. A temperature program was used in which the starting temperature (130°C) was raised at 8°C/min to 255°C and held at this temperature until all peaks had emerged.

4.2.3.2 Derivatization procedures

4.2.3.2.1 Anhydrous acetylation

The dried residue obtained at the end of each metabolic reaction was mixed with acetic anhydride (50 μl) and pyridine (25 μl) in a test tube which was capped tightly with screw cap before insertion in a metal heating module at 50°C for 30 min. The derivatized sample was allowed to cool to room temperature before the tube was opened and its content evaporated to dryness at room temperature under a stream of dry nitrogen. The residue was dissolved in toluene (100 μl) and aliquots (2 μl) were analyzed by gas chromatography (GC) as described in section 4.2.3.1.

4.2.3.2.2 Aqueous acetylation

The metabolism incubation reaction was terminated by cooling in an ice bath and basifying with solid $KHCO_3$ (350 mg). Internal standard (M-NMA; 18.24 nmol) was added followed by acetic anhydride (300 μl) and acetylation was allowed to proceed for 25 min at room temperature. The reaction mixture was extracted with the mixed organic

solvent (CH_2Cl_2:diethylether; 11:14 v/v; 3 ml x 3). The extracts were combined and evaporated under a stream of dry nitrogen and the residue was reconstituted in toluene (100 μl). Aliquots (2 μl) of this solution were used for GC analysis as described in section 4.2.3.1.

4.2.3.3 Quantitative analysis of metabolites

Calibration curves for M-NBA and HO-NBA were prepared by adding internal standard (M-NMA.HCl; 18.24 nmol) and varying amounts of analytes (3.31 – 49.57 nmol of M-NBA.HCl; 1.60 – 16.02 nmol of HO-NBA) to separate 0.5 ml volumes of incubation mixture, prepared as decribed in section 4.2.5.2.1. Each mixture was heated for 5 min at 37°C, then cooled in an ice bath prior to the addition of 25 % K_2CO_3 (100 μl) and CYP2D6 microsomal protein (0.20 mg). The mixture was then extracted and derivatized using the same procedures as those described for the substrate metabolism reaction. The ratios of M-NBA/M-NMA peak areas and ratios of HO-NBA/M-NMA peak areas were plotted against the quantities of M-NBA and HO-NBA, respectively.

Calibration curves for NBA, M-NEA, NEA, M-AM, AM, HO-NEA and HO-AM were prepared by the same procedure described for the preparation of the calibration curves of M-NBA and HO-NBA above, except that M-AM and AM were acetylated in aqueous medium.

4.2.4 Analysis of kinetic data

Apparent Michaelis Menten parameters, K_m and V_{max}, were calculated according to equation (1) by least-squares regression analysis of reaction velocities *vs* substrate concentration curves using the computer program "WinNonLin" (version 1.0; Scientific Consulting, Inc.). The appropriateness of the fit was determined by visual inspection of residual patterns and residual sums of squares.

$$V = \frac{V_{max} \cdot S}{K_m + S} \qquad (1)$$

4.2.5 *In vitro* metabolic experiments

NADPH generating system components: Separate stock solutions of each component were freshly prepared before use as follows: 20 μg/μl $NADP^+$in phosphate buffer; 20 μg/μl G6P in phosphate buffer; 6.7 μg/μl $MgCl_2.6H_2O$ in phosphate buffer; and G6PD stock solution: 50 U/ml of G6PD in phosphate buffer.

4.2.5.1 Metabolic reactions of 4-methoxy-N-alkylamphetamines

4.2.5.1.1 Metabolism of M-NBA

A typical incubation mixture contained M-NBA (10.09 nmol), $MgCl_2.6H_2O$ (50 μl stock solution), $NADP^+$ (25 μl solution), G6P (25 μl solution), G6PD (10 μl solution) and phosphate buffer to a volume of 460 μl. The mixture was preincubated at 37°C for 5 min in a shaking Magni Whirl® constant temperature water bath (Blue M Electric Co., Blue Island, IL). The enzyme reaction was started by the addition of CYP2D6 (0.20 mg). Additional buffer solution (20 μl) was used to rinse the protein container and this washing was also added to the incubation reaction. The final solution (500 μl) was incubated at 37°C for 15 min. The reaction was terminated by cooling in an ice bath, followed by the addition of K_2CO_3 solution (100 μl), which increased the pH of the incubation mixture to between 11 and 12. M-NMA (18.24 nmol) was added as an internal standard and the resulting solution was extracted with a mixture of CH_2Cl_2 and diethyl ether (11:14; 3 ml x 3) by vortexing vigorously for 1 min, shaking mechanically for 5 min and centrifuging for 5 min. The organic layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was anhydrously acetylated as described above in section 4.2.3.2.1 and derivatized sample was analyzed as described in section 4.2.3.1.

4.2.5.1.1.1 Time course study of M-NBA metabolism

A mixture of M-NBA (40.36 nmol), CYP2D6 (0.80 mg), stock solutions of $MgCl_2.6H_2O$ (200 μl), $NADP^+$ (100 μl), G6P (100 μl), G6PD (40 μl) and buffer to a volume of 2 ml was incubated as described in section 4.2.5.1.1, except that samples (250

μl) were withdrawn at various time intervals: 5, 10, 15, 20, 25 and 30 min. Each sample was cooled in an ice bath and basified by an addition of K_2CO_3 solution (50 μl). Buffer (300 μl) and internal standard (M-NMA; 9.12 nmol) were added prior to extraction. Dried extracts from each incubation were anhydrously acetylated (see "Derivatization procedures", section 4.2.3.2.1 above) and derivatized samples were assayed as described in section 4.2.3.1.

4.2.5.1.1.2 Kinetic study of M-NBA

The metabolism of M-NBA was repeated, as described in section 4.2.5.1.1, except that varying quantities of M-NBA.HCl (3.10; 4.97; 10.09; 17.08; 29.49; 49.67; 55.89 and 68.31 nmol.) were used. Each extract was anhydrously acetylated (see "Derivatization procedures", section 4.2.3.2.1 above). Acetylated samples were analyzed by GC as described in section 4.2.3.1.

4.2.5.1.1.3 Effect of varying enzyme concentration on the metabolism of M-NBA

Metabolic reactions of M-NBA were repeated, except that different quantities of CYP2D6 microsomal protein (0.1; 0.2; 0.3; 0.4 and 0.5 mg) were used. The quantity of M-NBA, cofactors and incubation time were the same as these described in the section 4.2.5.1.1. The final dried extracts were anhydrously acetylated ("Derivatization procedures", section 4.2.3.2.1), and the acetylated samples were analyzed by GC as described in section 4.2.3.1.

4.2.5.1.1.4 M-NBA inhibition study

The metabolism of M-NBA (10.09 nmol) with CYP2D6 (0.20 mg) as described in section 4.2.5.1.1 was repeated, except that quinidine (5.05 nmol) was added to the preincubated medium, before the enzymatic reaction was initiated by the addition of CYP2D6 solution.

4.2.5.1.1.5 Control metabolism of M-NBA

The metabolism of M-NBA was repeated as described in section 4.2.5.1.1 except that CYP2D6 enzyme solution was replaced with control microsomal protein (0.20 mg).

4.2.5.1.2 Metabolism of M-NEA

The incubation of M-NEA (10.09 nmol) with CYP2D6 (0.20 mg) at 37°C for 15 min was performed exactly as described for the metabolism of M-NBA in section 4.2.5.1.1. The final residue which contained substrate and metabolites was anhydrously acetylated (see "Derivatization procedures", section 4.2.3.2.1), and the dried acetylated product was reconstituted in toluene (100 μl). Aliquots (2 μl) were analyzed by GC as described in section 4.2.3.1.

4.2.5.1.3 Metabolism of M-AM

M-AM (10.04 nmol) was metabolized with CYP2D6 (0.20 mg) exactly as described for the metabolism of M-NBA in section 4.2.5.1.1, except that the incubation mixture was aqueously acetylated (see "Derivatization procedures", section 4.2.3.2.2). The dried acetylated product was dissolved in toluene (100 μl) and aliquots (2 μl) were analyzed by GC as described in section 4.2.3.1.

4.2.5.2 Metabolic reactions of N-alkylamphetamines

4.2.5.2.1 Metabolism of NBA

Enzymatic reaction of NBA (15.09 nmol) with CYP2D6 (0.5 mg) in the presence of cofactors and phosphate buffer was performed as described for the metabolism of M-NBA in section 4.2.5.1.1, except that the incubation mixture was incubated for 2 h at 37°C and the quantity of internal standard, M-NBA, used was 9.12 nmol. Derivatized sample was dissolved in toluene (100 μl) and a 2 μl aliquot was analyzed by GC as described in section 4.2.3.1. The incubation was also performed in the presence of the inhibitor, quinidine (5.05 nmol).

4.2.5.2.1.1 Kinetic study of NBA

The metabolism of NBA was repeated on various quantities of NBA (0.9; 2.02; 3.03; 4.98; 8.02; 11.05; 14.97 and 19.60 nmol/ incubation). Samples were acetylated and analyzed as described in sections 4.2.3.2.1 and 4.2.3.1.

4.2.5.2.2 Metabolism of NEA and AM

These metabolic reactions and their subsequent analyses were performed in the same manner as that described in section 4.2.5.1.1. Each enzymatic reaction contained NEA or AM (15.10 nmol), $MgCl_2$ (50 μl), $NADP^+$ (25 μl), G6P (25 μl), G6PD (10 μl) and CYP2D6 (0.50mg) in buffer (total volume = 500 μl). Incubation was continued at 37°C for 2 h. At the end of the NEA incubation period, internal standard (M-NMA, 9.12 nmol) was added and the resulting mixture was extracted, derivatized and GC analyzed as described in sections 4.2.3.2.1 and 4.2.3.1. At the end of the AM incubation, internal standard (M-NMA, 9.12 nmol) was added and the resulting mixture was subjected to aqueous acetylation as described in section 4.2.3.2.2, prior to GC analysis.

4.3 RESULTS

In this study, all incubation mixtures containing amphetamines and their metabolites were derivatized with acetic anhydride prior to GC analysis. Two derivatization procedures, aqueous and anhydrous acetylation, were employed. Aqueous acetylation was used at room temperature to derivatize AM and M-AM incubation mixtures prior to their extraction. This procedure converted basic and amphoteric compounds to neutral acetates that could be efficiently extracted into organic solvents. When the same aqueous acetylation procedure was performed on the N-butyl- and N-ethyl-amphetamines, complete acetylation of substrates and metabolites was never attained. In these instances, acetylations were satisfactory only when performed anhydrously.

When 4-methoxy-N-alkylated amphetamines were incubated with expressed CYP2D6 in a medium containing an NADPH generating system, the corresponding 4-

hydroxy-N-alkylamphetamines were formed at differing rates that depended on the length of the N-alkyl substituent. When M-NBA was the substrate, the formation of HO-NBA was a linear process over a 30 min incubation period (figure 4.2), at which time 90% of M-NBA was already O-demethylated to HO-NBA. Only one product, HO-NBA was detected in the GC trace of the incubation extract (figure 4.3). The formation of HO-NBA also increased linearly as the amount of CYP2D6 used was increased from 0.1 to 0.5 mg per incubation (figure 4.4). These data indicate that M-NBA is a very good substrate for CYP2D6.

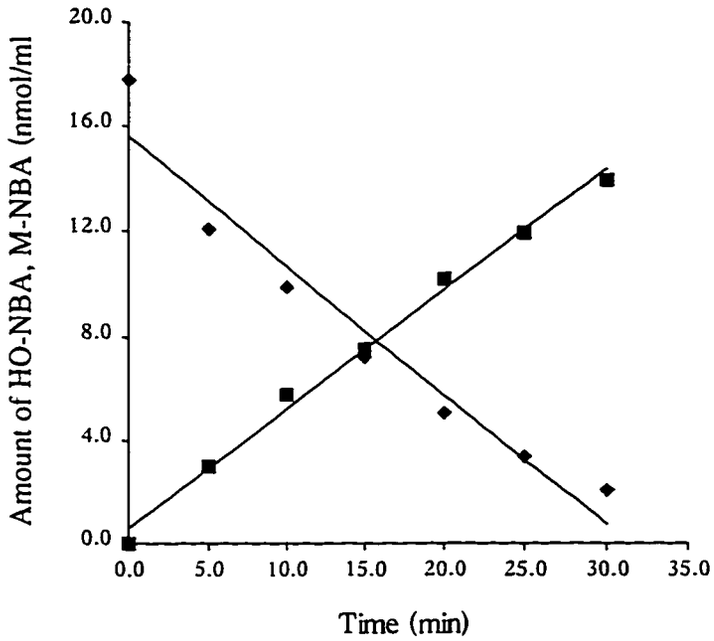


Figure 4.2 Time course study of M-NBA metabolism. Results are averages of two values which differed by less than 5%. Amount of HO-NBA (■) and recovered amount of M-NBA (◆).

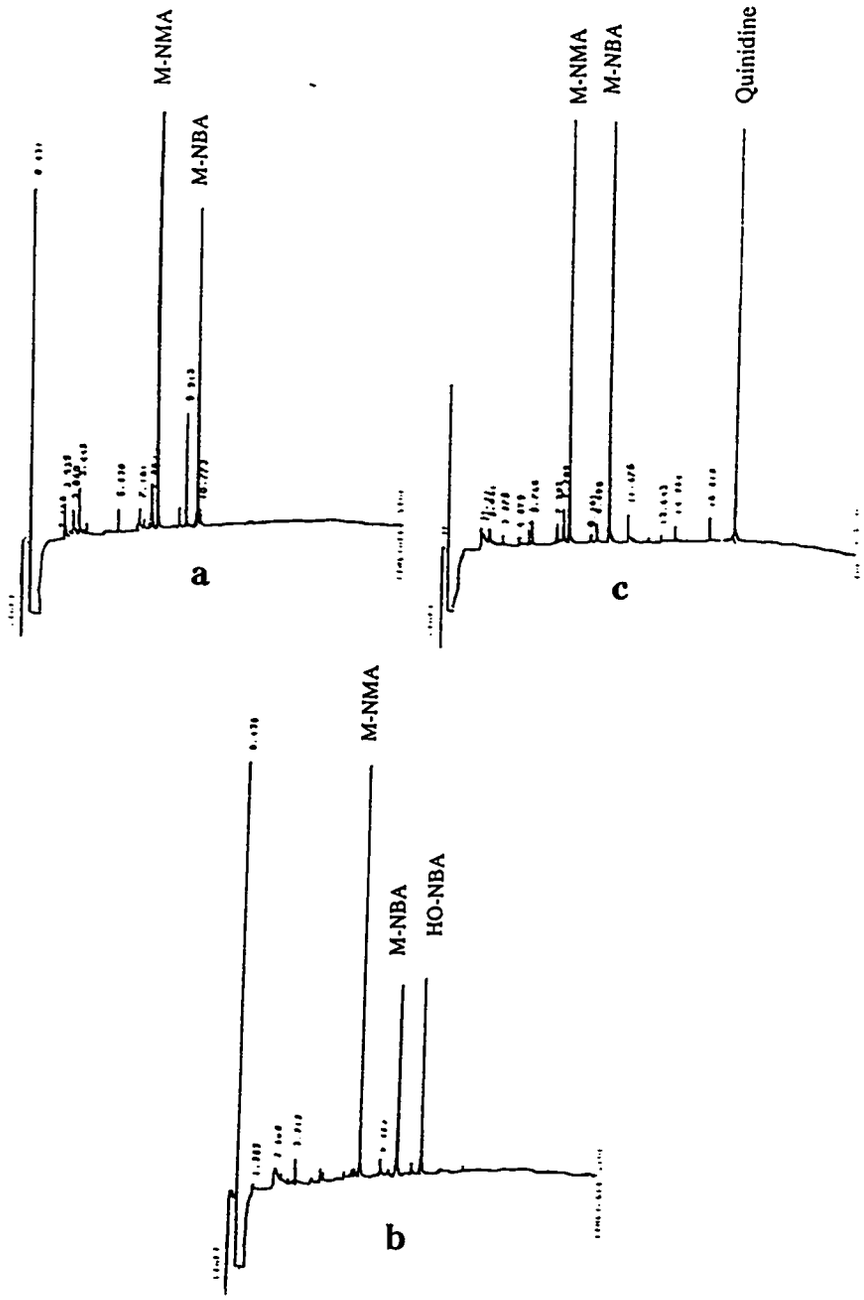


Figure 4.3 GC traces of acetylated dried extracts of M-NBA incubation with: a) control microsomal protein; b) CYP2D6 enzyme preparation; and c) CYP2D6 enzyme preparation to which quinidine was added. M-NBA: 10.09 nmol. Quinidine: 5.05 nmol. Protein solution: 0.20 mg. Incubation time: 15 min. Incubation volume: 0.5 ml.

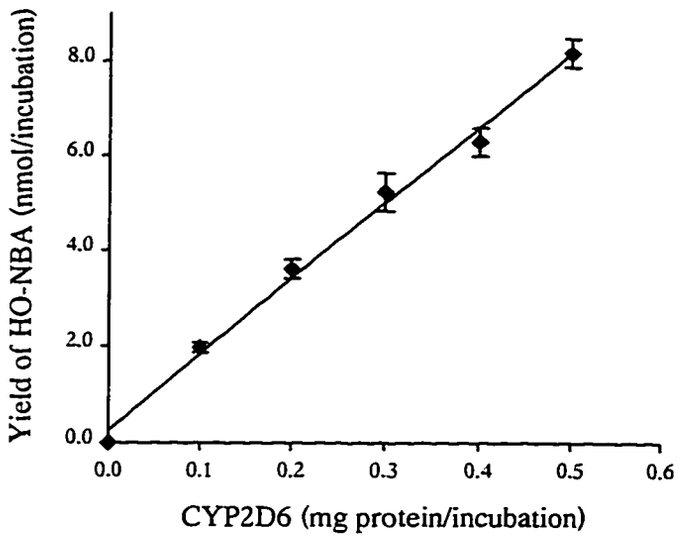


Figure 4.4 Effects of enzyme concentration on the yield of HO-NBA obtained by metabolism of M-NBA. Incubation time: 15 min. Volume of incubation: 0.5 ml; M-NBA: 10.09 nmol. Data represent means ± SD (n = 3).

Experiments were conducted in which a constant quantity of CYP2D6 was incubated with varying amounts of M-NBA ranging from 3.10 to 68.31 nmol/incubation to determine apparent K_m and V_{max} values. It can be deduced from figure 4.5 that CYP2D6 was saturated when the M-NBA concentration was > 60 μM. The formation of HO-NBA exhibited simple single enzyme Michaelis Menten kinetics and resulted in values of 11.9 ± 1.81 μM (mean ± SD; n = 3) and 121.5 ± 4.13 nmol/h/mg protein for K_m and V_{max}, respectively. No CYP2D6-mediated O-demethylation of M-NBA occurred when the control enzyme preparation was used in place of CYP2D6. O-Demethylation was also completely inhibited when the incubation medium also contained quinidine (10 μM).

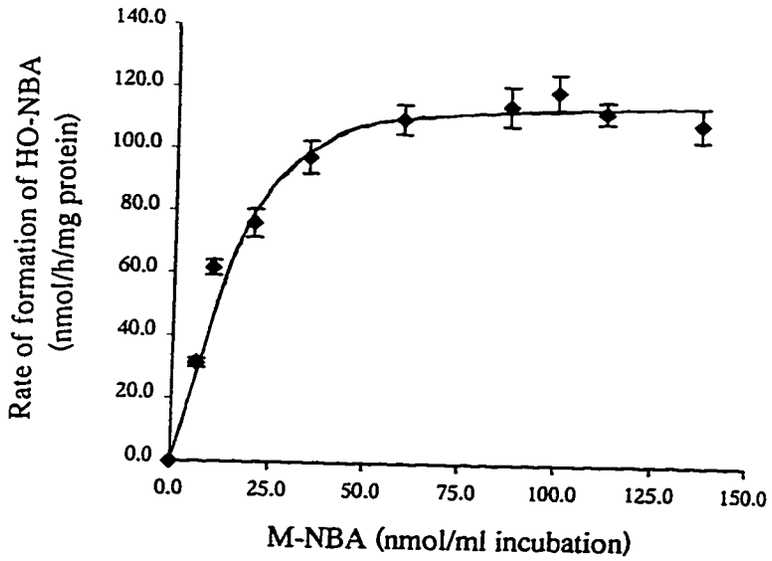


Figure 4.5 The kinetic saturation curve of M-NBA metabolism mediated by CYP2D6. Incubation time: 15 min. M-NBA: 6.2 to 136.6 nmol/ml. Data are means ± SD (n = 3).

When M-NEA and M-AM were individually incubated with CYP2D6 under conditions identical to those used for M-NBA, both substrates were also readily O-demethylated to their corresponding ring hydroxylated metabolites, HO-NEA and HO-AM, respectively. The yields of these metabolites decreased as the length of the N-alkyl group decreased (table 4.1).

Table 4.1 Yields of O-demethylation metabolites. Incubation time: 15 min. Protein : 0.20 mg/incubation. Values represent means of 3 determinations (± SD)

Substrate (10.09 nmol/incubation)	Metabolite (nmol/incubation)
M-NBA	HO-NBA: 3.81 ± 0.04
M-NEA	HO-NEA: 2.41 ± 0.14
M-AM	HO-AM: 1.36 ± 0.10

The incubation of N-alkylated amphetamines, NBA, NEA and AM with an expressed CYP2D6 enzyme preparation under the same conditions as those used to metabolize the analogous 4-methoxy-N-alkylated amphetamines (0.2 mg CYP enzyme/-incubation; duration of incubation: 15 min) provided only trace amounts of ring-hydroxylated metabolites. However, when the CYP enzyme concentration was increased to 0.5 mg/incubation and incubation time was extended to 2 h, ring 4-hydroxylated amphetamine metabolites were obtained. The products, 4-OH-NBA, 4-OH-NEA and 4-OH-AM, respectively (figure 4.1), and their acetylated derivatives had GC retention times that were identical to those of authentic reference compounds. The amount of HO-AM formed was less than one-third of the amounts of HO-NBA and HO-NEA formed (table 4.2).

A kinetic study of NBA's metabolism to 4-OH-NBA was performed, and apparent K_m and V_{max} values were calculated. The Michaelis Menten plot of the ring hydroxylation of NBA by CYP2D6 is provided in figure 4.6, and calculated metabolic constants were 3.68 ± 0.30 μM (mean ± SD; n = 3) and 0.62 ± 0.01 nmol/h/mg protein for K_m and V_{max}, respectively. The 4-hydroxylation of NBA mediated by CYP2D6 was completely inhibited by the inclusion of quinidine in the metabolism reaction. Intrinsic clearances (Cl_{int}) of M-NBA and NBA are listed on table 4.3.

Table 4.2 Yield of aromatic hydroxylation metabolites. Incubation time: 2 h. Protein: 0.50 mg/incubation. Values represent means of 3 determinations (± SD)

Substrate (15.09 nmol/incubation)	Metabolites (nmol/incubation)
NBA	HO-NBA: 0.58 ± 0.04
NEA	HO-NEA: 0.55 ± 0.03
AM	HO-AM: 0.16 ± 0.02

Table 4.3 Apparent K_m and V_{max} and Cl_{int} values of M-NBA and NBA metabolism by CYP2D6.

Metabolic reaction	Apparent K_m (μM)	V_{max} (nmol/h/mg protein)	Cl_{int} (μl/min/ mg protein)
NBA → HO-NBA	3.68 ± 0.30	0.62 ± 0.01	2.81
M-NBA → HO-NBA	11.9 ± 1.81	121.5 ± 4.13	170.4

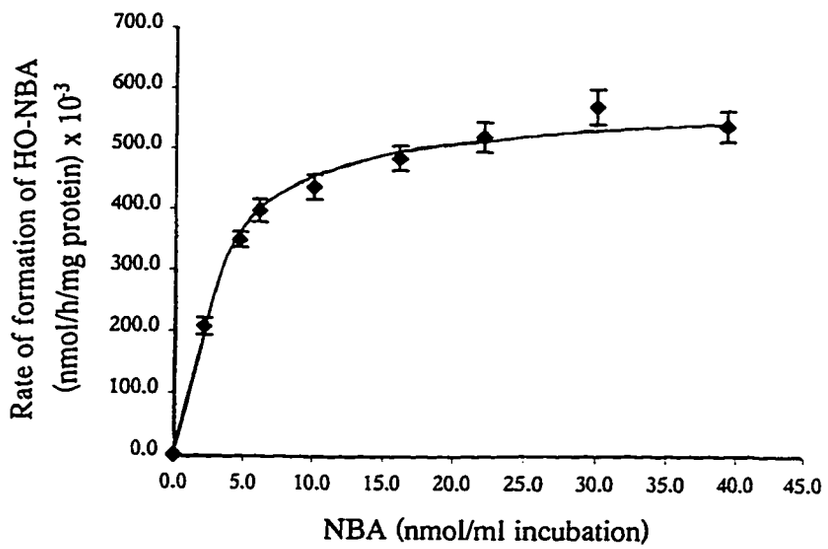


Figure 4.6 The kinetic saturation curve of NBA metabolism mediated by CYP2D6. Incubation time: 2 h. Concentration of NBA: 1.8 to 39.2 μM. Data are means ± SD (n = 3).

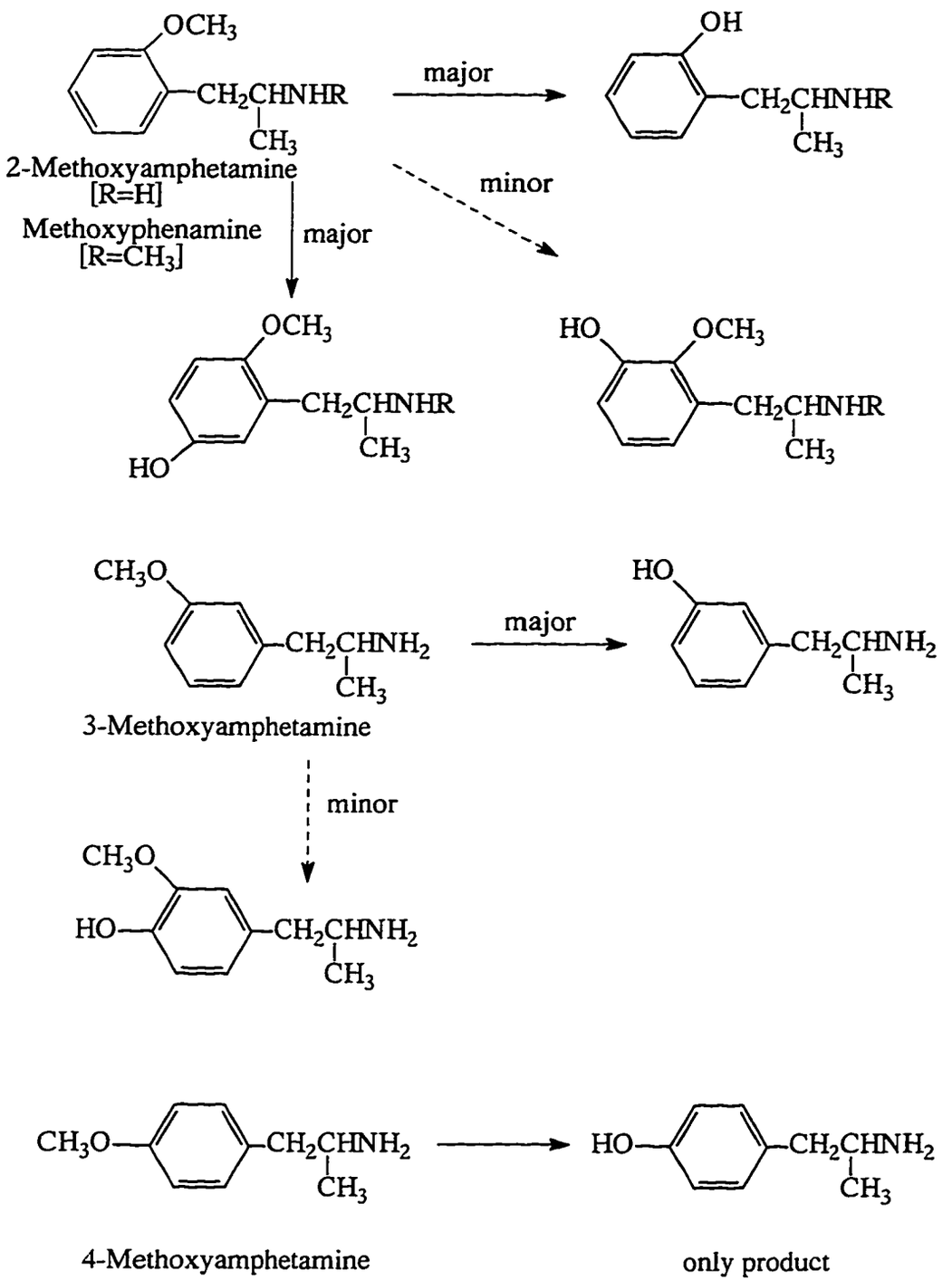


Figure 4.7 Structures of 2-, 3- and 4-methoxyamphetamine analogs and their metabolites in humans.

4.4 DISCUSSION

This study has shown that the 4-methoxy-N-alkylamphetamines, M-NBA, M-NEA and M-AM, all readily undergo O-demethylation to their corresponding 4-hydroxy-N-alkylamphetamines when incubated with a CYP2D6 enzyme system. The rates of formation of these phenolic amines are influenced by the size of alkyl groups: hydrogen < ethyl < butyl (table 4.1). CYP2D6 was not capable of catalyzing the ring hydroxylation of any of the three 4-methoxyamphetamines selected for this study (figure 4.3). This result contrasts with those obtained when 2-methoxyamphetamine and methoxyphenamine (a 2-methoxyamphetamine derivative) were metabolized *in vitro* with microsomal CYP2D6 or with CYP2D6-transfected intact human cells (Coutts *et al.* 1994; Geertsens *et al.* 1995). These substrates were not only ring-O-demethylated, but were also ring-hydroxylated, mainly on C-5 but also, to a small extent, on C-3. This inability to ring hydroxylate 4-methoxyamphetamines should also be compared with reported *in vivo* metabolic results of studies on 3-methoxyamphetamine. This substrate was O-demethylated (CYP enzyme not identified) in good yield to 3-hydroxyamphetamine, but only one ring-hydroxylated urinary metabolite, 4-hydroxy-3-methoxyamphetamine, was isolated as a minor metabolite (Midha *et al.* 1981). These observations show that whether ring hydroxylation occurs depends on the ring location of the methoxy group. If it does occur, the major sites of ring oxidation will be those *para* and, to a much lower extent, *ortho* to the methoxy group. The *para* position is much less sterically hindered (figure 4.7).

The current observation is in agreement with an earlier report that no ring hydroxylated metabolites was detectable in the urine of human who had digested 4-methoxyamphetamine (Kitchen *et al.* 1979).

Of the three 4-methoxyamphetamines examined in the present study, M-NBA was a very good substrate for CYP2D6. The size of the N-alkyl group influenced the extent of O-demethylation. As the size of the N-alkyl group increases, so does lipid solubility, and substrate concentration at the active site of the enzyme is a function of a substrate's lipid solubility (Duncan *et al.* 1983). Although CYP2D6 catalyzes the N-dealkylation of some amine compounds, no N-dealkylation was observed in the present study.

It was clear that CYP2D6 catalyzed the ring hydroxylation of NBA, NEA and AM as demonstrated by the formation of their 4-hydroxy N-alkylamphetamines and by the inhibition of the formation of these metabolites when the incubation mixture contained quinidine which is a specific inhibitor of CYP2D6 (Kobayashi *et al.* 1989). Comparison of the amount of HO-NBA and HO-NEA formed (see table 4.2) indicated that the catalytic activity of CYP2D6 appeared not to be affected by the size of N-alkyl substituents; however CYP2D6 activity was increased relative to the extent observed with AM. The formation of 4-HO-AM was less than 30% of the quantities of 4-HO-NEA or 4-HO-NBA formed by CYP2D6 (table 4.2).

CYP2D6 catalyzed the ring hydroxylation of N-alkylamphetamines, but its contribution to this pathway was not significant. As shown in table 4.3, the Cl_{int} values for NBA hydroxylation was much lower than that of M-NBA O-demethylation (2.81 μl/min *vs* 170.38 μl/min for NBA and M-NBA, respectively). In addition 3,4-dihydroxylated metabolites isolated in *in vivo* studies of NEA, NPA and NBA metabolism were not detected in this study. This indicates that enzyme(s) other than CYP2D6 is (are) involved in the formation of the catechol metabolites of N-alkylamphetamines.

In summary, it has been established that M-NBA, M-NEA and M-AM are excellent CYP2D6 substrates. It has also been shown that the CYP2D6-mediated metabolism of all three substrates produces only one metabolite in each instance, the corresponding O-demethylated products, HO-NBA, HO-NEA and HO-AM, and the production of each is an efficient process. It is also notable that the length of the N-alkyl side chain influences the activity of CYP2D6.

Unlike the 4-methoxy-N-alkylamphetamines, CYP2D6 play a very minor role in the ring hydroxylation of NBA, NEA and AM. Enzyme(s) other than CYP2D6 is (are) involved in the formation of ring hydroxylated metabolites of N-alkylamphetamines.

No N-dealkylated products were isolated from any of the metabolism reactions of N-alkylated-amphetamines or N-alkylated-4-methoxyamphetamines. Clearly, CYP2D6 was incapable of catalyzing the N-dealkylation of any of the examined substrates.

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Chapter 5

Metabolism of deprenyl analogs with CYP2D6 expressed in a human cell line

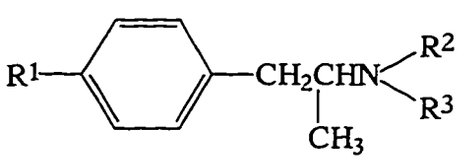
5.1 INTRODUCTION

When the metabolism studies on the involvement of CYP2D6 in the N-dealkylation of amphetamine derivatives, described in Chapter 4, were in progress, the irreversible monoamine oxidase type B inhibitor, deprenyl (DPR), was included in the compounds under investigation. Deprenyl (selegiline) is the N-propargyl (N-2-propynyl) derivative of (–)-N-methylamphetamine (NMA). We had examined two N-dealkylations (N-demethylation and N-depropargylation) of DPR before becoming aware of the study by Grace *et al.* (1994) became available to us. Grace and colleagues had also studied the *in vitro* metabolism of DPR with recombinant CYP2D6 and had found that this enzyme preparation catalyzed the N-dealkylation of DPR to both NMA and (–)-N-propargyl-amphetamine (PGA), and PGA formation was favored 13-fold over the NMA formation. Grace *et al.* described this metabolism of DPR as being an "atypical" dealkylation because they believed that this was the first example of N-dealkylation catalyzed by CYP2D6. However, in a review by Coutts *et al.* (1994), drugs whose N-dealkylation was known to be catalyzed by CYP2D6, at least to some extent, were identified as amiflamine, amitriptyline, desmethylcitalopram and imipramine. A further search of the literature (Coutts and Urichuk, 1997) has indicated that CYP2D6 is involved in the N-dealkylation of other basic drugs, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), deprenyl, haloperidol, mianserin, tomoxetine, trazodone, and venlafaxine, although in most instances its role is a minor one.

Again, while this study was in progress, a reinvestigation of the *in vitro* metabolism of DPR was reported by Wacher *et al.* (1996) who used dexamethasone-induced rat liver preparations and uninduced human liver microsomes. Incubations were performed in the absence of and in the presence of various inhibitors of CYP3A, CYP2D and CYP1A. These studies revealed that CYP3A plays a predominant role in the metabolism of DPR. N-Depropargylation appeared to be mediated almost exclusively by CYP3A. However, both CYP3A and, to a lesser extent, CYP2D isozymes played a role in the N-demethylation of DPR.

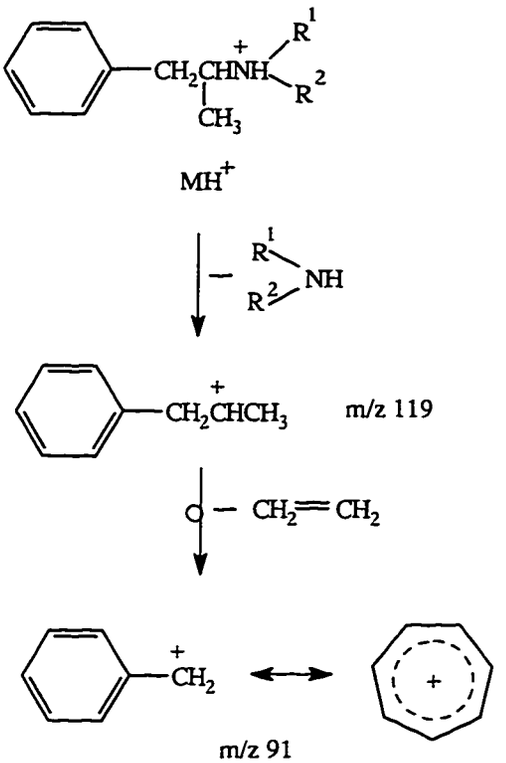
The publications by Grace *et al.* (1994) and by Wacher *et al.* (1996) somewhat reduced the significance of the initially planned project. However, metabolic studies on N-allylamphetamines were not included in these two recent publications, so the study in progress was expanded to include three additional N,N-dialkylated amphetamines: N-allyl-N-methylamphetamine (NAMA) (allyl = 2-propenyl), N,N-diallylamphetamine (NAAA), and N-methyl-N-propylamphetamine (NMPA). These three compounds were readily synthesized and their structures (figure 5.1) were confirmed by nuclear magnetic resonance (NMR) and electrospray mass spectrometry (ESMS). N-Allylamphetamine (NAA), a potential metabolite of NAMA and NAAA, was also synthesized and similarly characterized.

To determine whether N-allyl- and N-propyl-amphetamines were, like DPR, suitable substrates for CYP2D6-catalyzed N-dealkylation, the *in vitro* metabolism of NAMA, NAAA, NMPA, DPR and also benzphetamine (BPA), was studied using CYP2D6 expressed in a human cell line.



Compound	R^1	R^2	R^3
NAA	H	H	CH_2-CH=CH_2
NAMA	H	CH_3	CH_2-CH=CH_2
NAAA	H	CH_2-CH=CH_2	CH_2-CH=CH_2
NMA	H	H	CH_3
NPA	H	H	$(CH_2)_2CH_3$
NMPA	H	CH_3	$(CH_2)_2CH_3$
DPR	H	CH_3	CH_2-C≡CH
BPA	H	CH_3	$PhCH_2$

Figure 5.1 Structures of N,N-dialkylamphetamine analogs and N-dealkylated metabolites.



	R^1	R^2
NAA	$CH_2CH{=}CH_2$	H
NAMA	$CH_2CH{=}CH_2$	CH_3
NMPA	$CH_2CH_2CH_3$	CH_3
NAAA	$CH_2CH{=}CH_2$	$CH_2CH{=}CH_2$

Compound	m/z	% Relative Abundance		
		MH^+	m/z 119	m/z 91
NAA	176	100.0	36.1	63.0
NAMA	190	100.0	19.9	29.8
NMPA	192	100.0	13.5	18.0
NAAA	216	100.0	20.9	32.2

Figure 5.2 Characteristic fragment ions in the electrospray LC mass spectra of N-mono- and N,N-dialkylated amphetamines.

5.2 EXPERIMENTAL

5.2.1 Materials and Methods

5.2.1.1 Chemicals

Amphetamine sulfate was a gift from the former Smith, Kline and French Laboratories (Philadelphia) and (+)-benzphetamine HCl was kindly supplied by Prof. A.H. Beckett and Dr. D.A. Cowan, King's College, London. 4-Methoxy-N-methyl-amphetamine (M-NMA).HCl was synthesized (see Chapter 4). N-(n-Propyl)amphetamine (NPA).HCl and N-methylamphetamine (NMA).HCl were prepared by a previously

reported method (Coutts *et al.* 1976; 1978). Other chemicals were purchased from various commercial sources: allyl bromide, 1-bromopropane, analytical grade trifluoroacetic acid (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); R-(–)-deprenyl HCl (Research Biochemicals International, Natick, MA, U.S.A.); $NADP^+$ sodium salt from yeast, D-glucose-6-phosphate monosodium salt (G6P) and glucose-6-phosphate dehydrogenase (G6PD) type XII from Tortula yeast, Sigma Chemical Co. (St. Louis, MO, U.S.A.); K_2CO_3, methanol, n-hexane, HPLC grade acetonitrile, isopropanol, dichloromethane (CH_2Cl_2), diethyl ether, toluene (BDH, Toronto, Canada). All solvents were distilled before use. K_2CO_3 solution used in this study was a 25% w/v aqueous solution. The buffer solution used in all experiments was 100 mM, pH = 7.4.

5.2.1.2 Syntheses

5.2.1.2.1 (±) N-Allylamphetamine (NAA) and (±)-N,N-diallylamphetamine (NAAA)

(±)-Amphetamine sulfate (500 mg; 2.71 mmol) was suspended in CH_3CN (3 ml) and basified with K_2CO_3 solution (25% w/v; 1.5 ml; 2.71 mmol). The liberated amphetamine base was pipetted into another flask. The precipitate of K_2SO_4 was washed with CH_3CN (2.5 ml x 2) and the CH_3CN solutions were combined. To the combined organic solution, allyl bromide (117.45 µl; 1.36 mmol) was added, followed by K_2CO_3 solution (1.5 ml). The reaction mixture was left to stir at room temperature for 1 h during which time samples of the reaction mixture were examined by t.l.c. using 10% CH_3OH in CH_2Cl_2 as developing solvent. When the absence of allyl bromide was indicated, the reaction mixture was evaporated (rotary evaporator) and the residue obtained was dissolved in water (40 ml). This solution was basified with K_2CO_3 solution (1.5 ml) and extracted with CH_2Cl_2 (5 ml x 3). The combined extract was evaporated to dryness and the product was purified by silica gel column chromatography, using 2% CH_3OH in CH_2Cl_2 as eluting solvent. Two products were obtained. (±)-N,N-Diallylamphetamine (NAAA; 24.80 mg) eluted first, followed by (±)-N-allylamphetamine (NAA; 112.20 mg). Both were liquids. Total yield was 67.4%. NAA was converted to a salt by treating a

solution of it in diethyl ether with dry HCl gas, with cooling (solid CO_2 in acetone). The melting point of NAA.HCl (recrystallized from ethyl acetate) was 168.5 – 169.5°C.

NAA: ^{1}H NMR ($CDCl_3$) δ: 7.22–7.17 (m, 5H, **Ph**); 5.91–5.78 (m, 1H, allyl **CH**); 5.15–5.03 (m, 2H, terminal allyl CH_2); 3.36–3.29 (m, 1H) and 3.24–3.16 (m, 1H) (allyl N-CH_2); 3.00–2.90 (m, 1H, N-**CH**); 2.80–2.73 and 2.64–2.57 (two dd, 2H, J_{gem} 10 H_z, J_{vic} 5Hz; CH_2Ph); 1.07–1.05 (d, 3H, J 5 Hz, CH-CH_3).

The electrospray mass spectrum of NAA was consistent with its structure (figure 5.2)

When this reaction was repeated using excess allyl bromide (4 equivalents relative to the quantity of amphetamine), a single product, NAAA, was detected by t.l.c. The crude NAAA was subjected to silica gel column chromatography as described immediately above except that the solvent was a mixture of ethyl acetate and n-hexane (1:4 v/v ratio). The chromatographed product was converted to its HCl salt by bubbling HCl gas through a cooled (solid CO_2/acetone) solution of the product in dry diethyl ether. (±)-NAAA.HCl was obtained in 85% yield as a colorless solid, m.p. 162.0 – 163.0°C when recrystallized from ethyl acetate.

NAAA: ^{1}H NMR ($CDCl_3$) δ: 7.25–7.14 (m, 5H, **Ph**); 5.88–5.75 (m, 2H, two allyl **CH**); 5.22–5.08 (m, 4H, two terminal allyl CH_2); 3.22–3.06 (m, 5H, two allyl N-CH_2 overlapping N-**CH**); 2.96–2.90 (dd, 1H, J 8Hz and J 6Hz; CH_2Ph); 2.44–2.36 (dd, 1H, J 8Hz and J 3Hz, CH_2Ph); 0.95–0.93 (d, 3H, J 5 Hz, CH-CH_3).

The electrospray mass spectrum of NAAA was consistent with its structure (figure 5.2)

5.2.1.2.2 (±)-N-Allyl-N-methylamphetamine (NAMA)

The procedure immediately above for the synthesis of NAA was applied. (±)-N-Methylamphetamine (NMA.HCl; 200.0 mg; 1.08 mmol), suspended in CH_3CN (10 ml), was converted into its free base by the addition of K_2CO_3 solution (1 ml). To the isolated base, an excess of allyl bromide (2.69 mmol; 233 μl) was added, following by K_2CO_3 solution (1 ml). The resulting mixture was stirred at room temperature for 45 min at which time t.l.c. monitoring of the reaction mixture indicated that more than 90% of

NMA had been consumed. The residue obtained after solvent evaporation was dissolved in H_2O (10 ml) and basified with K_2CO_3 solution, then extracted into CH_2Cl_2. Chromatographic purification of the crude product on silica gel using 2.5% CH_3OH in CH_2Cl_2 as eluting solvent yielded (±)-NAMA base (224.0 mg; liquid) in 92.1% yield.

Dry HCl gas was bubbled through a cooled solution of (±)-NAMA (224.0 mg) in dry diethyl ether (8 ml) for 5 min. The reaction was then warmed to room temperature and the solvent was removed. The residue was recrystallized from ethyl acetate as a colorless solid, m.p.130.5 – 132°C.

NAMA: ^{1}H NMR ($CDCl_3$) δ: 7.30–7.16 (m, 5H, **Ph**); 5.95–5.82 (m, 1H, allyl **CH**); 5.30–5.12 (m, 2H, terminal allyl $\mathbf{CH_2}$); 3.17–3.15 (d, 2H, allyl N-$\mathbf{CH_2}$); 3.02–2.95 (m, 2H, one H of $\mathbf{CH_2}$Ph overlapping N-**CH**); 2.45–2.37 (dd, one H of $\mathbf{CH_2}$Ph); 2.30 (s, 3H, N-$\mathbf{CH_3}$); 0.96–0.94 (d, 3H, J 5 Hz, CH-$\mathbf{CH_3}$).

5.2.1.2.3 (±)-N-Methyl-N-propylamphetamine (NMPA)

This N-dialkylamphetamine was synthesized by following the same procedure used for the preparation of NAMA. The starting compound and halide reagent were (±)-N-methylamphetamine salt (NMA.HCl; 257.0 mg; 1.38 mmol) and 1-bromopropane (2.76 mmol; 251.66 μl), respectively. After one day of stiring at room temperature, a t.l.c trace of the reaction mixture developed with 7% CH_3OH in CH_2Cl_2 showed that almost no reaction had occurred, so additional amounts of 1-bromopropane (121 ml) and K_2CO_3 solution (1.02 ml) were made and the reaction was continued for another day. Crude product was placed on a silica gel column and eluted initially with 2%, and subsequently with 5% CH_3OH in CH_2Cl_2. Fractions were collected and examined by t.l.c. Those containing the desired product were combined and the resulting solution was evaporated to give NMPA as a liquid in modest yield (40%). NMPA.HCl salt was prepared as described for NAMA.HCl. It was a colorless solid, m.p. 113.0 – 114.0°C.

NMPA: ^{1}H NMR ($CDCl_3$) δ: 7.32–7.27 (m, 2H of Ph) and 7.22–7.17 (m, 3H of Ph); 3.10–2.86 (m, 2H, one H of $\mathbf{CH_2}$Ph overlapping N-**CH**); 2.46–2.37 (m, 3H, one H of $\mathbf{CH_2}$Ph and 2H of N-$\mathbf{CH_2CH_2}$CH$_3$); 2.32 (s, 3H, N-$\mathbf{CH_3}$); 1.58–1.44 (m, 2H, $\mathbf{CH_2}$CH$_3$); 0.95–0.89 (overlapping d and t, 6H, CH$\mathbf{CH_3}$ and $\mathbf{CH_2CH_3}$).

The electrospray mass spectrum of NMPA was consistent with its structure (figure 5.2).

5.2.1.3 Microsomal protein

Human CYP2D6 microsomal preparation used in this study was purchased from Gentest Corporation (Woburn, MA, U.S.A.). It was derived from a human AHH-1 TK+/- cell line transfected with complementary DNA that encoded human CYP2D6. Total protein content was 10 mg/ml in 100 mM potassium phosphate (pH = 7.4) and CYP2D6 content was 260 pmol/mg protein. Control microsomes obtained from the same human cell line that had not been transfected with specific cDNA, were purchased from the same source.

5.2.2 GC Assay procedure

5.2.2.1 Instrumental analysis

The gas chromatograph was an HP 5730A instrument (Hewlett Packard, PA, USA) equipped with a nitrogen-phosphorus detector and an HP-3396A integrator. It contained a DB-5 fused capillary column, 13 m x 0.25 mm ID x 0.25 μm film thickness (J&W Scientific, Folsom, CA). The carrier gas was ultrapure helium (Union Carbide, Edmonton, Canada) and its flow rate was adjusted to maintain a column head pressure of 10 psi. Make-up gas at the detector was a mixture of hydrogen (3 ml/min) and air (80 ml/min). The injector and detector temperatures were 260°C and 310°C respectively. Chromatographic peaks areas were measured with an HP 3396A integrator. Samples were analyzed by temperature programing. The initial temperature (110°C) was held for 2 min and then increased at 8°C/min to 240°C.

5.2.2.2 Quantitative analysis of metabolites

Calibration curves were prepared for three authentic metabolites, NMA, NAA and NPA by adding varying amounts of these metabolites (0.09 – 1.05 nmol) and a constant amount of internal standard (0.7 nmol of M-NMA). Peak area ratios of metabolite to

internal standard were plotted against concentrations of metabolite to produce calibration curves. The concentration of each metabolite in incubation mixtures was determined from the equation of the straight line derived from its calibration curve.

5.2.2.3 Derivatization procedure

The dried residue obtained at the end of each metabolic reaction in section 5.2.3 was mixed with trifluoroacetic anhydride (50 μl) and acetonitrile (25 μl) in a test tube which was capped tightly with screw cap before its insertion in a metal heating module at 60°C for 15 min. The reaction mixture was allowed to cool to room temperature before the tube was opened and its content evaporated to dryness at room temperature under a stream of dry nitrogen. The residue was dissolved in toluene (30 μl) and aliquots (2 μl) were analyzed by gas chromatography (GC) as described in section 5.2.2.1.

5.2.3 *In vitro* metabolic experiments

The NADPH generating system was prepared by mixing freshly prepared stock solutions of $NADP^+$ (1.3 mM; 1 mg/ml), G6P (3.3 mM; 1 mg/ml), $MgCl_2.6H_2O$ (3.3 mM; 0.67 mg/ml) and G6PD (50 U/ml buffer) in a 5:5:10:2 volume ratio.

5.2.3.1 Metabolism of N-allyl-N-methylamphetamine (NAMA)

An incubation mixture contained NAMA (6.9 nmol), CYP2D6 (12.5 μl; 0.125 mg) and phosphate buffer to a volume of 97.5 μl. The mixture was preheated at 37 °C for 5 min and the enzymatic reaction was started by addition of the NADPH generating system (27.5 μl). The resulting mixture (125 μl) was incubated at 37 °C for 2 h. The reaction was terminated by cooling the reaction flask in an ice bath and adding K_2CO_3 solution (100 μl). Internal standard (M-NMA, 0.7 nmol) was then added and the mixture was extracted with a a mixed organic solvent (2% v/v isopropanol in n-hexane; 2 x 3.5 ml) by vortexing vigorously for 1 min, shaking mechanically for 5 min and centrifuging for 5 min. The organic layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was anhydrously

trifluoroacetylated as described in section 5.2.2.3 and the derivatized sample was analyzed as described in section 5.2.2.1.

An identical incubation with control microsomes instead of CYP2D6 microsomes was also performed for comparison.

5.2.3.2 Metabolism of N-methyl-N-propylamphetamine (NMPA)

The metabolism of NMPA (6.9 nmol) by CYP2D6 (12.5 μl) in the presence of an NADPH generating system and in phosphate buffer was performed as described in section 5.2.3.1 for N-allyl-N-methylamphetamine.

5.2.3.3 Metabolism of N,N- diallylamphetamine (NAAA), deprenyl (DPR) and benzphetamine (BPA)

The incubation procedure described in section 5.2.3.1 for N-allyl-N-methylamphetamine was repeated, except that substrate NAMA was replaced with NAAA, DPR, and BPA (6.9 nmol).

5.3 RESULTS

The initial intent of the present study was to determine whether CYP2D6 played any role in the oxidative metabolism of the MAO-B inhibitor, DPR. The metabolism studies reported in previous chapters in this thesis suggested that both CYP2D6-catalyzed aromatic ring hydroxylation and N-dealkylation were likely to occur, at least to a small extent. One, two or three N-dealkylated products could theoretically be formed as the result of N-demethylation and/or N-depropargylation. When studies by others (Grace *et al.* 1994) on this subject appeared in print, the project was expanded to include analogs of DPR in which the N-propargyl group of DPR was replaced with an N-allyl and an N-propyl group. The proposed study, therefore, was expected to provide information on whether the introduction of a triple or a double bond into an N-propyl substituent had any effect on the extent of CYP2D6-catalyzed N-dealkylation.

The envisaged study required the synthesis of N-allylamphetamine, N-allyl-N-methylamphetamine and N-methyl-N-propylamphetamine. N,N-Diallylamphetamine was a byproduct of the synthesis of N-allylamphetamine, and was included in the study. The structures of these four compounds were confirmed by NMR and by mass spectrometry (MS). NMR data are provided and interpreted in the 'Materials and Methods' section. Electrospray LC-MS were recorded for interpretation. Diagnostic ions [MH^+, m/z 119 and 91] were present in the mass spectra of all compounds. Their formation and relative abundancies are presented in figure 5.2. Three of the spectra also contained additional fragments of low abundance that were consistent with the structures from which they were derived. These fragment ions are identified in table 5.1.

When five N,N-dialkylated amphetamines (NAMA, NMPA, NAAA, DPR and BPA) were individually incubated with CYP2D6 isozyme fortified with appropriate cofactors, no ring hydroxylation of any of these substrates was observed. N-Dealkylated metabolites, however, were detected in the incubation mixtures that contained NAMA, NMPA, NAAA and DPR. NAMA underwent two N-dealkylations to form the N-demethylation and N-deallylation products, NAA and NMA, respectively, which were isolated and characterized. Similarly, CYP2D6-catalyzed metabolism of NMPA produced NMA and NPA.

Table 5.1 Identities of minor fragment ions in the electrospray LC mass spectra of the synthesized N-mono- and N,N-dialkylated amphetamines.

Compound[1]	Fragment[2] (m/z; % rel. ab.)	Probable Identity
NAA	84 (5.8%)	$[MH^+–C_7H_8]$ $(CH_3CH=N^+HCH_2CH=CH_2)$
NMPA	74 (7.8%)	$[MH^+–C_6H_5CH=CHCH_3]$ $(CH_3CH_2CH_2N^+H_2CH_3)$
NAAA	174 (4.7%)	$[MH^+–CH_3CH=CH_2]$ $(C_6H_5CH_2C(CH_3)=N^+HCH_2CH=CH_2)$
NAAA	98 (7.2%)	$[MH^+–C_6H_5CH=CHCH_3]$ $(CH_2=CHCH_2N^+H_2CH_2CH=CH_2)$

[1]Structures identified in figure 5.1; [2]rel. ab. = relative abundance

Table 5.2 Yields of metabolites formed when various amphetamines were incubated with CYP2D6 and cofactors. Data are average values that differed by less than 5% in duplicated experiments.

Substrate (6.9 nmol/ incubation)	NMA (pmol/incubation)	Nor metabolite (pmol/incubation)
NAMA	98.0	279.0
NMPA	44.0	73.0
NAAA	(a)	594.0
DPR	624.0	(b)
BPA	0.0	0.0

(a) The metabolism of NAAA to NMA is not possible;
(b) Present but not quantified because of lack of a pure authentic sample.

In agreement with literature data, DPR was metabolized by N-demethylation to nordeprenyl and by N-depropargylation to NMA. NAAA was metabolized to NAA, but no dideallylated product was observed. The structures of these metabolites were confirmed by comparing their retention times with those of authentic reference samples of NMA, NPA and NAA and by interpretation of the mass spectrum of the N-demethylated metabolite of DPR after its trifluoroacetylation. This spectrum had two prominent ions of m/z 270 and 119 which are readily identified (figure 5.3). The quantities of metabolites formed are listed in table 5.2. N-Dealkylated metabolites were not detected in incubations of these substrates with control microsomes. This confirmed that CYP2D6 catalyzed these N-dealkylations. Typical GC traces of derivatized incubation mixtures of NAAA, NAMA, NMPA and DPR are shown in figure 5.4a and 5.4b.

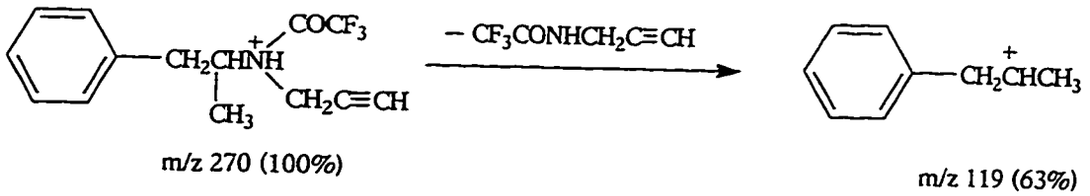


Figure 5.3 Diagnostic ions in the electrospray LC mass spectrum of the trifluoroacetylated metabolite of deprenyl. [Figures in brackets are % relative abundances].

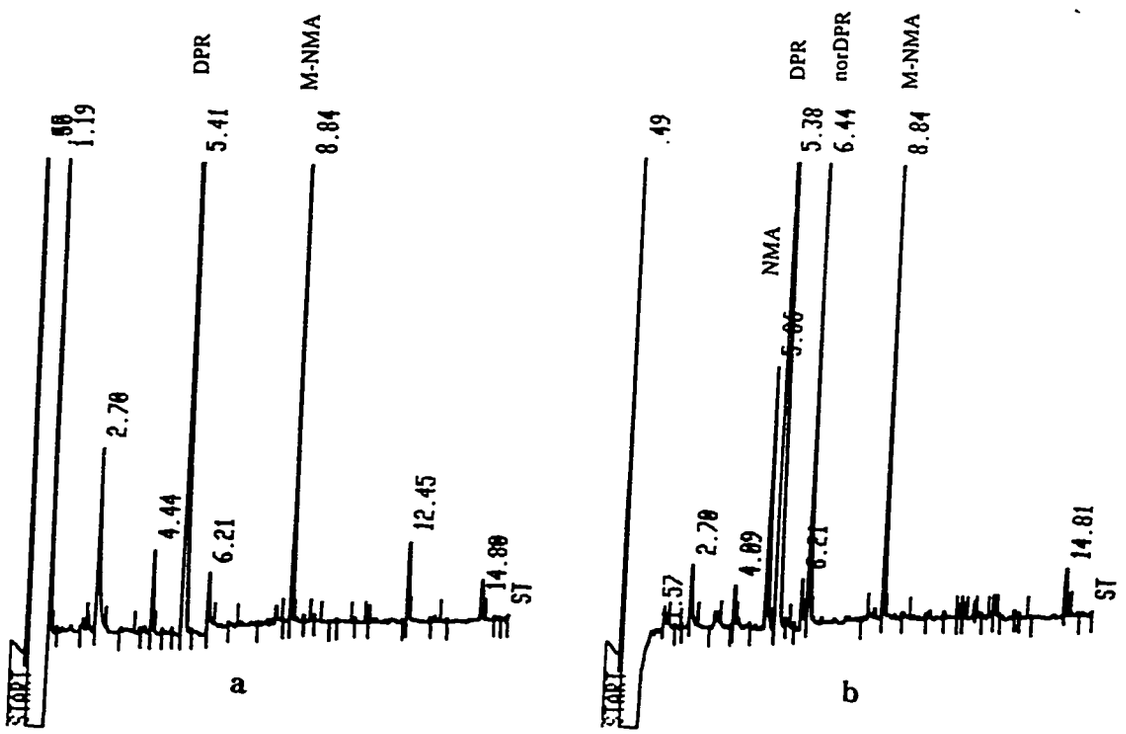


Figure 5.4a GC traces of trifluoroacetylated dried extracts of DPR incubation with: a) control microsomal protein; b) CYP2D6 enzyme preparation. M-NMA is the internal standard.

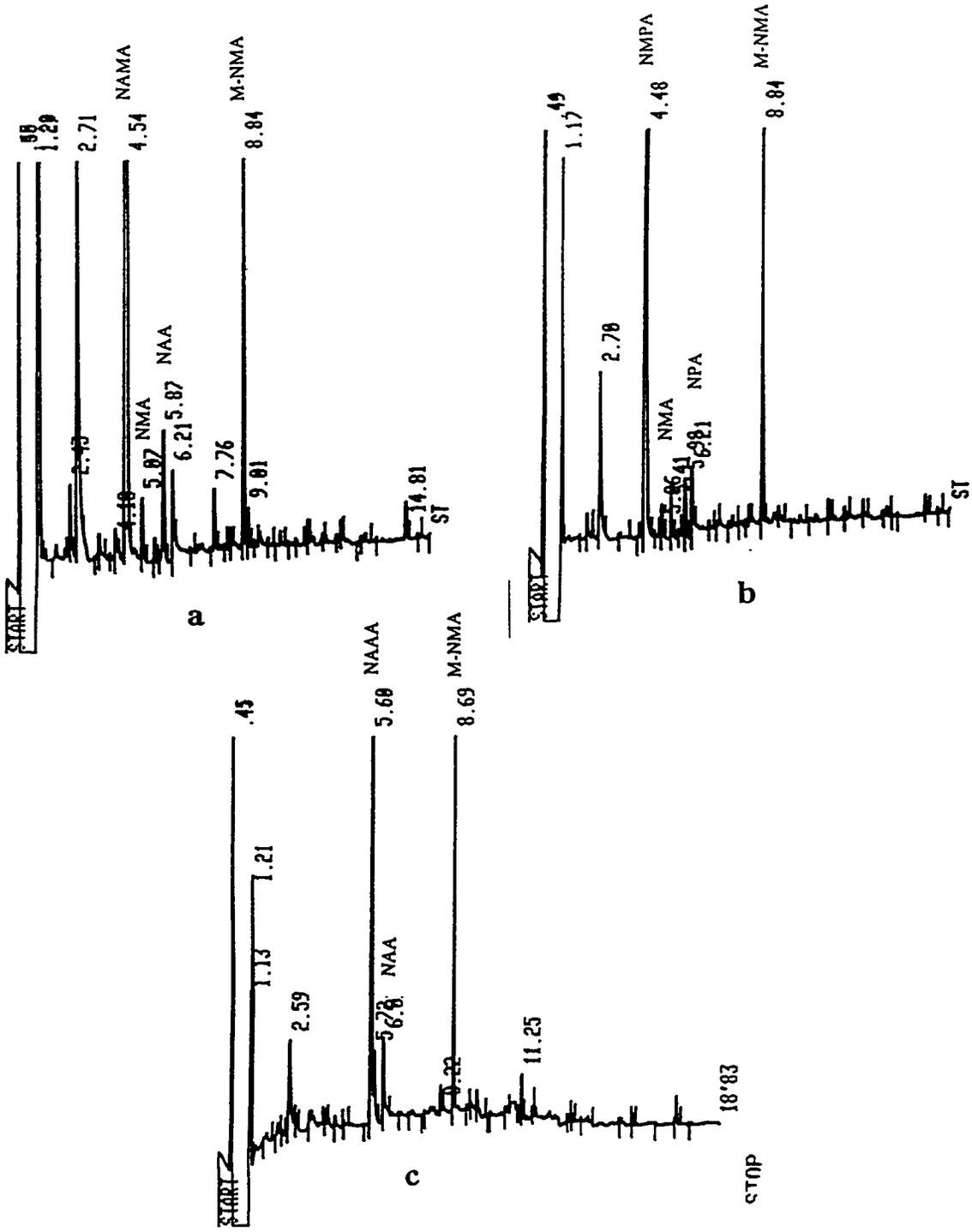


Figure 5.4b (a) GC trace of trifluoroacetylated dried extract of NAMA incubation with CYP2D6. (b) GC trace of trifluoroacetylated dried extract of NMPA incubation with CYP2D6. (c) GC trace of trifluoroacetylated dried extract of NAAA incubation with CYP2D6. Incubation mixtures were carried out under identical conditions. M-NMA is the internal standard.

5.4 DISCUSSION

The anticipated result of this study on AM derivatives in this study, was that the N,N-dialkylated AM bearing an N-methyl substituent (NAMA, NMPA, DPR and BPA) would at least be N-demethylated since this pathway is known to be catalyzed partially by CYP2D6 (Coutts and Urichuk, 1997). Ring hydroxylation was also expected because CYP2D6 is capable of mediating the ring oxidation of other AM such as N-n-butylamphetamine, N-ethylamphetamine and AM (Chapter 4). Three of the compounds investigated (NAMA, NMPA and DPR) were N-demethylated to varying extents (table 5.2) but, somewhat surprisingly, no CYP2D6-catalyzed N-dealkylation occurred when BPA was incubated with CYP2D6 and none of the substrates was ring hydroxylated. The major structural difference in the four N-methylated substrates, NAMA, NMPA, DPR and BPA, is the nature of the N-alkyl groups (figure 5.1). These N-substituents appeared to influence the catalytic activity of CYP2D6 in the N-dealkylation pathway. Of the four alkyl groups, allyl ($CH_2CH{=}CH_2$), benzyl ($PhCH_2$), propargyl ($CH_2C{\equiv}CH$) and n-propyl ($CH_2CH_2CH_3$), the presence of the propargyl group had the greatest effect on CYP2D6 activity. Indeed, DPR was found to be a very good *in vitro* substrate of CYP2D6 (figure 5.4b). The two N-dealkylated metabolites of DPR (NMA and nordeprenyl) were formed at different rates, indicating that CYP2D6 selectively mediated the cleavage of N-C bonds. When the propargyl group of DPR was replaced with an allyl group in NAMA, total N-dealkylation was much reduced and when the propargyl group was replaced with an n-propyl group in NMPA, a further reduction in the extent of N-dealkylation was observed. Finally, replacing the propargyl group with a benzyl moiety in benzphetamine resulted in a complete absence of CYP2D6-catalyzed metabolic N-dealkylation. It appears that when the N atom of AM contains both a 3-carbon substituent and a methyl group, the degree of unsaturation of the 3-carbon unit has an important influence on the extent of CYP2D6-catalyzed N-dealkylation. In addition, when the N-methyl group of NAMA was replaced by an allyl substituent in NAAA (which is N,N-diallylated), the extent of N-dealkylation was again increased (table 5.2). These findings suggest that the

affinities of N-methyl, N-propyl, N-allyl and N-propargyl groups for CYP2D6 vary considerably.

In summary, this study has shown that human expressed CYP2D6 enzyme is capable of catalyzing the N-dealkylation of four N,N-dialkylated amphetamines, namely NAMA, NMPA, NAAA and DPR, but does not mediate the N-dealkylation of benzphetamine. Rates of these N-dealkylation reactions was dependent upon the nature of N-substituents.

Results from the present study complemented those of previous investigators (Guengerich *et al.* 1986; Shet *et al.* 1993) who concluded that the N-demethylation of benzphetamine was catalyzed *in vitro* in humans by CYP3A4 and not by CYP2D6.

5.5 REFERENCES

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Chapter 6

General discussion and conclusions

6.1 GENERAL DISCUSSION and CONCLUSIONS

Many drugs possess one or more aromatic or aliphatic ring systems to which are attached sidechains that contain a basic primary, secondary or tertiary group. Common examples are the tricyclic antidepressants, e.g. amitriptyline and nortriptyline, and numerous phenylethylamines including many N-alkylated and/or ring-substituted amphetamines, e.g. N-ethylamphetamine, 4-methoxyamphetamine, N-propargyl-N-methylamphetamine (Deprenyl®), and many others. The aromatic and aliphatic rings in all such drugs are common sites of metabolic oxidation catalyzed by various cytochrome P-450 (CYP) enzymes which are found in many tissues and organs in mammals, including humans, but primarily in the liver. Many of the CYP enzymes found in human liver and livers of other mammals have been isolated and their amino acid sequences have been determined. Although there are many structural similarities in all CYP enzymes, no identical enzymes have been found in different mammalian species.

Different drugs are metabolically oxidized by different CYP enzymes in all mammalian species. In humans, 11 CYP enzymes are involved in most biochemical oxidations. These have been identified as CYP1A1, CYP1A2, CYP2A, CYP2B, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (DeVane, 1994; Guengerich, 1995; Spatzenegger and Jaeger, 1995; Daniel *et al.* 1996). It is important to establish which CYP enzymes catalyze the oxidative metabolism of all drugs. If these CYP enzymes are identified, harmful drug/drug interactions can be anticipated and avoided, and individuals who are deficient in one or more CYP enzyme and as a result, are likely to experience toxic side effects when taking certain drugs, can be identified. The CYP2D6 enzyme is of particular importance in the metabolism of many basic drugs while CYP3A4 is the most plentiful enzyme in humans and is involved in the metabolism of many drugs with diverse structures. Two other CYP enzymes, CYP1A2 and CYP2C19, are also extensively involved in the metabolism of various drugs consumed by humans (Guengerich, 1995).

There were two major objectives of the research described in this thesis. The first was to determine whether CYP2D6 had any catalytic role in the N-demethylation of

amitriptyline (AT) to nortriptyline (NT) and also to determine what other human CYP enzymes contributed to the biotransformation of AT to 10-hydroxyamitriptyline (10-HO-AT) and NT to 10-hydroxynortriptyline (10-HO-NT). Previous reports in the scientific literature on AT's metabolism were contradictory (Mellström and von Bahr, 1981; Mellström *et al.* 1983, 1986; Breyer–Pfaff *et al.* 1992). The second objective of the studies reported in this thesis was to determine whether the nature of N-alkyl groups in aromatic basic drugs had any influence on the extent of the CYP2D6-catalyzed metabolic ring oxidation of aromatic basic drugs to phenolic products by direct insertion of the phenolic group into the aromatic ring, or by the metabolic conversion of ring-methoxylated basic drugs to phenolic amines drugs. The drugs selected for the second objective were all simple derivatives of the CNS stimulant drug, amphetamine, and included some N-monosubstituted amphetamines; ring-methoxylated, N-monosubstituted amphetamines; and N,N-disubstituted amphetamines, including the monoamine oxidase (MAO)-B inhibitor, (–)-deprenyl [(-)-N-methyl-N-propargylamphetamine].

An argument is presented that metabolic N-dealkylation should *not* involve CYP2D6 since the distance between the protonated N atom and the adjacent C atom (the site of oxidation during the dealkylation reaction) is only about 0.15 nm, and not within the optimal 0.5 to 0.7 nm range. In spite of this restriction, it has been shown that CYP2D6 can catalyze many N-dealkylations, especially if the drugs are tertiary amines. This topic has been reviewed (Coutts, 1994; Coutts and Urichuk, 1997). In the studies described in this thesis, it was shown that CYP2D6 expressed in a human cell line was capable of catalyzing the N-demethylation of AT and 10-OH-AT to NT and 10-HO-NT, respectively, to some extent. However, both CYP3A4 and CYP1A2 were shown to be more efficient N-demethylating catalysts. The involvement of CYP1A2, CYP2D6 and CYP3A4 in the N-dealkylation of AT and 10-HO-AT was confirmed by the inclusion of specific or selective inhibitors of each of these CYP enzymes in the *in vitro* metabolism media. Additional information on the CYP enzymes involved in AT demethylation was obtained from additional *in vitro* studies with human liver microsomes. These studies again revealed that CYP3A4, CYP1A2 and CYP2D6 were all capable of catalyzing AT N-monodemethylation and that CYP3A4 was the most proficient enzyme. However,

significant correlations were also observed with CYP1A2, CYP2A6, CYP2D6, CYP3A4 and CYP4A, but, interestingly, not with CYP2C19 or CYP2E.

In all *in vitro* metabolic studies with expressed CYP enzymes that were conducted on AT and NT, only one enzyme, CYP2D6, was capable of catalyzing the 10-hydroxylation of both drugs.

Metabolism studies with expressed CYP2D6 enzyme were also conducted on amphetamine and some of its N-mono- and ring-methoxylated derivatives. Some of these substrates were synthesized for this purpose. In one study, amphetamine and two of its N-monosubstituted derivatives, N-ethylamphetamine and N-n-butylamphetamine, were examined. No N-dealkylation was observed. However, the two N-alkylated amphetamines and amphetamine itself were ring-hydroxylated in the 4-position, but only to a small extent. Various ring-methoxylated amphetamines are drugs of abuse. Three 4-methoxylated amphetamines were metabolized with expressed CYP2D6 enzyme, and all three were readily ring-O-demethylated. The extent of O-demethylation was greatest with the N-n-butyl derivative, less with the N-ethyl derivative and least with 4-methoxyamphetamine which contained no N-substituent. Once again, no N-dealkylation was observed. Finally, five N,N-dialkylated amphetamines were evaluated as substrates of expressed CYP2D6. The results obtained were unexpected. None of the five compounds was ring-hydroxylated and none underwent N,N-di-dealkylation. One compound, N-methyl-N-benzylamphetamine was not metabolized at all and N-methyl-N-n-propylamphetamine was biotransformed to N-methylamphetamine and N-n-propylamphetamine but yields of both products were low. Three compounds were efficiently metabolized by CYP2D6; N,N-diallylamphetamine was efficiently metabolized to N-allylamphetamine; N-methyl-N-propargylamphetamine gave two products, N-propargylamphetamine and N-methylamphetamine, and N-allyl-N-methylamphetamine was converted to the corresponding products, N-allylamphetamine and N-methylamphetamine. This preliminary study should be extended to include other N,N-dialkylated amphetamines. Clearly, the chemical nature of the N-substituents has a profound effect on the extent of N-dealkylation catalyzed by CYP2D6.

6.2 FUTURE STUDIES

Although, this study has identified most enzymes involved in the N-demethylation of AT to NT, further research still needs to be carried out that, perhaps, will provide the answers to questions that remain unanswered. One major area of study should be an evaluation of *in vitro* correlations of NT formation by two different sources of enzyme preparations – human enzymes and human recombinant enzymes. Relative contribution of each isoenzyme should be determined. Are the data generated by these two enzyme sources compatible with those obtained from *in vivo* data?. AT can serve as a good model to illustrate this point because AT elimination is mainly by one pathway, its conversion to NT. A second study that is worth conducting would be to apply the enzymatic methodologies developed for AT metabolism to other substrates that are biotransformed *in vivo* mainly by a single pathway in order to be able to draw general conclusions about *in vitro* and *in vivo* enzymatic correlations.

With regard to C-oxidations and N-demethylations that are catalyzed by CYP2D6, results from the present study of N-alkylated amphetamines and deprenyl analogs clearly showed that CYP2D6 has the ability to mediate both metabolic reactions. However, the involvement of this enzyme in the N-dealkylation reaction can be expanded further in studies on other dialkylated amines to investigate what are the required chemical structures for CYP2D6-catalyzed N-dealkylation to occur. A knowledge of CYP2D6–substrate relationships in the N-dealkylation procedure could be a very useful basis for prediction of other potential CYP2D6 substrates.

6.3 REFERENCES

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